Role of CD38 in chronic lymphocytic leukaemia cell motility

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by

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Abstract

Chronic lymphocytic leukaemia (CLL) is characterised by the proliferation of malignant B cells that progressively accumulate into lymphoid tissues and peripheral blood. Whereas CLL cells in the peripheral blood are mainly resting, proliferative and survival signals are provided to CLL cells within the lymphoid tissues in specific structures known as proliferation centres. Understanding the molecular basis for CLL cell migration and retention within lymphoid tissues is therefore essential to devise new treatment strategies for CLL. Expression of the surface molecule CD38 on CLL cells is a marker of poor prognosis. CD38 is a transmembrane ectoenzyme involved in Ca^{2+} mobilization, and although CD38 expression in CLL cells has been linked to cell migration, the underlying molecular mechanisms are unknown.

In this study, the role of CD38 in cell motility was investigated using a CD38 stably transfected CLL-derived cell line (MEC1) and primary CLL cell samples with different CD38 expression levels. CD38 expression markedly enhanced MEC1 cell basal migration and chemotaxis towards the chemokine CCL21. Additionally, CD38 expression increased MEC1 cell spreading on VCAM-1 and reduced their ability to crawl on and transmigrate through an endothelial cell monolayer. These results correlated with increased Rap1 GTPase activity observed in cells expressing CD38 compared to control cells, both in resting conditions and after CCL21 stimulation. An important finding was that CD38 expression increased intracellular basal Ca^{2+} levels in MEC1 cells. Knockdown and localisation studies in CD38-expressing MEC1 cells revealed that RasGRP2, a Ca^{2+}-regulated guanine nucleotide exchange factor for Rap1, may act as a critical signalling molecule in regulating the CD38-dependent migratory phenotype observed. Data obtained with primary CLL samples indicate that a similar mechanism could be responsible for the increased migration linked to CD38 expression in CLL cells.

In conclusion, this study reveals a link between CD38 and a RasGRP2-Rap1 signalling axis, which could contribute to our understanding of the role of CD38 in CLL cell motility and disease progression.

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Abbreviations

APC	antigen presenting cell
DAPI	4',6-diamidino-2-phenylindole
AID	activation-induced cytidine deaminase
ADPR	ADP-ribose
ATM	ataxia telangiectasia mutated
BAFF	B cell activating factor
BAFFR	B cell activating factor receptor
BCMA	B cell maturation antigen
BCR	B cell receptor
Bam32	B-lymphocyte adaptor molecule of 32 kDa
BSA	bovine serum albumin
Btk	Bruton tyrosine kinase
CCR	CC-chemokine receptor
CLL	chronic lymphocytic leukaemia
CSR	class-switch recombination
CDR	complementarity-determinant regions
C	constant
CXCR	CXC-chemokine receptor
cADPR	cyclic ADP-ribose
DOCK	dedicator of cytokinesis
DLEU	deleted in leukemia gene
DAG	diacylglycerol
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
D	diversity
DMEM	Dulbecco's modified Eagle's medium
ER	endoplasmic reticulum
EBI2	Epstein-Barr virus-induced molecule 2
ECM	extracellular matrix
ERK	extracellular regulated kinase
ERM	ezrin, radixin and moesin
FC	flow cytometry
FISH	fluorescence in situ hybridisation
FCS	foetal calf serum
FDC	follicular dendritic cell
FSC	forward scatter
Fab	fragment antigen binding
GPCR	G protein-coupled receptor
GC	germinal centre
GAP	GTPase-activating protein
GDI	guanine nucleotide dissociation inhibitor

GEF	guanine nucleotide exchange factor
HS1	hematopoietic lineage cell-specific 1
HEV	high endothelial venules
HUVEC	human umbilical vein endothelial cell
IF	immunofluorescence
Ig	immunoglobulin
ITAM	immunoreceptor tyrosine-based activation motif
IP_3	inositol trisphosphate
ICAM-1	intercellular adhesion molecule 1
IRM	interference reflection microscopy
IMDM	Iscove's modified Dulbecco's medium
IPTG	isopropyl β-D-1-thiogalactopyranoside
J	joining
JAM	junctional adhesion molecule
LFA-1	leukocyte function-associated 1
LB	Luria broth
MHC	major histocompatibility complexes
MRC	marginal reticular cell
MMP	matrix metallopeptidase
TRPM	melastatin-related transient receptor potential cation channel
MAPK	mitogen-activated protein kinase
mAb	monoclonal antibody
M-CLL	mutated CLL
MLC	myosin light-chain
NADP	NAD phosphate
NAD	nicotinamide adenine dinucleotide
NA	nicotinic acid
NAADP	nicotinic acid adenine dinucleotide phosphate
NFAT	nuclear factor of activated T-cells
NF-κB	nuclear factor-κB
NLC	nurselike-cell
PBD	p21-binding domain
PBMC	peripheral blood mononuclear cells
PMSF	phenylmethylsulfonyl fluoride
PBS	phosphate-buffered saline
PI3K	phosphatidylinositol 3-kinase
PI4K	phosphatidylinositol 4-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol-3,4,5-trisphosphate
PI5K	phosphatidylinositol-4-phosphate 5-kinase
PLC	phospholipase C
PC	plasma cell

PECAM-1	platelet/endothelial cell adhesion molecule-1
RalGDS	Ral guanine nucleotide dissociation stimulator
RBD	Rap binding domain
RIAM	Rap1-GTP-interacting adaptor molecule
RasGRP	Ras guanyl nucleotide-releasing protein
RAG	recombination-activating gene
ROCK	Rho-associated protein kinase
RYR	ryanodine receptor
SLO	secondary lymphoid organ
SSC	side scatter
SHM	somatic hypermutation
S1P	sphingosine 1-phosphate
S1PR	sphingosine 1-phosphate receptor
Syk	Spleen tyrosine kinase
SH2	Src homology 2
SMAC	supramolecular activation cluster
sIg	surface Ig
TCR	T cell receptor
TdT	terminal deoxynucleotidyl transferase
TEM	transendothelial migration
TACI	transmembrane activator and calcium modulator and cyclophilin ligand interactor
TBS-T	tris-buffered saline – Tween
TNF	tumour necrosis factor
ITIM	tyrosine-based inhibition motif
U-CLL	unmutated CLL
V	variable
VCAM-1	vascular cell-adhesion molecule 1
VLA-4	very late antigen 4
WAVE	WASP-family verprolin-homologous protein
WB	western blot
WASP	Wiskott-Aldrich syndrome protein
ZAP-70	ζ-chain-associated protein kinase 70 kDa

1. Introduction

1.1 The immune system

The immune system is a combination of cellular and molecular components with the main function of defending an organism against infections and toxic or allergenic substances that alter normal homeostasis. There are two main categories of response to pathogens. The first is the innate (natural) response, which occurs to the same extent irrespective of the type of infectious agent and of the number of times the infectious agent has been encountered. The second mechanism is the adaptive (acquired) response, which is able to improve after consecutive exposure to a specific infection (Delves and Roitt, 2000). The innate response is based on the recognition of molecular patterns shared by a large number of pathogens that are not present in the host. Because of this non-specificity, innate responses act rapidly after pathogen encounter and represent the initial host response. The cellular components of the innate immune system are phagocytic cells (neutrophils, monocytes and macrophages), natural killer cells, and cells that release inflammatory mediators (basophils, mast cells, and eosinophils) (Chaplin, 2010). In contrast, the adaptive responses are based on the recognition of specific foreign structures. This specificity is obtained through somatic rearrangement of the gene elements that encode for the antigen-binding molecules. Acquired immune responses involve the proliferation of antigen-specific B- and T- lymphocytes, which occurs upon the binding of their surface receptors to a specific antigen. Moreover, specialized antigen-presenting cells (APCs) participate in the acquired immune response by displaying on their surface antigens complexed with major histocompatibility complexes (MHCs) (Chaplin, 2010). The best characterised APCs are the dendritic cells, mostly present in secondary lymphoid tissues.

1.2 B-lymphocytes

B-lymphocytes are characterised by the expression of immunoglobulin (Ig) responsible for the recognition of specific antigen epitopes. Ig can be produced in two different forms: one soluble (antibody) and one bound to the cell surface (sIg) as a component of the B cell receptor (BCR). The Ig molecule consists of two identical heavy chains and two identical light chains bound together by disulphide bonds. The N-terminal region of each chain possesses a variable domain, V_H in the heavy chains and V_κ or V_λ in the light chains (Janeway et al., 2001). Each variable domain is composed of three hypervariable complementarity-determinant regions (CDR1, CDR2 and CDR3), which confer specificity and affinity for the cognate antigen. Therefore, each antibody possesses two identical antigen-binding arms (Fab) (Fig. 1.1). Conversely, the C-terminal domains of the heavy and light chains are constant within each Ig isotype (IgG, IgE, IgM, IgD and IgA). The constant region of the two heavy chains represents the Fc domain of the Ig, which mediates the binding to Fc receptors expressed on the cell surface of innate immune cells, such as mast cells, neutrophils, monocytes and macrophages (Delves and Roitt, 2000). These interactions trigger several effector responses, including the release of cytotoxic molecules, pro-inflammatory mediators and chemoattractant, as well as phagocytosis of immune complexes (e.g. antibody-coated microorganisms), depending on the immune cell involved (Nimmerjahn and Ravetch, 2008).

It has been estimated that B-lymphocytes are able to produce approximately 10^{15} different variable regions originating from 400 genes. This diversity is obtained by recombination processes that modify the variable gene products. The genes encoding the light chains κ and λ are located on chromosome 2 and 22, respectively. The heavy chain locus is encoded on chromosome 22 and it is composed of four segment regions: variable (V), diversity (D), joining (J) and constant (C). In contrast, the κ and λ light chain cluster contain only the V, J and C segments. A complete variable region is obtained through specific molecules expressed during the rearrangement step of developing B-lymphocytes. The recombination-activating proteins (RAG1 and RAG2) mediate the recombination process. Further diversity is obtained by the addition of non-gene-encoded sequences to the junction sites

mediated by the enzyme TdT (terminal deoxynucleotidyl transferase) (Janeway et al., 2001) (Pieper et al., 2013)(Fig. 1.2).

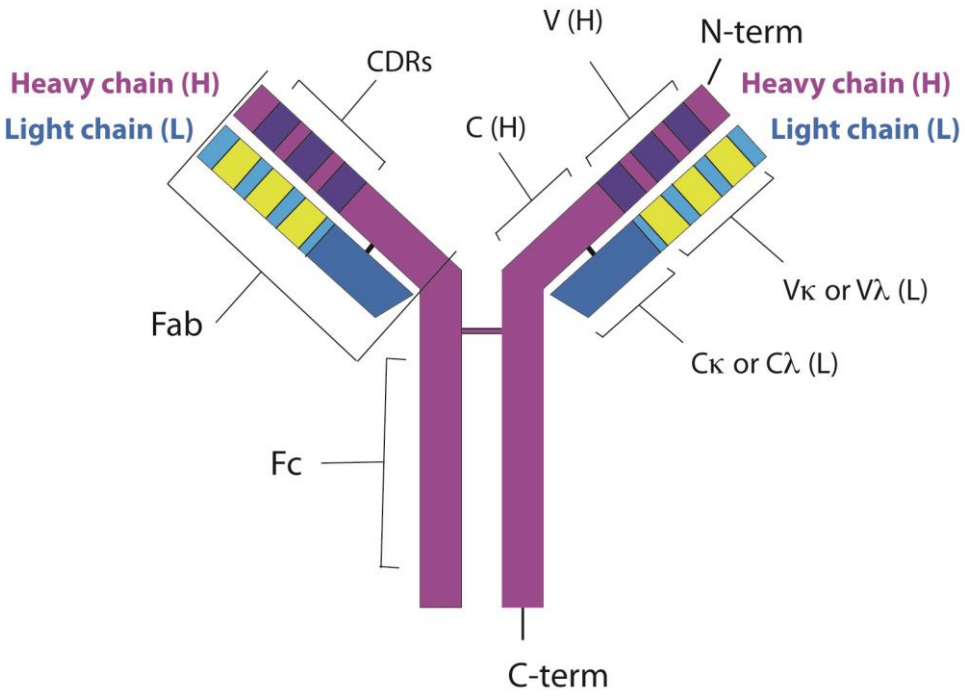

Figure 1.1 Schematic structure of the Immunoglobulin (Ig).
Ig molecules expressed by mature B cells consist of two identical heavy chains (purple) and two identical light chains (blue) bound together by disulphide bonds. The N-terminal domain of each heavy chain together with the full light chain represent the Fab (fragment, antigen-binding) region; each Ig contains two identical antigen-binding Fab regions. Each chain contains a constant domain (C_H for the heavy chain and Cκ or Cλ in the light chain) and a variable domain (V_H in the heavy chain and Vκ or Vλ in the light chain). Within the variable region, three hypervariable complementarity-determinant regions (CDR1, CDR2 and CDR3) mediate the antigen-binding affinity. The C-terminal domains of the two heavy chains form the Fc domain (constant within each Ig isotype), which mediates the binding to Fc receptors, contributing to the innate immune response.

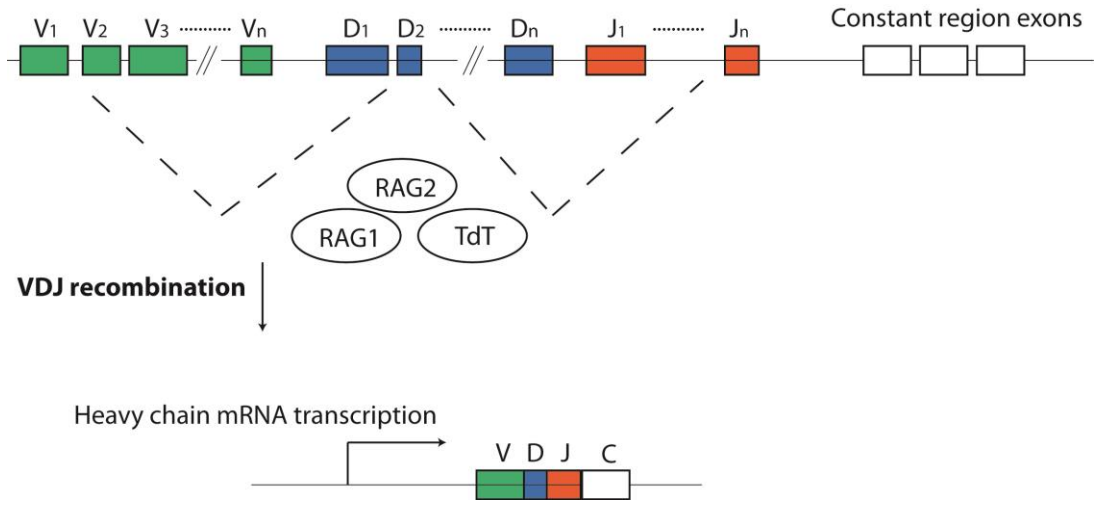

Figure 1.2 Schematic representation of VDJ recombination and generation of the Ig heavy chain locus.
The Ig heavy chain locus is generated by random rearrangement and assembly of genetic elements from among multiple germline variable (V), diversity (D) and joining (J) elements occurring during the pro-B cell maturation into pre-B cell. The recombination-activating gene (RAG1 and RAG2) protein complexes mediate the recombination process. The enzymatic activity of the terminal deoxyribonucleotidyltransferase (TdT) mediates incorporation of nucleotides and splicing inaccuracies, thus enhancing the variability.

1.2.1 Early B cell development

In adults, B-lymphocytes develop from pluripotent haematopoietic stem cells in specialized microenvironments of the bone marrow. Progenitors of B cells (pro-B cells) begin the Ig heavy chain rearrangement by joining a D element to a J element. At the successive developmental step, rearrangement of the V to the DJ elements occurs, providing a complete gene encoding the Ig heavy chain. Subsequently, the Ig heavy chain can associate with a surrogate Ig light chain, consisting of the λ5 and VpreB invariable components, to assemble a pre-BCR and determine the pre-B cell stage. Successful signalling through the pre-BCR and consequent proliferation of pre-B cells is required for the cessation of the surrogate light chain expression and initiation of the recombination of the elements of the κ and λ light chain clusters. Assembly of Ig heavy chain to the successfully rearranged Ig light chain leads to the expression of the IgM on the surface of the immature B cell (Janeway et al., 2001)(Fig. 1.3). The non-covalent association of transmembrane IgM to a heterodimeric Ig-α/Ig-

β (CD79a/CD79b) signalling complex form the BCR (Niiro and Clark, 2002). Signalling through the BCR is essential in determining the fate of developing and mature B cells. The BCR signal transduction pathway will be described in detail in Section 1.4.5.

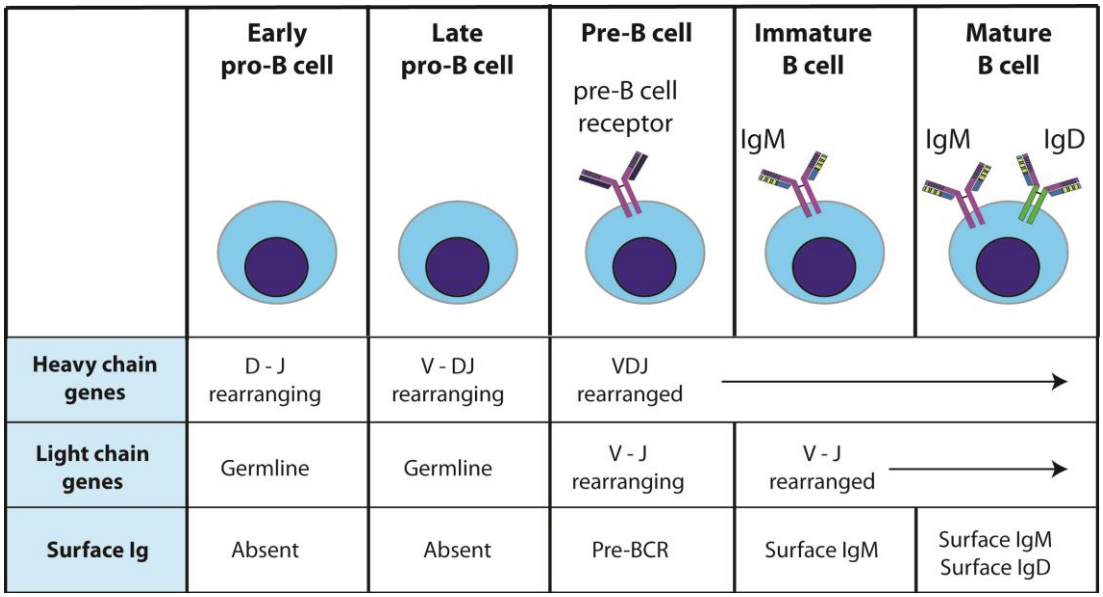

Figure 1.3 Schematic overview of B-lymphocyte development.
Rearrangement and expression of the components of the Ig determines the B cell developmental stages. Recombination of the heavy chain locus starts in early pro-B cells with the rearrangement of the D_H to a J_H genetic element, followed by the rearrangement of V_H to DJ_H during the late pro-B cell stage. If the VDJ_H rearrangement is successful, a complete Ig heavy chain can be expressed on the B cell surface together with a surrogate light chain as components of the pre-B cell receptor (pre-B cell stage). Recombination of the Ig light chain occurs during the pre-B cell stage, followed by the expression of a complete IgM molecule on the surface of immature B cells. Mature B cells, through alternative mRNA splicing, express surface IgD and IgM, which share the same antigen-binding specificity.

It has been suggested that 50-75% of B cells produced in the bone marrow are able to recognise self-antigens (autoreactive) and require to be silenced. Silencing of autoreactive B cells can be achieved by three different mechanisms (Janeway et al., 2001). One mechanism is receptor editing, resulting from the re-expression of RAGs in immature B cells and a further re-arrangement of a new Ig light chain. Other mechanisms are clonal deletion and B cell anergy. If receptor editing fails to eliminate

autoreactivity, self-reacting B cells undergo apoptosis (clonal deletion)(Niiro and Clark, 2002). Whereas high avidity to autoantigens favours receptor editing, low-affinity autoreactivity is thought to induce B cell anergy. Anergy can be defined as the intrinsic unresponsiveness to antigen. This phenomenon has been studied mainly with trangenic mouse models by inducing chronic interaction in B cells with fixed and controlled antigen-specificity, such as the hen-egg-lysozyme-specific, the DNA-specific and the arsonate-specific models (Cambier et al., 2007). Based on these studies, features of anergic B cells appear to be: reduced level of sIgM, decreased lifespan, no proliferation, no antibody secretion and altered anatomical localisation. However, these characteristics vary among different models (Cambier et al., 2007). Notably, a naturally occurring population of anergic B cells has been identified, both in mice and human (Duty et al., 2009; Merrell et al., 2006). The human anergic B cell fraction is characterised by a IgD^+ IgM^- $CD27^-$ phenotype, slightly higher amount of basal Ca^{2+} levels, reduced Ca^{2+} mobilisation and tyrosine phosphorylation in response to BCR cross-linking. Of note, chronic exposure to the antigen is required for the maintenance of anergy, which is considered a reversible state (Gauld et al., 2006).

1.2.2 The germinal centre reaction

Immature B cells that have successfully gone through developmental checkpoints leave the bone marrow and migrate to the peripheral lymphoid tissues. There they undergo a further developmental step and become mature B cells, expressing surface IgD, in addition to IgM, with identical antigen-specificity. Mature B cells, called naive B cells before antigen encounter, can eventually be activated upon binding of the BCR to the appropriate antigen and develop within the germinal centres (GCs) of spleen and lymph nodes into memory B cells or plasma cells (PC).

GCs are specialised structures that are generated in the B cell follicular compartments of secondary lymphoid organs (SLOs) in response to antigen challenge. Alongside antigen-experienced B cells, two types of accessory cells are found in GCs: follicular dendritic cells (FDCs) and follicular T helper cells (Gatto and Brink, 2010). FDCs are stromal cells that retain unprocessed antigen through Fc and complement receptors, acting as APCs. Follicular T helper cells constitute 5-20% of GC cells and provide

survival signals for GC B cells (Allen et al., 2007). BCR binding to the antigen on the surface of APCs leads to the internalization and processing of the antigen. Once the antigen has been processed, antigen-derived peptides can be loaded onto MHC molecules on the B cell surface and presented to helper T cells in the GCs.

GCs represent the primary site where antigen-experienced B cells undergo diversification and affinity maturation. Mature B cells constantly migrate within SLOs surveying for antigens. Antigen binding induces B cell activation and migration towards the T cell areas to encounter helper T cells. Interaction between the tumour necrosis factor (TNF) receptor family member CD40, constitutively expressed on the B cell surface, and its ligand CD40L (CD154) expressed by activated T cells together with T cell secreted cytokines, such as IL-21 and IL-4, drive B cell proliferation and GC formation (Gatto and Brink, 2010). During B cell proliferation, a high number of mutations are induced in the variable region of both heavy and light chain Ig genes by the activity of the enzyme activation-induced cytidine deaminase (AID), through a process known as somatic hypermutation (SHM) (Janeway et al., 2001). SHM leads to random variation in the BCR affinity and specificity to the immunising antigen. Following SHM, decreased antigen recognition induces GC B cells to undergo apoptosis, whereas increased affinity to the antigen induces clonal expansion. This is due to the fact that GC B cells are intrinsically predisposed to apoptosis and highly dependent on pro-survival signalling, due to their low expression level of the anti-apoptotic factors Bcl-2 and Bcl-X and elevated expression of surface death receptor (FAS) and the pro-apoptotic BIM (Gatto and Brink, 2010).

Subsequently, cytokines secreted from GC helper T cells induce immunoglobulin class-switch recombination (CSR), which like SHM also requires the enzyme AID. CSR is an irreversible somatic recombination process that induces the rearranged VDJ region to relocate into a new position upstream of alternative constant region, leading to the production of B cells expressing IgG, IgA and IgE with the same antigen specificity but with different effector functions (Janeway et al., 2001).

1.3 Regulation of immune cell trafficking

Trafficking and compartmentalisation of immune cells in secondary lymphoid organs, particularly in lymph nodes, are fundamental mechanisms that maximise the probability that rare antigen-specific B and T cells encounter their cognate antigen. *In vivo* analysis of murine lymphocyte migration patterns have shown that B cells spend approximately 24 hours 'exploring' a lymph node before migrating to another tissue (Tomura et al., 2008). During homeostasis, lymphocytes recirculate daily from the blood to the lymphoid organs and back to the blood.

1.3.1 Lymphocyte entry into the lymph nodes

B- and T-lymphocytes reach the lymph nodes mainly by crossing the endothelial barrier represented by the high endothelial venules (HEVs) through a mechanism defined as extravasation or transendothelial migration (TEM) (von Andrian and Mempel, 2003). Entry via afferent lymphatics has also been described for T cells (Braun et al., 2011). HEVs are a specific type of blood vessel that under physiological conditions are found exclusively in secondary lymphoid organs (excluding the spleen). Lymphocyte extravasation is a multi-step process, which includes the rolling of the lymphocyte on the lumen of the vessel, followed by the arrest of the lymphocyte and its transmigration through the endothelial barrier (diapedesis) (Fig. 1.4). The first level of interaction between lymphocytes and HEVs is mediated by the mucin-like glycoproteins, such as CD34, podocalyxin, endomucin and nepmucin, which are expressed by the endothelial cells and highly post-translationally modified (sulphated, fucosylated and sialylated) (Girard et al., 2012). Interaction of these molecules with the L-selectin expressed on the lymphocyte surface mediates B and T cell tethering and rolling along the endothelial lumen.

Rolling lymphocytes can then firmly arrest on the surface of the HEVs. This is mediated by the activation of lymphocyte integrins (described in detail in Section 1.4.4) and their binding to adhesion molecules expressed on the apical surface of endothelial cells. Activation and binding of the integrin leukocyte function-associated 1 (LFA-1; αLβ2-integrin) to the intercellular adhesion molecule 1 (ICAM-1) on HEVs has been shown to be crucial for lymphocyte TEM, as LFA-1 depletion strongly impairs murine lymphocyte homing to lymph nodes (Berlin-Rufenach et al., 1999). Further

interactions are mediated by vascular cell-adhesion molecule 1 (VCAM-1) on the HEVs, which is able to bind the α4-integrin subunit of the α4β7- and α4β1-integrin heterodimers (α4β1-integrin is also known as very late antigen 4; VLA-4) (Boscacci et al., 2010). It has also been proposed that VLA-4 could be involved in lymphocyte tethering and rolling (Alon et al., 1995).

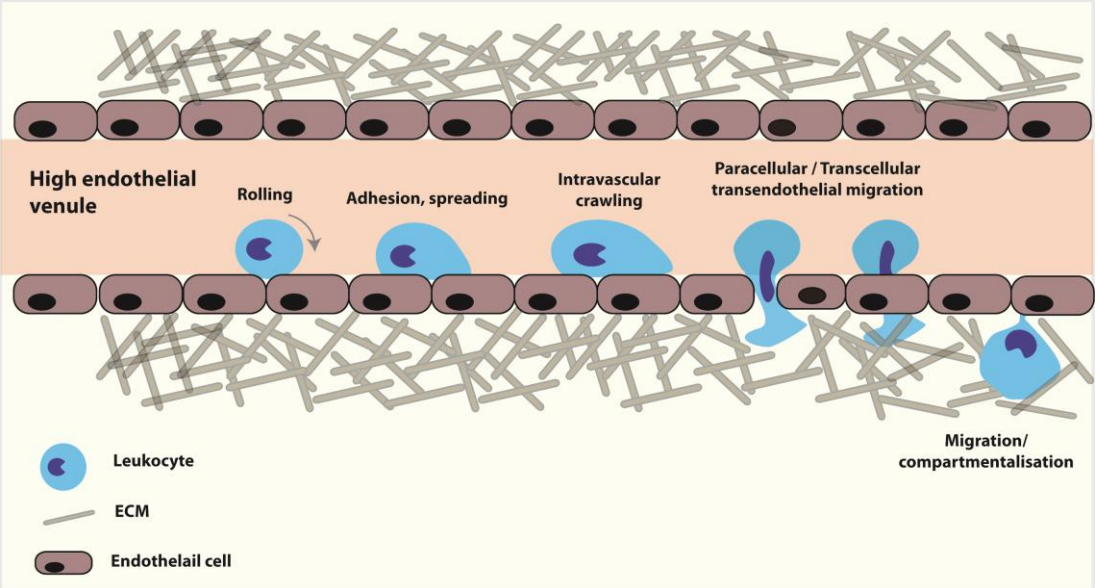

Figure 1.4 Schematic representation of the multi-step process of lymphocyte transendothelial migration (TEM).
In order to exit the blood stream and enter the lymph node, lymphocytes need to extravasate throught the high endothelial venules. The first step consists of lymphocyte rolling on the endothelium, followed by firm adhesion, which mediates its arrest. Subsequently, lymphocytes can crawl along the vessel wall before crossing the endothelium. Diapedesis can occur by a paracellular (between two endothelial cell) or by a transcellular route (across one single endothelial cells). Once in the lymphoid tissue, lymphocytes continue migrating through the extracellular matrix (ECM) and resident cells towards their specific compartment.

Integrin activation is dependent on the binding of chemokines, exposed on the apical surface of the endothelial cells, to the lymphocyte chemokine receptors (Fig. 1.5). The chemokines CCL21, CXCL12 and CXCL13 are constitutively expressed on the HEVs and mediate B-lymphocyte homing through their binding to the CC-chemokine receptor 7 (CCR7), CXC-chemokine receptor 4 (CXCR4) and CXCR5 respectively.

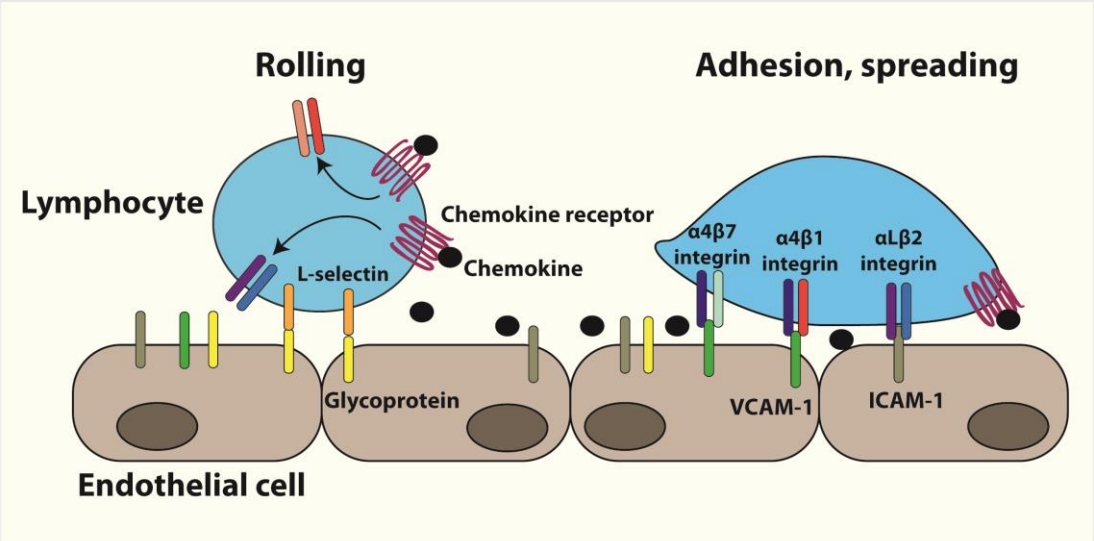

Figure 1.5 Lymphocyte-endothelium interactions during lymphocyte rolling and adhesion.
Lymphocyte rolling is mediated by highly modified glycoproteins expressed on the endothelial apical surface through an intermittent and reversible binding to L-selectin expressed on the lymphocyte. Subsequently, chemokine binding to the chemokine receptors expressed on the lymphocyte surface triggers a signalling cascade that activates integrins and allows firm adhesion and cell spreading. In B-lymphocytes, α4β1, α4β7 and αLβ2 are the major integrins involved in this process by mediating the binding to vascular cell-adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1).

B cell homing to lymph node is less efficient than T cells, possibly because of their lower expression of L-selectin (Tang et al., 1998). Intravital microscopy studies have shown that, after firm adhesion to the endothelial luminal surface, B-lymphocytes crawl, possibly in search of the optimal site, and cross the endothelium within 5 minutes of adhesion (Park et al., 2012).

1.3.2.Transendothelial migration

Several studies performed on leukocytes (general term for white blood cells including neutrophils, monocytes and lymphocytes) have addressed the transendothelial migration process, also known as diapedesis. *In vitro* studies have shown that adherent leukocytes induce the formation of endothelial cell projections that surround the leukocyte cell body and are enriched in vinculin, talin-1 and ERM

proteins (ezrin, radixin and moesin). These structures, defined as 'transmigratory cups' or 'docking structures', mediate the clustering of ICAM-1 and VCAM-1 around the leukocyte and are thought to facilitate the transmigration process (Barreiro et al., 2002; Carman and Springer, 2004).

Leukocyte TEM can occur through a paracellular or a transcellular route. Paracellular TEM involves the transient disruption of the endothelial cell-cell junctions mediated at least in part by the signalling triggered upon leukocyte adhesion. Formation of stress fibres in the endothelial cells could help to disrupt junctions by mechanical tension. Clustering of endothelial ICAM-1 and VCAM-1 has been shown to increase the activity of endothelial RhoA, which can induce stress fibre formation mediated by the activation of the Rho-associated protein kinase (ROCK) and the consequent myosin light chain (MLC) phosphorylation (Millán and Ridley, 2005).

Endothelial junction molecules, such as CD99, junctional adhesion molecules (JAMs) and platelet/endothelial cell adhesion molecule-1 (PECAM-1; CD31) can directly participate in leukocyte diapedesis (Muller, 2009). PECAM-1 is a member of the immunoglobulin superfamily localised mainly at the junctions of endothelial cells. Leukocytes express PECAM-1 with a diffuse localisation, and homophilic interaction between leukocyte and endothelial PECAM-1 was reported to be involved in TEM (Muller et al., 1993). After interaction with neutrophils, endothelial PECAM-1 is translocated from a lateral border recycling compartment, a parajunctional reticulum of vesicles, to the endothelial surface. Inhibition of this mechanism impairs neutrophil TEM (Mamdouh et al., 2003). Homophilic binding of CD99 expressed by leukocytes and endothelial cells has also been shown to mediate leukocyte TEM (Schenkel et al., 2002).

Leukocyte TEM can also occur through the body of an endothelial cell, a mechanism known as transcellular TEM. This mechanism has been described rarely in HEVs, where the paracellular route appear to be the most common form of TEM (Muller, 2009). As for the paracellular pathway, transcellular TEM has been shown to be mediated by an initial clustering of ICAM-1 on the endothelial apical surface in contact with the leukocyte. ICAM-1 is then translocated to the endothelial basal plasma membrane through caveolin- and F-actin-rich regions, resulting in the formation of channels that allow the passage of the leukocyte (Millan et al., 2006).

Transcellular TEM also requires the trafficking through the lateral border recycling compartment of the same molecules involved in paracellular TEM, such as CD99, PECAM-1 and JAMs. An enrichment of PECAM-1 at the site of leukocyte adhesion has also been reported (Carman et al., 2007; Carman and Springer, 2004; Mamdouh et al., 2009), and its contribution to transcellular TEM appears to be fundamental, as blocking PECAM-1 with blocking antibodies is able to inhibit this process. Inhibition of CD99, but not JAM-A, is also able to inhibit transcellular TEM (Mamdouh et al., 2009).

1.3.3 Lymphocyte migration within lymph nodes

After entering the lymph node, lymphocytes need to traffic and strategically compartimentalise to facilitate the encounter with their cognate antigen. Naive B-lymphocytes localise in primary follicles around the T cell zone and move among a network of stromal cells mainly represented by FDCs and marginal reticular cells (MRCs) (Mueller and Germain, 2009) (Fig 1.6). Both these stromal cell types express the chemokine CXCL13, which has been shown to have a critical role in guiding B cell access to the follicles by binding to the chemokine receptor CXCR5 expressed by all mature B cells (Forster et al., 1996). Beside its chemotactic role, CXCL13 also upregulates the B cell expression of the cytokine lymphotoxin α1β2, which promotes a positive feedback by contributing to FDC maturation and CXCL13 expression (Ansel et al., 2000). Within the follicles, B cells are highly motile with an approximate speed of 6 µm per minute (Miller et al., 2002). Lymphocyte migration within lymph nodes shows frequent directional changes and has been defined as a 'random walk', as it appears to be independent of chemoattractant gradients. However, inhibition of the chemokine receptor pathway was shown not only to impair B cell access to the follicles, but also to reduce their migration speed within the follicles, indicating a possible role for chemokines in this process (Han et al., 2005).

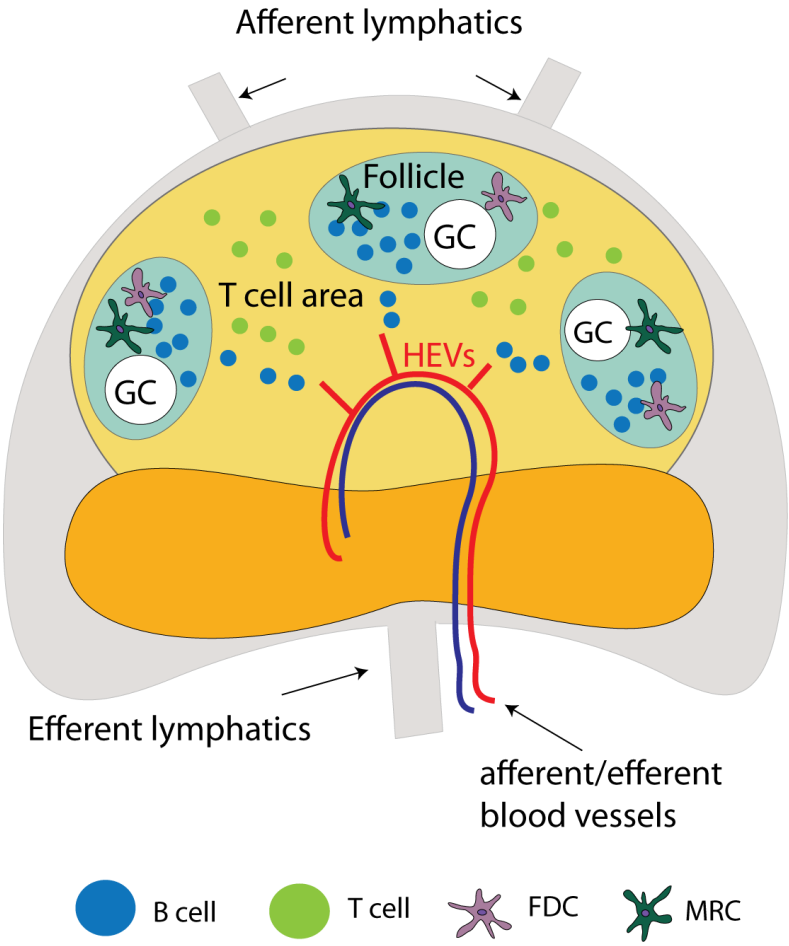

Figure 1.6 Compartmentalisation of lymphocytes within the lymph node.
B and T cells enter the lymph nodes mainly by crossing the high endothelial venules (HEVs), although entry via afferent lymphatics has also been observed for T cells. Chemokine gradients guide B and T cells to localise in the follicles and in the T cell area respectively. Within the follicles, B cells migrate among a network of stromal cells, such as follicular dendritic cells (FDCs) and marginal reticular cells (MRCs) surveying for antigens. Antigen encounter induces B cell activation and the generation of a germinal centre (GC) within the follicle.

It has been reported that B cell migration within the follicles could be guided and constrained by the network of FDC processes, leading to the hypothesis of a molecular signature on the FDC surface involving chemokines and adhesion molecules expressed by FDCs such as ICAM-1 and VCAM-1 (Bajenoff et al., 2006). The chemoattractant-type receptor Epstein-Barr virus-induced molecule 2 (EBI2) has also been shown to be involved in the compartmentalisation of B cells in the follicles (Pereira et al., 2009). Unlike CXCR5, the expression of which stays stable during B cell trafficking, EBI2 levels are increased after B cell activation and this process is crucial

for GC formation. The CCR7 receptor has also been shown to guide B cell distribution in the follicles. Mature B cells express CCR7 at a low level and are able to respond to its ligands CCL21 and CCL19. The CCL21 concentration within the lymph node is high in the T cell zone and diffuses with a gradient that reaches the follicles (Pereira et al., 2010). After antigen-recognition, B cells rapidly upregulate CCR7 expression and migrate towards the T cell zone, positioning at the border of the follicle (Ekland et al., 2004) (Fig. 1.7). The close contact with the T cell zone allows B cells to encounter T cells with cognate-antigen specificity and receive pro-survival signals (Okada et al., 2005). Naive B cells that fail to encounter their cognate antigen leave the lymph node through efferent lymphatics. Studies on the effects of the immunosuppressant FTY720 on normal B cells identified sphingosine 1-phosphate (S1P) as a key molecule involved in lymphocyte exit from lymph nodes. S1P acts by binding to five G-protein coupled receptors: S1PR1 – S1PR5 (Takuwa et al., 2012). Modulation of S1PR1 expression on recirculating lymphocytes is essential for their trafficking, as ligand-induced downregulation of SP1R1 is required to prevent the immediate exit of lymphocytes from the lymph nodes (Matloubian et al., 2004). S1PR2 on the other hand regulates the survival and the clustering of the B cells in germinal centres (Green et al., 2011).

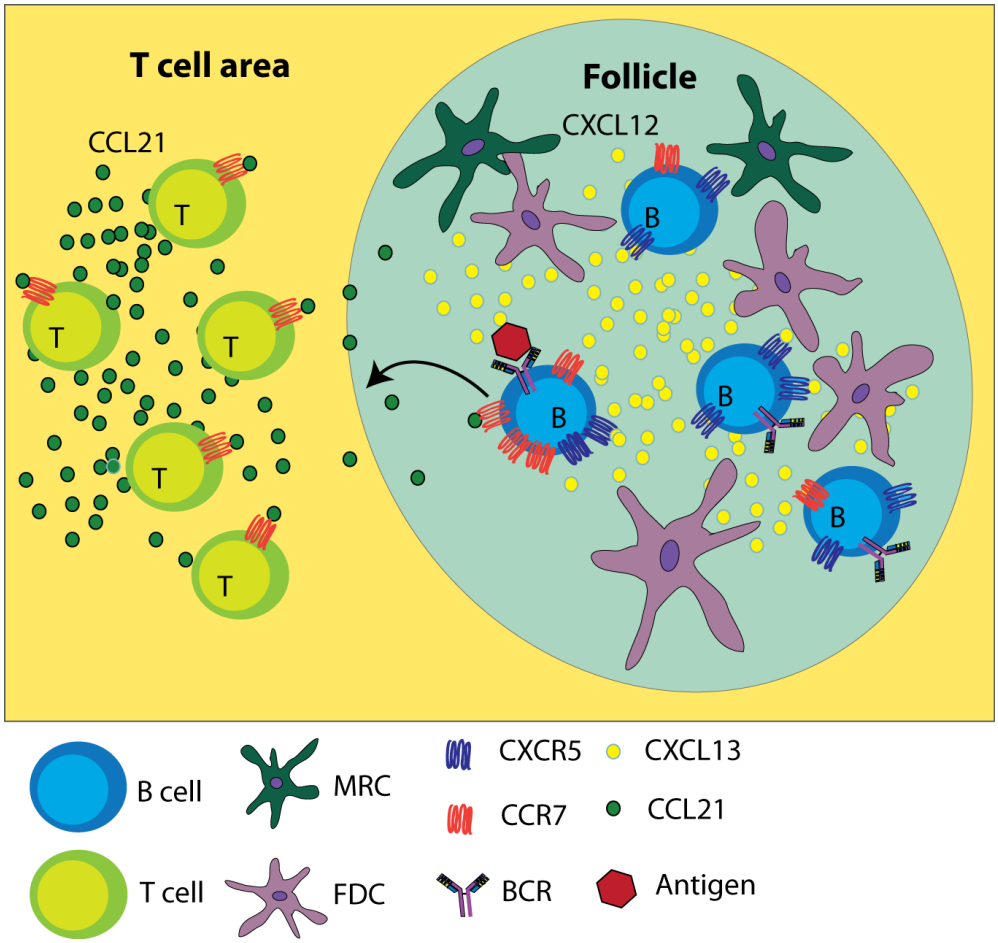

Figure 1.7 Chemokine-mediated B cell migration within the lymph node.
Figure 1.7 The chemokine CXCL13 expressed by FDCs and MRCs guides CXCR5-expressing B cells toward the follicular compartment. The high concentration of CCL21 within the T cell area diffuses with a gradient reaching the follicles. Expression of CCR7 in B cells is low in resting conditions but it is rapidly upregulated after antigen recognition. This allows B cells to migrate towards the border of the follicle and interact with cognate T cells.

1.4 Molecular regulation of lymphocyte motility

Lymphocytes are characterised by an outstanding ability to move rapidly, and most of their maturation and activation steps depend on the dynamical regulation of migration, local arrest and positioning within the tissues. The Rho family of small GTPases are crucial players in the coordination of signalling pathways involved in lymphocyte adhesion and locomotion on different substrates (Friedl and Weigelin, 2008; Tybulewicz and Henderson, 2009). They also contribute to other cellular processes including survival and cell cycle progression (Coleman et al., 2004). In B-lymphocytes, *in vitro* and *in vivo* experiments have shown that Rho GTPases together with their regulatory and effector molecules are involved in B cell development, B cell receptor signalling and cell migration (Tybulewicz and Henderson, 2009). Rho GTPases are part of the Ras superfamily, which also include the Ras, Rab, Arf and Ran families (Vega and Ridley, 2008). The Rap GTPases, a subgroup of the Ras family, are also involved in B cell function.

1.4.1 Rho and Rap GTPases

Most Rho and Rap GTPases, including the best-characterized Rho family members RhoA, Cdc42 and Rac1/2, switch between an inactive GDP-bound and an active GTP-bound form. This mechanism is finely regulated by two major classes of regulatory protein: the guanine nucleotide exchange factors (GEFs), which facilitate GDP dissociation with the consequent binding to GTP, and the GTPase-activating proteins (GAPs), which stimulate the hydrolysis of bound GTP to GDP (Fig. 1.8). GTP-bound Rho and Rap GTPases interact with and activate multiple downstream effectors. Some Rho family members, for example RhoH, are unable to hydrolyse GTP and are constitutively GTP-bound. They are defined as atypical and their activity is mainly regulated by protein expression, phosphorylation and stability (Heasman and Ridley, 2008).

Further regulation of Rho and Ras GTPase activity is mediated by their subcellular localisation and interactions with distinct membrane compartments. Rho and Ras proteins can be post-translationally modified, by the addition of a lipid group by prenylation to the CAAX motif (C=Cys, A=aliphatic, X=any amino acid) and/or palmitoylation of different cysteine residues at their C-terminus, facilitating their

membrane association and interaction with downstream effectors (Roberts et al., 2008; Vega and Ridley, 2008). Guanine nucleotide dissociation inhibitors (GDIs) can negatively regulate this mechanism by binding to the prenyl group and promoting cytosolic sequestration of several Rho GTPases (Dovas and Couchman, 2005).

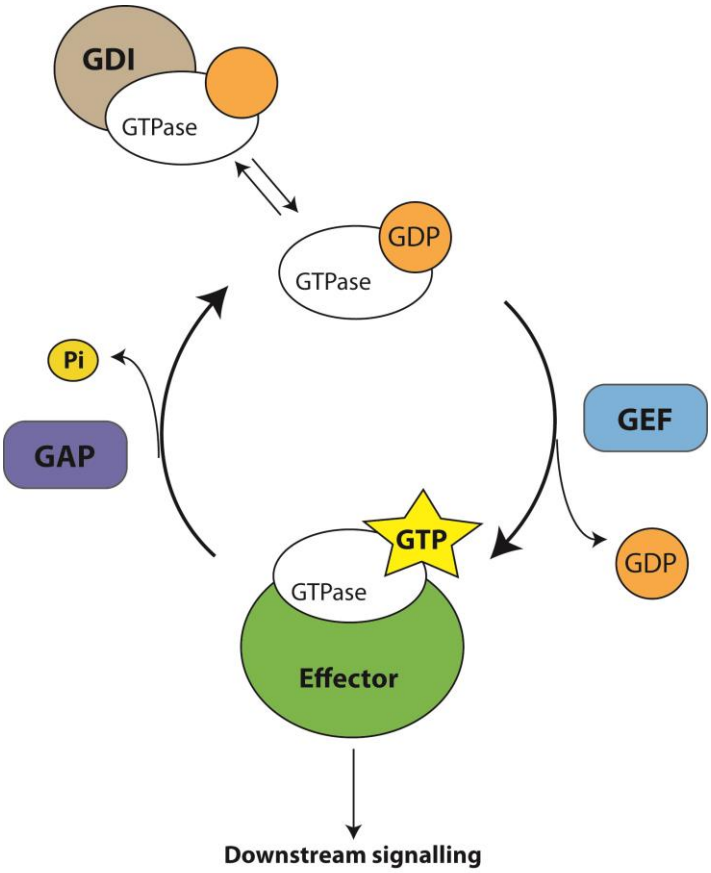

Figure 1.8 The Rho and Ras GTPase cycle.
Most Rho and Ras GTPases cycle between a GDP-bound (inactive) to a GTP-bound (active) form. Guanine nucleotide exchange factors (GEFs) facilitate the dissociation of the GDP, allowing a GTP molecule to bind the unbound GTPase. GTP-bound GTPases can bind their effectors and mediate multiple signalling cascades. GTPase activating proteins (GAPs) enhance the intrinsic GTPase activity and the hydrolysis of GTP to GDP, leading to inactivation of the GTPase. A third mechanism of regulation is represented by the guanine nucleotide dissociation inhibitor (GDI), which can bind prenylated GTPases and extract them from the membrane compartment to the cytosol, therefore impairing their activity.

1.4.2 Roles of Rho and Rap GTPases in chemokine-mediated signalling

Chemokine receptors are G protein-coupled receptors (GPCRs). After the chemokine binds to its cognate receptor, the heterotrimeric G-protein dissociates into the Gα- and the Gβγ-subunits that are then able to activate downstream signaling cascades that differ depending on the subunits involved. Several α,β, and γ subunits have been identified. Gα and associated Gβγ subunits have been grouped into the Gi, Gq, Gs, and G12/13 subfamilies, based on their heteromeric combination (Kehrl, 2006). Chemokine receptors mainly associate with the Gi and Gq subfamily members. Inhibitors of Gi, such as pertussis toxin, have been shown to block the binding of leukocytes to HEVs and their homing into lymph nodes (Bargatze and Butcher, 1993; Cyster and Goodnow, 1995). Many different pathways are activated after chemokine receptor stimulation, which ultimately lead to the cytoskeleton reorganisation required for the lymphocyte to polarise and migrate (Thelen and Stein, 2008). The phosphatidylinositol 3-kinase (PI3K) pathway is known to be involved in the chemokine response downstream of the Gβγ subunit. There are 3 classes of PI3Ks, the Class I PI3Ks are best characterized for their roles in B cells. The Class I PI3Ks consist of heterodimers containing a catalytic subunit p110 and a regulatory subunit (Cain and Ridley, 2009). The p110δ subunit in particular has been shown to have a crucial role in B-lymphocyte motility, as p110δ depletion or treatment with specific pharmacologic inhibitors impairs B cell chemokine responses (Reif et al., 2004).

Chemokine receptor stimulation also induces the activation of phospholipase C (PLC), which mediates the cleavage of membrane phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$ or PIP_2) to produce two intracellular second messengers: diacylglycerol (DAG) and inositol trisphosphate (IP_3). IP_3 mobilises Ca^{2+} from intracellular stores and from extracellular compartments. In B cells, PLCγ2 has been reported to be the PLC isotype involved in this mechanism, probably downstream of the Bruton tyrosine kinase (Btk) (de Gorter et al., 2007). Btk-deficient B cells showed an impaired PLCγ2 activation, correlating with reduced adhesion and migration in response to CXCL12 and CXCL13. It has been proposed that the residual B cell motility could be controlled by the PI3K pathway.

Rho and Rap GTPases are critical signal transducers of chemokine-mediated pathways (Fig. 1.7). RhoA has been proposed to be involved in the B cell chemokine-response through its GEF LSC (also known as ARHGEF1). LSC is able to bind the GPCR-associated Gα12/13 subunit through its regulator of G protein signalling (RGS) domain, and down-regulate the GPCR signalling (Tybulewicz and Henderson, 2009). LSC-depleted marginal zone B cells showed increased ability to migrate in response of S1P, indicating that LSC and possibly RhoA are involved in the regulation of the S1PR1-mediated lymph node egress of B cells in mice (Girkontaite et al., 2001). Moreover, LSC-depleted marginal zone B cells showed a reduced ability to detach from ICAM-1 during their *in vitro* migration, probably due to a requirement for LSC-mediated RhoA activation during cell detachment from integrin ligands (Rubtsov et al., 2005).

Rac has also been shown to be involved in B cell chemokine responses. Depletion of Rac1 and Rac2 impairs *in vivo* B cell homing to lymph nodes and spleen, in agreement with their decreased migration *in vitro* in both basal conditions and upon stimulation with CXCL12, CXCL13 and CCL21 (Croker et al., 2002; Henderson et al., 2010). Several GEFs can be involved in the regulation of Rac activation during chemokine responses, depending on the cell type. In B cells, the Rac-specific GEF DOCK2 has been shown to have a critical role in the activation of Rac in the migratory response to CXCL12, CXCL13 and S1P (Nombela-Arrieta et al., 2004; Nombela-Arrieta et al., 2007). DOCK2 deficiency in B-lymphocytes was shown to partly reduce chemokine-mediated Rap1 activation, indicating that this Rac GEF and possibly Rac itself could be involved in the activation of Rap1 during this process (Fig 1.9).

Rap proteins (Rap1a,b and Rap2a,b,c) have been shown to have a crucial role in chemokine responses. Studies performed in primary murine lymphocytes have shown that CXCL4 and CCL21 stimulation induces rapid GTP-loading of Rap1 (Shimonaka et al., 2003), and CXCL12 stimulation of B cells has been shown to induce activation of both Rap1 and Rap2 through PLC (McLeod et al., 2002). Inhibition of Rap proteins through the expression of RapGAPII was shown to inhibit CXCL12-mediated chemotaxis in B cell lines. Notably, spontaneous migration was also reduced after Rap inhibition in a murine B cell line (McLeod et al., 2002). In agreement with these

data, *in vivo* studies have shown that Rap1b depletion reduces murine B cell homing to lymphoid organs (Chen et al., 2008b; Chu et al., 2008).

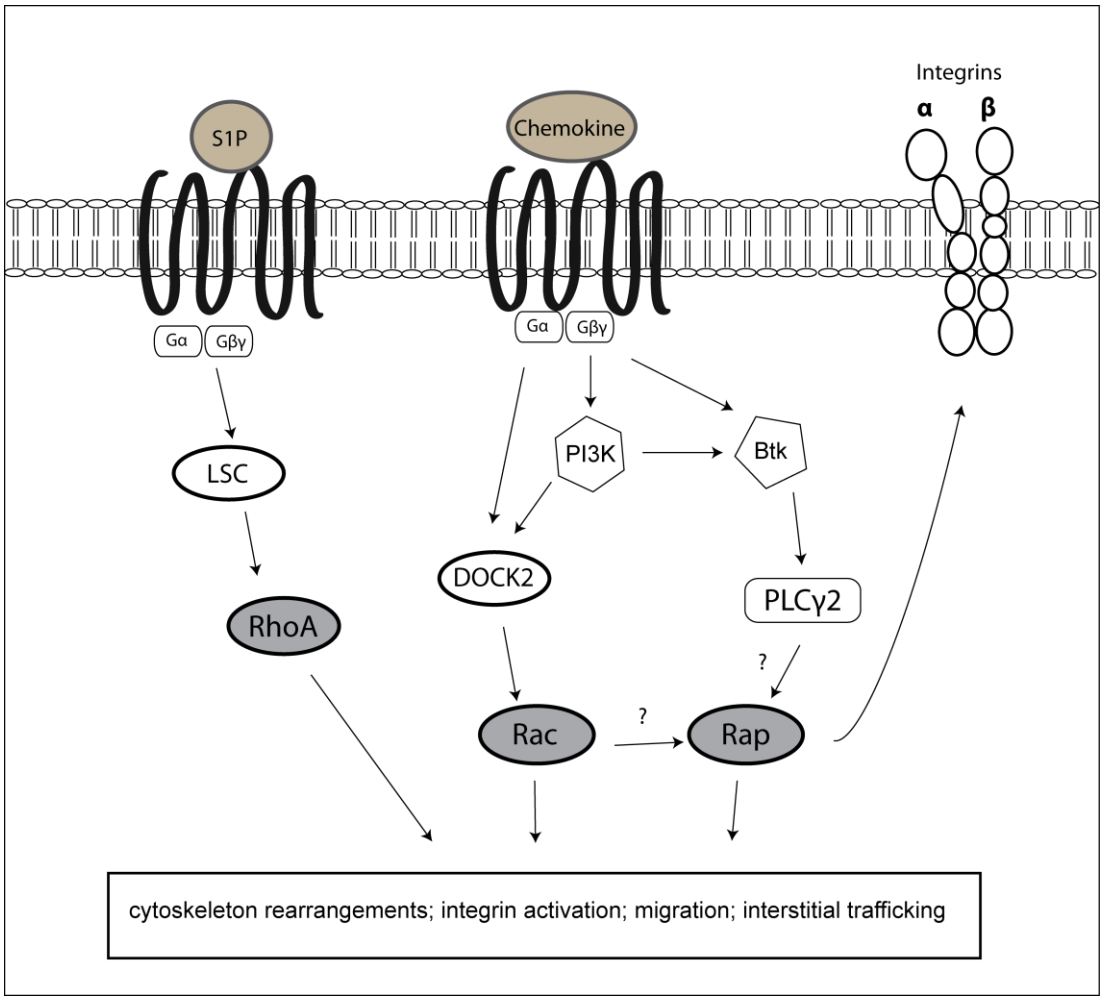

Figure 1.9 Roles of Rho and Rap GTPases in B cell chemokine-mediated signalling. Chemokine signalling is mediated through binding of chemokines to their cognate receptors on the surface of B cells. Chemokine receptors are associated with heterotrimeric G-proteins that, after chemokine binding, dissociate and trigger several signalling cascades. The GTPases RhoA, Rac and Rap have key roles in the cytoskeleton rearrangements and integrin activation necessary for B cells to migrate and traffic in response to chemokines. Chemokine receptors expressed by B cells such as CCR7, CXCR5, CXCR4 guide them towards the lymphoid organs, whereas the S1P receptors (S1PRs) are involved in the B cell exit from lymphoid organs. PLCγ2, phospholipase C-γ2; DOCK2, dedicator of cytokinesis 2; PI3K, phosphatidylinositol 3-kinase; Btk, Bruton tyrosine kinase.

1.4.3 Lymphocyte polarisation and locomotion

Cell polarity is a pre-requisite for cell migration. Whether migration is directed by a chemoattractant gradient (chemotaxis) or random directionality in the presence of a uniform chemokine concentration (chemokinesis), cells need to acquire functional and morphological asymmetry to generate forward movement (Lauffenburger and Horwitz, 1996). Polarised leukocytes lose their round morphology by acquiring a broad protruding leading edge called a lamellipodium and a short cylindrical-shaped structure at the rear defined as a uropod (Friedl and Weigelin, 2008). Studies performed *in vitro* have shown that human T-lymphocytes stimulated with CCL19 form several lamellipodia, one of which is subsequently stabilised and this leads to the formation of a uropod on the opposite cellular pole (Real et al., 2007). The formation and maintenance of the leading edge is mediated by an asymmetric distribution of the actin cytoskeleton. Actin polymerarisation at the front of the cell induces the formation of a broad branched network of actin filaments. This process is mediated by actin nucleators, such as the Arp2/3 complex, which stimulates the addition of actin monomers to pre-existing actin filaments and whose activity is modulated by adaptor proteins such as the Wiskott-Aldrich syndrome proteins (WASPs) and the WASP-family verprolin-homologous proteins (WAVEs) (Campellone and Welch, 2010). Rac1 and Rac2 have been shown to be key regulators of lamellipodium formation in several cell types (Ridley, 2011). Studies performed using GTPase biosensors in migrating fibroblasts have shown that active RhoA, Cdc42 and Rac1 are localised at the leading edge. RhoA appears to initiate the formation of lamellipodial protrusions, while Cdc42 ad Rac1 have a subsequent stabilising role (Machacek et al., 2009). In T cells, RhoA was shown to be active at the front during TEM (Heasman et al., 2010).

GTP-bound Cdc42 has been shown to bind and activate WASP and N-WASP, inducing activation of the Arp2/3 complex and chemotaxis in T-lymphocytes (Goley and Welch, 2006). In B cells, WASP deficiency induced impaired adhesion, migration and homing, suggesting that a similar mechanism could mediate B-lymphocyte motility (Westerberg et al., 2005). In agreement with this observation, Rac2 depletion in murine B cells significantly decreases the level of basal and chemokine-induced actin polymerisation (Henderson et al., 2010).

Rap proteins are also important players in lymphocyte polarisation. Constitutively active Rap1 expression in B cells was shown to enhance both spontaneous and chemokine-induced cell polarisation (Shimonaka et al., 2003). TEM was also enhanced in the presence of constitutively active Rap1, although this required the application of shear stress (Shimonaka et al., 2003).

In order for the cell to move forward, the actin polarisation at the leading edge must be coordinated with a contractile force at the trailing edge. This force is generated in the uropod by actomyosin contraction triggered by phosphorylation of the myosin light-chain (MLC). This mechanism is controlled by the activation of RhoA and its effector ROCK (Lee et al., 2004).

Cell polarisation also includes an asymmetrical distribution of both intracellular and plasma membrane receptors. For example, polarised B cells show redistribution of chemokine receptors at the leading edge whereas CD44 is mainly found in the uropod. This phenomenon has been observed in B cells polarised upon chemokine stimulation but also in B cells polarised upon constitutively active Rap1 expression (Shimonaka et al., 2003). Interestingly, *in vivo* studies have suggested that antigen-bearing B cells in the lymph nodes appear to transport the antigen on their uropod during their migration (Carrasco and Batista, 2007).

1.4.4 Lymphocyte adhesion: the role of integrins

Arrest of migrating lymphocytes requires the activation of adhesion receptors on the lymphocyte surface and a subsequent binding to counter-receptors found in the extracellular matrix or surrounding cells (Dixit and Simon, 2012). Integrins, a family of proteins with a double role of adhesion molecules and receptors, are key players in this process. Integrins are transmembrane heterodimers, with one α- and one β-subunit; a total of 18 α-subunits and 8 β-subunits have been identified; association of these subunits allows the formation of at least 24 known integrins (Kinashi, 2005). Integrins are in equilibrium between different conformational and activity states. In resting cells, integrins are mainly in an inactive conformation, consisting of a bent shape of their extracellular domain that is unable to bind ligand (Hogg et al., 2011). Signalling cascades triggered by other receptors (inside-out signalling) induce conformational changes in the integrin extracellular domains leading to clustering of

integrins at the cell membrane and an intermediate affinity for their ligands (Takagi et al., 2002). This mechanism is mediated by cytoplasmic proteins that bind to the carboxy-terminal tail of the integrin α- and β- subunits inducing a conformational change and providing connection to the cytoskeleton, namely talins and kindlins. Upon binding to the appropriate ligand, integrins acquire a high-affinity conformation state, possibly requiring the application of external forces such as shear flow (Alon and Dustin, 2007; Zhu et al., 2008). The signalling cascade leading to adhesion strengthening mediated by ligand binding to the integrin is defined as outside-in signalling (Hogg et al., 2011). Several kinases have been shown to be involved in the outside-in signalling cascade; in T cells these include the tyrosine kinases of the Src family, Syk and the ζ-chain-associated protein kinase 70 kDa (ZAP-70) (Evans et al., 2011; Mocsai et al., 2006). In B cells, the molecular components involved in integrin outside-in signalling have not been elucidated, but it has been proposed that this mechanism could mediate B cell spreading upon BCR engagement (Arana et al., 2008a). Mature B cells express high levels of LFA-1 and VLA-4 integrins. Their ligands ICAM-1 and VCAM-1 are expressed on the surface of several cell types, such as endothelial cells, leukocytes, dendritic cells and FDCs (Freedman et al., 1990; Harwood and Batista, 2010; Koopman et al., 1991). Integrin-mediated adhesion has been shown to have a crucial role in B cells, being involved in B cell recruitment to tissues and lymphoid organs (Berlin-Rufenach et al., 1999; Lo et al., 2003), interaction with APCs during the antigen-recognition process (Carrasco and Batista, 2006; Carrasco et al., 2004), long-term compartmentalisation and retention in lymphoid tissues (Lu and Cyster, 2002).

Rap proteins are major regulators of cell adhesion. Although it was initially postulated that the role of Rap1 is to act as a Ras antagonist, because of its ability to bind Ras effectors forming non-productive complexes, later studies showed that Rap1 has a fundamental Ras-unrelated role in controlling cell adhesion through integrin activation and cell polarisation (Bos et al., 2001). Rap1 has been shown to regulate integrin activity in leukocytes mainly by its effectors RAPL and Rap1-GTP-interacting adaptor molecule (RIAM) (Hogg et al., 2011). Integrin activation by the Rap1-GTP-RIAM complex has been proposed to be mediated by talin. RIAM has been shown to immunoprecipitate with talin and increase integrin activity (Lafuente et al., 2004).

Moreover, Rap1 has been shown to modulate LFA-1 localisation through the formation of a complex with RAPL and the threonine/serine kinase Mst-1 linked to the vesicular redistribution of LFA-1 to the leukocyte leading edge (Katagiri et al., 2006). It still needs to be defined if Rap1 found in vesicular complexes is in an active state or if its activation is restricted to the plasma membrane compartment (Hogg et al., 2011). B cells from Rap1a and Rap1b knockout mice showed impaired *in vitro* adhesion to VCAM-1 and ICAM-1 (Chu et al., 2008; Duchniewicz et al., 2006). Moreover, B cells from RAPL-deficient mice exhibit impaired *in vitro* adhesion, polarisation and migration as well as *in vivo* homing to lymphoid compartments, indicating that this Rap1 effector is essential in mediating Rap1 function in B-lymphocytes (Katagiri et al., 2004). Notably, integrin inactivation has been shown to have a milder effect compared to Rap1 depletion on B-lymphocyte motility. In agreement with this observation, interstitial migration has been proposed to be an integrin-independent mechanism that nevertheless requires Rap1 (Ebisuno et al., 2010; Lämmermann et al., 2008). These data suggest an additional role for Rap1 in lymphocyte motility that will require further investigations.

It still needs to be defined which GEFs are involved in the chemokine-mediated activation of Rap proteins in B-lymphocytes. Several GEFs such as C3G (RAPGEF1), Ras guanyl nucleotide-releasing proteins (RasGRP2, also known as CALDAG-GEFI, and RasGRP3), EPAC, PDZ-GEFs and RasGEF1 can induce Rap activation in different cell types (Gloerich and Bos, 2011). A key role for RasGRP2 in the activation of Rap1 has been shown in immune cells (Bergmeier et al., 2007; Cifuni et al., 2008; Ghandour et al., 2007). RasGRP2, together with the other RasGRP proteins, contains one DAG- and two Ca^{2+}-binding motifs. However, there is no clear evidence to date on how these two second messengers activate RasGRP2 (Stone, 2011). It has been hypothesised that RasGRP2 activation is mediated through PLCγ production of DAG and Ca^{2+} upon chemokine receptor stimulation. RasGRP2 is required for the Rap1-mediated activation of αLβ2- and αIIβ3 in T cells and platelets respectively (Cifuni et al., 2008; Ghandour et al., 2007). Moreover, it has been shown that murine RasGRP2-depleted neutrophils and platelets fail to activate Rap1 in response to different stimuli and exhibit functional defects in β1, β2 and β3 integrins (Bergmeier et al., 2007).

1.5 B cell receptor signalling

As described above in this Chapter, the fate of B cells is highly dependent on the BCR signalling, which coordinates multiple pathways leading to cell differentiation and proliferation. Several of these pathways involve Rho and Rap GTPases (Fig 1.10).

1.5.1 B cell receptor-induced proliferation

The BCR is composed of an IgM or IgD associated with Ig-α/Ig-β complex. Both Ig-α and Ig-β contain a single immunoreceptor tyrosine-based activation motif (ITAM) in their intracellular domains that mediates BCR signalling. Antigen binding to the complementarity-determining regions of the IgM or IgD causes cross-linking, and subsequent phosphorylation of the Ig-α and Ig-β ITAM tyrosine residues by the tyrosine kinase of the Src family, Lyn. This phosphorylation generates docking sites necessary for the association and activation of Src homology 2 (SH2)-domain containing proteins with the BCR complex (Niiro and Clark, 2002). The Spleen tyrosine kinase (Syk) recruitment and activation plays a key role in this signalling cascade, as deficiency in Syk has been shown to compromise BCR signalling (Saijo et al., 2003). Proximal events involve the formation of a signalling complex that involve the B cell linker protein (BLNK), PI3K, Btk, Vav and PLCγ2. Increase of intracellular Ca^{2+} and production of DAG downstream of PLCγ2 activate the protein kinase C (PKC), which in turn stimulates various pathways including the mitogen-activated protein kinase (MAPK)-family kinases (extracellular regulated kinase (ERK), p38 and JNK) and several transcription factors including nuclear factor-κB (NF-κB), which all contribute to B cell proliferation (Niiro and Clark, 2002; Rickert, 2013). Moreover, Ca^{2+} induces activation of Ca^{2+} -dependent molecules such as the serine-threonine phosphatase calcineurin that activates the transcription factor NFAT (nuclear factor of activated T-cells) by dephosphorylating it (Crabtree and Schreiber, 2009).

PI3K mediates BCR-signalling by phosphorylating phosphoinositides and thus generating the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP_3). PIP_3 in turn can bind and regulate downstream effectors, including the serine/threonine kinase Akt and several GEFs (Cain and Ridley, 2009).

RhoA is also involved in the BCR signalling cascade. Inhibition of RhoA in B cell lines reduced the BCR-induced release of Ca^{2+} from intracellular stores and impaired cell

proliferation by regulating production of the PLCγ substrate PIP_2 (Saci and Carpenter, 2005). RhoA might mediate this function by regulating the localisation or either directly activating phosphatidylinositol 4-kinase (PI4K) and phosphatidylinositol-4-phosphate 5-kinase (PIP5K), required for the synthesis of PIP_2 (Mele et al., 2014).

Vav proteins, well characterized GEFs for several Rho GTPases, including Cdc42 and Rac, play an important role in the proliferative signalling triggered by the BCR. (Tybulewicz and Henderson, 2009) Vav1-deficient B cells show reduced activation and proliferation upon BCR stimulation (Fujikawa et al., 2003), and combined Vav1 and Vav2 depletion strongly inhibits BCR-induced proliferation (Tedford et al., 2001). A similar phenotype to Vav depletion is observed after Rac1 and Rac2 depletion, indicating the requirement of these GTPases in BCR responses. Vav proteins, Rac and Cdc42 could affect BCR signalling by promoting BCR endocytosis together with the B-lymphocyte adaptor molecule of 32 kDa (Bam32) (Malhotra et al., 2009; Niiro et al., 2004).

BCR signalling can be reinforced through co-receptor-mediated signalling. Co-receptor molecules in mature B cells are expressed as a complex consisting of CD19, CD21 and CD81 (Kurosaki, 2002). CD21 is a receptor for the C3d fragment of complement, which after activation is able to induce phosphorylation of the cytoplasmic domain of CD19 with the consequent recruitment of PI3K.

Furthermore, several molecules act as negative regulators of BCR signalling. For example, CD22, CD5, CD72 and the Fc receptor FcγRIIB are receptors that contain distinct motifs called immunoreceptor tyrosine-based inhibition motifs (ITIMs) (Kurosaki, 2002). ITIMs are phosphorylated by the tyrosine kinase Lyn upon BCR stimulation, leading to the recruitment of one or other of the SH2 domain-containing inhibitory phosphatases, the tyrosine phosphatases SHP-1 and the inositol-5'-phosphatase SHIP (Brauweiler et al., 2000; Doody et al., 1995). Activation of SHP-1 induces the dephosphorylation of Syk, Btk, Lyn and BLNK, whereas SHIP activity directly counteracts the activity of lipid kinases (e.g. PI3K) downstream of the BCR (Kurosaki, 2002). Mechanisms of negative regulation and co-signalling are essential for the modulation of the BCR response and allow differential biological outcomes during B cell development such as proliferation, apoptosis or anergy (Gauld et al., 2006).

1.5.2 B cell receptor signalling to the cytoskeleton

It has been shown that early events in the BCR activation include cytoskeletal rearrangements and inside-out integrin activation, both of which are required for the antigen-recognition process (Arana et al., 2008a). B cells and APCs form an immunological synapse in which the BCR and adhesion molecules are clustered. Upon contact with APCs, B cells undergo a rapid response by spreading and contracting along the antigen-containing surface, activating their integrins in order to adhere to the APCs followed by spatial segregation of antigen-bound BCR into a central supramolecular activation cluster (cSMAC) surrounded by a peripheral SMAC (pSMAC) of active LFA-1 (Arana et al., 2008a; Mele et al., 2014).

Several GTPases have been linked to this process. Rap1 and Rap2 GTPases are activated upon BCR engagement and have been reported to play a key role in the spreading of the B cells after BCR stimulation. Rap activation is also essential to the formation of a functional immune synapse (Lin et al., 2008). This probably reflects the ability of Rap to activate LFA-1 integrin through inside-out signalling leading to integrin clustering as well as increased affinity for their ligands. The RapGEF involved in the Rap activation in response to BCR engagement has not yet been identified. As mentioned above, both Rac1 and Rac2 are involved in the BCR-mediated signalling. However, only Rac2, and not Rac1, is required for the immunological synapse formation between B and APC cells (Arana et al., 2008b). Rac2 appears to act upstream of Rap1 to induce LFA-1-mediated adhesion, although the links between Rac2 and Rap1 are not known.

In addition to Rac2, Btk and PLCγ2 have been implicated upstream of Rap activation. Btk activation is necessary for the inside-out activation of the integrin VLA-4/α4β1 upon BCR stimulation (Spaargaren et al., 2003). PLCγ2 acts downstream of Btk to mediate VLA-4 activation, and Rap1 activation following BCR stimulation requires PLCγ2 (McLeod et al., 1998; Mele et al., 2014).

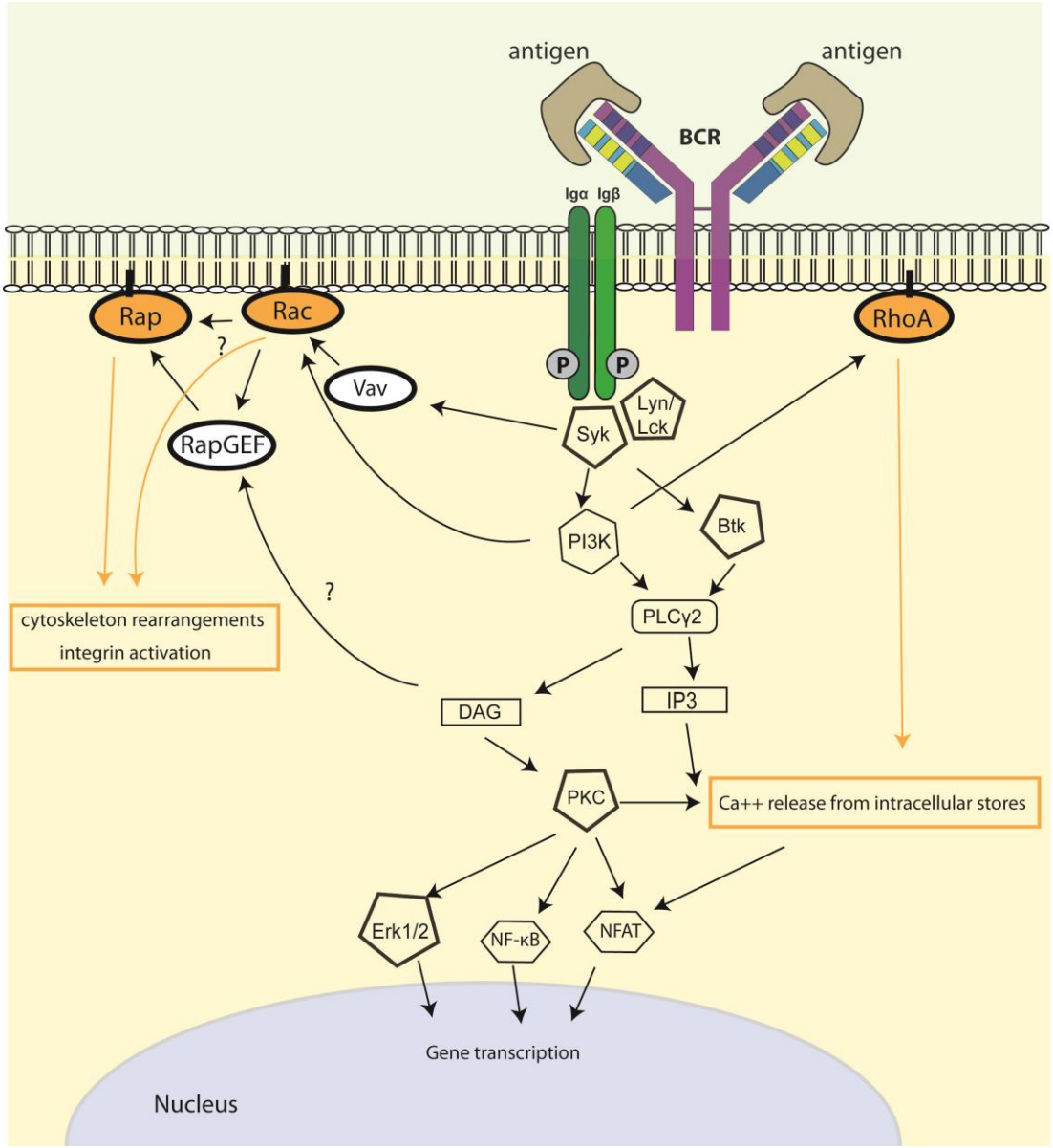

Figure 1.10 B cell receptor signalling.
Upon antigen binding, Igα and Igβ are phosphorylated by the Src family kinases Lyn and Lck, leading to the formation of a signalling complex that involves PI3K, Btk, Vav and PLCγ2. Activation of Rac, mediated by its GEF Vav or downstream of PI3K signalling, is necessary for the B cell cytoskeletal reorganisation. Downstream of PI3K, Btk and PLCγ2, Rap is activated through an unknown GEF and contributes to integrin activation and cytoskeletal rearrangement. The BCR signalling cascade also leads to PI3K-mediated activation of RhoA, which contributes to the Ca^{2+} release from intracellular stores. This mechanism is implicated in the regulation of transcription factors mediated by activation of several signalling pathways including ERK1/2, NF-κB and NFAT.

1.6 B cell malignancies: Chronic Lymphocytic Leukaemia

Chronic lymphocytic leukaemia (CLL) is one of the most common forms of leukaemia in adults. It is characterized by the progressive accumulation of neoplastic monoclonal B-lymphocytes in the blood, bone marrow, lymph nodes and other lymphoid organs. Malignant CLL cells are characterized by the expression of CD5, CD19 and CD23. Typically, CLL cells express IgM and IgD surface antibodies with a variable density. However, class switch recombination has been reported in CLL cells and a minor subset of CLL cells express IgG and IgA (Efremov et al., 1996; Malisan et al., 1996).

Symptomatic disease can include lymph node enlargement, hepatosplenomegaly, bone marrow failure and recurrent infections because of the reduced production of functional immune cells (Chiorazzi et al., 1995). Initially, CLL was considered a tumour of lymphocytes with a minimal proliferation index, which accumulate because of a faulty apoptotic process. However, findings in recent years have challenged this model. Although CLL cells in the peripheral blood are mainly resting, structures found in the bone marrow and lymph nodes known as proliferation centres contain a small fraction of actively proliferating CLL cells. The CLL proliferation centres are characterised by a pseudofollicular structure, vaguely nodular regions observed by histopathological analysis and representing a hallmark of CLL. Aggregates of proliferating leukaemic cells, characterised by the expression of the proliferation marker Ki67, can be found In these structures and they differ from the healthy proliferating B cells by lacking the expression of reactive GC B cells markers, such as CD10 and Bcl-6, and expressing the anti-apoptotic factor Bcl-2 (Stevenson and Caligaris-Cappio, 2004).

Heavy-water experiments have demonstrated a definable turnover of CLL cells in each patient, with a proportion of new cells generated per day up to 1% of the entire clone (Messmer et al., 2005). It has also been proposed that the small fraction of CLL cells in the peripheral blood with a proliferative phenotype represents the proportion of CLL cells that have recently divided and exited the lymphoid compartments (Calissano et al., 2011).

1.6.1 Prognostic factors in CLL

CLL is characterized by a marked degree of heterogeneity. Patients suffering from CLL can be affected by an indolent form of the disease without requiring any treatment or instead they can present with a rapidly progressive disease and fatal clinical course. The variable course of CLL progression reflects heterogeneity in the disease biology. Because of this heterogeneity, staging systems like Rai (Rai et al., 1975) and Binet (Binet et al., 1981) have been developed to help clinicians assess patient prognosis and plan therapeutic intervention. Nevertheless, there are still many difficulties in the prediction of the clinical outcome if the disease is diagnosed at early stages. In the past decade many genetic, molecular and cellular features have been identified as good markers that can be linked to the tumour aggressiveness, some of which are described here.

1.6.1.1 IGHV mutational status

CLL cases are classified into 2 main subsets, based on the degree of somatic hypermutation of the IGHVs. Unmutated CLL cases (U-CLL) have >98% IGHV sequence homology with the germline sequence, whereas mutated CLL cases (M-CLL) have <98% sequence homology (Burger and Chiorazzi, 2013). U-CLL is associated with a more aggressive clinical course and shorter survival, conversely M-CLL cases typically show longer survival and slow disease progression (Damle et al., 1999). It has been proposed that the 2 subsets could arise from different B cell lineages and therefore they should be considered as 2 different diseases, at least from a developmental point of view (Stevenson et al., 2011). It has been hypothesised that U-CLL could be generated from a B cell before initiation of IGHV hypermutation, whereas M-CLL would derive from a B cell that transited through the GC (Section 1.2). Initially, gene expression profile studies reported that CLL cells were more related to memory B cells compared to CD5+ B cells isolated from neonatal cord blood (Klein et al., 2001), leading to the hypothesis that both U-CLL and M-CLL cells can be considered antigen-experienced 'memory-like' B cells that arose from a typical GC reaction (M-CLL) or that failed to rearrange their antigen-combining site (U-CLL) (Chiorazzi and Ferrarini, 2011). However, in a more recent study, comparison of CLL cells with normal naive B cells, IgM+ memory B cells, adult CD5+ B cells and class-switched B cells showed that

both U-CLL and M-CLL cells are very similar to normal CD5+ B cells found in the peripheral blood (Seifert et al., 2012). IGHV mutation analysis showed that this subset of normal B cells, although mostly IGHV unmutated (Brezinschek et al., 1997) include a small distinct subset of CD27+ (generally considered as marker for memory B cells (Agematsu et al., 2000)) CD5+ B cells with mutated IGHVs (Seifert et al., 2012). Based on this findings, the current hypothesis id that CLL could be a malignancy derived from CD5+ B cells. U-CLL would originate from CD5+ CD27- B cells with unmutated IGHV, whereas M-CLL would derive from CD5+ CD27+ B cells with mutated IGHV (Zhang and Kipps, 2014) (Fig 1.11).

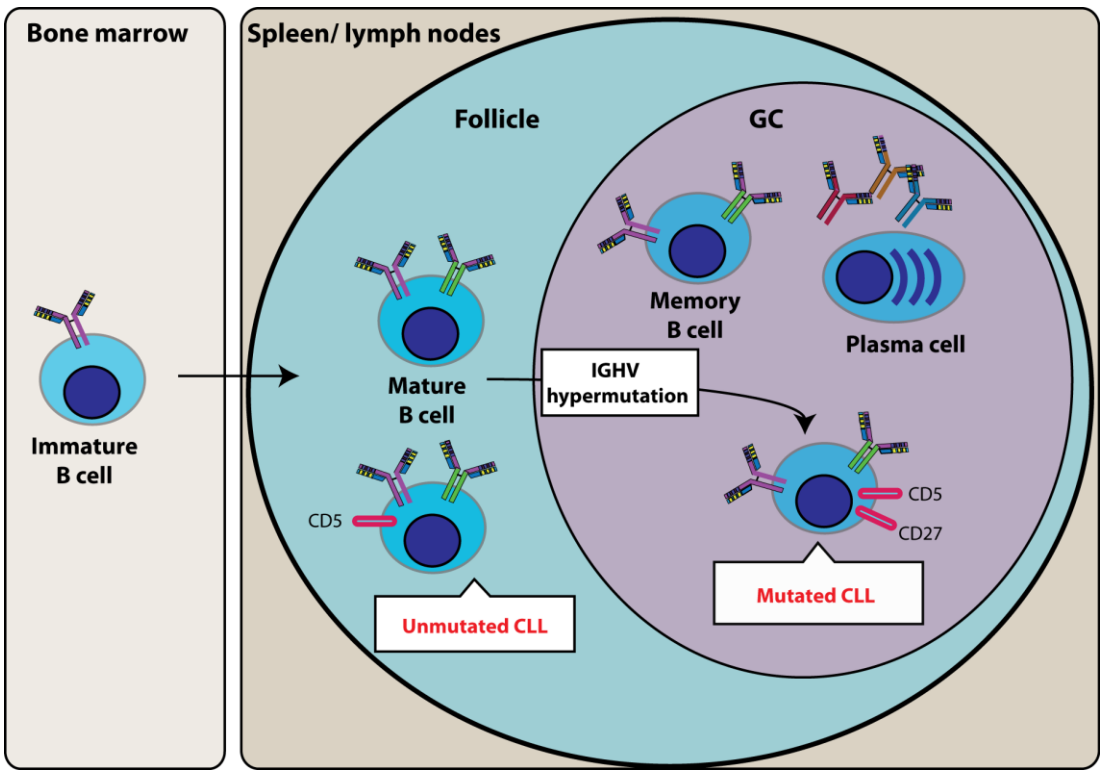

Figure 1.11 Proposed origin of CLL cells during B cell differentiation.
Generation of the two subtypes of CLL has been associated with different stages of B cell development. The unmutated type of CLL (U-CLL) could derive from mature CD5+ B cells, which have not undergone the IGHV hypermutation. The mutated CLL type might be generated from CD5+ CD27+ B cells.

1.6.1.2 Cytogenetic abnormalities

The low mitotic activity of CLL cells has held back the analysis of genomic abnormalities achievable with standard chromosome banding studies. However,

stimulation of CLL cell proliferation and techniques able to detect chromosomal abnormalities irrespective of the cell mitotic phase, such as fluorescence in situ hybridisation (FISH) with specific probe sets, allowed genomic aberration to be identified in approximately 80% of CLL cases (Zenz et al., 2011). The most recurrent mutations identified include the deletion of chromosomal regions on 13q, 11q, 17p and the trisomy of chromosome 12. The presence of these aberrations has been shown to be associated with different rates of disease progression and clinical outcome. Deletion of 13q14.3 is considered the most common genetic abnormality in CLL. It has been reported to be present in a total of 55% CLL patients, including CLL cases where multiple aberrations are observed. CLL cases with the sole loss of 13q14.3 as genetic aberration (36% of CLL patients, based on FISH data) have a favourable prognosis and show a slow disease progression (Dohner et al., 2000). The 13q14.3 region contains the deleted in leukemia-1/2 genes (DLEU1 and DLEU2), which are long non-protein coding RNAs. The DLEU1 gene contains the microRNAs miR-15a and miR-16-1, which have been implicated in CLL pathogenesis (Calin et al., 2002). Experiments performed in mouse models have shown that deletion of the miR-15a/16-1 is sufficient to induce the development of lymphoproliferative disorders mimicking features of CLL (Klein et al., 2010). The effect observed is likely to be due to the tumour suppression function linked to miR-15a/16-1, which appear to inhibit the expression of multiple genes involved in the regulation of cell growth and apoptosis, including BCL2.

Approximately 15% of CLL patients exhibit trisomy 12 (Dohner et al., 2000). Whereas initial studies associated trisomy 12 with intermediate progression, more recent data suggest that this genomic lesion predicts shortening of the time to the first treatment but overall favourable survival (Hallek et al., 2010). The candidate genes involved in the pathogenesis of CLL with trisomy 12 have not been identified. Interestingly, a recent study has shown that expression of the RAP1B gene, located in the 12q15 region of chromosome 12, is up-regulated in CLL cases with trisomy 12 compared to other CLL subgroups and normal B cells (Riches et al., 2014). Moreover, CLL cases with trisomy 12 show increased expression of CD49d, RasGRP2 and RAPL compared to other CLL cases (Riches et al., 2014; Zucchetto et al., 2013). These genes are not on chromosome 12 and the mechanisms involved in their up-regulation have not

been defined. Trisomy 12 has also been associated with increased incidence of NOTCH1 mutation (Balatti et al., 2012). NOTCH1 mutations have been identified in approximately 10% of CLL cases at the time of diagnosis and associated with shortened survival (Fabbri et al., 2011; Puente et al., 2011). Studies on a cohort of CLL patients with trisomy 12 showed that *NOTCH1* mutation had a frequency of 24% among this subgroup, correlating with a 2.4 fold increase of poor prognosis (Del Giudice et al., 2012). The NOTCH1 gene encodes for the Notch1 transmembrane protein that is activated by ligands of the Jagged/Delta family. Upon binding to its ligands, Notch1 is proteolytically cleaved and its intracellular domain translocates to the nucleus, where it regulates the expression of several genes involved in lymphocyte development and function (Radtke et al., 2013). Notch1 mutations in CLL disrupt the intracellular C-terminal domain involved in regulating the protein turnover, resulting in an impaired Notch1 degradation (Gaidano et al., 2012).

Deletion of 17p13 in CLL patients correlates with the most adverse prognosis and is detected in 4-9% CLL cases (Dohner et al., 2000). This region includes the gene for the tumour suppressor factor p53. Whereas mutation of p53 has been observed only in 5% of CLL cases with no 17p13 deletion, 80% of CLL patients with monoallelic 17p13 deletion show inactivating mutations of p53 on the remaining allele (Zenz et al., 2010).

CLL patients with deletion of 11q22-q23 show a fast disease progression and remarkable lymphadenopathy (Dohner et al., 2000). This cytogenetic abnormality is found in approximately 25% of CLL patients with advanced disease and in most of the cases it affects the ataxia telangiectasia mutated (ATM) gene (Stilgenbauer et al., 2002). The product of this gene is a protein kinase involved in cell cycle checkpoint and DNA repair after damage. The loss of ATM therefore leads to genomic instability (Braggio et al., 2012).

Notably, intraclonal genetic heterogeneity as a result of clonal evolution (Greaves and Maley, 2012) contributes to CLL progression and clinical outcome, representing a further level of complexity (Landau et al., 2013; Schuh et al., 2012).

1.6.1.3 ZAP-70

The tyrosine kinase ZAP-70 mainly expressed in T-lymphocytes where it plays a key role in the T cell receptor (TCR) signalling response (Kane et al., 2000). In T cells ZAP-70 is distributed throughout the cytoplasm, but following TCR engagement and ITAM phosphorylation it is recruited to the plasma membrane and becomes active via tyrosine phosphorylation (Sloan-Lancaster et al., 1997). Expression of ZAP-70 has been reported in activated B-lymphocytes, but not in resting B cells, indicating that its expression is associated with the cell stimulation processes (Cutrona et al., 2006; Scielzo et al., 2006). Expression of ZAP-70 is also detected in a subset of CLL patients. Its expression highly correlates with the unmutated status of IGHV (Wiestner et al., 2003) and it has been proposed as a surrogate marker for U-CLL (Crespo et al., 2003), because of the relatively easy and inexpensive methods of detecting it. However, ZAP-70 has been shown to have high prognostic value independently to the IGHV mutation status (Rassenti et al., 2008); CLL patients with >20% leukaemic cells expressing ZAP-70 show a faster disease progression. ZAP-70 expression in CLL has been shown to be associated with enhanced BCR signalling (Chen et al., 2002). However, the tyrosine kinase activity of ZAP-70 does not seem to be involved in this mechanism, suggesting that it could possible act as an adaptor protein in the BCR induced signalling pathway (Chen et al., 2008a). Moreover, it has been shown that expression of ZAP-70 in CLL is associated with increased response to CCL19, CCL21 and CXCL12 chemokines, with a consequent increased migration and survival signalling (Calpe et al., 2011; Richardson et al., 2006).

1.6.1.4 CD38

CD38 is a transmembrane protein member of the ADP ribosyl cyclase family. CD38 expression is considered ubiquitous in the lymphoid tissues but it is particularly regulated during B cell development. Precursor cells in the bone marrow show a high CD38 expression that decreases in circulating mature B-lymphocytes; expression levels increase again after their differentiation into plasma cells (Malavasi et al., 2008) (Fig. 1.12). Expression of CD38 can be detected in a subset of CLL patients and it has been shown to predict poor clinical outcome when expressed in >30% CLL cells. This expression refers to the presence of the protein on the cell surface only as

detected by flow cytometry (Damle et al., 1999; Del Poeta et al., 2001; Hamblin et al., 2002). However, different cutoff values have been proposed such as 20% (Durig et al., 2002; Ibrahim et al., 2001) and 7% (Krober et al., 2002; Thornton et al., 2004). CD38 expression in CLL has been associated with shorter overall survival, shorter time to the first treatment and high incidence of lymph node involvement. As absence of mutations in the IGHV gene often correlates with CD38 expression in CLL patients, CD38 was initially proposed to be predictive of U-CLL cases (Damle et al., 1999). However, this correlation was not observed in subsequent studies (Hamblin et al., 2002; Jelinek et al., 2001), suggesting that CD38 can be considered as an independent poor prognostic factor in CLL.

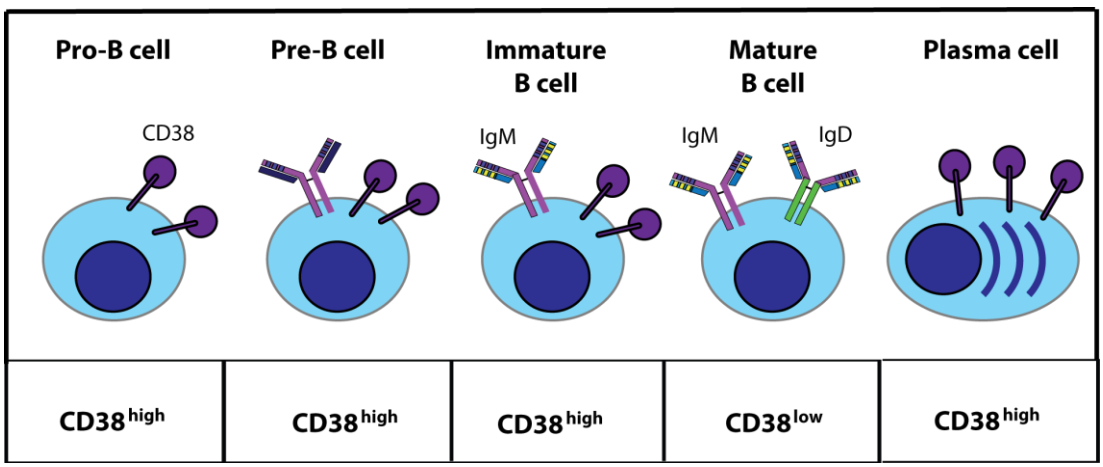

Figure 1.12 CD38 expression through the stages of human B cell development. CD38 expression is tightly regulated during B cell maturation. It is expressed at high levels in pro-B, pre-B and immature B cells. It expression decreases in mature lymphocytes and then increases again in plasma cells.

1.6.1.5 CD49d

CD49d, the α4 subunit of the integrin VLA-4, is variably expressed between CLL patients. Even though CD49d expression in CLL often correlates with poor prognostic markers such as ZAP-70 and CD38, multivariate analysis of large cohorts of CLL patients performed by two independent groups suggested that CD49d could be an independent predictor of poor prognosis using a surface CD49d expression cut-off of 30% (Gattei et al., 2008) and 45% (Shanafelt et al., 2008) relative to the percentage of CLL cells expressing CD49d detected with flow cytometry. Subsequent studies

confirmed the relevance of CD49d expression as a prognostic factor in CLL that predicts shorter treatment-free survival and shorter overall survival (Majid et al., 2011; Nuckel et al., 2009). Moreover, recent meta-analysis studies performed on a large cohort of CLL cases (2,972 CLL samples) indicated that CD49d expression (cut-off of 30%) is the strongest flow-cytometry-based prognostic factor, when compared to CD38 and ZAP-70, to predict shorter treatment-free and overall survival (Bulian et al., 2014)

1.6.2 Role of the microenvironment in CLL

Specific interactions of CLL cells with cellular and matrix components within the microenvironmental niches in the lymphoid organs are crucial for the leukaemic clone survival, proliferation and therefore for the disease progression (Fig 1.13). The high apoptotic rates of CLL cells in *in vitro* cultures have highlighted the importance of these interactions. Direct physical contact of CLL cells with bone marrow-isolated stromal cells has been shown to significantly reduce CLL cell apoptosis *in vitro* and extend their survival up to several weeks (Lagneaux et al., 1998; Panayiotidis et al., 1996). Endothelial cells are also important players of the CLL microenvironment. Co-culture with endothelial cells has been shown to prevent both spontaneous and chemotherapy-induced apoptosis of CLL cells (Maffei et al., 2012), upregulating the expression of antiapoptotic proteins such as Bcl-2, Mcl-1, and Bcl-XL possibly through NF-κB activation (Buggins et al., 2010). Interestingly, binding of β1- and β2-integrin expressed on CLL cells to their respective ligands VCAM-1 and ICAM-1 on the endothelial surface is essential to the pro-survival effect. Soluble factors are also involved in CLL cell survival; conditioned media from endothelial cells was partly able to reduce the apoptotic rate of CLL cells (Maffei et al., 2012).

FDCs can also be found associated with CLL cells in lymph nodes, and *in vitro* co-culture of CLL cells and a FDC cell line has been shown to protect leukaemic cells from spontaneous apoptosis through the up-regulation of the anti-apoptotic protein Mcl-1 at least partly mediated by stimulation of the cell surface receptor CD44 expressed by CLL cells (Fedorchenko et al., 2013; Pedersen et al., 2002).

Notably, $CD4^+$ T-lymphocytes are present in the pseudofollicles and particularly in close proximity to proliferating $Ki67^+$ CLL cells (Ghia et al., 2002). Several $CD4^+$ T-lymphocytes in pseudofollicles express CD40L, suggesting that they are in an active state and leading to the hypothesis that they can have a role in supporting the proliferation of the leukaemic clone (Ghia et al., 2008). CD40 stimulation with CD40L was able to induce a gene expression signature in CLL cells *in vitro* resembling that found in lymph node CLL samples. Moreover, combined stimulation of CLL cells with CD40L and IL-21 was shown to induce CLL cell proliferation *in vitro* (Pascutti et al., 2013).

Monocyte-derived adherent cells have been observed in ex vivo cultures of peripheral blood derived from CLL patients (Burger et al., 2000) as well as healthy donors (Tsukada et al., 2002). These cells have been named 'nurselike-cells' (NLCs), because of their similarities with thymic nurse cells that nurture developing thymocytes (Wekerle et al., 1980). Co-culture of NLCs with CLL cells is able to protect CLL cells from spontaneous and drug-induced apoptosis. NLCs, characterised by the expression of CD45, CD14 and CD68, can also be found in the secondary lymphoid organs of patients with CLL (Tsukada et al., 2002), and they resemble the CD68+ tumour-associated macrophages found in solid tumours and other B cell lymphomas (Burger, 2011; Giannoni et al., 2014). Several molecular mechanisms involved in the NLC-CLL cell cross-talk have been identified. One is mediated by the two ligands of the TNF family expressed by NLCs, BAFF (B cell activating factor) and APRIL (a proliferation-inducing ligand). Binding of these ligands to their receptors BAFFR (BAFF receptor), TACI (transmembrane activator and calcium modulator and cyclophilin ligand interactor) and BCMA (B cell maturation antigen) expressed by CLL cells has been shown to protect CLL cells from apoptosis through activation of the NF-κB pathway (Endo et al., 2007; Nishio et al., 2005).

Notably, chemokines and chemokine-receptor signalling have been shown to support CLL cell survival, beside their role in the trafficking of CLL cells. CXCL12 and CXCL13 are both expressed by NLCs and they were shown to be able to reduce the apoptosis rate of isolated CLL cells expressing their receptors CXCR4 and CXCR5, possibly through a prolonged activation of ERK 1/2 (Burger et al., 2000; Burkle et al., 2007).

Another molecular mechanism involved in the support of CLL cell survival is represented by CD100 (semaphorin-4D), expressed on the membrane of CLL cells, and its receptor Plexin-B1. *In vitro* stimulation of CLL cells with Plexin-B1 increases CLL cell survival and proliferation (Granziero et al., 2003). Notably, Plexin-B1 is expressed by several components of the CLL microenvironment, including NLCs (Deaglio et al., 2005) BM stromal cells, activated T-lymphocytes and FDCs (Granziero et al., 2003).

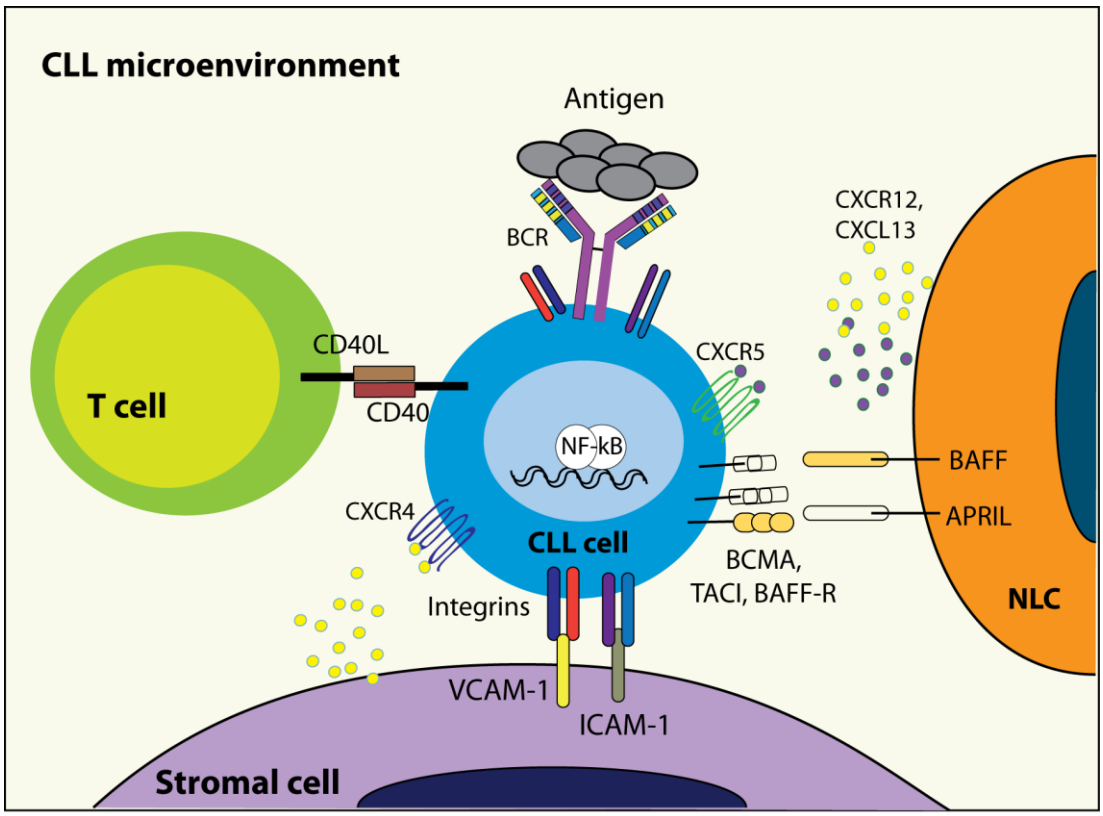

Figure 1.13 Molecular cross-talk in the CLL microenvironment.
Pro-survival signalling provided from the non-malignant component of the CLL microenvironment can be mediated by the binding of adhesion molecules such as VCAM-1 and ICAM-1, expressed by stromal and endothelial cells, to integrins expressed by CLL cells. Activated T cells support the proliferation of CLL cells by binding through CD40L. The binding of BAFF and APRIL expressed by NLCs to their receptors BCMA, TACI and BAFF-R activates the NF-κB pathway and protect CLL cells from apoptosis. Chemokine produced from NLCs and stromal cells are further anti-apoptotic factors. Moreover, self- or antigen-mediated BCR stimulation can induce the proliferation of the leukaemic cells.

1.6.3 BCR signalling in CLL

BCR signalling is a key player in the pathogenesis of several B cell malignancies, including CLL. Somatic mutations leading to constitutive activation of components of the BCR pathway have been observed in B cell lymphomas, such as mutations of the signalling adaptor CARD11 (caspase recruitment domain 11) and the ITAMs in the activated B cell-like subtype of diffuse large B cell lymphoma (ABC DLBCL) (Davis et al., 2010). However, no activating mutation appears to be involved in the BCR pathway activation in CLL. Gene expression profiling data have shown that BCR-associated signalling pathways are enhanced in CLL cells isolated from lymph nodes compared to peripheral blood and bone marrow (Herishanu et al., 2011). Activation of the BCR pathway can be antigen-dependent or antigen-independent (also known as 'tonic'); both mechanisms have been reported in CLL.

Several antigens have been shown to be recognised by CLL cells. Igs produced by CLL cells can bind to apoptotic T and B cell lines *in vitro*, recognising epitopes belonging to native intracellular molecules relocated to the cell surface during apoptosis or neo-epitopes produced by oxidative mechanisms linked to the apoptosis (Catera et al., 2008; Lanemo Myhrinder et al., 2008). Recognition of antigens from bacterial origin was also reported in CLL cases (Lanemo Myhrinder et al., 2008). The immunoglobulin repertoire within CLL cases is biased. Several studies reported the presence of subsets of CLL cases sharing closely homologous, or even identical (stereotyped) CDR3 sequences on IGH and light (L) chains (Ghiotto et al., 2004; Messmer et al., 2004; Stamatopoulos et al., 2007; Widhopf et al., 2004) (Fig. 1.2). As previously mentioned (Section 1.2), the CDR3 sequence is unique for each B cell and confers the specificity of the BCR towards an antigen, and the statistical probability that different CLL clones share the same sequence by chance is very low. These observations suggest that an antigen-mediated selection could be involved in CLL pathogenesis.

The antigen-independent BCR activation in CLL cells has been proposed to be mediated by the binding to an intrinsic IGHV motif. *In vitro* studies have shown that transferring CLL BCRs into mouse cells, lacking endogenous components of the BCR, leads to an increase of intracellular Ca^{2+} levels mediated by the binding of the heavy chain CDR3 region to an internal epitope of the BCR (Duhren-von Minden et al., 2012).

CLL cells generally co-express surface IgM and IgD at a lower level compared to naive B cells (Section 1.5). Moreover, a further reversible downregulation of surface IgM expression, but not IgD, has been reported (Mockridge et al., 2007). Consistent with the expression levels, most CLL cases show responsiveness to IgD stimulation *in vitro*. In contrast, response to IgM stimulation has been shown to be variable between CLL patients. A subset of CLL samples stimulated *in vitro*, with anti-IgM as a surrogate antigen, appear to be unresponsive to surface IgM stimulation. This is most common in M-CLL cases (Lanham et al., 2003). Moreover, among the subset responsive to IgM engagement, variability has been observed in the activation of the downstream signalling pathways. A low surface level of IgM correlates with a lack of IgM response and it has been proposed that this could reflect a repeated antigen stimulation and down-regulation of surface IgM *in vivo* (Mockridge et al., 2007). Moreover, IgM unresponsiveness in CLL is also associated with a constitutive activation of ERK and NF-kB, thus resembling features of B cell anergy (Muzio et al., 2008). Notably, it has been shown that *in vitro* incubation of CLL cells is able to increase sIgM expression level and signalling capacity both in responsive and unresponsive CLL cases, providing evidence for the presence of a signal operating *in vivo* able to maintain CLL cells in an anergic state (Mockridge et al., 2007).

Differences in the BCR response observed between U-CLL and M-CLL cases could reflect the differences observed in their clinical behaviour. It has been proposed that M-CLL cells could derive from a B cell clone able to bind with high affinity to restricted and rare types of antigens, such as the Fc-tail of IgG (rheumatoid factors) and the β-(1,6)-glucan found in yeast, leading to a slower rate of clonal expansion. Conversely, U-CLL clones have been shown to be polyreactive and bind with low affinity to several exogenous and autoantigens, such as myosin and vimentin found in the CLL microenvironement (Burger and Chiorazzi, 2013). That could therefore imply a more frequent BCR engagement in U-CLL clones and increased signalling ability.

Heterogeneity in sIgM expression level and responsiveness can also been found within each CLL sample. By grouping CLL cells into sub-groups with different levels of IgM expression, it has been shown that within individual CLL samples the sub-group with low sIgM expression also had reduced expression level of CXCR4 and contains the $Ki67^{+}$ positive cells (Coelho et al., 2013). This sub-group could therefore identify

the fraction of cells that have recently left the tissue microenvironment where exposure to the antigen down-modulated their IgM surface level. In contrast, the sub-group with relatively high sIgM expression have a higher level of CXCR4 and could represent the fraction of cells which left the microenvironment earlier in time and are possibly ready for homing back to the lymphoid tissues. The mechanisms regulating CLL cells trafficking have not bee fully elucidated, but their homing into secondary lymphoid organs has been proposed to be mediated by the same mechanisms used by normal B cells (Davids and Burger, 2012) and described earlier in this Chapter (Section 1.3).

1.6.4 Targeted therapy in CLL

Treatment with alkylating agents such as chlorambucil, which mediates anti-tumour activity mainly by crosslinking the DNA and inhibiting its replication (Begleiter et al., 1996), was considered the 'gold standard' therapy for CLL for several decades and this agent is still a good option in some CLL cases because of its low toxicity. The introduction of purine analogues led to improved results in the treatment of CLL, particularly the compound fludarabine, which inhibits several enzymes involved in DNA synthesis by competing with the physiological substrate deoxyadenosine 5'-triphosphate (dATP) (Ricci et al., 2009). Fludarabine was shown to produce a better response compared to treatment with alkylating agents, inducing a complete remission in 7-40% patients, even when used as a sole agent (Hallek, 2005). Humanised monoclonal antibodies, such as anti-CD20 (rituximab and ofatumumab) and anti-CD52 (alemtuzumab) also show efficacy in the treatment of CLL. A combination of chemotherapeutic compound with monoclonal antibodies has been shown to have a synergistic cytotoxic effect. The addition of rituximab to the combined chemotherapy fludarabine and cyclophosphamide (FCR) led to an overall response rate of 95%, with a complete remission in 72% of cases when tested in previously untreated CLL patients (Tam et al., 2008). Nevertheless, these treatments are not suitable for all CLL patients and they are less effective in cases of disease relapse. Approximately 20% of patients fail to achieve complete disease control with these therapies, mainly because of resistance mechanisms to cytotoxic agents such as loss of p53 function (Pleyer et al., 2009).

The identification of the critical role of BCR signalling in CLL pathology led clinical research to focus on inhibitors of the BCR pathway. In the past 6-8 years, new inhibitors of key components of the BCR signalosome such as ibrutinib (Btk inhibitor), idelalisib (PI3Kδ inhibitor) and fostamatinib (Syk inhibitor) have been showing promising clinical responses (Wiestner, 2012) (Fig 1.14). These kinase inhibitors cause rapid shrinkage of the lymph nodes and a transient increase in the number of lymphocytes in the blood in most patients (lymphocytosis), probably due to the egress of the malignant cells from the lymphatic tissues.

Ibrutinib (PCI-32765, also known as Imbruvica) irreversibly inhibits the Btk active site by forming a covalent bond with cysteine 481 (C481). Experiments performed *in vitro* and *in vivo* in mouse models have shown that treatment with ibrutinib inhibits not only CLL cell survival, but also their signalling, adhesion and chemotaxis in response to CXCL12, CXCL13 and CCL19 chemokines (de Rooij et al., 2012; Ponader et al., 2012). These data, in agreement with the clinical effects observed in CLL patients, indicates that these inhibitors affect not only BCR-mediated pro-survival signalling but also the chemotactic and motility properties of CLL cells.

Similar results were obtained with SYK and PI3Kδ inhibitors. Studies *in vitro* show that fosfamatinib and Idelalisib are not only able to stop the pro-survival signal from BCR stimulation in CLL cells but also reduce their chemotaxis toward CXCL12 and CXCL13 and impair their integrin-mediated adhesion and their transmigration beneath stromal cells (Buchner et al., 2010; Herman et al., 2010; Hoellenriegel et al., 2011; Quiroga et al., 2009).

Notably, extremely positive results obtained using Ibrutinib in recent clinical trials led to the accelerated approval of this compound by the US Food and Drug Administration for the treatment of CLL patients who received at least one previous treatment (approved on February 2014), with a further extension to CLL patients with deletions on 17q (approved on July 2014), which normally exhibit poor responses to standard treatments.

However, treatment with ibrutinib does not completely eliminate the leukaemic clone and mechanisms of resistance in CLL patients have been reported. Single point mutation of C481 in Btk and gain of function mutations in PLCγ2, a downstream target of Btk (Fig 1.14), have been identified in a subset of CLL patients who showed

progressive disease during ibrutinib treatment (Furman et al., 2014a; Woyach et al., 2014). Moreover, although the surge in blood lymphocyte count is transient in most of the CLL patients, approximately 20% of cases show persistent lymphocytosis, increasing the opportunity for the development of resistant leukaemic clones (Young and Staudt, 2014). The marked clinical and biological heterogeneity of CLL cases and their therapeutic outcome indicate the need for patient-specific treatments, able to address the differences in the molecular pathways active in leukemic cells.

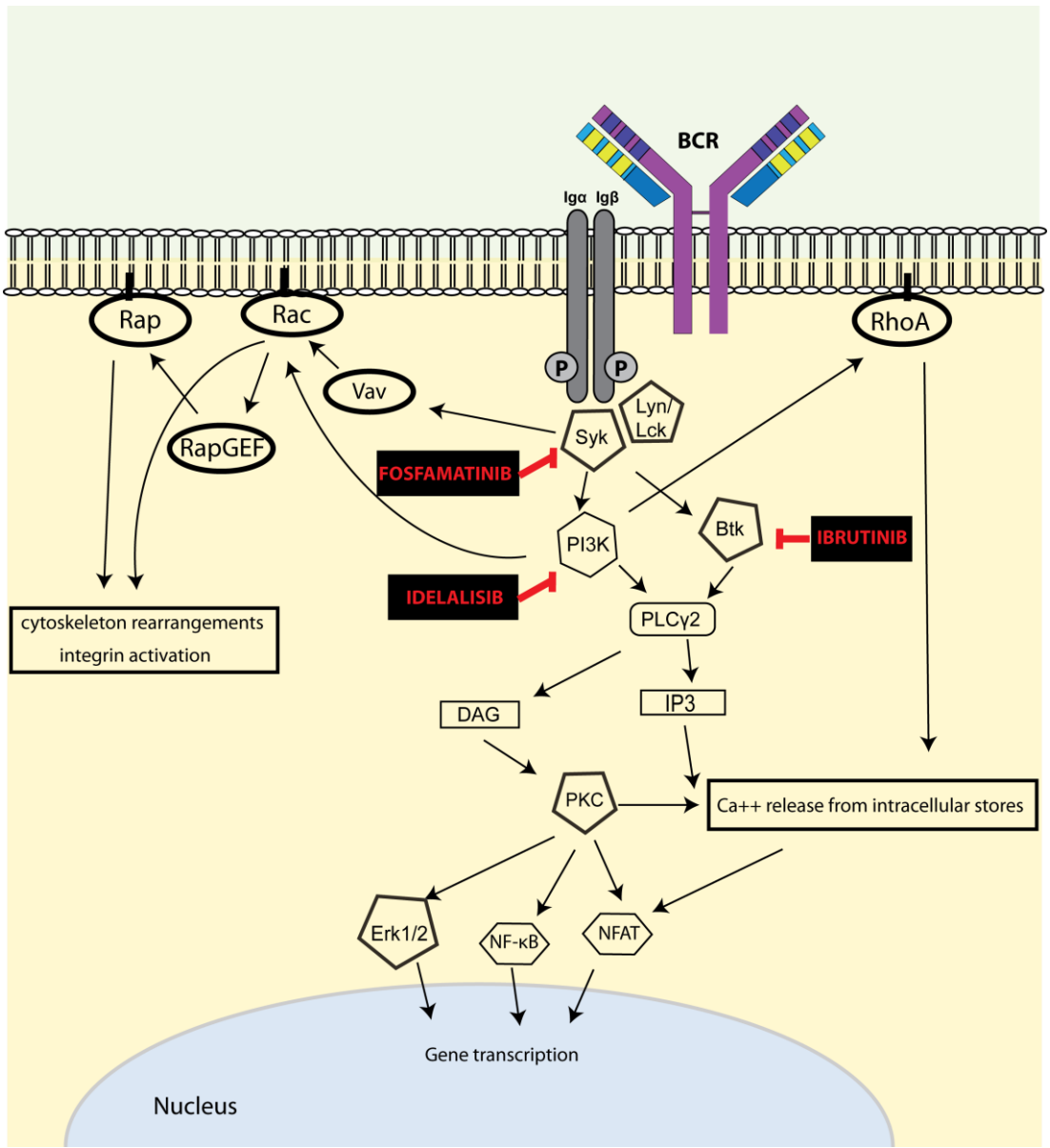

Figure 1.14 Inhibitors of BCR signalling as therapies for CLL.

Kinase inhibitors able to interfere with the BCR signalling pathway have shown promising results for the treatment of CLL in clinical trials. Targets of these inhibitors are proximal components of the BCR signalling pathway such as Syk, PI3K and Btk.

1.7 CD38 and its role in CLL

1.7.1 CD38 structure and enzymatic function

CD38 is a 45 kDa type II transmembrane protein containing a large extracellular C-terminal domain of 256 amino acid residues, a hydrophobic transmembrane domain and a short intracellular 21 amino acid residue N-terminal tail (Jackson and Bell, 1990). CD38 was firstly identified by immune typing of lymphocytes (Reinherz et al., 1980). Sequence comparison of CD38 to the ADP-ribosyl cyclase family member *Aplysia* cyclase showed that these proteins share 30% sequence homology, suggesting a possible enzymatic role for CD38 (States et al., 1992). Subsequent studies confirmed that the extracellular domain of CD38 is an ectoenzyme that can synthesize cyclic ADP-ribose (cADPR) from the substrate nicotinamide adenine dinucleotide (NAD) (Howard et al., 1993). However, only a fraction of the substrate is converted in cADPR, with the remaining being hydrolysed to ADP-ribose, due to the ability of CD38 to catalyse the hydrolysis of its own product cADPR to ADP-ribose. Moreover, it was shown that NAD phosphate (NADP) is a substrate of CD38. In the presence of nicotinic acid (NA), CD38 can catalyse the exchange of the nicotinamide group of NADP with NA to generate nicotinic acid adenine dinucleotide phosphate (NAADP)(Aarhus et al., 1995). This reaction seems to be predominant under acidic conditions (Lee, 2006)(Fig. 1.15). It has been suggested that CD38 molecules associate to form homodimers and tetramers (Umar et al., 1996). The crystallographic structure of the extracellular domain of CD38 indicated that a CD38 tetrameric complex is formed via dimer-of-dimers assembly (Hara-Yokoyama et al., 2012; Liu et al., 2005). This conformation seems to be necessary for CD38 catalytic

activity and facilitates its localisation into specialised membrane microdomains enriched in cholesterol and sphingolipids (lipid rafts) (Hara-Yokoyama et al., 2012).

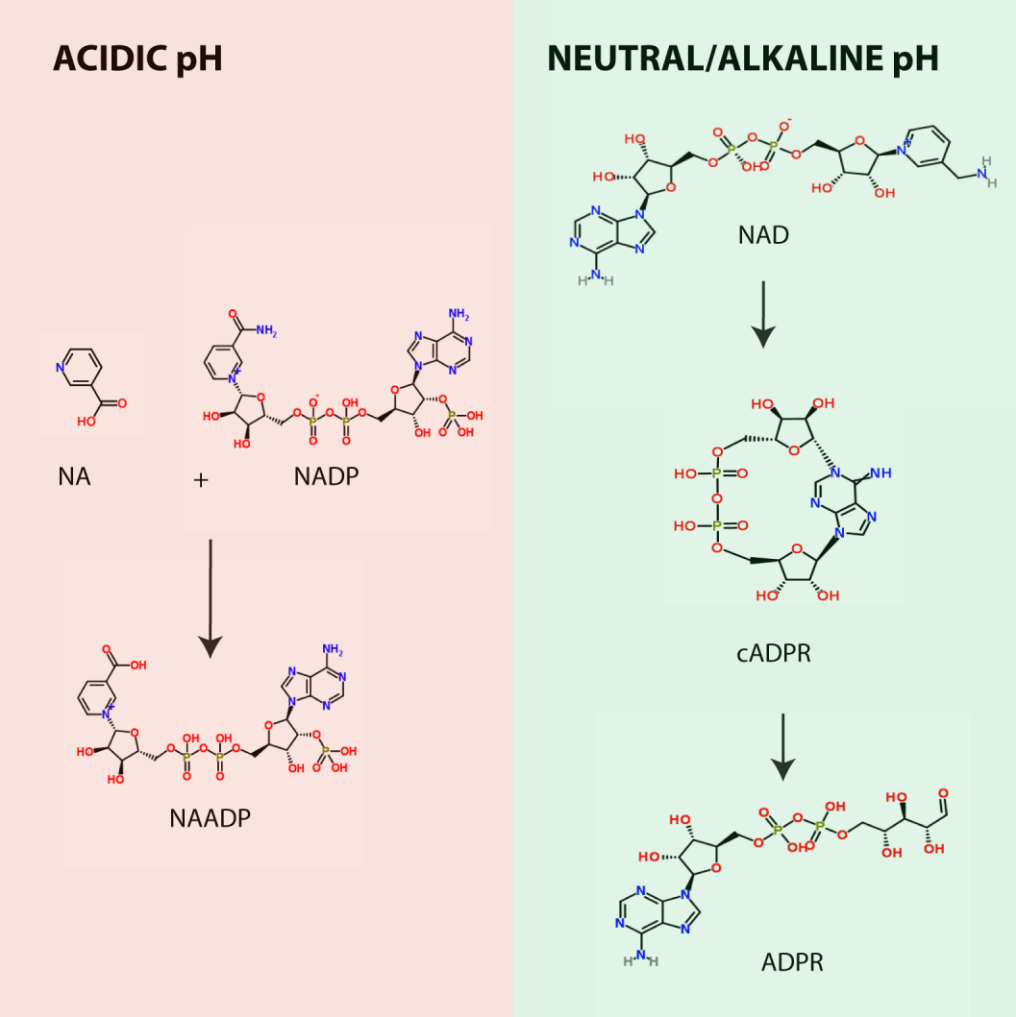

Figure 1.15 Enzymatic reactions catalysed by CD38 at neutral or acidic pH.
At neutral pH, CD38 mainly produces cADPR (as intermediate product) and ADPR from NAD. At acidic pH (found for example in lysosomes and late endosomes), CD38 produces NAADP from NA and NADP. Abbreviations: nicotinamide adenine dinucleotide, NAD; cyclic ADP-ribose, cADPR; ADP-ribose, ADPR; NAD phosphate, NADP; nicotinic acid, NA; nicotinic acid adenine dinucleotide phosphate, NAADP.

The enzymatic products of CD38 (cADPR, NAADP and ADPR) are all involved in Ca^{2+} mobilisation (Galione, 2002) (Fig 1.16). Pharmacological studies have shown that NAD, through the enzymatic conversion into cADPR, induce Ca^{2+} mobilisation from the endoplasmic reticulum (ER). This mechanism is independent from IP_3, as inhibiting IP_3 has been shown to have no effect on the Ca^{2+} release induced by cADPR

(Dargie et al., 1990). It has been proposed that the intracellular calcium channels ryanodine receptors (RyRs) are involved in the cADPR-mediated Ca^{2+}- mobilisation (Galione et al., 1991). It still needs to be defined whether cADPR can directly bind to RyRs or if other molecules are involved in this mechanism (Copello et al., 2001; Guse, 2004). NAADP has also been linked to Ca^{2+} release from the ER as well as from acidic organelles like lysosomes (Churchill et al., 2002). The dependence on an acidic pH condition for NAADP metabolism and its activity on acidic cell compartments could therefore suggest a specific role for NAADP as a Ca^{2+} messenger related to cellular endocytic pathways (Malavasi et al., 2008). A role for NAADP in the Ca^{2+} influx mediated by the two-pore channels (TPCs), voltage-gated ion channels expressed on endosomal and lysosomal membranes, has also been reported (Brailoiu et al., 2009; Calcraft et al., 2009). Moreover, the further CD38 enzymatic product, ADPR, has been shown to have a role in Ca^{2+} influx by activating the melastatin-related transient receptor potential cation channel (TRPM2) in U937, Jurkat and EOL1 immune cell lines (Sano et al., 2001).

The localisation of the CD38 catalytic domain in the extracellular space presents a paradox because its main substrate (NAD) is located intracellularly. In order for the catalytic reaction to happen, NAD should therefore be transported outside of the cell to allow it to be cyclised into cADPR, which in turn must be transported back into the intracellular compartments to mediate Ca^{2+} mobilisation from the endoplasmic reticulum (ER). Two mechanisms have been proposed that could explain this paradox. Transmembrane transporters could be involved in the passage of NAD to the extracellular space. In agreement with this hypothesis, connexin 43 hemichannels have been proposed to mediate the transport of NAD from the cytosol to the active site of CD38 (both extracellular and intravesicular) (De Flora et al., 2004) and to import cADPR from the extracellular compartment into the cell (Song et al., 2011). Nucleoside transporters could also transfer cADPR back into the cytosol (Guida et al., 2002). It has been proposed that CD38 itself could act as a unidirectional transporter of cADPR (Zocchi et al., 1999).

An alternative hypothesis has been suggested based on the observation that a portion of the total CD38 expressed in the cell membrane seems to have an opposite orientation, with the catalytic domain situated in the cytosol (Zhao et al., 2012). The

co-existence of these two different orientations could represent a further mechanism of regulation of CD38 activity.

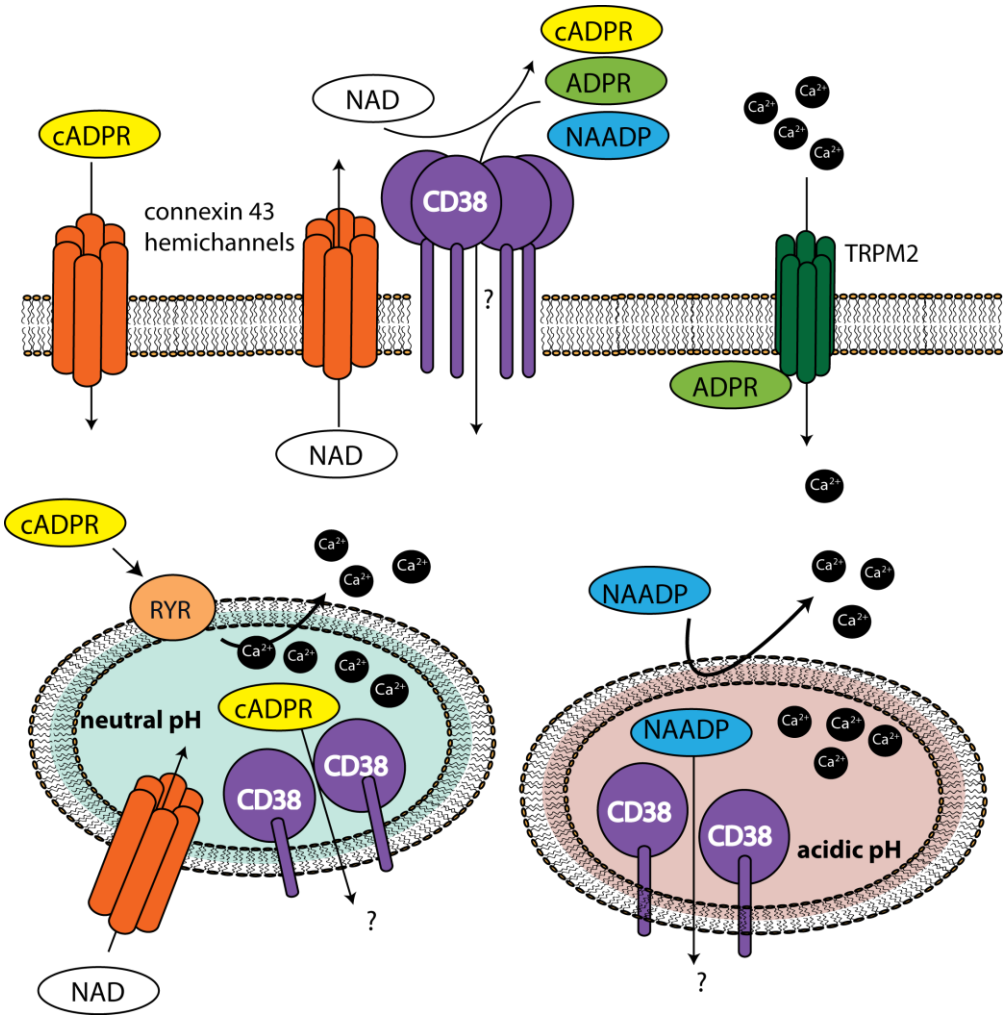

Figure 1.16 Postulated mechanisms of Ca^{2+} mobilisation mediated by CD38 enzymatic products.
The CD38 enzymatic products, NAADP, cADPR and ADPR are involved in Ca^{2+} mobilisation from intracellular stores and extracellular compartment. NAADP induces Ca^{2+} release mainly from acidic cellular compartments, such as lysosomes. The Ca^{2+} release induced by cADPR is mainly mediated by the ryanodine receptors (RyRs) found in the ER and in endosomes. ADPR mediates Ca^{2+} influx by binding to the cation channel TRPM2. The mechanisms mediating the intracellular and subcellular localisation of these molecules have not been fully elucidated. CD38 homodimers and homotetramers could act as a unidirectional transporter. The connexin 43 hemichannels might transport the CD38 substrate NAD to the active site of CD38 as well as transferring cADPR from the extracellular compartment into the cell.

1.7.2 Non-enzymatic functions of CD38

Several studies, mainly performed using monoclonal antibodies (mAb) anti-CD38, have supported the hypothesis that CD38 could also act as a receptor. Firstly, stimulation with anti-CD38 mAb was shown to have proliferative effects in human peripheral blood mononuclear cells (PBMC) (Funaro et al., 1990) and to induce an increase in intracellular Ca^{2+} levels in murine B-lymphocytes (Santos-Argumedo et al., 1993).

Interestingly, studies in human B-lymphocytes showed that *in vitro* ligation of CD38 can induce different cellular responses based on the developmental stage of the cells. Whereas CD38 ligation prevents apoptosis of germinal centre (GC) B cells (Zupo et al., 1994), it inhibits B-lymphopoiesis and induces apoptosis in B cell progenitors (Kumagai et al., 1995).

It was reported that CD38 antibody ligation induced tyrosine phosphorylation of and activation of several signalling molecules such as Syk and PLCγ (Silvennoinen et al., 1996).

The identification of CD31 (PECAM-1) as a potential physiological ligand of CD38 supported the hypothesis that CD38 could also have a function as a receptor. The binding of CD38 with CD31 was reported to recapitulate the signalling events observed after ligation with anti-CD38 mAb (Deaglio et al., 1998). It was also proposed that CD38 could directly mediate a weak adhesion of T- and B-lymphocytes to the endothelial cells (Dianzani et al., 1994). This adhesion would be mediated by CD38 binding to CD31, as using blocking CD31 mAb was able to inhibit the adhesion of several CD38-expressing cell lines.

However, the mechanism by which CD38 could mediate its function as a receptor has not been elucidated. Based on the observation that CD38 ligation on B cell progenitors did not induce any detectable difference in the levels of NAD, cADPR and ADPR, and addition of these molecules was not able to induce the same inhibitory effects in B cell progenitors, it was proposed that CD38 enzymatic functions are not involved in this mechanism (Kumagai et al., 1995). Hower, as interactions with putative ligands could interfere with CD38 catalytic activity and deletion of the catalytic domain of CD38 impairs the binding with these agonistic antibodies, the involvement of CD38 enzymatic activity in these signalling events cannot be

completely excluded (Deaglio et al., 2008; Hoshino et al., 1997; Munshi et al., 2000). Notably, the signalling cascade observed after CD38 mAb stimulation of murine pro-B cells transfected with human CD38 lacking its cytoplasmic domain was comparable to that obtained with the wild-type protein (Kitanaka et al., 1999), indicating that CD38 does not act as a direct mediator of intracellular signalling but its effect probably requires accessory molecules. Supporting this hypothesis, lateral association of CD38 with several key signalling molecules organised in membrane lipid rafts have been reported in different cellular systems. In particular, CD38 has been shown to interact with the TCR/CD3 complex in T-lymphocytes and with the BCR/CD19 complex in B-lymphocytes (Malavasi et al., 2008).

1.7.3 CD38 in CLL biology

1.7.3.1 CD38 in CLL cell proliferation

The relevance of CD38 as a prognostic factor in CLL (Section 1.5.1.4) led to investigations on its potential functional role in the disease. Several studies aimed to investigate whether CD38 expression in CLL cells has a role in the pathophysiological features of the leukaemic clones, as opposed to being considered merely a prognostic marker. It was shown that CD38 positive subpopulations of CLL clones are enriched in Ki-67+ cells (Damle et al., 2007). Moreover, analysis of CLL cell turnover in the peripheral blood of CLL patients identified CD38-expressing cells within individual CLL clones as the recently proliferated ones (Messmer et al., 2005). Consistently with these data, co-expression of CD38 and Ki-67 has been observed in the proliferation centres of CLL patient lymph nodes, where CD38 expression is up-regulated by CLL cell cross-talk with the tumour microenvironment, as suggested by the induction of CD38 expression observed in CLL cells cocultured *in vitro* with autologous activated CD40L-expressing T cells (Patten et al., 2008). Taken together, these observations suggest that CD38 expression within individual CLL clones can be modulated by microenvironmental stimuli and it is associated with CLL cell proliferation, despite not providing evidence for a direct role of CD38 in CLL cell proliferation. An active role of CD38 in CLL cell proliferation was suggested by experiments performed using anti-CD38 mAb. CD38 ligation in the presence of IL-2 induced an increase in intracellular Ca^{2+} and CLL cell proliferation (Deaglio et al., 2003). However, analysis of

this mechanism using CD31, the putative physiological ligand of CD38, showed discordant results. Co-culture of CD31-overexpressing fibroblasts or CD31-expressing NLCs was reported to induce an increased survival and proliferation of CD38-expressing CLL cells. The specificity of this mechanism was shown by the use of an antagonist anti-CD31 mAb, which abrogated this effect (Deaglio et al., 2005). However, studies performed by another group showed no effect on CLL cell survival and proliferation using co-cultures of CD31-transfected fibroblasts or endothelial cells in the presence or absence of either anti-CD31 or anti-CD38 blocking mAbs (Tonino et al., 2008). Therefore, whether CD38 expression is directly involved in CLL cell proliferation still needs to be defined.

1.7.3.2 CD38 in CLL cell motility

Several lines of evidence have shown that CD38 expression correlates with increased motility properties in CLL cells. Firstly, it was shown that CD38-positive CLL samples (with a cut-off of 20% CD38-expressing CLL cells) had increased ability to migrate towards the chemokine CXCL12. However, the same study reported a correlation between CD38 and ZAP-70 expression in the CLL patient samples analysed and it was proposed that the CD38-mediated effect on chemotaxis required ZAP-70 expression, as discordant cases (CD38+/ZAP-70-) did not show the same migratory ability compared to CD38+/ ZAP-70+ cases (Deaglio et al., 2007a). Subsequent studies confirmed the enhanced CXCL12-mediated chemotactic properties of CD38-expressing CLL cells, which was inhibited by antagonist anti-CD38 mAb and enhanced by stimulation with agonistic anti-CD38 mAb. Moreover, ectopic expression of CD38 in CLL cells was able to increase their CXCL12-mediated chemotaxis (Vaisitti et al., 2010).

These results suggested that CD38 plays an important role in CLL cell chemokine responses, therefore suggesting that it could be involved in leukocyte trafficking. However, the role of CD38 in CLL cell trafficking is still controversial. Whereas some studies showed that blocking anti-CD38 antibodies could reduce CLL cell homing to the spleen and bone marrow in an *in vivo* xenograft mouse model (Vaisitti et al., 2010), other research performed using the same model showed that CD38 expression was dispensable for CLL cell homing to the bone marrow and the use of a different

blocking anti-CD38 antibody did not confirm the previous results (Brachtl et al., 2011). Notably, enhanced engraftment of CD38-expressing CLL samples in murine spleen has been linked to the CD38-dependent proliferation occurring in the lymphoid tissues (Aydin et al., 2011), thus adding complexity to the identification of the potential role of CD38 in homing.

CD38 expression in CLL cells has been shown to correlate with the expression of other molecules involved in cell migration, including the α4- and β2-integrin subunits (Herens et al., 2006; Pittner et al., 2005; Zucchetto et al., 2012), making it difficult to ascribe the enhanced migratory properties exclusively to CD38. Furthermore, a lateral association of CD38 with molecules which are involved in cell migration such as CD44, the matrix metallopeptidase 9 (MMP-9) and α4-integrin (Buggins et al., 2011; Vaisitti et al., 2013; Zucchetto et al., 2012) has been described, together with the association with BCR-related components such as CD19 observed also in normal B-lymphocytes (Deaglio et al., 2007b).

Recent studies have reported that MMP-9 expression and activity is variable between CLL patients and correlates with CD38 expression (20% CD38-expressing cells used as cut-off value) (Vaisitti et al., 2013). Moreover, analysis of CLL samples with bimodal CD38 expression showed the CD38-positive subclones displayed enhanced MMP-9 mRNA levels compared to the CD38-negative subpopulations within individual CLL patients (Vaisitti et al., 2013). Notably, MMP-9 enzymatic activity has been shown to be involved in CLL cell TEM and invasion through matrix components and its expression can be up-regulated in CLL cells following α4β1-integrin or CXCR4 stimulation (Redondo-Muñoz et al., 2006). Moreover, MMP-9 has been shown to induce pro-survival signalling in CLL cells through a mechanism that does not involve its catalytic activity, but it is mediated by its binding to a docking structure containing α4β1-integrin and a 190kD CD44 variant (Redondo-Muñoz et al., 2010).

Finally, it has been proposed that CD38 could have a role in CLL cell adhesion. In this context, the correlation between CD38 and CD49d expression in CLL cells causes difficulties in the analysis of the role of each single component. However, studies performed using discordant CLL cases (cut-off of 30% positive cells for both antigens) showed that discordant CLL cases (CD38-/CD49d+) have reduced adhesion to VCAM-1 compared to CD38+/CD49d+. This result was confirmed by experiments performed

ectopically expressing CD38 in the CLL-derived MEC1 cell line, which increased their adhesion to VCAM-1 and the induction of filopodium-like protrusions (Zucchetto et al., 2012).

Whether the effects of CD38 in CLL cell motility is linked to its enzymatic function or to its proposed role as a receptor is currently being investigated. A recent study showed that ectopic expression of wild-type CD38 in MEC1 cells induces an increase in their adhesion to VCAM-1 and their chemotaxis towards CXCL12, CCL19 and CXCL10. However, ectopic expression of the enzymatic deficient version of CD38, with a single amino-acid mutation, failed to induce the same effects (Vaisitti et al., 2014). This suggests that the catalytic function of CD38 is probably involved in the role of CD38 in modulating CLL cell adhesion and motility.

In conclusion, although it seems obvious that CD38 is not just a marker of an aggressive form of CLL, its contribution to CLL progression is still unclear.

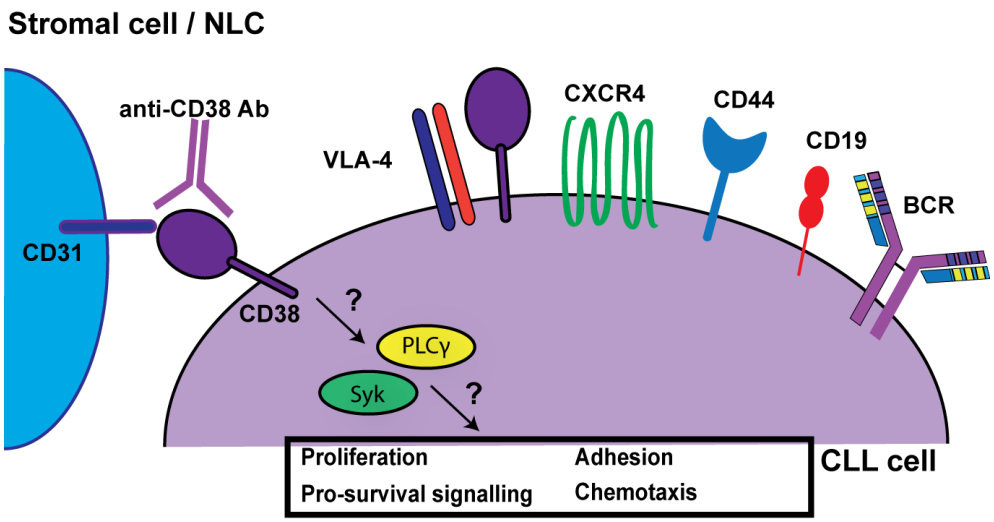

Figure 1.17 Postulated roles of CD38.
CD38 signalling, induced by ligation with agonistic anti-CD38 antibodies or following binding to its postulated physiological counter-receptor CD31, leads to an increased protein tyrosine phosphorylation. This supports the hypothesis that CD38 could act as a receptor involved in cell proliferation, survival, adhesion and chemotaxis. However, the molecular mechanisms regulating these events still need to be determined. Of note, in CLL cells, CD38 laterally associated with key receptor molecules such as the integrin VLA-4, CD44, CXCR4 and the BCR/CD19 complex.

1.8 Aims of the project

CLL cell trafficking between blood and lymphoid organs is crucial to the pathophysiology of CLL, as leukaemic cell survival and proliferation is remarkably dependent on the interactions with the lymphoid microenvironment. CD38 expression in CLL cells is a marker of unfavourable prognosis and correlates with enhanced CLL cell motility properties, possibly affecting CLL cell trafficking dynamics. However, the molecular mechanism involved in the CD38-mediated effect on CLL cell migration has not been elucidated. This project had three main aims:

1) To investigate the role of CD38 expression in the different steps involved in CLL cell motility. Ectopic expression of CD38 in a CLL-derived cell line, MEC1, was the strategy adopted in order to evaluate the role of CD38 expression in the adhesion, transendothelial migration and chemotaxis of CLL cells.

2) To investigate the signalling pathways involved in the different migration properties of the CD38-expressing cells. Particularly, because of their critical roles in cell motility, the activity of GTPases and their regulators was investigated.

3) The final aim was to analyse the distinctive signalling mechanisms identified in the CD38-expressing CLL-derived MEC1 cell line in a cohort of primary CLL samples with differential CD38 expression levels.

2. Materials and methods

2.1 Materials

Chemicals for solutions were purchased from Sigma-Aldrich unless otherwise stated.

2.1.1 Reagents and kits

Table 2.1 Reagents and kits

Reagent/kit	Supplier
0.4% Trypan Blue solution	Sigma-Aldrich
1kb-Ladder	Life Technologies
7-AAD Viable Staining Solution	BioLegend
AlexaFluor®-conjugated phalloidin	Life Technologies
Alkaline Phosphatase, Calf Intestinal (CIP)	New England Biolabs
Amersham Protran 0.45 Nitrocellulose membrane	GE Healthcare Life Science
Bovine Serum Albumin (BSA) Fraction V, pH 7.0	PAA Laboratories
4',6-diamidino-2-phenylindole (DAPI) - Nucleic Acid Stain	Life Technologies
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich
DNA-free Kit	Life Technologies
DNA sample buffer (6x)	Life Technologies
Dried skimmed milk	Marvel
Enhanced chemiluminescence (ECL) detection kit	GE Healthcare Life Science
EDTA-free Protease Inhibitor Cocktail Tablets	Roche Applied Science
EndoFree Plasmid Maxi Kit	Qiagen
Endothelial basal medium-2	Lonza
Endothelial cell growth supplements	Lonza
Ethidium Bromide Solution	Life Technologies
Fibronectin from human plasma	Sigma-Aldrich
Fluorescence mounting medium	Dako Cytomation
FuGENE® 6 Transfection Reagent	Roche Applied Science

GIBCO® Dulbecco's modified Eagle's medium (DMEM)	Life Technologies
GIBCO® Iscove's modified Dulbecco's medium (IMDM)	Life Technologis
GIBCO® Dulbecco's Phosphate-Buffered Saline (DPBS) (-) CaCl2 (-) MgCl2	Life Technologies
GIBCO® Dulbecco's Phosphate-Buffered Saline (DPBS) (+) $CaCl_2$ (+) $MgCl_2$	Life Technologies
GIBCO® Foetal Bovine Serum	Life Technologies
GIBCO® Penicillin-Streptomycin	Life Technologies
GIBCO® RPMI 1640	Life Technologies
GIBCO® trypsin/EDTA 0.05%	Life Technologies
Glass bottom culture dishes	Corning Life Science
Glutathione Sepharose™ 4 Fast Flow	GE Healthcare Life Science
Human B Cell Nucleofector® Kit	Lonza
Human recombinant CCL21	R&D systems
Human recombinant CXCL12/SDF1	R&D systems
Human recombinant CXCL13	R&D systems
HUVEC Nucleofector® Kit	Lonza
Ibrutinib (PCI-32765)	Selleckchem
Indo1-AM	Life Technologies
Ionomycin, calcium salt	Cambridge Bioscience
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Sigma-Aldrich
Restriction Endonuclease	New England Biolabs
Luria Broth (LB) Tablet microbial growth medium	Sigma-Aldrich
Lysozyme from chicken egg white	Sigma-Aldrich
NEB buffers	New England Biolabs
NuPAGE® 4 - 12% Bis-Tris Gel	Life Technologies
NuPAGE® LDS sample buffer (4×)	Life Technologies
NuPAGE® MES SDS running buffer (20×)	Life Technologies
Precision PlusProtein™ standards	Bio-Rad

QIAprep Miniprep Kit	Qiagen
QIAquick gel extraction kit	Qiagen
Super RX medical X-ray film	Fuji
SuperScript® VILO Kit	Life Technologies
T4 DNA ligase	New England Biolabs
T4 DNA ligase reaction buffer	New England Biolabs
TNF-α/TNFSF1A	R&D systems
Transwell™ Permeable Supports, 5 µm pore size, 6.5 mm	Costar
Trypan Blue Stain 0.4%	VWR International
VCAM-1 human recombinant	R&D systems
Whatman 3MM chromatography paper	Whatman

2.1.2 Buffers and solutions

Table 2.2 Buffers and solutions

Buffer	Composition
Flow cytometry (FC) buffer	3% Bovine Serum Albumin in DPBS (+) $CaCl_2$ (+) $MgCl_2$
Lysis buffer	20 mM Tris pH 7.5, 150mM NaCl, 1mM EDTA, 1% NP-40, 0.1% SDS - supplemented with 25mM NaF, 100 µM Na_3VO_4
Transfer Buffer	25 mM Tris HCl pH 8.3, 192 mM Glycine, 20% MeOH
Tris-buffered saline – Tween (TBS-T)	5 mM Tris HCl pH 7.6, 50 mM NaCl, 2.5 mM KCl, 0.1% Tween-20
Stripping buffer	100 mM Glycine, 1% SDS, 0,1% NP-40, pH 2.2
Bacterial lysis buffer	50 mM Tris HCl pH 7.6, 50 mM NaCl, 5 mM $MgCl_2$ supplemented with 1 mM DTT, 1 mg/ml lysozyme, Protease Inhibitor Cocktail Tablets
Beads washing buffer	Tris HCl 50 mM pH 7.6, NaCl 50 mM, $MgCl_2$ 5 mM
Pull-down lysis buffer	Tris HCl 50 mM pH 7.4, NaCl 500 mM, $MgCl_2$ 10 mM, Triton X-100 1%, Deoxycholic acid 0.5%, SDS 0.1%, EGTA 2.5 mM supplemented with Protease Inhibitor Cocktail Tablets, NaF 50 mM, Na_3VO_4 1 mM, PMSF 1 mM

TAE buffer	Tris 40mM, acetic acid 20 mM, EDTA 1 mM, pH 8.4

2.1.3 Antibodies

Table 2.3 Primary antibodies for Western Blot (WB) and Immunofluorescence (IF)

Antigen	Species	Dilution	Supplier
Cdc42	rabbit	**(WB)** 1:1000	Cell Signalling
Rac1 (clone 23A8)	mouse	**(WB)** 1:1000	Upstate Millipore
Rap1A/Rap1B (clone 26B4)	rabbit	**(WB)** 1:1000	Cell Signalling
Rap1A	goat	**(WB)** 1:250	Santa Cruz Biotechnology
Rap1B (clone 36E1)	rabbit	**(WB)** 1:1000	Cell Signalling
Phospho-p44/42 MAPK (Erk1/2)	rabbit	**(WB)** 1:2000	Cell Signalling
p44/42 MAPK (ERK1/2)	rabbit	**(WB)** 1:2000	Cell Signalling
IQGAP1 (clone AF1)	mouse	**(WB)** 1:1000	Life Technologies
CD38	rabbit	**(WB)** 1:500	Santa Cruz Biotechnology
CD38	rabbit	**(IF)** 1:100	Abcam
RasGRP2	rabbit	**(WB)** 1:1000 **(IF)** 1:200	Thermo Scientific
RasGRP3	rabbit	**(WB)** 1:1000	Cell Signalling
α-tubulin (clone DM1A)	mouse	**(WB)** 1:10000	Sigma-Aldrich
GAPDH	mouse	**(WB)** 1:10000	Upstate Millipore

Table 2.4 Primary antibodies for FC and IgM stimulation

Antigen	Species	Conjugate (if applicable)	Dilution	Supplier
CD38 (clone HB7)	mouse	APC/PE	2.5 µg/10^6cells	BD Biosciences
CCR7 (clone 150503)	mouse	n.a.	2.5 µg/10^6cells	R&D systems
CD49d (integrin α4)	mouse	n.a.	2.5 µg/10^6cells	AbD Serotec
CD29 (integrin β1)	mouse	PE-Cy5	2.5 µg/10^6cells	BD Biosciences
Integrin β1 (12G10)	mouse	n.a.	2.5 µg/10^6cells	Abcam
IgM	goat	n.a.	20 µg/ml	AbD Serotec

Table 2.5: Secondary antibodies

Antigen	Species	Conjugate	Diluition	Supplier
Mouse IgG	Sheep	HRP	1:2000-1:10000	GE Healthcare
Rabbit IgG	Goat	HRP	1:2000-1:5000	GE Healthcare
Goat IgG	Rabbit	HRP	1:2000	GE Healthcare
Rabbit IgG (H+L)	Goat	AlexaFluor® 546	1:200	Life Technologies
Rabbit IgG (H+L)	Goat	AlexaFluor® 647	1:200	Life Technologies
Mouse IgG (H+L)	Goat	AlexaFluor® 546	1:200	Life Technologies

2.1.4 Oligonucleotides

Table 2.6: siRNA Oligonucleotides

Gene Symbol	Sense strand sequence	Supplier	Serial Number	Oligo number
RasGRP2	GUGCAAGGAUCGCCUGUCA	Dharmacon	D-009365-04	#1
RasGRP2	CCAAUUCCCUGCAGGUGAA	Dharmacon	D-009365-05	#2
RasGRP3	GGAGAAAGCUGCAAUGAAU	Dharmacon	D-008517-01	#1
RasGRP3	GAAUGCCUCUCACCACUUA	Dharmacon	D-008517-03	#2

Rap1A	GAUAGAAGAUUCCUACAGA	Dharmacon	D-003623-01	#1
Rap1A	CAAUAAAUGUGACCUGGAA	Dharmacon	D-003623-01	#2
Rap1B	GAACAACUGUGCAUUCUUA	Dharmacon	D-010364-01	#1
Rap1B	CAAUGAUUCUUGUUGGUAA	Dharmacon	D-010364-03	#2
IQGAP1	GGAAAGCUCUGGCAAUUUA	Dharmacon	D-004694-01	#1
IQGAP1	GAACGUGGCUUAUGAGUAC	Dharmacon	D-004694-03	#2

Table 2.7: Primers

Primer	Sequence
XhoI-pEGFP-FOR	5’- AATAGACCTCGAGGACACCATGGTGAGCAAGGGCGAGG -3’
XhoI-pEGFP-REV	5’ - AAGCTTGAGCTCGAGATCTGAGTCCGGACTTG -3’
RasGRF1-FOR	5’- GACGTGGCTCAAAGTCTCT -3’
RasGRF1-REV	5’- CGGAGAGAAGACTCGTAGAG -3’
RasGRF2-FOR	5’- TGGAGATCACCTCGGCCTTA -3’
RasGRF2-REV	5’- TACTGTGCGACCTTTGGCTG -3’
RasGRP1-FOR	5’- CCTGTGTCGAAGTAACCAACTG -3’
RasGRP1-REV	5’- GTCACGGGCATTGATTTGAG -3’
RasGRP2-FOR	5’- CACCCTGGACCTGGACAA -3’
RasGRP2-REV	5’- TGAGAGGAGGGGATGTACCA -3’
RasGRP3-FOR	5’- CACTAACATCGCTGACTTGC -3’
RasGRP3-REV	5’- TGGCATTTCGATACATGCAGAG -3’

Table 2.8: Sequencing primers

Vector Sequenced	Primer	Sequence	Supplier
pEGFP-C1-FOR	pEGFP-C1-for	5’- GATCACTCTCGGCATGGAC -3’	Eurofins
pEGFP-C1-REV	pEGFP-C1-rev	5’- CATTTTATGTTTCAGGTTCAGGG -3’	Eurofins
mCherry-N1	CMV-for	5’- CGCAAATGGGCGGTAGGCCTG -3’	Eurofins
pLentiSEW	CD38-for	5’-GAGATGAGACATGTAGACTGCC -3’	Eurogentec
pLentiSEW	CD38-rev	5’- ATGTGCAAGATGAATCCTCAG -3’	Eurogentec
pLentiSEW	pEGFPN1-rev	5’- GTCCAGCTCGACCAGGATG -3’	Eurofins

2.2 Methods: cell biology

2.2.1 B-lymphocyte cell lines.

The human B-lymphocyte MEC1 cell line was a kind gift of Prof. John Gribben (Queen Mary University of London, UK). The MEC1 cell line was established from the peripheral blood of a patient diagnosed with chronic B cell leukaemia (B-CLL in prolymphocytoid transformation to B-PLL) (Stacchini et al., 1999). Cells were maintained in IMDM containing 2 mM glutamine and supplemented with 10% heat inactivated foetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 µg/ml). Cell density was maintained between 5 X 10^5 and 2 X 10^6 cells/ml. The Burkitt lymphoma B cell line Ramos (kind gift of Prof. Hannah Gould, King's College London, UK) was maintained in ATCC-formulated RPMI-1640 and supplemented with 10% heat inactivated FCS, penicillin (100 U/ml), and streptomycin (100 µg/ml). Cell density was maintained between 2 X 10^5 and 1 X 10^6 cells/ml.

2.2.2 Primary CLL cells.

PBMCs collected from patients with a confirmed diagnosis of CLL were isolated by Ficoll density gradient centrifugation and cryopreserved in aliquots. Cryopreserved aliquots and clinical data, including CD38 expression, were provided from the Haematology tissue bank, King's College Hospital. Cells were cultured in RPMI-1640 supplemented with 10% heat inactivated FCS, 1% bovine serum albumin, penicillin (100 U/ml) and streptomycin (100 µg/ml). Cell viability was determined by adding 10 µl of a 0.4% Trypan Blue solution to 10 µl of the cell suspension at the desired dilution. Cells were then loaded into a haemocytometer and examined immediately under a microscope, the ratio of blue staining cells (non-viable) and the total cells was then calculated.

2.2.3 Endothelial cells.

Human umbilical vein endothelial cells (HUVECs) (PromoCell, UK) were cultured in flasks coated with 10 µg/ml fibronectin in endothelial basal medium-2 supplemented with 2% FCS and endothelial cell growth supplements. Cells were used between passages 1 and 5.

2.2.4 HEK 293T cells

Due to its high transfectability, the HEK 293T cell line was used for virus packaging. Cells were cultured in DMEM with 10% FCS, penicillin (100 U/ml), and streptomycin (100 µg/ml).

2.2.5 Cell treatments

2.2.5.1 TNF-α treatment

HUVECs were stimulated over night with 10 ng/ml TNF-α for 16 h, medium was replaced with fresh warm medium (without TNF-α) before cells were used for experiments.

2.2.5.2 BTK inhibitor

MEC1 and primary CLL cells were treated with 1 µM Ibrutinib (PCI-32765 - Selleck Chemicals) for 1 h before being used for experiments. As a control, cells were treated in parallel with the same volume of DMSO as a vehicle control.

2.2.5.3 Calcium depletion

To decrease the intracellular calcium concentration, 1 x 10^7 MEC1 and primary CLL cells were resuspended in (DPBS) (-) $CaCl_2$ (-) $MgCl_2$ for 10 min before the addition of the calcium ionophore ionomycin (0.5 µM) and a further incubation of 5 min. As a control, cells were resuspended in DPBS (+) $CaCl_2$ (+) $MgCl_2$ and treated in parallel with the same volume of DMSO.

2.2.6 Lentivirus generation and infection of MEC1 cells

Lentiviral vectors expressing CD38 and GFP (pLentiS38W or pLentiSEW respectively (Pearce et al., 2010); a kind gift of Dr Paul Brennan, Cardiff University, Cardiff, UK) or the fusion protein EGFP-CD38 (pLentiSEGFP-CD38W) were amplified in DH10β *Escherichia coli* (*E.coli*) strain. The gag-pol plasmid (pΔ8.91) and the VSVG-encoding plasmid (pMD2-G) were amplified in DH5α *E. coli* strain. All plasmids were purified using the EndoFree Plasmid Maxi Kit For lentivirus production, 30 µl of Fugene 6 reagent and 300 µl of Optimem medium were mixed and incubated at room temperature for 5 min. Plasmid DNA was added to the mix (vector plasmids: 5 µg; pMDG: 1.75 µg; pΔ8.91: 3.25 µg) and then added to a T25 flask of 293T cells (70%

confluent) containing 2 ml of complete medium. Supernatant containing viral particles was collected after 24 and 48 h, filtered through a 0.45 µm filter and stored at -80°C. MEC1 cells (2 x 10^6) were resuspended in 1 ml of complete medium in a 15 ml falcon tube and 1 ml of viral supernatant was added. The cells and the viruses were then centrifuged for 1 h at 200 g. Medium was then removed and replaced with 2 ml of fresh complete IMDM medium. After 7 days, cell cultures were tested for the presence of infectious viral particles in the supernatant. 1x10^7 pLentiS38W-transduced MEC1 cells were labelled under sterile conditions using a PE-conjugated anti-CD38 antibody (HB7). All cell populations were collected (1x10^7) and resuspended in 1 ml of sterile PBS supplemented with 2% bovine serum albumin (BSA), 100 U/ml penicillin, and 100 µg/ml streptomycin and subsequently sorted using a FACScan (Beckman-Coulter EPICS Elite flow cytometer) under sterile conditions. Cells were sorted based on the PE (pLentiS38W-transfected) or GFP (pLentiSEW and pLentiSEGFP-CD38-tranfected) fluorescence intensity. Sorted cells were grown in complete IMDM medium for one week before being used for experiments.

2.2.7 siRNA transfection of MEC1 cells

MEC1 cells (5 x 10^6) were mixed with 1.2 µM siRNA (see TABLE 2.6) in 100 µl Human B Cell Nucleofector® Kit and transferred to an electroporation cuvette. Cells were nucleofected using an Amaxa Nucleofector apparatus (programme C-016), according to the manufacturer's instructions. After nucleofection, cells were immediately transferred to 3 ml of complete medium. After 48 h cells were pelleted and resuspended in 3 ml of fresh complete medium. Experiments were performed 72 h after nucleofection.

2.2.8 DNA transfection of HUVECs

HUVECs were detached using trypsin/EDTA, counted and 1 x 10^6 cells were resuspended in 100 µl HUVEC Nucleofector® Kit. Plasmid DNA (5 µg) was added to the solution and cells were nucleofected using an Amaxa Nucleofector apparatus (programme A-034), according to the manufacturer's instructions. After nucleofection, cells were resuspended in 1 ml of complete medium and 5 x 10^5 cells were added per well on coverslips pre-coated with 10 µg/ml fibronectin. After 24 h

medium was replaced with complete fresh medium supplemented with 10 ng/ml TNF-α. After 16 h MEC1 cells (3 x 10^5 /well) were seeded on top of the HUVEC monolayer and allowed to adhere and transmigrate for 30 minutes before being fixed with 4% paraformaldehyde in PBS for 15 minutes. Coverslips were then mounted onto slides using fluorescent mounting medium, and visualised using a LSM 510 laser scanning confocal microscope (Zeiss).

2.2.9 Chemotaxis assays

MEC1 (3 x 10^5 cells in 100 µl) and primary CLL cells (5 x 10^5 cells in 100 µl) were added to VCAM-1-coated (5 µg/ml) Transwell™ filters (6.5 mm diameter; 5 µm and 3µm pore size for MEC1 and primary CLL cells respectively). Lower chambers were filled with 600 µl of fresh medium containing the desired concentration of chemokine (CCL21, CXCL13 and CXCL12) (Figure 2.1A). After 2 or 4 h (for MEC1 and primary CLL cells respectively), transmigrated cells were collected and counted using a Casy® cell counter (Schärfe System GmbH). Medium without chemokine was added in the bottom chamber to test basal migration.

2.2.10 Transendothelial migration Transwell™-based assay

HUVECs were grown to confluency on Transwell™ filters (6.5 mm diameter, 5 µm pore size) and stimulated with 10 ng/ml TNF-α prior experiments. 3 x 10^6 MEC1 cells were pelleted and resuspended in 1 ml of fresh medium, and then 100 µl of the cell suspension were added per filter. Lower chambers were filled with 600 µl of medium containing 500 ng/ml of CCL21 and cells were allowed to transmigrate for 4 h at 37°C and 5% CO_2 (Figure 2.1B). Cells from the lower chamber were counted using a CASY® cell counter (Schärfe System GmbH).

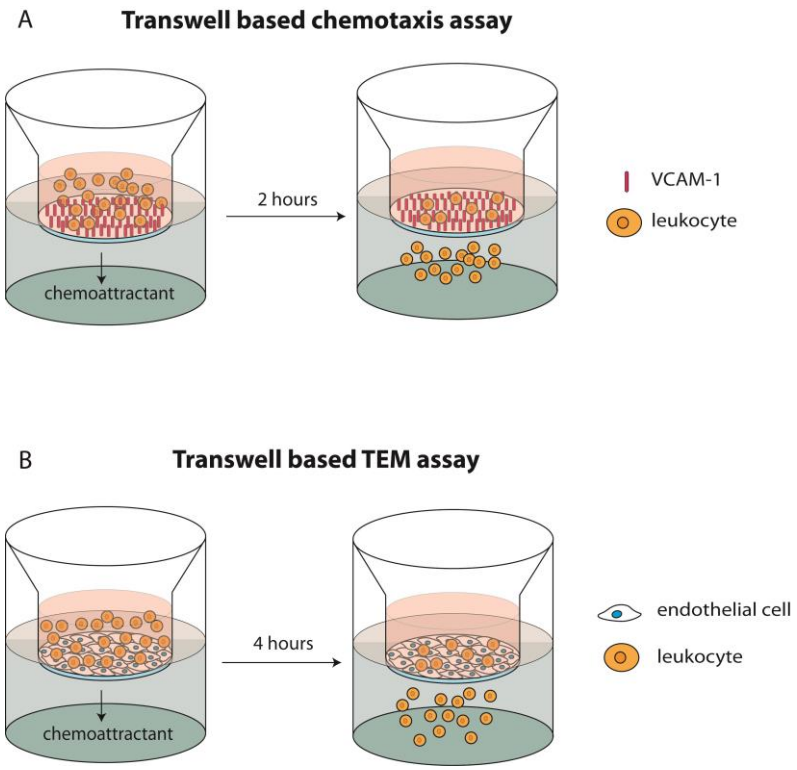

Figure 2.1 Schematic representation of transwell based assays.
Leukocytes were seeded on a permeable support (top chamber) coated with VCAM-1 (A) or on top of an endothelial monolayer (B). Medium containing chemoattractant was added in the bottom chamber; transmigrated cells were collected and counted after 2 h (in the absence of endothelial monolayer) or 4 h (transendothelial migration assay).

2.2.11 Time-lapse microscopy

MEC1 or primary CLL cells (1 x 10^5) were seeded on a HUVEC monolayer or a VCAM-1 coated (5 µg/ml) well and filmed for 2 h in a conditioned atmosphere (37°C, 5% CO_2). HUVECs (1.5 x 10^5) were previously grown to confluency on FN-coated wells and treated for 16 h with 10 ng/ml TNF-α. Images were acquired every minute using Metamorph software (Molecular Devices) on a Nikon Eclipse TE2000 microscope with 10x or 20× objectives. Time-lapse images from the duration of the experiment were then displayed as a movie, and cells from each frame tracked using Manual Tracking plugin of ImageJ software (http://rsb.info.nih.gov/ij/). Mean cell speed was obtained in each experiment by measuring the mean speed of n ≥ 30 cells for each condition using the Chemotaxis Tool plugin of ImageJ software.

2.2.12 Cell spreading

For cell spreading quantification, 2 x 10^5 cells were seeded on 5 µg/ml VCAM-1-coated glass plates. After 15 min, fresh medium with or without 100 ng/ml of CCL21 was added to the well and cells were allowed to spread for 5 min before being fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and washed carefully three times with PBS before being imaged. Interference reflection microscopy (IRM) technique was used to produce an image containing only the regions of close contact between the cell and the adhesive surface (0–200 nm from the surface) (Figure 2.2 A and B). Images of at least 100 cells from three independent experiments were acquired using a LSM 510 laser scanning confocal microscope (Zeiss) and analysed using ImageJ software. The attachment area of each cell was drawn using the Polygon selection tool and quantified as μm^2 using the tool Analyse - Measure (Figure 2.2 C).

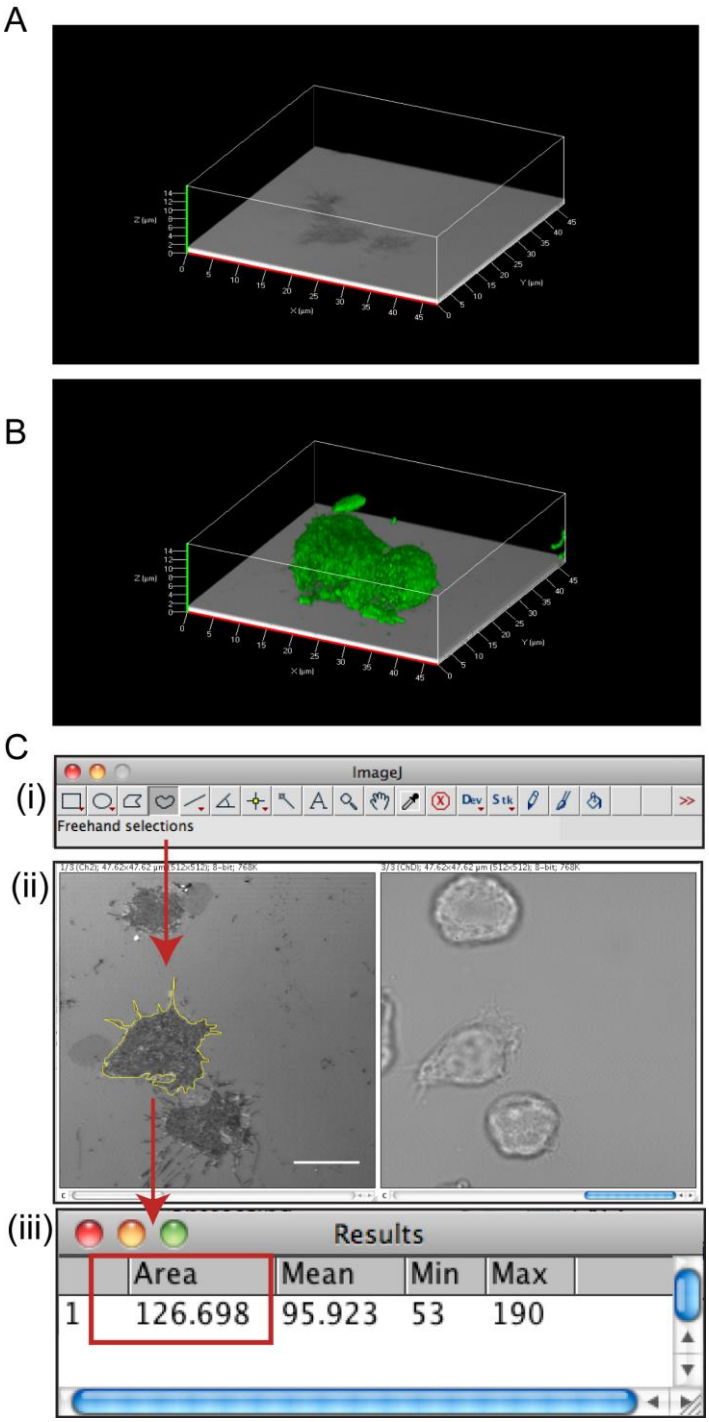

Figure 2.2 Interference reflection image acquisition and analysis.
3D projection of z-stacks of a GFP-expressing MEC1 cell seeded on VCAM-1-coated glass imaged with IRM technique (A) or fluorescence microscopy (B). IRM imaging is restricted to the region of close contact between the cell and the surface (0–200 nm). (C) Representative attachment area measurement of one MEC1-CD38H cell. Perimeter of the area was drawn on the IRM image using the Poligon selection tool (Freehand selection - C, i and ii) and quantified as Area, expressed as μm^2, using the tool Analyse - Measure (C, iii). Scale bar (C, ii), 10 µm.

2.2.13 Flow cytometry

To assess surface antigen expression in lymphocytes, cells were analysed by flow cytometry. 1 x 10^6 cells were harvested, pelleted by centrifugation at 200 g for 5 min and washed in PBS before being incubated with unconjugated primary antibodies (see TABLE 2.4) or fluorochrome-conjugated antibodies or with the appropriate isotype control in 100 µl FC buffer on ice in the dark for 30 min. Cells were then washed 3 times in PBS and fixed with 1% paraformaldehyde in PBS for 30 minutes. When unconjugated primary antibodies were used, a 1/200 dilution of fluorochrome-conjugated secondary antibody was added to the cells in 100 µl FC buffer, incubated on ice for 20 min and then washed 3 times in PBS before fixation. Cells were resuspended in FC buffer prior to analysis. Flow cytometry analysis was performed with either a BD LSRFortessa™, or BD FACSCanto™ II flow cytometer (BD Biosciences). To assess levels of surface antigen expression, 10,000 events were recorded per sample and analysis was performed on data obtained after gating cells on forward scatter/side scatter (FSC/SSC) using FlowJo (Tree Star, Inc.) software.

2.2.14 Calcium detection assay

To measure intracellular Ca^{2+} concentration and changes in intracellular Ca^{2+} after chemokine or IgM stimulus, the ratiometric Ca^{2+} detector Indo1-AM was used. Indo1-AM is a cell permeant dye, which can passively diffuse across the cell membrane, but it becomes impermeant once intracellular esterases cleave its acetyl group (MacFarlane et al., 2010). Indo1 is UV-excitable (peak excitation ≈345 nm) with a maximum emission that can shift between two different wavelengths depending on whether it is bound to Ca^{2+} (≈410 nm) or unbound (≈485 nm). The ratio of these two wavelengths therefore reflects the intracellular Ca^{2+} concentration. For the assay, 5 x 10^6 cells/ml cells were incubated in growth medium supplemented with 1 µM Indo1-AM for 1 h at 37°C and 5% CO2. Cells were washed, resuspended in fresh medium and incubated at 37°C and 5% CO2 for 30 min then stored on ice before being used for experiments. For each acquisition, 3 x 10^6 cells resuspended in 1 ml of medium were used. For dead cell exclusion 5 µl of the 7-AAD Viability Staining Solution was added to each sample and incubate for 10 min in the dark before data acquisition; cells were warmed to 37°C for 5 min before performing the analysis. Flow

cytometric data were acquired using FACS Diva software; a bivariate plot of the ratio between the two fluorescence intensities at the desired emission wavelengths (420/510) against time was created. After recording basal calcium fluorescence for 1 min, the tube was removed and CCL21 (100 ng/ml) or anti-human IgM (20 µg/ml) was added to the cells Samples were then mixed and replaced on the platform, events were recorded up to 300 sec. Ionomycin at a concentration of 1 µM was used to induce calcium influx as a positive control. The stimulation protocol was optimised using the Ramos B-cell line; a representative recording obtained with primary CLL cells is shown in Figure 2.3. Flow cytometric data obtained were analysed using the kinetic application of the FlowJo software (Tree Star, Inc.). Viable cells were gated and a new parameter (Ratio Indo1) was derived as the ratio between the two Indo1 emission wavelengths (420 nm/510 nm), then a bivariate plot of the median of the fluorescence intensity ratio against time was produced. In order to provide a quantitative measure for the cellular response after the stimulus and subsequent comparison between experiments, the background fluorescence threshold intensity was established at the 85^{th} percentile of basal fluorescence ratio of unstimulated cells (Mockridge et al., 2007). The percentage of cells that exhibited an increase in the fluorescence intensity ratio above the threshold following treatment with CCL21 and anti-IgM was then calculated by applying the statistic tool (FloJo) to the defined time lines. In order to compare the basal fluorescence ratio of samples acquired within the same experiment, graph overlays of flow cytometry data were generated and the median of the fluorescence intensity ratio was calculated.

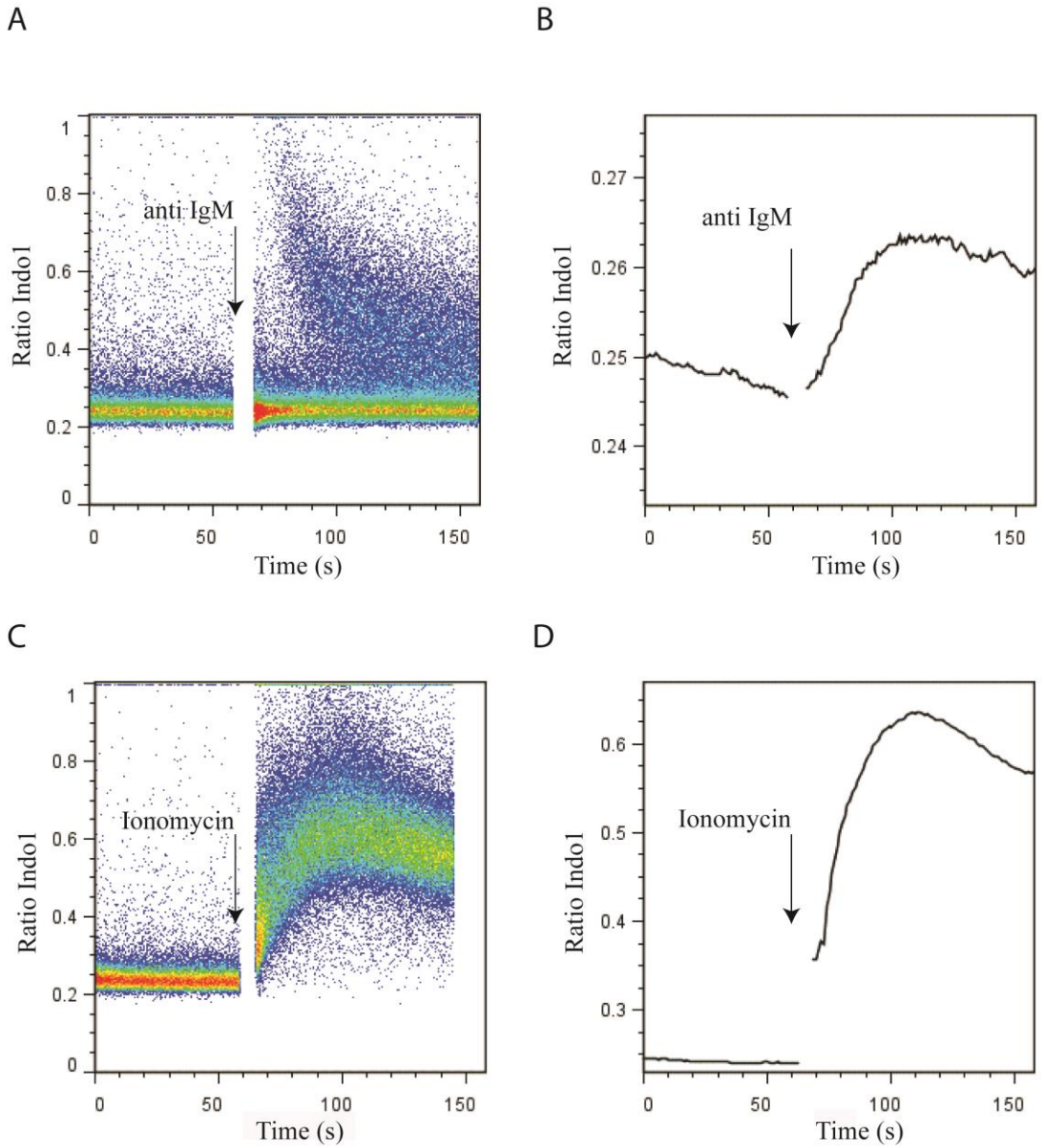

Figure 2.3 Representative plots obtained by calcium detection assay in primary CLL cells.

Cells were loaded with 1 µM Indo1 and analysed by flow cytometry. Basal fluorescence ratio of Indo1 (Ratio Indo1) bound and unbound to calcium (420/510 nm) was recorded for 1 min, followed by the addition of the stimulus (arrows) and a total recording of minimum 150 sec. Representative dot plots (a, c) and kinetic plot (b, d) show the calcium influx upon addition of IgM (a, b) or Ionomycin (c, d).

2.2.15 Immunofluorescence microscopy

For immunofluorescence labelling, 2-5 x 10^5 cells were seeded and allowed to adhere for 15 min on coverslips previously coated overnight with 5 µg/ml VCAM-1 in PBS or 30 min with 0.01% poly-L-lysine solution. Cells were then fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and washed carefully three times with PBS to remove the non-adherent cells before being permeabilised with 0.1% Triton X-100 in PBS (5 min, 4°C). A blocking step was performed by incubating the samples with 3% BSA in PBS (45-60 min). Coverslips were then incubated with primary antibodies at the appropriate dilution (see TABLE 2.3) for 2 h at room temperature or at 4°C overnight. Coverslips were washed a minimum of three times with PBS before being incubated with fluorophore-conjugated IgG secondary antibodies (see TABLE 2.5) in 3% BSA in PBS. To visualise F-actin, cells were counterstained with AlexaFluor 488-, 546- or 633-conjugated phalloidin. Coverslips were mounted onto slides using fluorescent mounting medium, and visualised using a LSM 510 laser scanning confocal microscope (Zeiss).

2.2.16 Analysis of subcellular protein localisation

Images were analysed using ImageJ software. To quantify the polarised distribution of the fluorescence intensity obtained by immunofluorescence analysis of primary CLL cells, the Oval Profile plug-in was used. A circular selection around the cell was drawn and a Radial Sum profile plot was generated for each cell (minimum 30 cells per sample analysed) representing the interpolated pixel intensities along 360 radii from the circle centre to the periphery. Values obtained were then sorted from the lowest to the highest and the slope of the curve obtained was then derived. A slope score equal to 0 (straight horizontal line) represents an absence of variation within the 360 radii, hence a uniformly distributed fluorescence signal (low polarisation), and a highly positive slope score represents a high variation of the fluorescence localisation along the radii (high polarisation) (Fig 2.4).

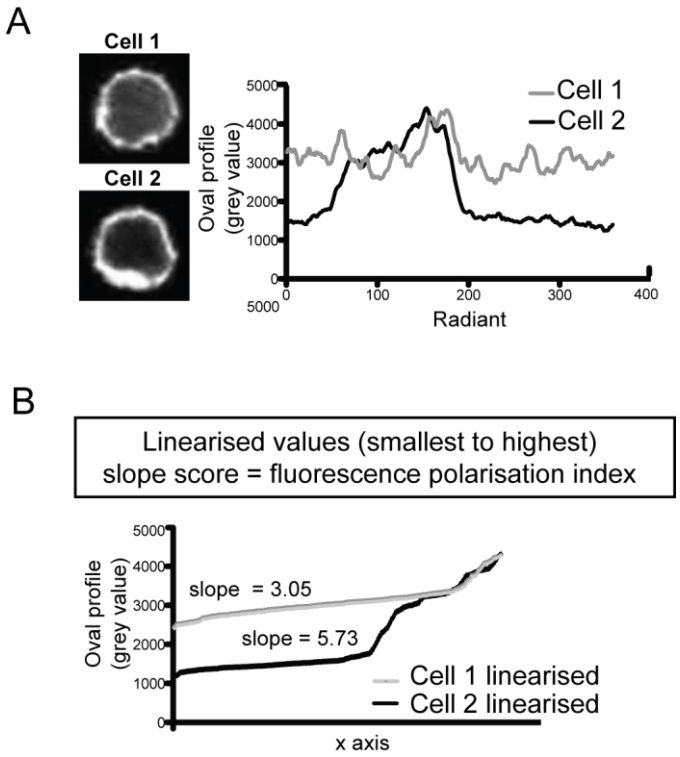

Figure 2.4 Representative quantification of fluorescence intensity distribution.
(A) Immunofluorescence analysis was performed using ImageJ software (Oval Profile plug-in). Radial sum profile plot obtained for the 2 representative CLL cells is shown. Curves represent the pixel intensities along 360 radii calculated for each cell. The 360 values obtained were then sorted from the smallest to the highest to obtain a new curve (B). The slope of the curve was then calculated and correlates with the variation of the fluorescence intensity along the radii.

2.3 Methods: biochemistry

2.3.1 Preparation of cell lysates

Cells (5-10 x 10^6) were washed in PBS and pelleted by centrifugation (250 g for 5 min) before being lysed on ice in an appropriate volume of lysis buffer (100 µl per 2.5 x 10^6 cells). Lysates were then sonicated on ice for 10 sec with a 30% amplitude using a Vibra-Cell™ VCX130 (Sonics and Materials, Inc.), and clarified by centrifugation at 17,000 g at 4°C for 30 min. The supernatant was transferred to a fresh tube, an appropriate volume of 4x sample buffer with 5% β-mercaptoethanol was added and the lysate boiled at 100°C for 5 min. Samples were either used immediately or stored at –20°C.

2.3.2 Western blotting

Proteins extracted from cell lysates were separated on NuPAGE® 4 – 12% Bis-Tris gels at a constant voltage of 150 V in NuPAGE® MES running buffer. After electrophoresis proteins were transferred to a nitrocellulose membrane for western blotting. Protein transfer was carried out for 120 min at a constant voltage of 100 V in transfer buffer. Membranes were then blocked in TBS-T and either 5% non-fat dried milk or 5% BSA for 1 h at RT, incubated with primary antibody either for 2 h at RT or for 16 h at 4°C. Membranes were then washed with TBS-T before being incubated with HRP-conjugated anti-mouse, anti-rabbit or anti-goat secondary antibodies for 1 h at RT. After three washes with TBS-T (10 min each), immunodetection was performed using an ECL detection kit according to the manufacturer's instruction and signal detected by membrane exposure to Super RX medical X-ray film. Western blots were quantified by densitometry using ImajeJ software (Tree Star, Inc.).

2.3.3 Stripping of nitrocellulose membranes

To remove primary and secondary antibodies from nitrocellulose membranes and allow subsequent rounds of western blot detection, membranes were incubated with stripping buffer (TABLE 2.2) for 20 min at 56°C with agitation. After three washes with TBS-T (10 min each), membranes were blocked in 5% non-fat dried milk or 5% BSA for 1 h at room temperature and then blotted with the appropriate primary antibody according to the described western blot procedure.

2.3.4 GTPase activity assay

To detect GTPase activity in treated and untreated MEC1 and primary CLL cells, GST-fusion proteins of the p21-binding domain (PBD) of PAK was used for Rac1 and Cdc42 pull-down assays whereas the GST-fusion protein of the Rap binding domain (RBD) of Ral guanine nucleotide dissociation stimulator (RalGDS) was used for the pull-down of active Rap1.

2.3.4.1 GST-fusion protein extraction and bead-conjugation

E. coli transformed with plasmids encoding the GST-fusion proteins were inoculated in 10 ml of LB medium containing 100 µg/ml ampicillin and grown for 16 h at 37°C

with agitation. The overnight culture was diluted 1/20 into fresh LB medium with ampicillin and grown at 37°C to an optical density of 0.7 (OD600). IPTG was then added at a concentration of 0.3 mM for 2 h at 37°C with agitation to induce protein expression. Bacterial cultures were then centrifuged at 600 g for 30 min at 4°C and pellets were either stored at -40°C for future use or used the same day. Bacterial pellets derived from 250 ml of culture were resuspended in 10 ml of ice-cold bacterial lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mg/ml lysozyme, 1 mM dithiothreitol (DTT) and complete EDTA-free protease inhibitors. The lysate was then clarified by centrifugation at 6000 g for 30 min at 4°C. Sepharose beads were washed three times with ice-cold pull-down bead washing buffer before being added to the clarified lysate and incubated at 4°C for 1 h with rotation. The supernatant was removed and the beads washed three times with pull-down bead washing buffer and resuspended in pull-down lysis buffer. Protein-conjugated sepharose beads were either used the same day or stored at -80°C after the addition of 10% glycerol.

2.3.4.2 Pull-down assay

MEC1 or primary CLL cells (1 x 10^7 cells per condition) were either untreated or treated with 100 nM CCL21 for 1 and 5 min, pelleted and lysed with pull-down lysis buffer for 5 min on ice. Lysates were centrifuged for 10 min at 18,500 g and 40 µl were kept as total cell lysate to determine levels of total GTPases. The remaining lysate was incubated with the protein-conjugated sepharose beads for 30 min at 4°C in rotation. The supernatant was removed and beads were washed 3 times with pull-down washing buffer. After washing, 30 µl of 2X sample buffer with 5% β-mercaptoethanol was added to the beads and samples were boiled for 5 min. Both pull-down and total lysate samples were separated by gel electrophoresis and protein levels were detected by western blotting.

2.4 Methods: molecular biology

2.4.1 Generation of mCherry-PECAM1 vector

PECAM-1-encoding cDNA was subcloned from the pAcGFP1-N1 construct (kind gift of Dr Jaime Millán, Universidad Autònoma de Madrid, Spain) to a pmCherry-N1 vector. Both vectors were digested using KpnI. After purification and extraction of the DNA from an agarose gel, the PECAM-1-coding DNA fragment was ligated into the pmCherry-N1 vector. Competent DH5α strain of *E. coli* was transformed with 10 µl of the ligation reaction and transformed clones in which PECAM-1 cDNA was inserted in the correct direction were selected by the difference in the plasmid digestion pattern with EcoRI (Figure 2.5). The correct construction of PECAM-1-mCherry was further confirmed by DNA sequencing.

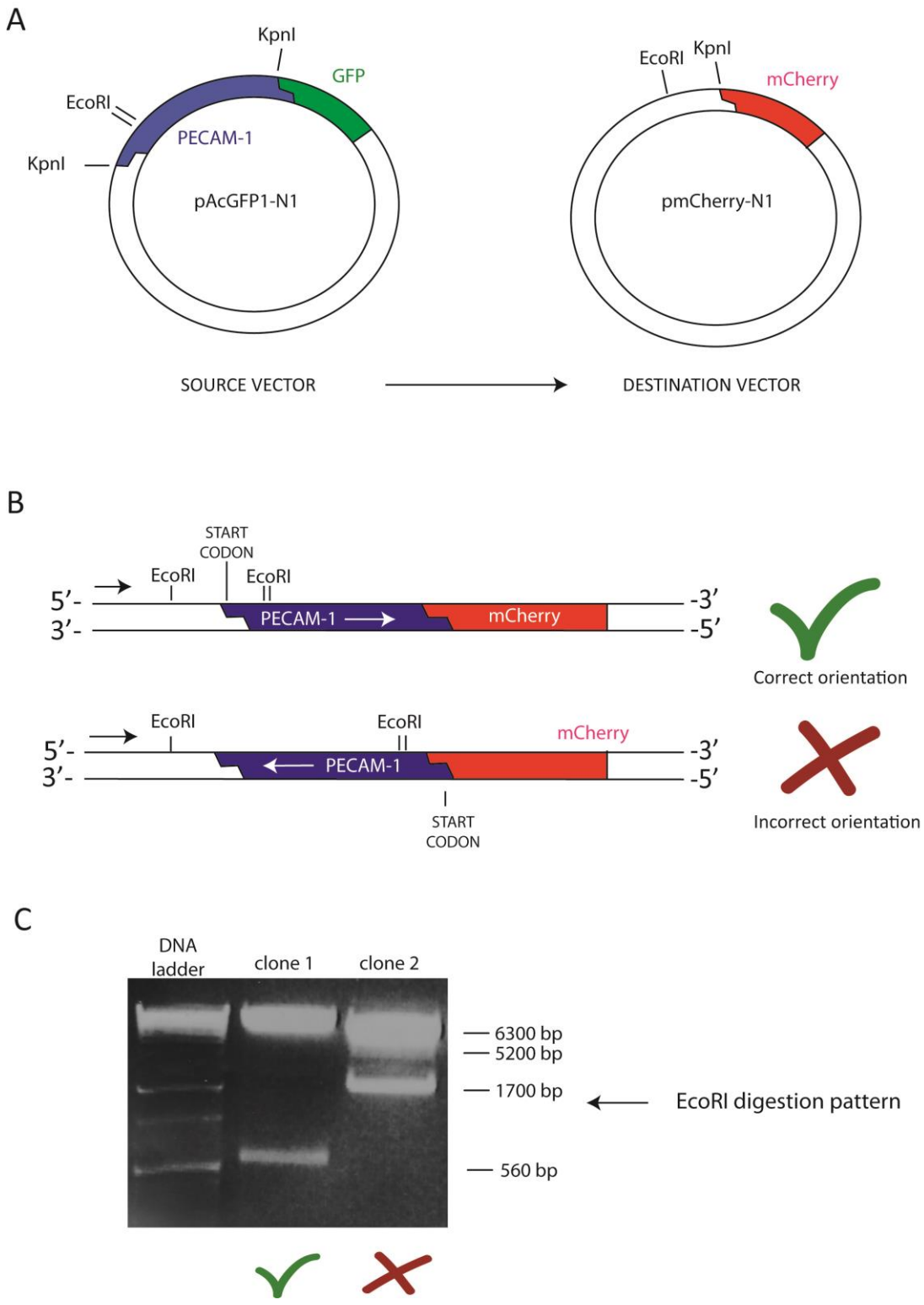

Figure 2.5 Schematic diagram of PECAM-1 subcloning into pmCherry-N1.
(A) All vectors were digested with KpnI. PECAM-1 was ligated into pmCherry-N1 using T4 ligase. The two different directions of insertion are shown (B). Clones in which PECAM-1 fragment was inserted in the desired direction were identified through the difference in the plasmid digestion pattern with EcoRI (C).

2.4.2 Generation of pEGFP-CD38 vector

CD38 cDNA was subcloned from the pLentiS38W vector to a pEGFP-C1 vector. Both vectors were digested using XhoI and KpnI. After purification and extraction of the DNA from an agarose gel, the CD38-coding DNA fragment was ligated into the pEGFP-C1 vector. Competent DH5α strain of *E. coli* was transformed with 10 µl product of ligation reaction, and clones were verified by plasmid digestion using XhoI and KpnI followed by DNA sequencing.

2.4.3 Generation of lentiviral pLENTISEGFP-38W vector

EGFP cDNA was cloned into pLENTIS38W to obtain pLENTISEGFP-38W. Nucleotide sequence recognised from XhoI (CTCGAG) was added to the 5' end of the forward and reverse primer used to amplify the EGFP-encoding cDNA from the pEGFP-C1 construct. EGFP cDNA and pLENTIS38W vector were digested using XhoI. After purification and extraction of the DNA from an agarose gel, the EGFP-coding fragment was ligated into pLENTIS38W vector (Figure 2.6). Competent DH10ß strain of *E. coli* was transformed with 10 µl of ligation reaction, and clones were verified by plasmid digestion with XhoI followed by DNA sequencing.

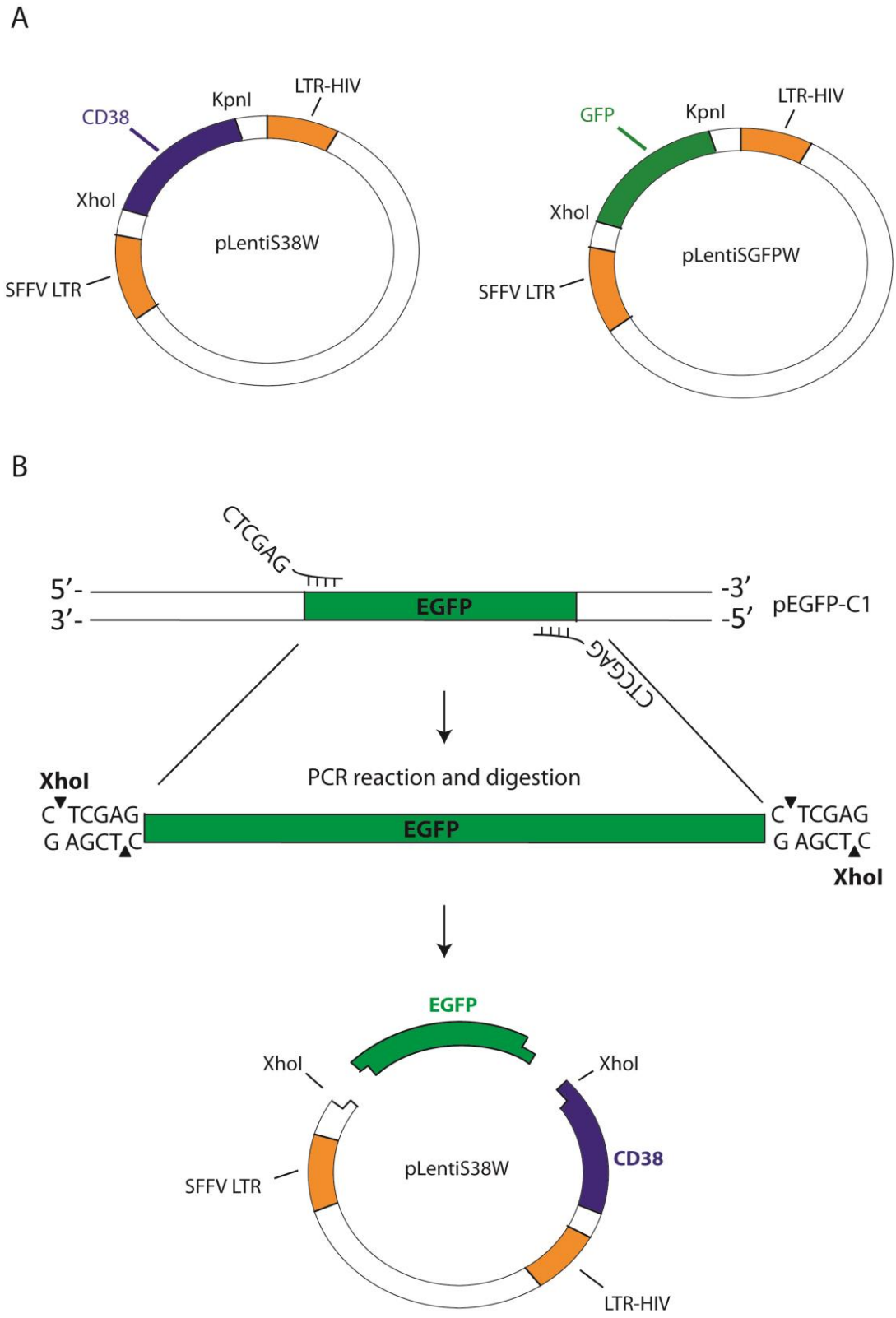

Figure 2.6 Schematic diagram of EGFP cloning into pLentiS38W.
(A) Schematic representation of the lentiviral vectors pLentiS38W and pLentiSGFPW. (B) An XhoI restriction enzyme recognition site (CTCGAG) was added to the 5' end of the primer designed to amplify the EGFP-coding fragment. The pLentiSGFPW vector and the amplified fragment were digested with XhoI and ligated using the ligase T4.

2.4.4 Primer design and PCR

Primers were designed using PerlPrimer software (http://perlprimer.sourceforge.net/). Standard DNA amplification was obtained via PCR reactions using the DNA Taq Polymerase Kit. A 50 µl PCR reaction contained 50 ng of DNA template, 5 µl of 10 x buffer, 0.75 µl of 50 mM $MgCl_2$, 0.5 µl of 10 µM of each primer, 0.25 µl of Taq polymerase in dH_2O.

Standard PCR reactions were carried out with an initial 1 min denaturation at 94°C followed by 35 cycles of 45 sec at 94°C (denaturation), 30 sec of oligo annealing (50-60°C depending on the cDNA sequence) and 72°C for 2 min of extension. The Pfu DNA Polymerase was used to generate PCR products for cloning. The reaction mixture was heated to 94°C for 1 min before the addition of 1.25 U (0.5 µl) of Pfu DNA Polymerase.

2.4.5 Enzymatic digestion of DNA

DNA (1 µg) was digested with 10 units of the appropriate restriction enzyme (EcoRI, XhoI and/or KpnI) in the NEB buffer as recommended by the manufacturer for up to 1 h at 37°C. To prevent self-ligation destination vectors were incubated with 10 units of calf intestinal alkaline phosphatase (CIP) for 30 min at 37°C.

2.4.6 DNA separation and purification

To separate DNA fragments and vectors, electrophoresis of the samples was carried out in 1% (w/v) agarose gels. Agarose was dissolved in TAE buffer and 0.5 µg/µl ethidium bromide was added to visualize DNA fragments using a UV- transilluminator (wavelength ≈312 nm). After complete polymerization, DNA samples were loaded on the gel and separated by electrophoresis at 100 V in TAE buffer. The DNA fragments of interest were excised under a UV-transilluminator and purified using the QIAquick gel extraction kit according to the manufacturer's instructions.

2.4.7 DNA ligation and bacterial transformation

Ligation of the DNA fragments to the appropriate destination vector was carried out by using 200 units of T4 DNA ligase in the supplied ligation buffer at 16°C for 16 h. A molar ratio of 3:1 of insert to destination vector was used for the reaction. Following the ligation, 10 µl of the reaction product were mixed with 100 µl of competent

bacterial cells and incubated on ice for 30 min. The bacterial suspension was heat-shocked for 45 sec at 42°C and placed again on ice for 2 min. Pre-warmed LB (900 µl) was then added to the bacteria and incubated at 37°C for 1 h with agitation. Finally, 100 µl of the cells were plated onto an LB agar plate supplemented with the appropriate antibiotic (100 µg/ml ampicillin or 50 µg/ml kanamycin) and incubated for 16 h at 37°C. Colonies obtained were then cultured and plasmid DNA content was verified by plasmid DNA purification.

2.4.8 Purification of plasmid DNA from bacteria

For plasmid DNA purification from a small-scale bacterial culture (2-5 ml), a Qiagen QIAprep Miniprep Kit was used. LB supplemented with the appropriate antibiotic (100 µg/ml ampicillin or 50 µg/ml kanamycin) was inoculated with a single colony from an agar plate and incubated overnight at 37°C. The plasmid DNA was purified following the Miniprep kit manufacturer's instructions and the DNA eluted in 50 µl ddH_20. For plasmid DNA purification from a large-scale bacterial culture (100-200 ml), an EndoFree Maxiprep Kit was used following manufacturer's instructions, plasmid DNA was resuspended in an appropriate volume of ddH_20 to obtain a minimum DNA concentration of 2 µg/µl.

2.4.9 DNA sequencing

Purified plasmid DNA (100 ng) and 2 pmol/µl of sequencing primers (see TABLE 2.8) were resuspended in a minimum volume of 15 µl H_2O and sent to MWG (www.eurofinsdna.com) for DNA sequencing.

2.5 Statistical analysis

Statistical analysis was carried out, where three or more independent experiments were performed, using IBM SPSS Statistic or GraphPad Prism software. Two-tailed Student's t-test was performed to compare two sets of data and analysis of variants (ANOVA) with Tukey post-hoc test was performed to compare three or more independent sets of data.

3. Role of CD38 in MEC1 cell motility

3.1 Introduction

Understanding the migration properties characterizing different CLL cases is of crucial importance in order to understand the mechanism behind the high heterogeneity that marks CLL clinical course. In CLL, disease progression involves the accumulation of leukaemic cells in lymph nodes and other lymphoid tissues, where microenvironmental stimuli induce CLL cell proliferation (Gaidano et al., 2012) (Chapter 1.5.2). Homing and trafficking of leukocytes is a fine orchestrated process that involves many steps controlled by various signalling pathways (Millán and Ridley, 2005). The complex biology that controls CLL cells recruitment, retention and release from the lymphoid stroma is far from been elucidated (Davids and Burger, 2012).

CD38 is considered a negative prognostic marker in CLL and its expression on leukaemic cells has been associated with enhanced cell migration. Increased chemotactic response to the chemokine CXCL12 has been shown in patients with high CD38 expression (Deaglio et al., 2007a; Vaisitti et al., 2010) and in CLL cells with ectopic CD38 expression (Vaisitti et al., 2010) but the molecular mechanism involved in this process has not been clarified. CD38 expression in CLL cells correlates with the expression of other molecules involved in cell motility, such as α4- and β2- integrin subunits (Herens et al., 2006; Pittner et al., 2005; Zucchetto et al., 2012). Moreover, in CLL cells CD38 is part of a multimeric complex that includes key molecules of the migration process such as VLA-4 integrin, CD44 and MMP-9 (Buggins et al., 2011; Zucchetto et al., 2012). Because of this interrelationship, it is difficult to ascribe the enhanced cell migratory properties exclusively to CD38.

The adhesion molecule PECAM-1 has been reported as a non-substrate CD38 ligand and it has been suggested that these two molecules are involved in the adhesion process between lymphocytes and endothelium (Deaglio et al., 1998). Previous work has shown that PECAM-1 is required for leukocyte TEM (Mamdouh et al., 2009; Muller et al., 1993) but the role of its interaction with CD38 during this process has not been investigated.

Studying the individual role of differentially expressed molecules in primary CLL cells is problematic because of the high heterogeneity between CLL samples and the strong association between several different markers, which makes it difficult to identify molecule-specific functions. To overcome this problem, genetic modulation of selected components represents a helpful approach. However, this strategy is particularly challenging in primary CLL cells, partly because of their high spontaneous *in vitro* apoptosis rate, and also because of their resistance to most transfection techniques (Buggins et al., 2010; Lagneaux et al., 1998; Seiffert et al., 2007). Stable cell lines, although they may differ from their primary counterparts because of their growth in culture, are an indispensable tool to study molecule-specific functions. Conversely to other hematopoietic malignancies, only a few number of CLL-derived cell line are available, mainly because of the resistance of primary CLL cells to viral transformation by Epstein-Barr virus (Takada et al., 1980). The human CLL-derived MEC1 cell line, generated in 1993 from a 58-year-old Epstain-Barr virus positive patient with refractory CLL (Stacchini et al., 1999), is the most well characterised CLL cell line and it has previously been used as a tool to investigate CLL biology (Buchner et al., 2009; Capitani et al., 2012; Scielzo et al., 2010).

Here, to study the effect of CD38 on CLL cell migration, CD38 was ectopically expressed in MEC1 cells and several steps of the adhesion cascade were investigated. Differential properties of CD38-expressing cells interacting with endothelial cells were analysed by time-lapse and immunofluorescence microscopy during lymphocyte crawling and TEM. Chemokine-directed and random migration of CD38-expressing MEC1 cells was analysed using transwell assays. The localisation of ectopic CD38 during cell migration was also investigated using live imaging.

3.2 Generation of stable CD38-expressing MEC1 cell populations

It has been reported that MEC1 cells do not express CD38 (Zucchetto et al., 2012). Here, to assess CD38 expression, flow cytometry analysis was performed. In agreement with the previous studies, MEC1 cells showed a barely detectable endogenous CD38 expression level (Fig. 3.1, A). This data was then confirmed by Western blot analysis (Fig. 3.1, B). The T-cell acute lymphoblastic leukaemia (T-ALL) Jurkat cell line was used as a positive control because of its reported CD38 expression (Morra et al., 1998; Zubiaur et al., 1999), and here it showed high CD38 expression in both flow cytometry and Western blot analysis (Fig. 3.1, A and B).

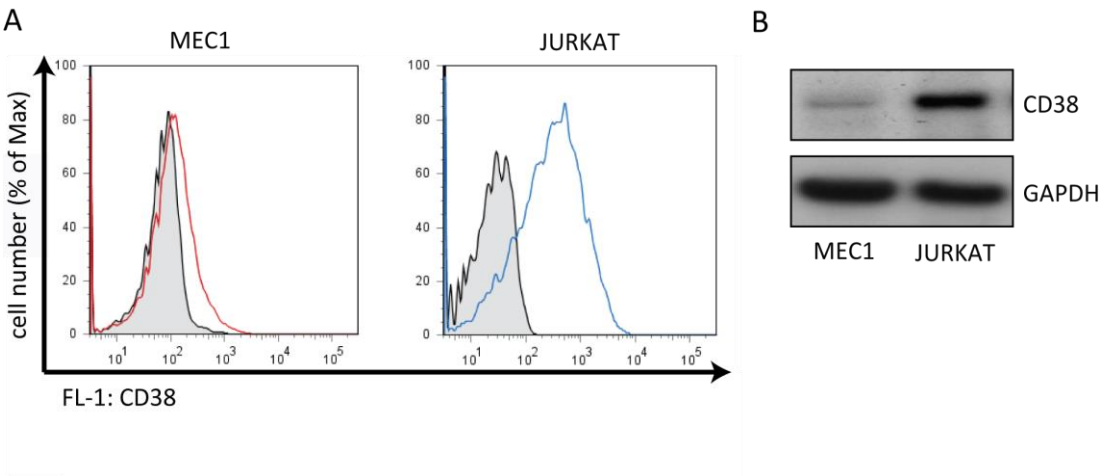

Figure 3.1 Analysis of endogenous CD38 expression in MEC1 cells.
(A) Surface levels of CD38 were investigated by flow cytometry analysis on MEC1 and Jurkat cells (positive control). 1x10^6 cells were stained with a PE-conjugated anti-CD38 antibody and analysed by flow cytometry. (B) Total CD38 expression in both cell lines was analysed by western blot.

Stable CD38-expressing MEC1 cell populations were generated using a lentiviral-based transduction system. Two different vectors were used to generate lentiviral particles; the CD38-expressing pLenti-S38W and the GFP-expressing pLenti-SEW vector as a control (Pearce et al., 2010). Lentiviral particles were generated in 293T cells and transfection was monitored by GFP fluorescence in 293T cells for the GFP-expressing vector. The supernatant of 293T cells, containing the lentiviral particles, was then used to infect MEC1 cells before cell sorting was performed by FACS

(fluorescence-activated cell sorting) based on CD38 and GFP expression. The CD38-expressing pLenti-S38W and the GFP-expressing pLenti-SEW vectors showed a similar efficiency of infection: 40.5% and 43% of MEC1 cells were infected respectively (data not shown). One population of GFP-expressing MEC1, called MEC1-GFP, was obtained by sorting cells with a mean fluorescence intensity (MFI) between 10^3 and 10^4 in the FITC channel (Fig.3.2, A). Two distinct populations of CD38-expressing MEC1 cells were sorted based on the fluorescence intensity detected with a PE-conjugated anti-CD38 antibody. One population, called MEC1-CD38L (low-expression of CD38), was obtained by sorting cells with a mean fluorescence intensity (MFI) between 10^3 and 10^4. Another population, called MEC1-CD38H (high expression of CD38), was obtained by sorting cells with a MFI between 10^4 and 10^5 (Fig.3.2, B). At least 5 X 10^5 cells per population (MEC1-GFP, MEC1-CD38L and MEC1-CD38H) were obtained by FACS and expanded in culture. To verify that the expression of CD38 was stable over time, post-sorting flow cytometry and western blot analysis were performed; representative results are shown (Fig. 3.3).

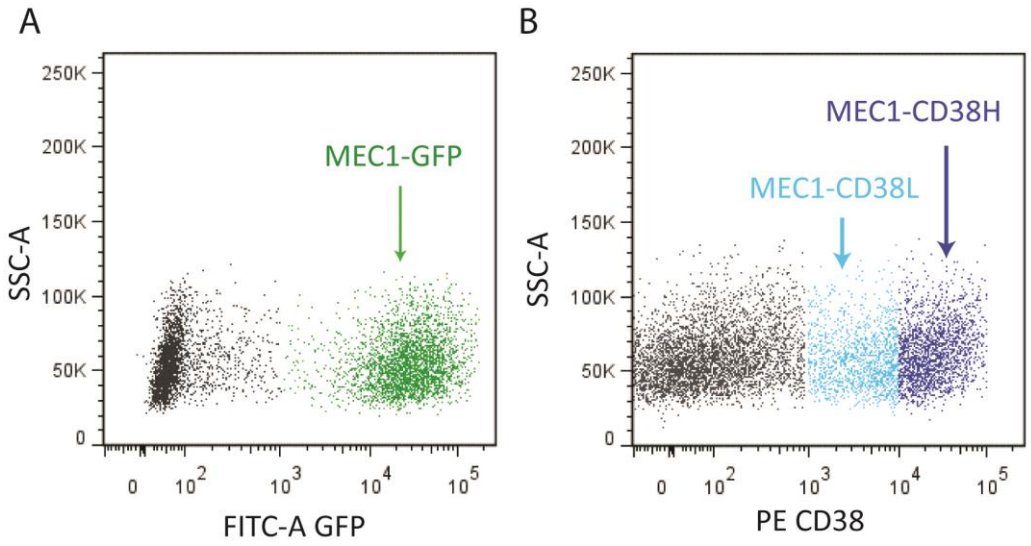

Figure 3.2 FACS sorting of stable GFP- and CD38-expressing MEC1 cell populations. MEC1 cells ($1x10^7$) were infected with lentiviral vectors pLenti-SEW and pLenti-S38W expressing GFP and CD38 respectively. (A) pLenti-SEW transfected MEC1 cells were analysed by FACS and GFP-expressing cells with a fluorescence intensity interval between 10^4 and 10^5 (MEC1-GFP) were sorted. (B) pLenti-S38W-transfected MEC1 cells stained with a PE-conjugated antibody to CD38 and analysed by FACS. Two populations were sorted based on the fluorescence intensity on the PE channel, MEC1-CD38L with a fluorescence interval between 10^3 and 10^4 and MEC1-CD38H with between 10^4 and 10^5 based on a mean fluorescence intensity in the PE channel.

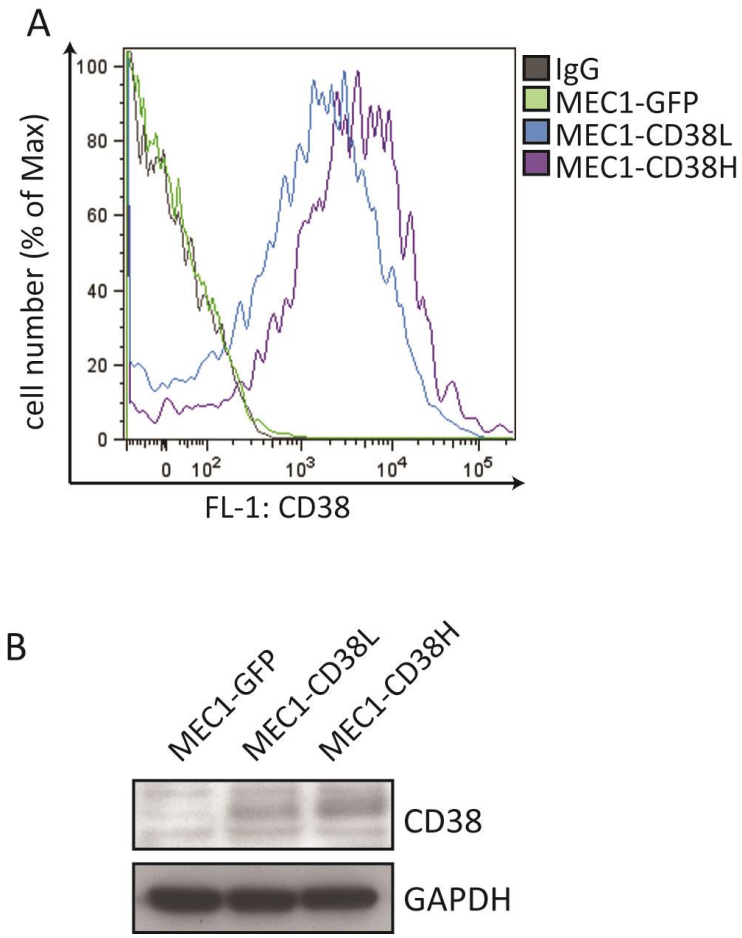

Figure 3.3 Analysis of post-sorting CD38 expression in MEC1 cells.
GFP- and CD38-expressing cells obtained by FACS sorting were analysed for their CD38 expression. (A) Surface levels of CD38 were investigated by staining MEC1-GFP, MEC1-CD38L and MEC1-CD38H with a PE-conjugated anti-CD38 antibody and subsequent analysis by flow cytometry. A PE-conjugated IgG isotype control was used to detect background signal in both populations. (B) Total CD38 expression in both cell lines was analysed by Western blot.

3.3 Role of CD38 in MEC1/endothelial cell interaction

3.3.1 CD38 expression impairs MEC1 cell crawling on endothelial cells

In order for leukocytes to exit the blood stream and home to the lymphoid organs, they have to cross the endothelial barrier forming the wall of blood vessels. Before extravasation, leukocytes crawl inside the blood vessels, which is thought to help them to find the optimal site for TEM (Ley et al., 2007; Phillipson et al., 2006). In order to investigate the role of CD38 during the crawling process, the interaction of MEC1 cells with endothelial cells was observed by time-lapse microscope. Primary human umbilical vein endothelial cells (HUVECs) were used as a model system. HUVECs were grown to confluency and stimulated with TNF-α to induce upregulation of leukocyte adhesion molecules such as ICAM-1 and VCAM-1 (Mackay et al., 1993; Osborn et al., 1989). MEC1-GFP, MEC1-CD38L and MEC1-CD38H cells were added to the endothelial monolayer and images of the co-culture were acquired every 60 sec for 2 h (Supplementary movies 1, 2 and 3). Mean cell speed was calculated from migration tracks of each cell analysed. CD38-expressing cells had a significantly lower migration speed compared to MEC1-GFP control cells. MEC1-GFP cells crawling on the endothelium had a mean migration speed of 7.70 µm/min whereas MEC1-CD38L and MEC1-CD38H cells had a mean migration speed of 6.44 µm/min and 6.75 µm/min respectively (Fig 3.4).

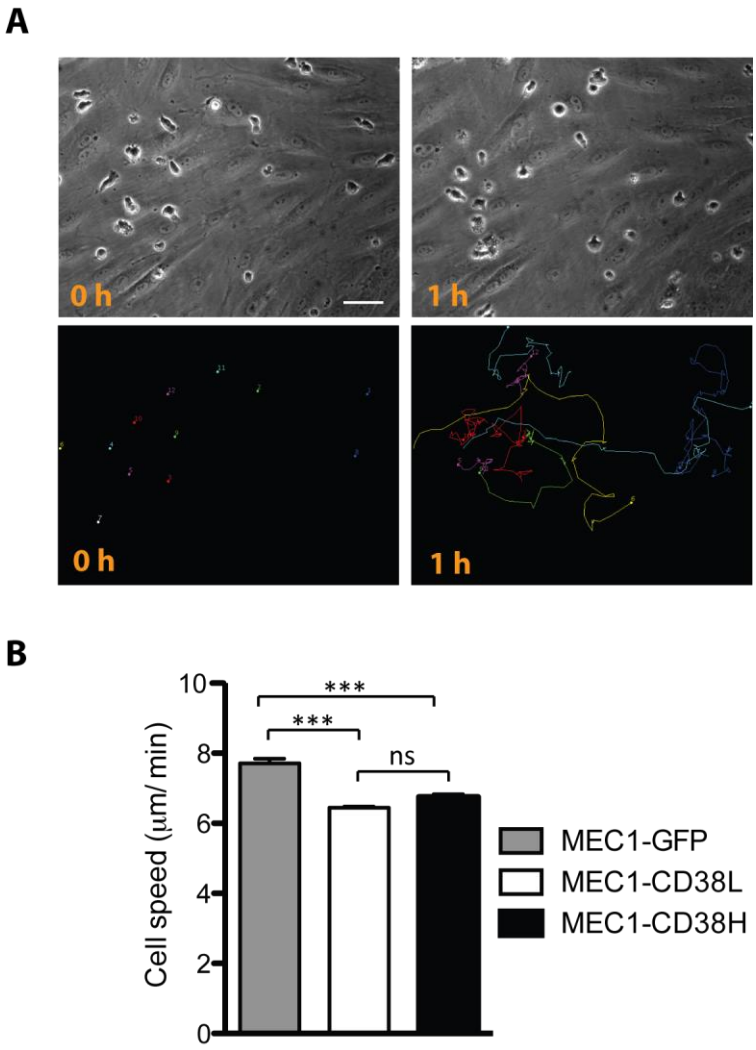

Figure 3.4 CD38 expression reduces MEC1 cell crawling speed on endothelial cells. Confluent HUVECs were stimulated with 10ng/ml TNF-α for 16 hours. MEC1-GFP, MEC1-CD38L and MEC1-CD38H cells were added to the HUVEC monolayer and imaged every 1 min for 2 hours. Time-lapse movies were analysed by tracking cells from each frame using ImageJ Manual Tracking plugin. Only cells that did not undergo cell division or TEM and stayed in the recorded field for at least half the duration of the movie were included in the analysis. (A) Representative images of cells and cell tracks at 0 and 1 hour are shown (see Supplementary movies 1, 2 and 3). Scale bar, 50 µm. (B) Tracks obtained were analysed using ImageJ Chemotaxis Tool plugin; n ≥ 30 cells per population were tracked each experiment, data shown are mean of three independent experiments ± SEM. *** $p<0.001$ determined by two-way analysis of variance (ANOVA) followed by Tukey post-hoc test; ns, not significant.

3.3.2 MEC1 cell TEM is reduced by CD38 expression

To investigate the effect of CD38 expression on MEC1 cell TEM, an *in vitro* model based on the Transwell™ assay was used. HUVECs were grown to confluency on a transwell filter and stimulated with TNF-α. MEC1-GFP, MEC1-CD38L and MEC1-CD38H cells were added to the HUVEC monolayer and the chemokine CCL21 was added to the bottom chamber as a chemoattractant. As the filters used in the assay were not optically transparent, F-actin staining on HUVECs was performed after the assay and analysed by fluorescence microscopy to verify the confluency of the HUVEC monolayer (data not sown). Both MEC1-CD38L and MEC1-CD38H cells showed a decreased TEM compared to MEC1- GFP cells (Fig 3.5).

As no significant differences in the properties of the MEC1-CD38L cells compared to the MEC1-CD38H population were detected in this study, all subsequent sections of this thesis focus on the comparison between MEC1-GFP and MEC1-CD38H cells only.

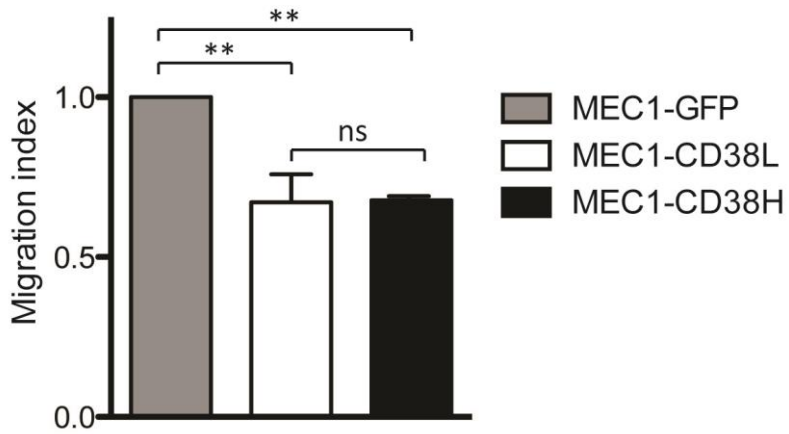

Figure 3.5 CD38 expression impairs MEC1 cell TEM.
HUVECs were seeded on Transwell™ filters and stimulated with 10ng/ml TNF-α for 16 hours. MEC1-GFP, MEC1-CD38L and MEC1-CD38H cells were added to the HUVECs and CCL21 (500ng/ml) was added to the bottom chamber. After 4 hours, cells that had transmigrated through the HUVECs into the lower well were counted using a Casy counter. A migration index was obtained by normalising the values obtained to the MEC1-GFP control cells. Data shown are mean of three independent experiments ± SEM. ** $p<0.01$; ns = not significant, determined by two-way analysis of variance (ANOVA) followed by Tukey post-hoc test.

3.4 CD38 expression increases MEC1 cell spreading on VCAM-1

Adhesion is one of the key events during the trafficking of leukocytes in and out of lymphoid organs. Adhesive interactions between leukocytes and endothelial cells allow the transendothelial migration process to occur. A transient, dynamic and tightly regulated interaction between integrins expressed on the leukocyte surface, mainly LFA-1/α4β1 and VLA-4/αLβ2, and their respective ligands ICAM-1 and VCAM-1 expressed on the endothelial cells allow leukocytes to firmly arrest to the blood vessel wall despite the blood flow (Hogg et al., 2011). Before extravasation, leukocytes undergo morphological changes by remodelling their actin cytoskeleton and spreading on the endothelium (Ley et al., 2007).

Recent studies have shown that CD38 expression in CLL cells can enhance VLA-4-mediated adhesion (Zucchetto et al., 2012). Nevertheless, the role of CD38 in cell spreading has not been investigated so far. Here the effect of CD38 expression on MEC1 cell spreading to a VCAM-1 coated surface was tested. MEC1-GFP and MEC1-CD38H cells were left adhere for 15 min before the addition of media with or without the chemokine CCL21. After 5 min cells were fixed and imaged simultaneously with bright field and Interference reflection microscopy (IRM). The IRM technique allowed imaging exclusively of the region of close contact between the cell and the surface (0–200 nm) thus providing a visualization of the cell attachment area. In IRM images, the brightest reflection comes from the cell-free areas of the sample whereas dark reflections are generated from the negative interference of the light waves occurring at the glass/cell interface (Barr and Bunnell, 2009). The site of close contact between the cell and the substratum therefore coincide with the dark, high contrast areas of the image (Fig 3.6). Cell attachment area was calculated using ImageJ software as described in Chapter 2 (Section 2.2.12). Data obtained showed that MEC1-CD38H cells had a larger area of cell attachment compared to MEC1-GFP cell, both with and without CCL21 stimulation. MEC1-CD38H cells showed a mean contact area of 53.94 ± 3.97 μm^2 whereas MEC1-GFP showed a mean contact area of 31.15 ± 2.50 μm^2 ($p<0.001$). The addition of CCL21 did not significantly change the cell attachment area in any of the two cell populations (Fig. 3.6).

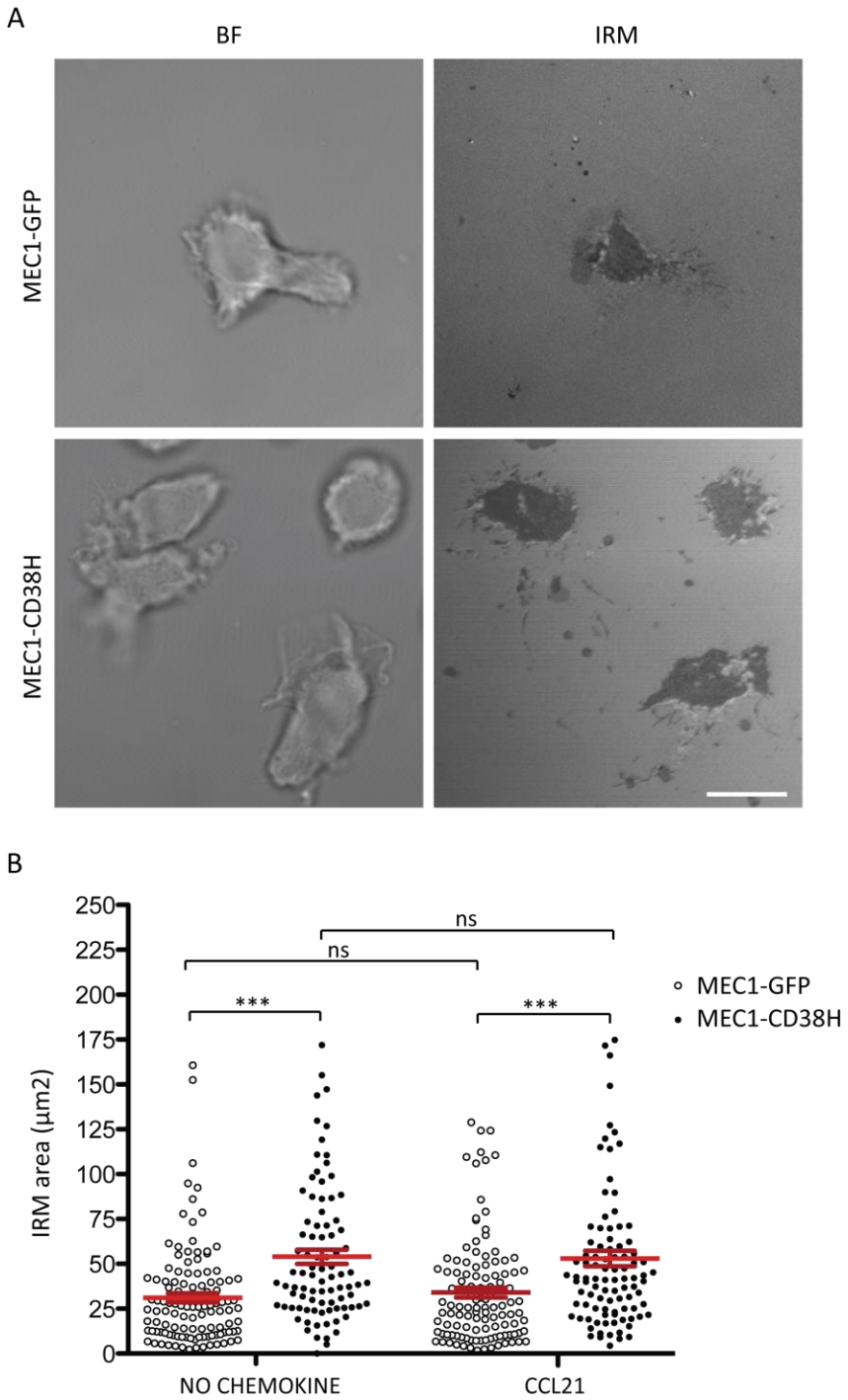

Figure 3.6 CD38 expression increases MEC1 cell spreading on VCAM-1.
(A) Representative bright field (BF) and Interference reflection microscopy (IRM) images of MEC1-GFP and MEC1-CD38H cells seeded on VCAM-1 for 15 min. In IRM, images dark areas correspond to cell contact with the surface. Scale bar, 10 µm. (B) Quantification of the cell spreading area of MEC1-GFP and MEC1-CD38H cells seeded on VCAM-1 coated glass wells with and without CCL21 stimulation (100 ng/ml for 5 min) was performed using ImageJ on at least 100 cells from three independent experiments (≈ 30 cells/experiment). ***p<0.001 determined with two-way analysis of variance (ANOVA) followed by Tukey post-hoc test; ns, not significant.

3.5 CD38 does not affect α4-β1 integrin expression levels in MEC1 cells

CD38 expression on CLL cells is known to correlate with the expression of the α4 subunit of the VLA-4 integrin, CD49d (Herens et al., 2006; Zucchetto et al., 2012), which has been recognised as another CLL negative prognostic marker (Majid et al., 2011; Shanafelt et al., 2008). The two proteins co-localise in the plasma membrane of CLL cells within a macromolecular complex (Buggins et al., 2011; Zucchetto et al., 2012). In order to assess if CD38 expression affected the level of expression of the VLA-4 integrin, the total surface levels of the α4 and β1 subunits were analysed by flow cytometry in the MEC1-GFP and MEC1-CD38H populations. Moreover, for the analysis of the β1 subunit, an antibody able to bind the ligand-induced binding site of this subunit was used in order to assess potential differences in the β1 integrin activation status in both cell populations. Cells were harvested and fixed in suspension in resting condition to keep unaltered the integrin structure before being processed for the immunofluorescence staining and performing the flow cytometry analysis. No significant differences were observed between MEC1-GFP and MEC1-CD38H cells in the total surface levels of α4 and β1 integrin subunits (Fig 3.7 A, B). The level of the active form of β-1 integrin also did not show significant differences between MEC1-GFP and MEC1-CD38H cells (Fig 3.7 C) but a high degree of variability between experiments was observed.

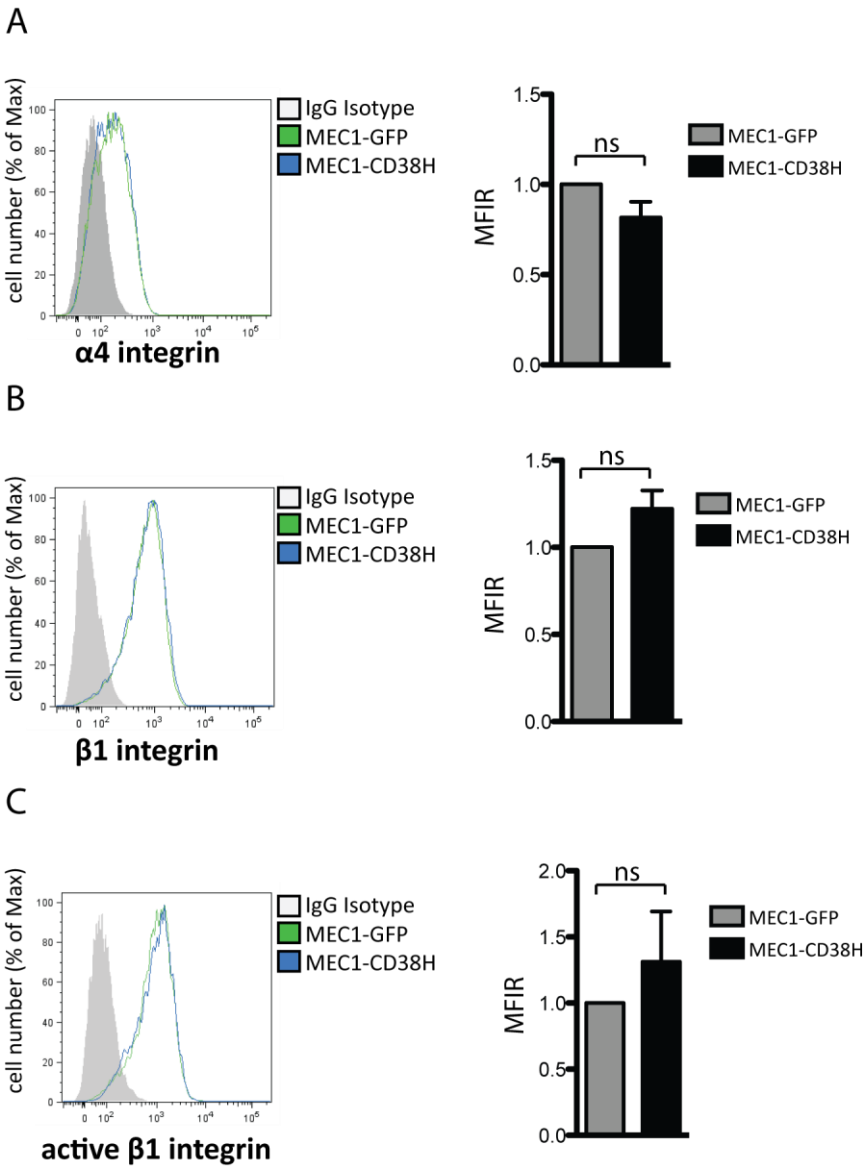

Figure 3.7 CD38 expression does not affect VLA-4 surface expression.
Surface levels of (A) α4 and (B) β1 integrin expression were tested in MEC1-GFP and MEC1-CD38H cells. Cells were stained with the respective antibody and subsequently analysed by flow cytometry. IgG isotype control was used to detect non-specific signal in both populations. (C) Surface level of active β1 integrin was tested in MEC1-GFP and MEC1-CD38H cells. Mean of the fluorescence intensity ratio (MFIR) obtained by normalizing intensity values of MEC1-CD38H against MEC1-GFP in three independent experiments ± SEM are shown. ns, not significant.

3.6 CD38 enhances migration and chemotaxis of MEC1 cells

The ability of CLL cells to migrate towards a chemokine gradient and thereby reach specific microenvironments contributes to the development of CLL (Burger, 2010). Several chemokine receptors and respective ligands have been shown to be involved in CLL cell migration and recruitment to secondary lymphoid organs, including CCR7 and its ligands CCL21 and CCL19 (Lopez-Giral et al., 2004; Till et al., 2002), CXCR4 and its ligand CXCL12 (Lopez-Giral et al., 2004), CXCR5 and its ligand CXCL13 (Burkle et al., 2007). Here, the ability of CD38 to affect MEC1 cell migration properties in the absence and presence of a gradient of CCL21, CXCL12 and CXCL13 was investigated using the Transwell™ assay. Filters were pre-coated with VCAM-1 to allow the adhesion of MEC1 cells and the subsequent migration to the bottom chamber to increasing concentrations of each chemokine. Both MEC1-CD38H and MEC1-GFP cells showed a concentration-dependent chemotactic response when CCL21 was used as a chemoattractant. Chemotaxis of MEC1-CD38H cells was significantly enhanced compared to MEC1-GFP cells at all the concentrations tested (Fig. 3.8, A). Interestingly, MEC1-CD38 cells showed also an increased basal migration, tested by allowing the cells to migrate in the absence of the chemoattractant. These data indicate that CD38 expression is involved in enhancing intrinsic cell motility as well as chemokine responses. The CCL21 concentration that gave the biggest difference chemotaxis between MEC1-GFP and MEC1-CD38 cells was 100 ng/ml and it was therefore used in subsequent experiments. It appeared that both MEC1-CD38H and MEC1-GFP cells are not able to respond to CXCL12 and CXCL13, as both populations did not show increased migration to CXCL12 and CXCL13 (Fig. 3.8, B and C). As only two independent experiments were performed with these two chemokines, more experiments would be needed to draw any conclusion. However, previous studies performed in MEC1 have shown similar results when using CXCL12 as chemoattractant (Kaucka et al., 2013; Vaisitti et al., 2014).

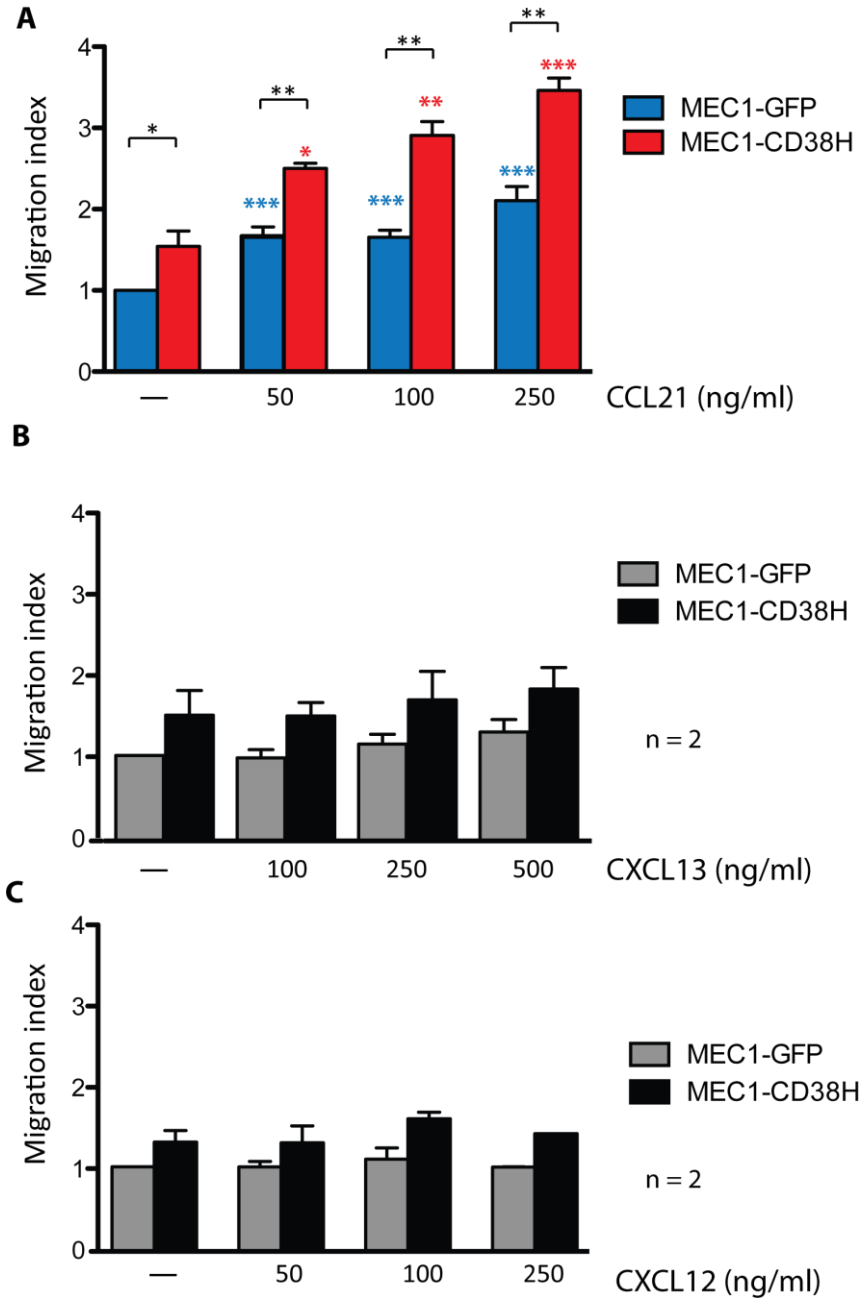

Figure 3.8 CD38 expression affects MEC1 cell migration and CCL21-directed chemotaxis.

MEC1-GFP and MEC1-CD38H cells were added to Transwell™ filters previously coated with VCAM-1. Different concentrations of CCL21 (A), CXCL13 (B) or CXCL12 (C) were added to the bottom chamber. After 2 hours, cells that had transmigrated through the filters into the lower wells were counted using a Casy™ counter. A migration index was obtained by normalising the values to the MEC1-GFP control cell. Data shown in (A) are mean of three independent experiments ± SEM; data shown in (B and C) are mean of two independent experiments ± SEM. *p<0.05, **p<0.01, *** p<0.001 determined by two-tailed Student's t-test. Black asterisks represent statistical differences between the two cell populations in each condition. Blue and red asterisks represent statistical differences between basal migration and migration observed at different CCL21 concentrations within each population.

3.7 CCR7 expression levels are not affected by CD38 expression

Previous studies have demonstrated the importance of the chemokine receptor CCR7 in the pathology of CLL (Till et al., 2002). CCR7 expression is increased in circulating CLL cells when compared to circulating normal B cells (Richardson et al., 2006). CCR7 expression levels in CLL samples not only correlate with an enhanced *in vitro* response to the chemokines CCL21 and CCL19 but it is also associated to a clinical lymphadenopathy (Lopez-Giral et al., 2004; Till et al., 2002). To investigate whether CD38 modulates the expression of CCR7 and to assess if the increased chemotaxis towards CCL21 observed in CD38-expressing MEC1 cells was mediated by an increase in the expression level of the chemokine receptor, the surface level of CCR7 was determined. MEC1-GFP and MEC1-CD38H cells were stained in suspension with an anti-CCR7 antibody and fixed before being analysed by flow cytometry. No significant difference was observed in the expression of CCR7 between the two populations (Fig 3.9). It is therefore unlikely that the increased response to CCL21 observed in MEC1-CD38H cells is due to changes in the expression of its receptor.

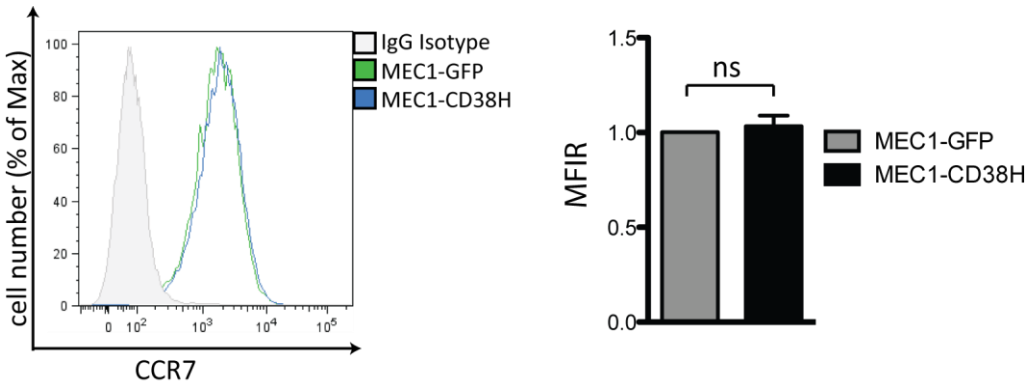

Figure 3.9 CD38 expression does not affect CCR7 surface expression.
Surface level of CCR7 was tested in MEC1-GFP and MEC1-CD38H. Cells were stained with a anti-CCR7 antibody and subsequently analysed by flow cytometry , IgG isotype control was used to detect non specific signal in both populations. Mean of the fluorescence intensity ratio (MFIR) obtained by normalizing intensity values of MEC1-CD38H against MEC1-GFP in three independent experiments ± SEM are shown; ns, not significant.

3.8 CD38 localisation during MEC1 cell migration

In order to study the distribution and subcellular localisation of CD38 during MEC1 cell migration, CD38 was subcloned into the pEGFP-C1 plasmid to generate a vector encoding an N-terminal EGFP-tagged CD38 (Chapter 2.4.2). MEC1 cells were transiently transfected with this vector and 24 h after transfection live imaging was performed on cells seeded on VCAM-1 coated wells. VCAM-1 was chosen as a substrate as MEC1 cells showed optimal adhesion properties to VCAM-1, whereas very few cells were able to adhere to the other substrates tested (ICAM-1 and fibronectin; data not shown). The distribution of EGFP-CD38 during cell crawling on VCAM-1 was followed by time-lapse movies microscopy; 5 sec per frame movies were acquired (Supplementary movie 4). EGFP-CD38 was enriched at the plasma membrane but it also accumulated in vesicle-like structures mainly at the rear of the cell (Fig. 3.10).

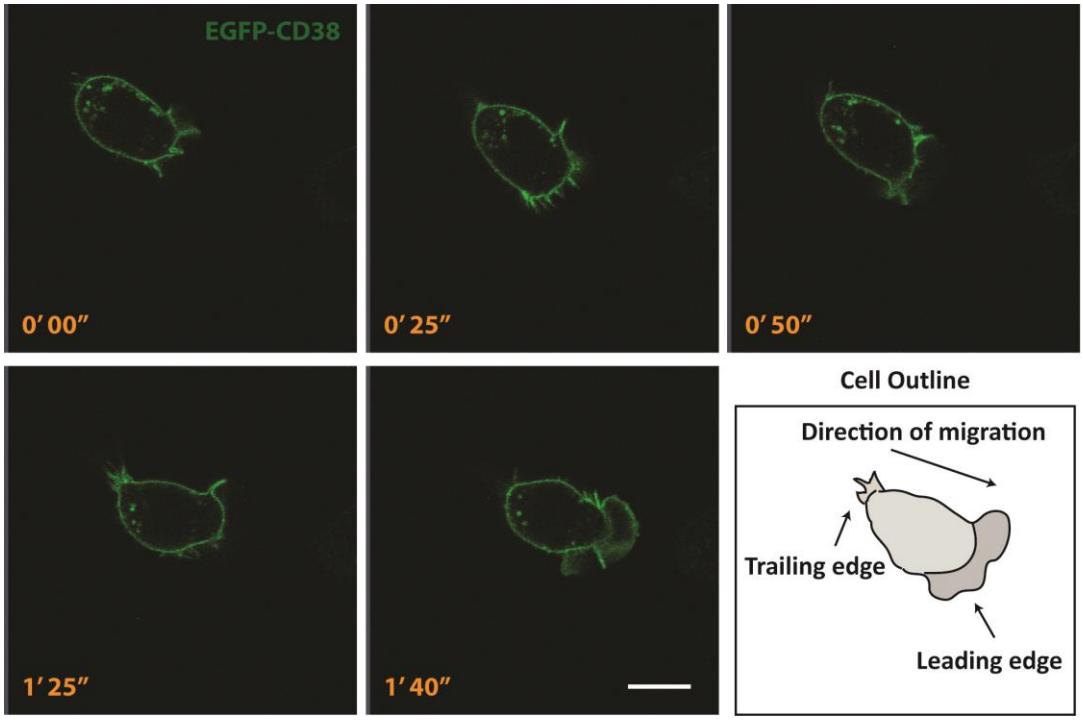

Figure 3.10 EGFP-CD38 localisation during MEC1 cell crawling.
MEC1 cells were transiently transfected with 30 µg pEGFP-CD38 plasmid. After 24 h cells were plated on a VCAM-1-coated plate. Live cell imaging was performed to observe the localisation of EGFP-CD38 during MEC1 cell migration. Time is shown as min:sec. In the cell outline panel, direction of migration, leading and trailing edge of the representative cell are indicated. Scale bar, 10 µm. Images are representative of three independent experiments.

3.9 Analysis of CD38-PECAM-1 interaction

The platelet-endothelial-cell adhesion molecule (PECAM-1 or CD31) is a protein belonging to the Ig superfamily diffusely expressed on leukocytes and concentrated at the cell-cell junctions in endothelial cells (Ley et al., 2007; Newman et al., 1990; Wong and Jackson, 2004). PECAM-1 is required for leukocyte TEM (Mamdouh et al., 2003; Muller et al., 1993). Homophilic interaction between the N-terminal extracellular region of the endothelial PECAM-1 and of leukocyte PECAM-1 is necessary for transmigration to occur (Mamdouh et al., 2003). PECAM-1 has been reported to be a physiological ligand of CD38 (Deaglio et al., 1998; Tonino et al., 2008). It has been hypothesised that CD38 and PECAM-1 interaction could occur and have a role during leukocyte TEM.

In order to visualise the possible interaction between the two proteins during TEM, microscopy analysis was performed. To visualise PECAM-1 exclusively on the endothelial cells, a C-terminal mCherry-tagged PECAM-1-expressing vector (Chapter 2.4.1) was transiently transfected into HUVECs. Initial IF experiments performed on HUVECs and MEC1-CD38H cell co-cultures using anti-CD38 antibody showed signal in both cell types (data not shown). To visualise CD38 localisation specifically on the leukaemic cells, EGFP-CD38 fusion protein was expressed in MEC1 cells. Because the low efficiency of the transient transfection in MEC1 cells did not allow to perform this assay, a stable EGFP-CD38-expressing MEC1 cell population was generated (MEC1-EGFP-CD38). A vector expressing the fusion protein EGFP-CD38 was generated by cloning the EGFP cDNA at the N-terminus of the CD38 cDNA into the pLenti-S38W and used to generate lentiviral particles in 293T cells. Supernatant of infected 293T cells was then used to infect MEC1 cells and the MEC1-EGFP-CD38 cell population was selected by FACS sorting based on EGFP fluorescence intensity. MEC1-EGFP-CD38 cells were seeded on a TNF-α stimulated PECAM-1-Cherry-expressing HUVEC monolayer and fixed after 30 minutes before performing microscopy analysis. PECAM-1-mCherry localised mainly at endothelial intercellular borders as the endogenous PECAM-1. As reported in previous studies PECAM-1 was weakly associated with the cuplike structures surrounding transmigrating leukocytes (Carman and Springer, 2004) (Fig. 3.11). No enrichment of EGFP-CD38 was observed

in these regions. EGFP-CD38 showed a diffuse membrane distribution during MEC1-EGFP-CD38 cell transmigration across the endothelial monolayer. Localisation of EGFP-CD38 was also investigated in MEC1-EGFP-CD38 cells adhered on top of the HUVEC monolayer but not performing TEM, no co-localisation between EGFP-CD38 and PECAM-1-cherry was observed (Fig. 3.12).

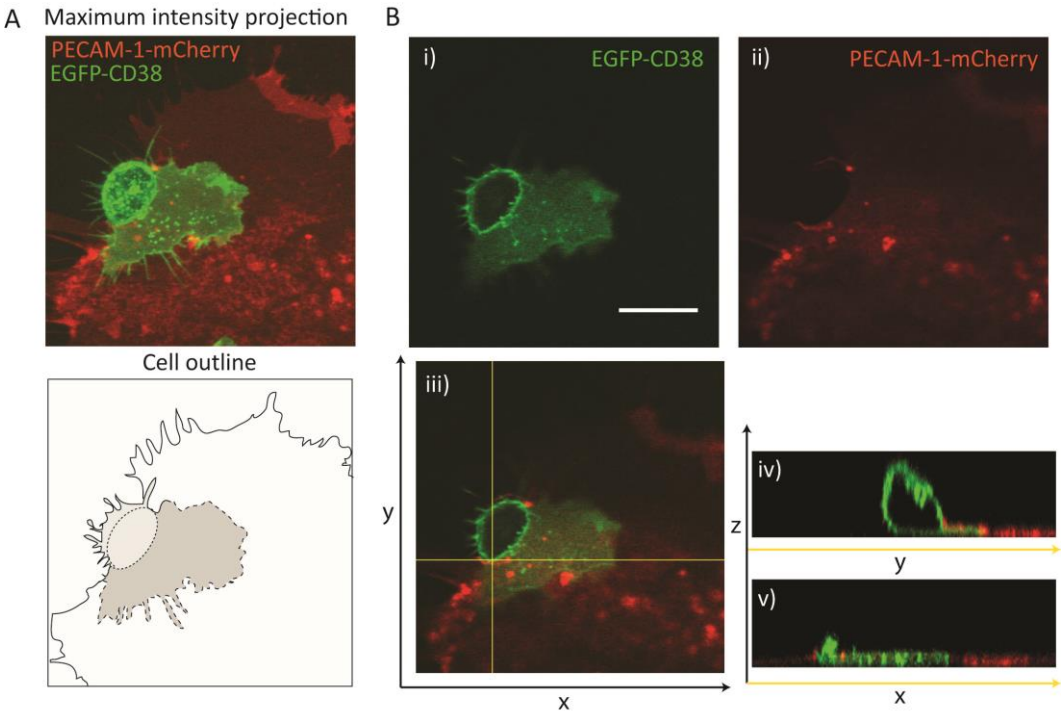

Figure 3.11 EGFP-CD38 and PECAM-1-mCherry localisation during MEC1 cell TEM. HUVECs were transiently transfected with a PECAM-1-Cherry expressing vector, seeded on a FN-coated coverslip and TNF-α-activated. MEC1-EGFP-CD38 cells were seeded on the HUVEC monolayer, fixed and observed by confocal microscopy. (A) Top view projection of all z-series sections of a representative MEC1-EGFPCD38 cell partly transmigrated underneath an endothelial cell through the endothelial borders. Dotted and dashed lines shown in the cell outline panel indicates the TEM passage and the edges of the sub-endothelial leukocyte membrane respectively. (B) Top view of one confocal section showing EGFP-CD38 (B, i) and PECAM-1-cherry (B, ii) localisation in the TEM passage. The merged of the two channels is shown (B, iii). (B, iv and v) Side view projection of cross sections in (B, iii) depicting MEC1-EGFP-CD38 cell body partly transmigrated underneath the endothelial cell. Scale bar, 10 µm.

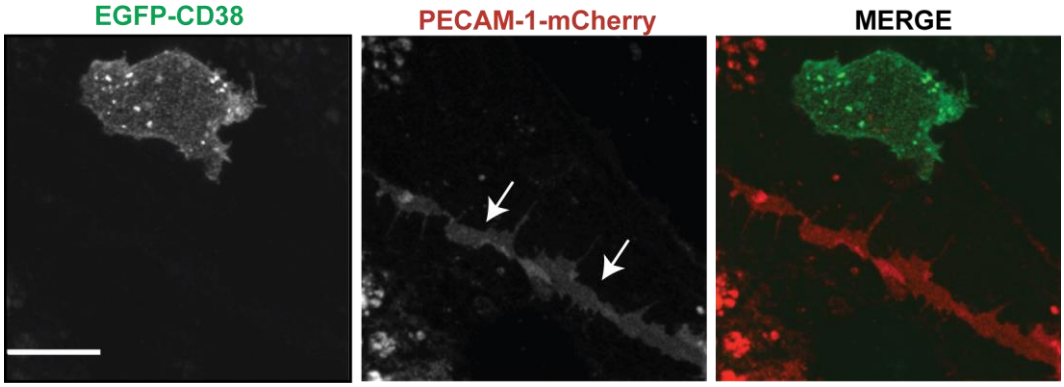

Figure 3.12 EGFP-CD38 and PECAM-1-mCherry localisation during MEC1 cell adhesion on HUVECs.

Top view projection of all z-series sections acquired of a MEC1-EGFP-CD38 cell adhering onto HUVECs transiently transfected with a PECAM-1-Cherry expressing vector, seeded on a FN-coated coverslip and TNF-α-activated. Arrows indicate HUVEC cell-cell junctions. Image is representative of three independent experiments. Scale bar, 10 µm.

3.10 Discussion

The clinical course of CLL is very heterogeneous and CD38 is a surface marker strongly associated with poor prognosis (Damle et al., 1999; Del Poeta et al., 2001; Hamblin et al., 2002). Distinct features of primary CLL cells expressing high levels of CD38, particularly their co-expression of other prognostic factors that can alter cell motility properties, has made it particularly challenging to define its role in the migration of CLL cells. The aim of the research described in this chapter was to clarify whether expression of CD38 directly affected CLL cell motility. In order to do that, different populations of stable CD38-expressing MEC1 cells were generated, providing a tool to compare the migratory behaviour of the same system in which the only variable was CD38 expression. Several steps of the leukocyte adhesion cascade involved in the cell trafficking into lymphoid organs were analysed.

Previous studies attempted to identify a role of CD38 in CLL cell trafficking but the results are controversial (Brachtl et al., 2011; Vaisitti et al., 2010). Here, CD38 expression in MEC1 cells decreased their migration speed on HUVECs and decreased their ability to cross the endothelial monolayer. *In vivo* studies, performed with real-time and time-lapse intravital video-microscopy of neutrophils within microvessels, have shown that when crawling is disabled the transmigration process is delayed, probably due to migration across suboptimal transmigration sites (Phillipson et al., 2006). It is therefore possible that the decrease in the crawling speed of CD38-expressing cells and their decreased TEM are interrelated effects. Time-course experiments should be performed in order to better understand if the decreased TEM could be due to a delay in the process rather than an actual impairment of the transmigratory ability of cells. One possible explanation of the CD38-mediated migratory phenotype is that the increased spreading properties led to the reduced speed crawling and TEM. A balance between strength of adhesive contacts and cytoskeletal rearrangements mediate adhesion disassembly and turnover that controls cell migration speed (Friedl et al., 2001; Kuijpers et al., 1993; Svensson et al., 2010). Notably, the experiments were performed under static conditions, thus lacking the shear stress continuously applied from the blood stream on migrating lymphocytes in physiological conditions. *In vitro* studies have shown that shear forces

promote leukocyte transmigration across the endothelium (Cinamon et al., 2001). Further experiments should be performed under flow conditions to determine if the expression of CD38 affects TEM of MEC1 cells in this context.

When tested for chemotaxis in the absence of endothelial cells, MEC1-CD38H cells showed an enhanced CCL21-directed migration compared to MEC1-GFP cells. These data are in agreement with previous studies that showed increased chemotactic properties toward CXCL12 in primary CLL cells that ectopically expressed CD38 (Vaisitti et al., 2010). The enhanced chemotaxis observed in the MEC1-CD38H population was not due to an enhanced surface level of the CCL21 receptor, as no differences was observed in CCR7 expression between MEC1-GFP and the MEC1-CD38H cells. This data suggest that the increased chemotactic response observed in CD38-expressing cells is due to molecular events that act downstream of the chemokine-chemokine receptor interaction.

Interestingly, CD38-expressing cells showed an increased migration through transwells even in the absence of a chemokine gradient. Whereas previous studies had already linked the enhanced chemotactic ability of CLL cells to CD38 expression (Vaisitti et al., 2014; Vaisitti et al., 2010), its effect on intrinsic cell motility has not been observed so far. Notably, B-lymphocytes within lymphoid organs are highly motile and their crawling shows a 'random-walk' pattern along scaffolding elements, such as follicular dendritic cells, promoted only partly by chemokines (Cahalan and Parker, 2006; Cyster, 2010). At present, no studies have been performed on the motility of CLL cells within lymphoid organs and the role of intrinsic motility in CLL has not been elucidated. The enhanced basal motility observed in the CD38-expressing cells suggests that the migration of CLL cells inside the lymphoid organs could be increased by CD38 expression, therefore affecting CLL cell localisation and trafficking.

Analysis of cell spreading revealed that MEC1-CD38H cells had an increased contact area on VCAM-1 coated surfaces compared to MEC1-GFP cells. Cell spreading requires a cytoskeletal rearrangement that allows cell flattening and mediates an increase in the cell/surface contact (Gauthier et al., 2011). The data indicate that

CD38 is directly involved not only in cell adhesion, as previously shown by other groups (Zucchetto et al., 2012), but also in the cytoskeletal rearrangement involved in the generation of sustained interactions between cells or with the ECM. Interestingly, a close correlation between CD38 expression in CLL cells and the enrichment of VCAM-1-expressing stromal and endothelial cells has been reported in bone marrow biopsies of CLL patients, which is linked to the survival of the malignant cells (Zucchetto et al., 2009). The enhanced interaction between VCAM-1 and the CD38-expressing MEC1 cells observed here suggests that CD38 could play a role in the enhanced retention of CLL cells in the lymphoid tissues.

The expression of CD38 has been found to correlate with the expression of the α4 subunit of VLA-4, CD49d (Herens et al., 2006; Zucchetto et al., 2012) in CLL patients. Here, no significant differences in the surface levels of either α4 or β1 integrins between MEC1-GFP and MEC1-CD38H populations were observed, and thus the enhanced spreading was probably not due to an increased level of VLA-4. Integrins are cell surface receptors that in resting conditions are in an inactive conformation but several signals can rapidly change their affinity status for their ligands (Hogg et al., 2011). Levels of active β-1 integrin detected in MEC1-CD38H cells did not show significant difference to the ones detected in MEC1-GFP cells, however, the assay showed a high intrinsic variability. Notably, experiments were performed only in cells in resting conditions and therefore it cannot be excluded that CD38 could have a role in the transient integrin activation induced by signals such as chemokines.

At present, PECAM-1 is the only physiological non-enzymatic CD38 ligand identified (Deaglio et al., 1998). PECAM-1 is known to be involved in leukocyte TEM (Muller et al., 1993). It was therefore hypothesised that the interaction of CD38 with PECAM-1 could be relevant during CLL cell TEM. Endothelial ectopic PECAM-1 showed a modest localisation in the region surrounding the transmigrating MEC1 cells, in agreement with other studies, which have shown a minor enrichment of PECAM-1 compared to other adhesion molecules, such as VCAM-1 and ICAM-1, that have been shown to cluster in the docking site of transmigrating leukocytes (Carman and Springer, 2004; Millan et al., 2006). The localisation of ectopic CD38 during MEC1 TEM was diffuse and not enriched in these regions, suggesting that any interaction between the two

proteins is unlikely to have any role during TEM. No co-localisation of CD38 and PECAM-1 was observed during MEC1-EGFP-CD38 cell adhesion on HUVECs. Nevertheless, it is still possible that CD38 and PECAM-1 interaction could occur in other steps of the adhesion cascade, for example during leukocyte rolling as hypothesised previously (Malavasi et al., 2008).

Taken together, data presented in this chapter indicate that CD38 affects the migratory behaviour of CLL cells by increasing cell spreading and enhancing intrinsic and directed cell motility. CD38 expression also decreased MEC1 cell crawling speed on HUVECs and their TEM, possibly as a result of enhanced adhesive interactions. The molecular mechanisms that link CD38 expression with cell migration have not been defined to date and will be the subject of the following chapters.

4. CD38-mediated signalling in MEC1 cells

4.1 Introduction

CD38 is a multi-functional enzyme involved in the production of multiple Ca^{2+}-mobilising compounds (Malavasi et al., 2008) (Chapter 1.6.1). Studies performed in neutrophils and dendritic cells have shown that these compounds are involved in several functions in immune cells, including cell motility (Partida-Sánchez et al., 2001; Shi et al., 2007). Despite the evidence of CD38 involvement in cell migration, the signalling pathways associated to this process have not being elucidated so far. Particularly in CLL, the effects of CD38 on the signalling machinery involved in cell migration remain obscure.

Small GTPases have a key role in the regulation of cytoskeletal dynamics and are therefore involved in several cellular processes such as cell adhesion, polarity establishment and cell migration (Heasman and Ridley, 2008; Tybulewicz and Henderson, 2009). Most GTPases are able to switch between an inactive GDP-bound form to an active GTP-bound form which interacts with downstream effectors. This mechanism is finely regulated by GEFs (guanine nucleotide exchange factors), which activate GTPases, and GAPs (GTPase-activating proteins), which stimulate the hydrolysis of bound GTP to GDP. *In vitro* and *in vivo* experiments have shown that several small GTPases, together with their regulatory and effector molecules, are involved in B-lymphocyte trafficking and homing to the lymphoid organs as well as B-cell development and B-cell receptor-mediated signalling (Henderson et al., 2010; Tybulewicz and Henderson, 2009) (Chapter 1.4).

For example, Rac1 and Rac2 have been shown to have a role in the B-lymphocyte chemokine-directed migration and lymph node trafficking (Henderson et al., 2010). Cdc42, another member of the Rho GTPase family, has been shown to have a critical role in B-cell development and function, but its role in B cell trafficking has not been analysed (Guo et al., 2009).

The GTPase Rap1 is known for its role in stimulating integrin activation and cytoskeletal rearrangement in lymphocytes. Several studies have reported that Rap1 is involved in different processes in B cell signalling, including not only B cell development and antigen responses (Ishida et al., 2006; Lin et al., 2008) but also migration and trafficking (Chen et al., 2008b; Dustin et al., 2004; Ebisuno et al., 2010; McLeod and Gold, 2001). Notably, Rap1 has been linked to invasiveness in B-cell malignancies and is involved in chemokine-induced adhesion, migration and transendothelial migration of malignant B cells (Kometani et al., 2004; Lin et al., 2010).

Results obtained in the previous chapter showed that CD38 expression can affect the migratory behaviour of CLL cells, increasing both intrinsic and chemokine-directed cell motility. In this chapter, the effect of CD38 expression on the activity of key members of the Rho and Ras GTPase families and their regulators was investigated. Motility-related signalling pathways were compared between MEC1-GFP and MEC1-CD38H cells. In order to identify the molecules involved in the CD38-mediated enhanced migratory phenotype, protein down-regulation was performed using siRNA-mediated knockdown and migration properties were tested.

4.2 Investigating the role of Rho and Rap GTPases in MEC1-GFP and MEC1-CD38H cells

4.2.1 CD38 expression does not affect basal and CCL21-induced Rac1 and Cdc42 activity

It has been reported that Rac1 and Cdc42 may have a role in the chemokine-induced activation of integrin LFA-1 (αLβ2) in CLL cells (Montresor et al., 2009). However, Rac1 and Cdc42 seem to regulate integrin activity only in a subset of CLL cases. In the remaining cases, chemokine-induced activation of LFA-1 appears to be insensitive to Rac1 or Cdc42 inhibition. These data indicate a possible alteration of Rac1 and Cdc42 signalling in a subset of CLL patients and suggest there is variability in the GTPase signalling among different CLL clones.

In order to investigate if CD38 expression may modify Rac1 and Cdc42 activity, GST pull-down assays were performed. Active forms of the two Rho GTPases were isolated using a GST-fusion protein of the p21-binding domain (PBD) of PAK, common downstream effector of both Rac1 and Cdc42 (Burbelo et al., 1995). Pull-down assays were performed on lysates obtained from MEC1-GFP and MEC1-CD38H cells in suspension, untreated or treated for 1 and 5 minutes with CCL21.

No differences in the basal level of Rac1-GTP were observed between MEC1-GFP and MEC1-CD38H cells. Stimulation with CCL21 did not show any significant change in the level of active Rac1 at any of the time points tested in any of the two populations (Fig 4.1).

Similarly, it was not detected a substantial up-regulation of active Cdc42 upon treatment with CCL21 for 1 and 5 minutes in any of the two populations (Fig. 4.2). A small difference was observed in the basal Cdc42 level, which in MEC1-CD38H cells appeared to be enhanced. However, as this experiment was performed only twice, these results need to be repeated to draw definitive conclusions on the effect of CD38 on Cdc42 activity.

To determine whether CCL21 could induce signalling in MEC1 cells, phosphorylation of ERK was monitored (Otero et al., 2006). A small increase in ERK phosphorylation

was observed after 1 minute, and stronger after 5 minutes of treatment with CCL21, indicating that CCL21 can signal in MEC1 cells (Fig. 4.3).

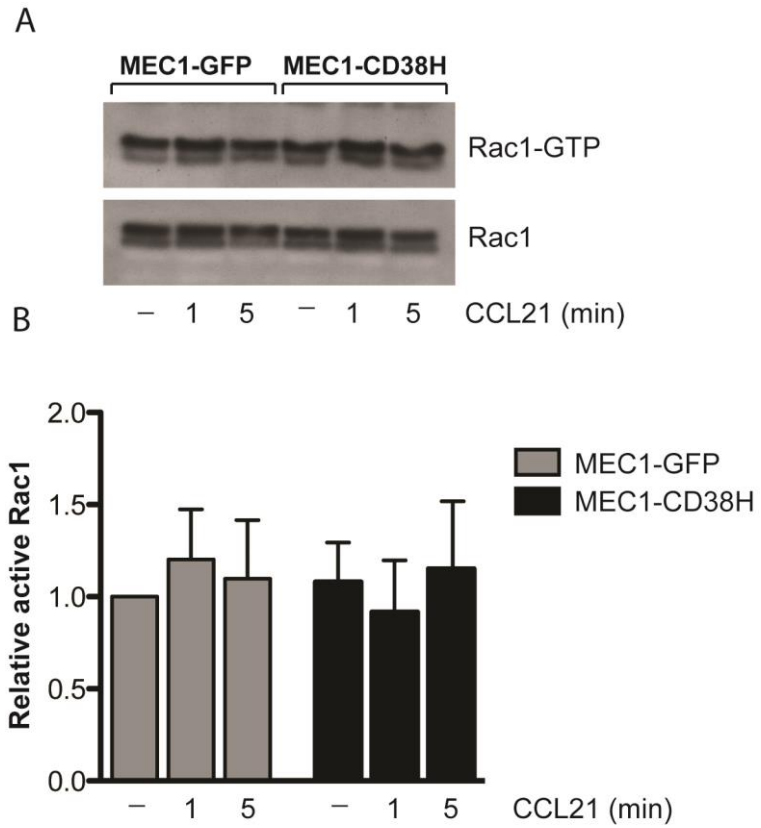

Figure 4.1 CD38 expression does not affect Rac1 activity.
Cell lysates obtained from MEC1-GFP and MEC1-CD38H, untreated or treated with CCL21 (100 ng/ml) for 1 or 5 minutes, were incubated with GST-PBD fusion protein and then analysed by western blotting with anti-Rac1 antibody. (A) Representative western blot is shown. (B) Levels of active Rac1 were quantified by densitometry using ImageJ and normalised to total Rac1. Relative Rac1 levels shown were obtained by normalising to the untreated MEC1-GFP control cells. Data shown are the mean of three independent experiments ± SEM.

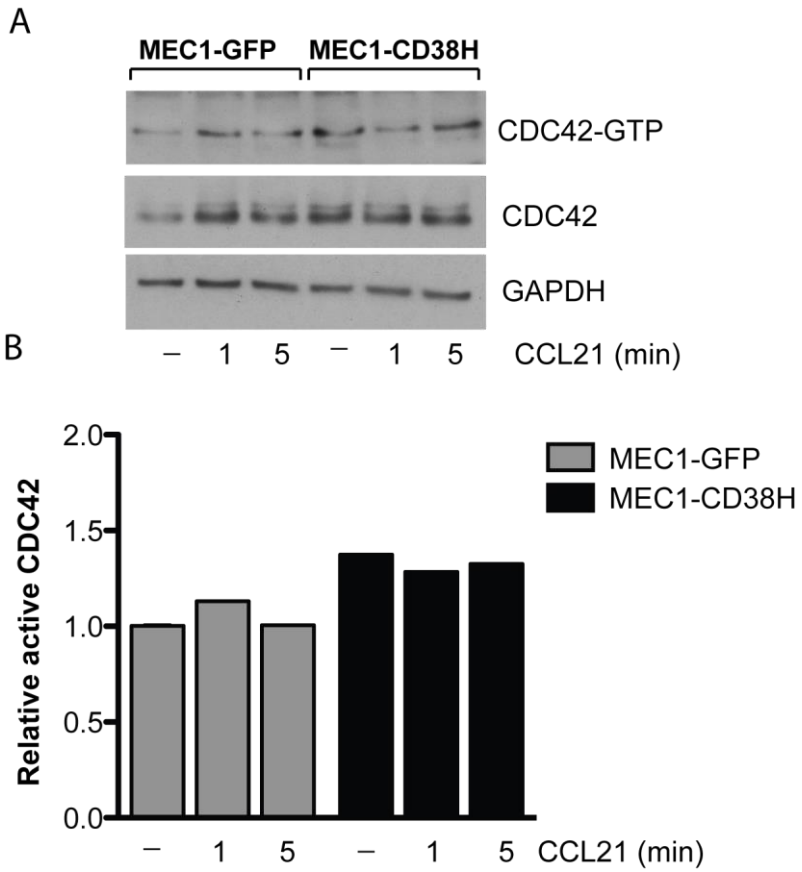

Figure 4.2 Cdc42 activity in MEC1-GFP and MEC1-CD38H cells.
Cell lysates obtained from MEC1-GFP and MEC1-CD38H, untreated or treated with CCL21 for 1 or 5 minutes, were incubated with GST-PBD fusion protein and then analysed by western blotting with anti Cdc42 antibody. (A) Representative western blot is shown. (B) Levels of active Cdc42 were quantified by densitometry using ImageJ and normalised to total protein. Relative Cdc42 levels shown were obtained by normalising to the untreated MEC1-GFP control cells. Data shown are the mean of two independent experiments.

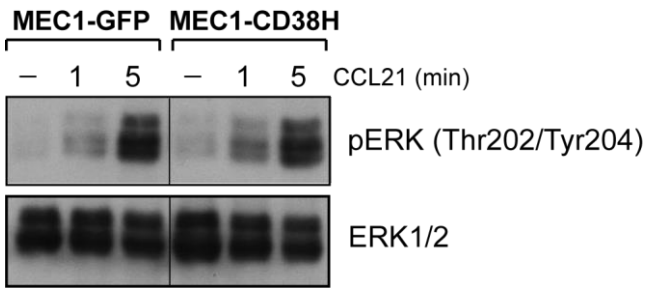

Figure 4.3 Detection of ERK phosphorylation upon CCL21 treatment.
Cell lysates obtained from MEC1-GFP and MEC1-CD38H, untreated or treated with 100 ng/ml of CCL21 for 1 or 5 minutes, were analysed by western blotting with antibody against pERK (Thr202/Tyr204) and ERK1/2. Image shown is representative of three independent experiments.

4.2.2 CD38 expression enhances basal and chemokine-induced Rap1 activity

Very little is known about the role of Rap1 in CLL. Its involvement in chemokine-mediated integrin activation in CLL has been investigated *in vitro* (Till et al., 2008). It was shown that CXCL12-, CXCL13- and CCL21-mediated Rap1 activation is variable between patients. In most of the CLL patient samples Rap1-GTP level fail to increase upon chemokine stimulation and cells showed a defective polar clustering of the integrin LFA-1 (αLβ2). In contrast, in normal B-cells and a small percentage of CLL samples Rap1-GTP increases after chemokine treatment and lead to LFA-1 clustering. It has been proposed that the lack of response to chemokine could be due to a defective endosomal recycling reported in some CLL cases (Pye et al., 2013).

In order to assess if CD38 expression could affect Rap1 activity, levels of active Rap1 were investigated in MEC1-GFP and MEC1-CD38H cells, untreated or treated with CCL21. Active Rap1 (Rap1-GTP) was isolated using a GST-fusion protein of the Rap binding domain (RBD) of Ral guanine nucleotide dissociation stimulator (RalGDS) (Chapter 2.3.4).

Treatment with CCL21 for 1 minute induced a transient increase of Rap1 activity, which then decreased at 5 minutes. This activation pattern was observed in both MEC1-GFP and MEC1-CD38H cells. However, MEC1-CD38H cells showed a significantly enhanced level of active Rap1 both in unstimulated conditions and after CCL21 stimulation (1 min) compared to MEC1-GFP (Fig 4.4). No difference was observed in the total level of Rap1 expression between the two populations (Fig 4.4)

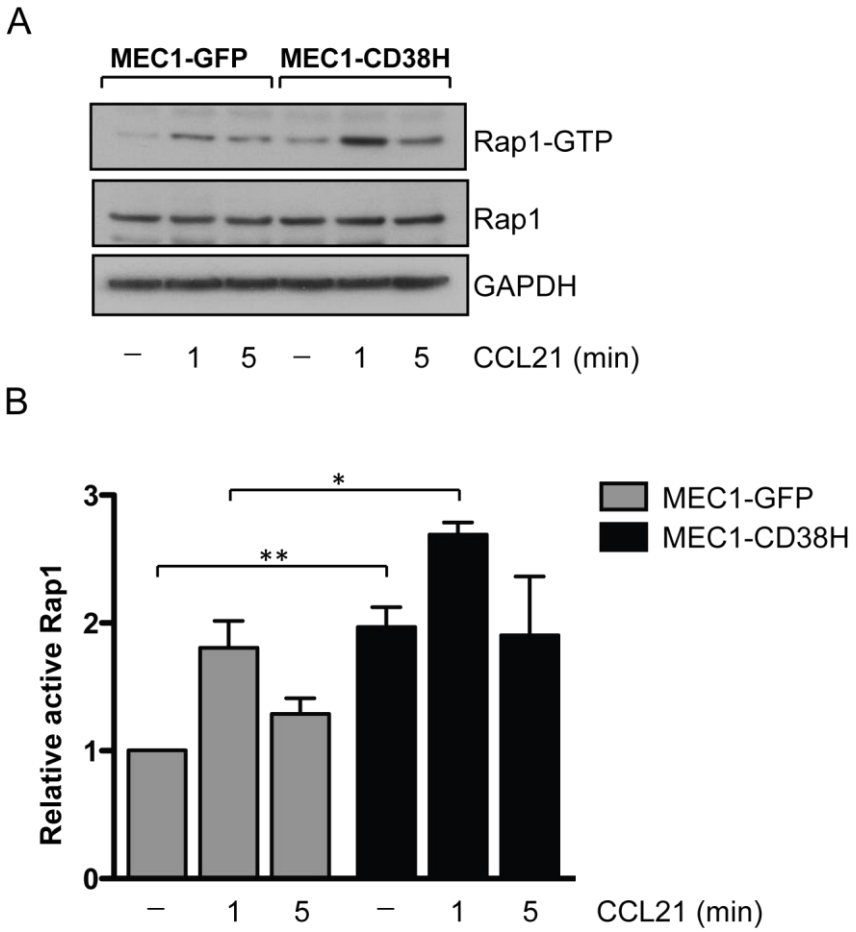

Figure 4.4 CD38 expression enhances basal and CCL21-induced Rap1 activity. Cell lysates obtained from MEC1-GFP and MEC1-CD38H cells, untreated or treated with CCL21 (100 ng/ml) for 1 or 5 minutes, were incubated with GST-RalGDS fusion protein and then analysed by western blotting with anti-Rap1 antibody. (A) Representative western blot. (B) Levels of active Rap1 were quantified by densitometry using ImageJ and normalised to total protein. Relative Rap1 levels shown were obtained by normalising each value to the untreated MEC1-GFP control cells. Data shown are the mean of three independent experiments ± SEM. *p<0.05, **p<0.01 determined by two-tailed Student's t-test.

4.2.3 Basal migration and chemotaxis of MEC1 cells is impaired by Rap1a and Rap1b knockdown

Mammalian have two closely related Rap1 genes encoding for the two proteins Rap1a and Rap1b, which are 95% identical at the amino acid level (Bokoch, 1993). It has been reported that Rap1b could be the most prominent Rap1 protein expressed in B cells (Chen et al., 2008b; Chu et al., 2008). Nevertheless, both Rap1a and Rap1b have been shown to be involved in B-lymphocyte adhesion and migration (Chen et al., 2008b; Duchniewicz et al., 2006).

To investigate if the enhanced Rap1 activity was linked to the increased migratory properties of MEC1-CD38H cells (Chapter 3.6), the effect of Rap1a and Rap1b depletion on MEC1 migration and chemotaxis was tested. Knockdown of Rap1a and Rap1b were performed using two independent siRNAs for each gene. The efficiency of protein depletion was assessed by western blotting, using antibodies specific for each Rap1a and Rap1b (Fig. 4.5).

The migration of MEC1-CD38H and MEC1-GFP cells depleted of Rap1a or Rap1b was investigated using Transwell^{TM} assays in the absence and in the presence of a gradient of CCL21.

Both Rap1a and Rap1b knockdown induced a significant decrease in basal migration and CCL21-directed chemotaxis in both MEC1-GFP and MEC1-CD38H populations (Fig 4.6 and Fig 4.7). Interestingly, the decrease in MEC1-CD38H cells was more pronounced compared to MEC1-GFP cells, indicating that the higher basal and CCL21-directed migration of MEC1-CD38H cells described in Chapter 3 could be linked to their enhanced level of active Rap1.

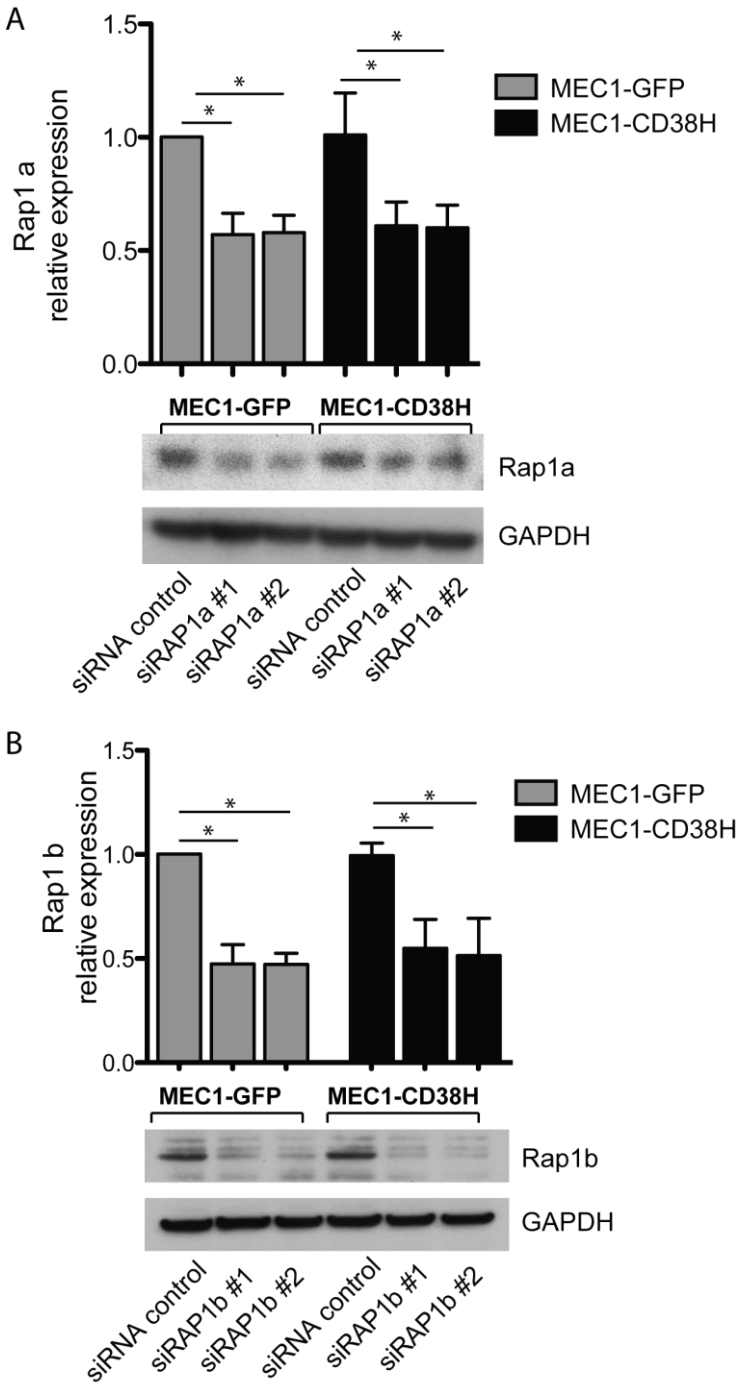

Figure 4.5 Efficiency of siRNA-mediated Rap1a and Rap1b down-regulation.
MEC1-GFP and MEC1-CD38H cells were transfected with two individual siRNAs targeting Rap1a (siRAP1a#1 and siRAP1a#2), RAP1b (siRAP1b#1 and siRAP1b#2), or with siRNA control. Cell lysates were obtained 72 h after transfection and protein levels were analysed by western blotting with antibodies against (A) Rap1a and (B) Rap1b. Representative images are shown. Levels of Rap1a and Rap1b were quantified by densitometry using ImageJ and normalised to GAPDH. Relative Rap1 levels shown were obtained by normalising each value to the MEC1-GFP control cells. Graphs show the mean of three independent experiments ± SEM. *$p<0.05$, determined by two-tailed Student's t-test.

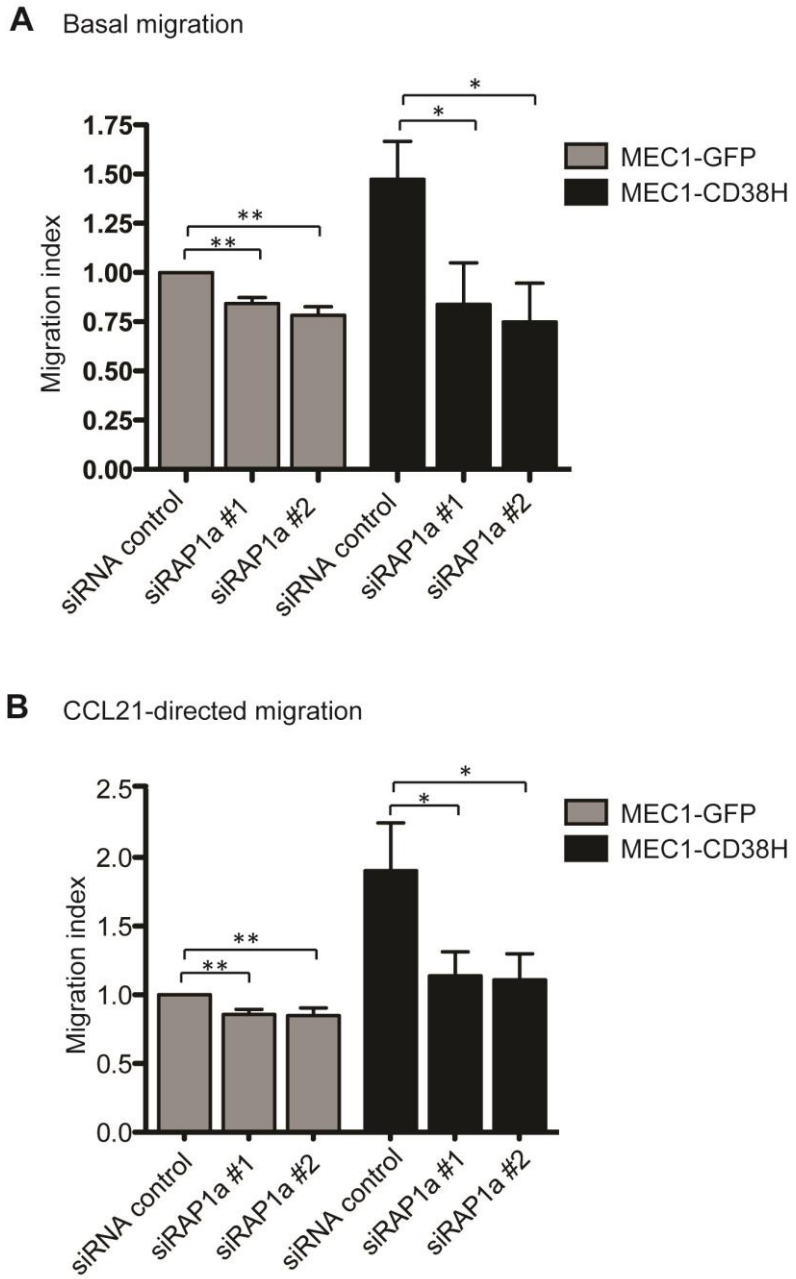

Figure 4.6 Rap1a down-regulation reduces basal cell migration and chemotaxis. MEC1-GFP and MEC1-CD38H cells were transfected with siRNAs targeting Rap1a (siRap1a#1 and siRap1a#2) or siRNA control. After 72 hours, cells were added to Transwell™ filters previously coated with VCAM-1. After 2 hours cells that had transmigrated through the filters into the lower wells in the absence of chemoattractant (A) or in the presence of CCL21 (100 ng/ml) added to the bottom chamber (B) were counted using a Casy™ counter. A migration index was obtained by normalising all the values to the MEC1-GFP cell transfected with siRNA control. Data shown are mean of four independent experiments ± SEM. *p<0.05, **p<0.01, determined by two-tailed Student's t-test.

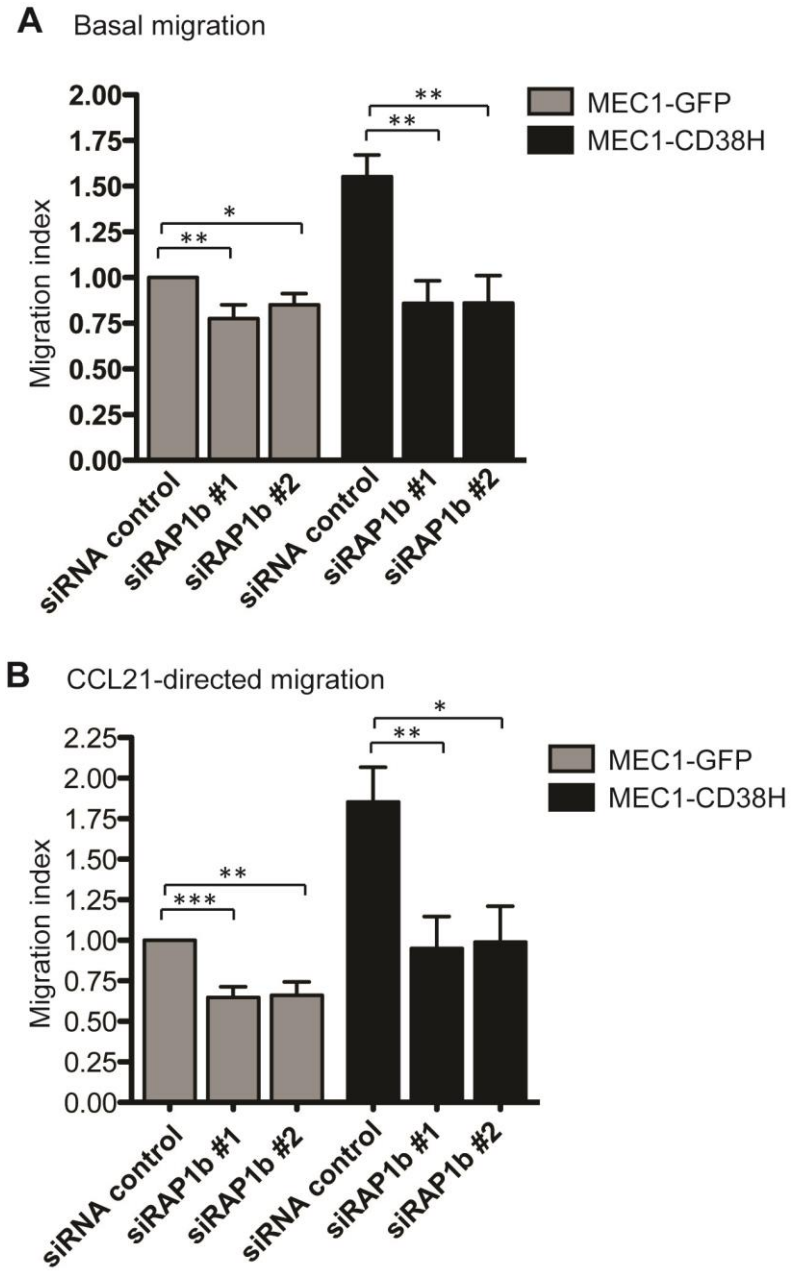

Figure 4.7 Rap1b down-regulation reduces basal cell migration and chemotaxis. MEC1-GFP and MEC1-CD38H cells were transfected with siRNAs targeting Rap1b (siRap1b#1 and siRap1b#2) or siRNA control. After 72 hours, cells were added to Transwell™ filters previously coated with VCAM-1. After 2 hours cells that had transmigrated through the filters into the lower wells in the absence of chemoattractant (A) or in the presence of CCL21 (100 ng/ml) added to the bottom chamber (B) were counted using a Casy™ counter. A migration index was obtained by normalising all the values to the MEC1-GFP cell transfected with siRNA control. Data shown are mean of four independent experiments ± SEM. *p<0.05, **p<0.01, ***p<0.001, determined by two-tailed Student's t-test.

4.3 CD38 and Ca^{2+} signalling

4.3.1 Intracellular basal [Ca^{2+}] is increased in MEC1-CD38H cells

CD38 has been established as a multi-functional enzyme involved in the production of cADPR, NAADP and ADPR. These molecules are ligand for receptor/channel involved in the Ca^{2+} mobilisation from different stores (Malavasi et al., 2008) (Chapter 1.6.1). Studies performed on CD38-deficient mice have shown that CD38-dependent cADPR production is necessary for the Ca^{2+} mobilisation and chemotaxis toward bacterial chemoattractants in neutrophils (Partida-Sánchez et al., 2001), as well as chemokine response in dendritic cells (Partida-Sánchez et al., 2004). At present, information on the role of CD38 on chemokine-mediated calcium signalling in B cells, and particularly in CLL, is limited (Chapter 1.6).

Here, the effect of CD38 expression on the intracellular [Ca^{2+}] was investigated. The ratiometric fluorescent calcium indicator Indo-1 was used to measure calcium levels in MEC1-GFP and MEC1-CD38H cells (Chapter 2.2.14). Both basal calcium levels and inducible [Ca^{2+}] increase upon stimulation with CCL21 were investigated. The basal calcium level in MEC1-CD38H cells was reproducibly higher compared to the MEC1-GFP population (Fig. 4.8, A), in agreement with data recently published (Vaisitti et al., 2014). In order to compare the CCL21-induced Ca^{2+} response between the two populations, the difference observed in the basal [Ca^{2+}] was eliminated by establishing a fluorescence threshold intensity at the 85^{th} percentile of basal fluorescence ratio of unstimulated cells (Fig. 4.8, B). The CCL21 response was then calculated as the percentage of cells that exhibited an increase of fluorescence above the threshold following treatment with CCL21. The increase of intracellular [Ca^{2+}] generated in both populations upon CCL21 stimulation was minimal. The average percentage of responding cells in the MEC1-CD38H population was slightly higher compared to the MEC1-GFP cells, although this difference was not statistically significant (Fig. 4.8, B).

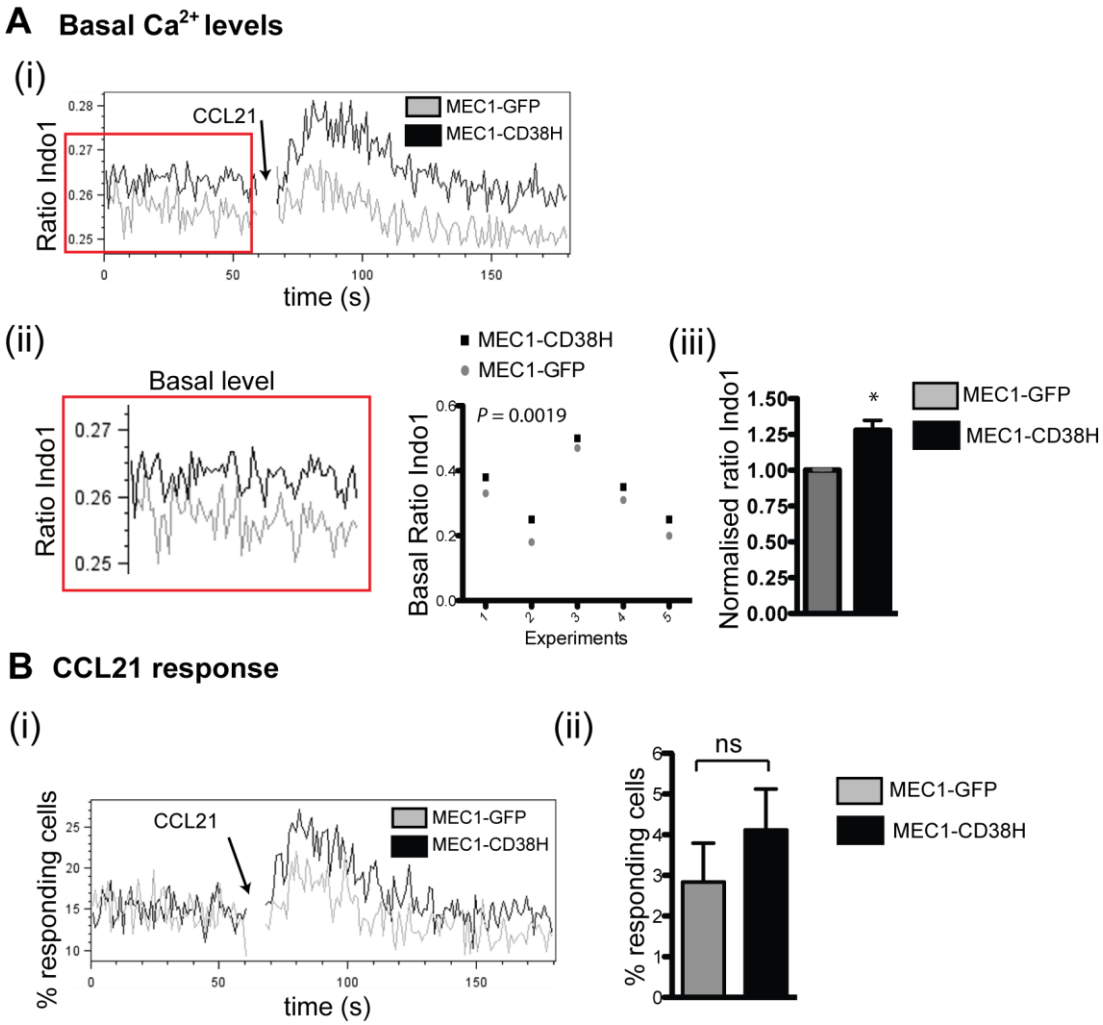

Figure 4.8 Analysis of intracellular [Ca^{2+}] in MEC1-GFP and MEC1-CD38H cells.
MEC1-GFP and MEC-1-CD38H cells were incubated with the ratiometric Ca^{2+} detector Indo-1 and analysed by flow cytometry. The basal fluorescence level was recorded for 1 minute before stimulation with CCL21 (100 ng/ml) and for a further 2 minutes. (A, i) Representative bivariate plot of the median of fluorescent intensity ratio (Indo-1 emission wavelengths - 420/510 nm) against time. Red box indicates the basal fluorescence level. (A, ii) The median of the basal fluorescence intensity was obtained in each of the 5 independent experiments for each cell population using FlowJo Kinetic statistic tool. P value was determined by Student's t-test. (A, iii) Graph shows the mean of the basal fluorescence intensity ratio of 5 independent experiments normalised to MEC1-GFP cells. *p<0.05, determined with two-tailed Student's t-test. (B, i) Representative bivariate plot obtained by establishing the 85th percentile of basal fluorescence ratio intensity as a threshold. The % of cells with a fluorescence intensity ratio above the threshold (% responding cells) after stimulation with CCL21 was obtained with FlowJo Kinetic statistic tool. Data shown in (B, ii) are mean of 5 independent experiments ± SEM. ns = not significant, determined by Student's t-test.

4.3.2 CD38 does not affect B-cell receptor-mediated response in MEC1 cells

BCR signalling has a key role in the survival and proliferation of CLL cells (Woyach et al., 2012). Two main subsets of CLL patients have been identified, based on the in vitro response to BCR, with one-half of CLL cases lacking the ability to initiate a phosphorylation cascade upon surface IgM stimulation (Apollonio et al., 2013). This unresponsiveness was shown to be associated with a constitutive activation of ERK1/2 and MAPK/ERK kinases 1/2 (MEK1/2). In addition, failure to mobilise intracellular Ca^{2+} upon BCR triggering has been shown in unresponsive CLL clones and is associated with a low surface level of IgM and the clone mutational status, with mutated CLL (M-CLL) showing the highest percentage of unresponsive cases (Mockridge et al., 2007) (Chapter 1.5.3).

The direct involvement of CD38 in the BCR response has not been fully elucidated and conflicting results have been reported. Studies on BCR-mediated signalling in CLL cases with bimodal expression of CD38, in which two discrete $CD38^{neg}$ and $CD38^{pos}$ populations could be identified within the same CLL patient sample, reported that $CD38^{pos}$ populations show constitutively higher basal protein tyrosin phosphorylation and an increased ability to signal when stimulated with anti-IgM compared to their $CD38^{neg}$ counterparts (Pepper et al., 2007). These data were not confirmed in later studies when comparing BCR responsiveness in CLL case with bimodal CD38 expression (Mockridge et al., 2007). Moreover, despite an overall association of CD38 with BCR responsiveness mainly due to the fact that responsive unmutated CLL samples (U-CLL) are generally CD38 positive, CD38 expression did not appear to have a significant impact in the BCR response in M-CLL samples (Mockridge et al., 2007).

Here, in order to assess if CD38 expression could lead to an increase in the BCR-mediated signalling response, stimulation with anti-IgM was performed in MEC1-GFP and MEC1-CD38H cells and the responsiveness, both in terms of Ca^{2+} mobilisation and ERK1/2 phosphorylation, was assessed.

Intracellular [Ca^{2+}] was measured in MEC1-GFP and MEC-1-CD38H cells before and after IgM stimulation using Indo-1. The Human B-lymphocyte Ramos cell line was used as a positive control, because it is able to respond to IgM stimulation (Calpe et

al., 2011). As observed above, the basal [Ca^{2+}] of the MEC1-CD38H cells was higher compared to the MEC1-GFP population (Fig. 4.9, A). MEC1-GFP and MEC1-CD38H cells showed only a partial response to IgM stimulation, compared to Ramos cells (Fig. 4.9, B). No difference was observed in the intracellular [Ca^{2+}] in response to IgM between MEC1-GFP and MEC1-CD38 cells (Fig. Gig. 4.9, C), supporting the hypothesis that CD38 might not be directly involved in the BCR-mediated calcium response.

In order to investigate if CD38 could be involved in the phosphorylation of ERK upon BCR stimulation, ERK phosphorylation levels were analysed in MEC1-GFP and MEC1-CD38H cells treated with anti-IgM for 10 or 30 minutes. It has been previously reported that MEC1 cells show a constitutive ERK phosphorylation, with features resembling those of unresponsive CLL clones (Apollonio et al., 2013). Here, in agreement with these data, a minimal response to IgM stimulation was detected in MEC1-GFP and MEC1-CD38H cells at the time points tested (Fig. 4.10), particularly if compared to the clear induction of ERK phosphorylation observed upon stimulation with CCL21 (Fig 4.3). No difference was observed in ERK1/2 phosphorylation levels between MEC1-GFP and MEC1-CD38H cells, both in resting conditions and after IgM stimulation, in two independent experiments performed (Fig. 4.10).

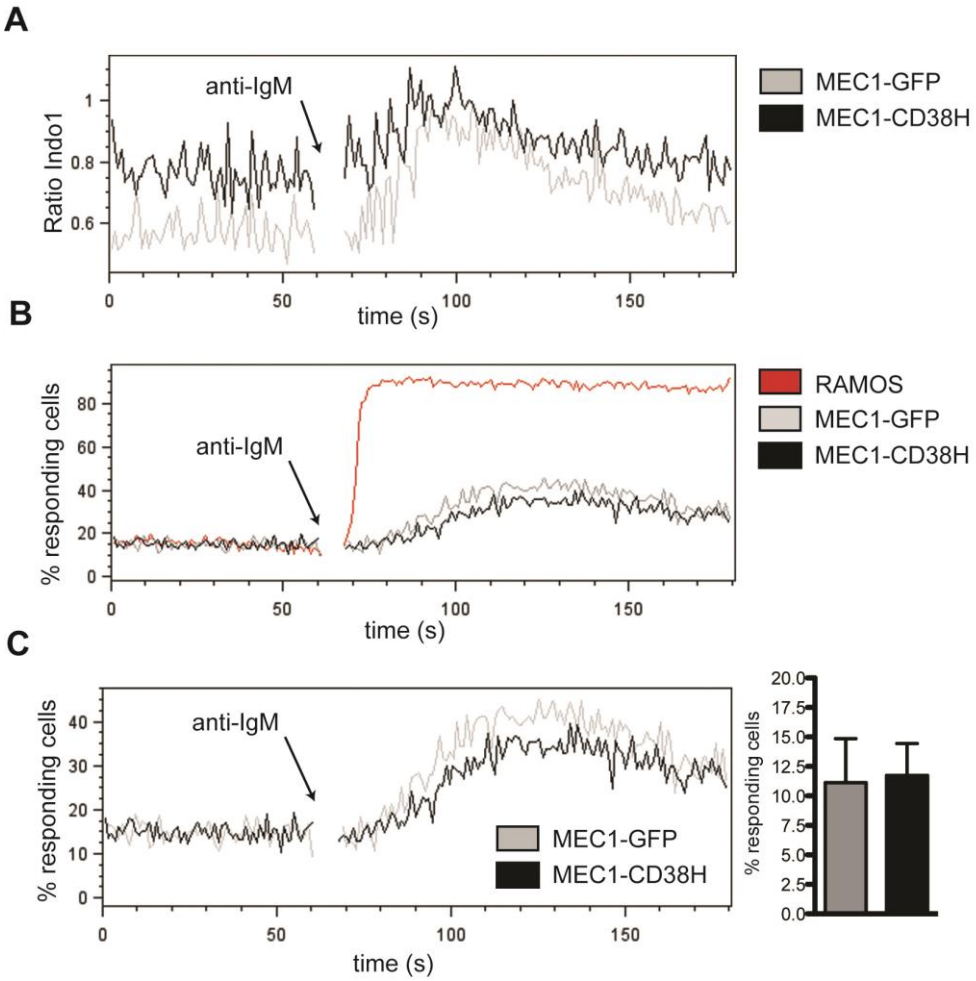

Figure 4.9 CD38 expression does not affect the BCR-induced Ca^{2+} signalling.
MEC1-GFP and MEC-1-CD38H cells were incubated with the ratiometric Ca^{2+} detector Indo-1 and analysed by flow cytometry. The basal fluorescence level was recorded for 1 minute before stimulation with IgM (20 µg/ml) and then for a further 2 minutes. (A) Representative bivariate plot of the median of fluorescent intensity ratio (Indo-1 emission wavelengths - 420/510 nm) against time. (B) Representative bivariate plot obtained by establishing the 85th percentile of basal fluorescence ratio intensity as a threshold. Ramos cells were used as a positive control. (C) The % of cells with a fluorescence intensity ratio above the threshold (% responding cells) after stimulation with IgM was obtained with FlowJo Kinetic statistic tool. Graph shows the mean of 3 independent experiments ± SEM.

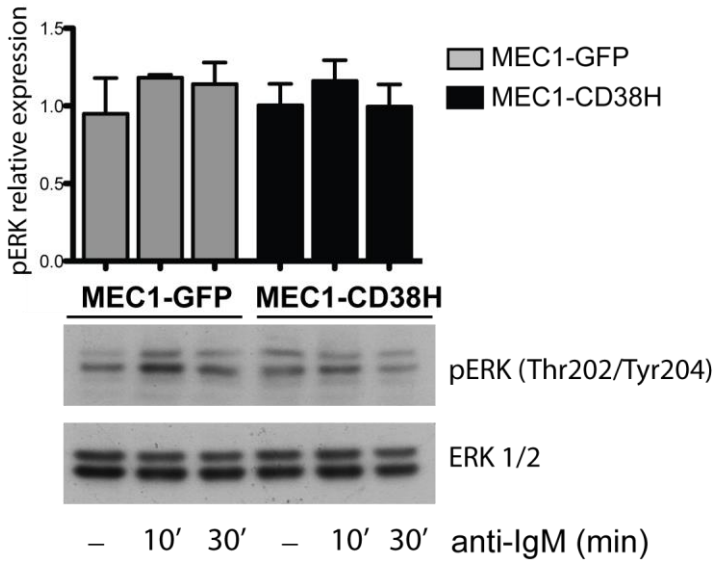

Figure 4.10 Analysis of ERK phosphorylation in response to IgM stimulation
Cell lysates obtained from MEC1-GFP and MEC1-CD38H, untreated or treated with 20 μg/ml of human anti-IgM antibody for 10 or 30 minutes, were analysed by western blotting with antibody against pERK and ERK1/2. Levels of pERK were quantified by densitometry using ImageJ and normalised to the corresponding ERK1/2 levels. Graph shows the mean of two independent experiments ± SEM. Representative image is shown.

4.3.3 Calcium depletion reduces Rap1 activity in MEC1-CD38H cells

In order to investigate if the enhanced Rap1 activity in MEC1-CD38H cells was related to the higher basal calcium level, depletion of intracellular calcium was performed (Chapter 2.2.5.3) in MEC1-GFP and MEC1-CD38H cells and the level of Rap1-GTP was investigated. Calcium depletion decreased the amount of active Rap1 in MEC1-CD38H cells, whereas no effect was observed in MEC1-GFP cells (Fig. 4.11). This suggests that the enhanced basal calcium level may be responsible for the enhanced Rap1 activity detected in CD38-expressing cells.

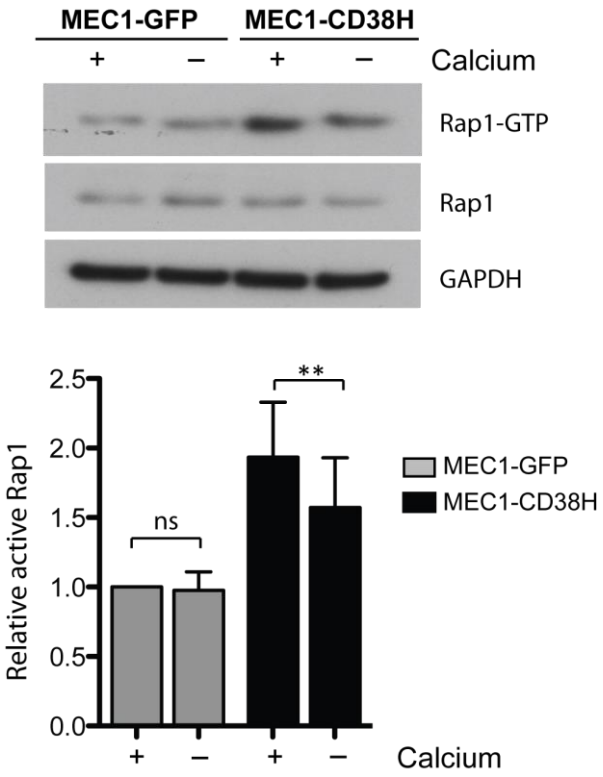

Figure 4.11 Calcium depletion reduces Rap1 basal activity in MEC1-CD38H cells. MEC1-GFP and MEC1-CD38H cells were resuspended in $CaCl_2$- and $MgCl_2$- free PBS and treated with the calcium ionophore ionomycin (0.5 μM) for 5 minutes. Control cells were resuspended in PBS supplemented with $CaCl_2$ and $MgCl_2$ and treated in parallel with the same amount of DMSO as vehicle control. Cells were lysed and lysates were incubated with GST-RalGDS fusion protein and then analysed by western blotting with anti-Rap1 antibody. Levels of active Rap1 were quantified by densitometry using ImageJ and normalised to total Rap1. Representative image is shown. Graph shows the mean of four independent experiments ± SEM. **p<0.01; ns, not significant, determined by two-tailed Student's t-test applied on data normalised to the respective control population.

4.4 RasGRP GEFs in MEC1 cell motility

4.4.1 RasGRP2, and not RasGRP3, affects MEC1-CD38H cell motility

The Ras guanine nucleotide releasing protein (RasGRP) family is composed of four GEFs that possess a DAG-binding domain and two Ca^{2+}-binding domains. Two members of this family, RasGRP2 and RasGRP3, have been shown to activate Rap1, whereas the other two members appear to be selective Ras GEFs (Stone, 2006). The structured domains of RasGRPs have led to the hypothesis that these proteins could integrate the cellular response to Ca^{2+} and DAG. How these second messengers activate RasGRPs has not been elucidated so far (Stone, 2011).

Given that MEC1-CD38H cells showed higher basal Intracellular [Ca^{2+}], which correlated with enhanced Rap1 activity and cell migration properties, the role of the calcium-activated Rap1 GEFs was investigated. RasGRP2 and RasGRP3 were depleted in MEC1-GFP and MEC1-CD38H cells using two independent siRNAs (Fig. 4.12, A and B) and their ability to migrate in the absence or in the presence of a CCL21 gradient was investigated.

RasGRP2 depletion in MEC1-CD38H cells significantly decreased both basal migration and CCL21-directed chemotaxis (Fig. 4.13, A and B). Conversely, no effect was observed in MEC1-GFP cells after RasGRP2 depletion, both in basal migration and chemotaxis (Fig. 4.13, A and B). When RasGRP3 was down-regulated, no significant effect was observed in the basal migration and chemotaxis of either cell population (Fig 4.14, A and B).

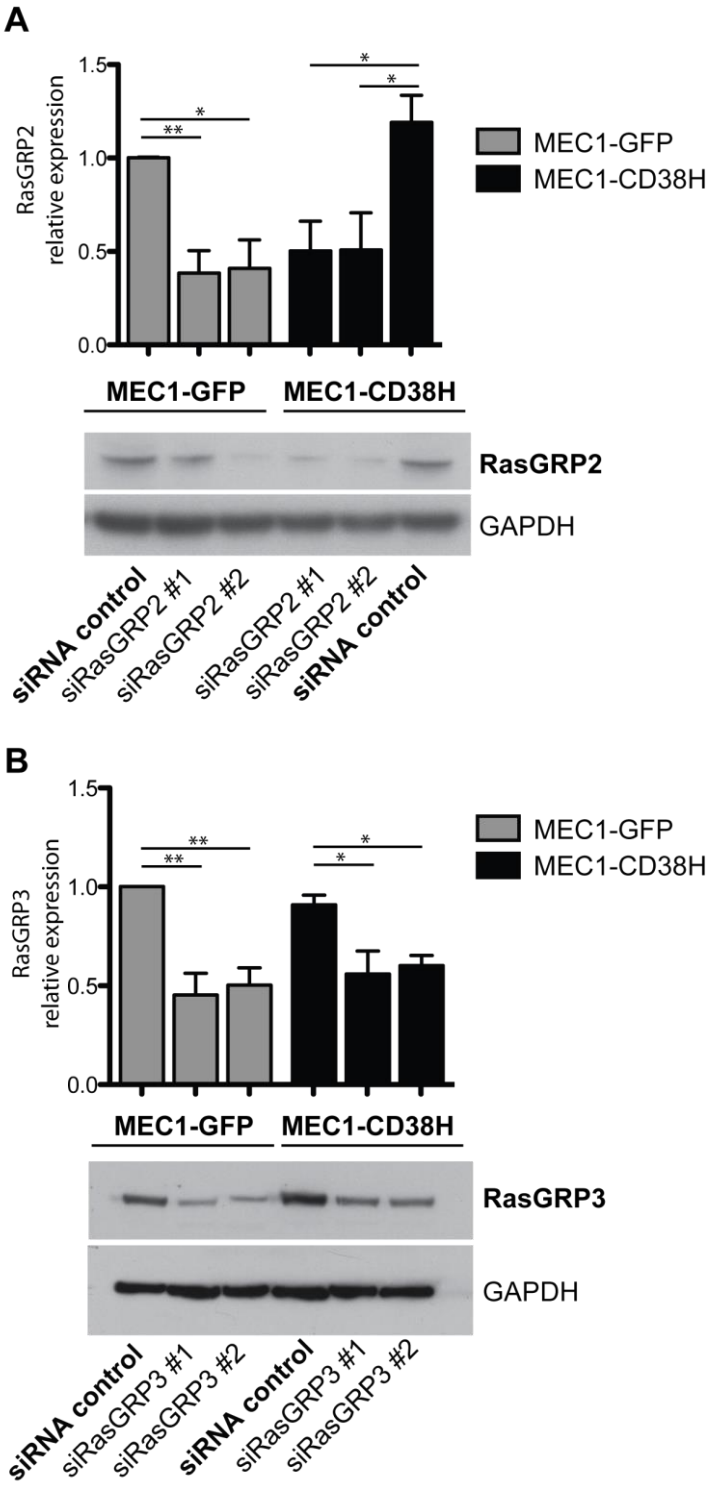

Figure 4.12 Efficiency of siRNA-mediated RasGRP2 and RasGRP3 down-regulation. MEC1-GFP and MEC1-CD38H cells were transfected with two individual siRNAs targeting RasGRP2 (siRasGRP2#1 and siRasGRP2#2), RasGRP3 (siRasGRP3#1 and siRasGRP3#2) or with siRNA control. Cell lysates were obtained 72 h after transfection and protein levels were analysed by western blotting with antibodies against (A) RasGRP2 and (B) RasGRP3. GAPDH was used as a loading control. Representative images are shown. Graphs show the mean of three independent experiments ± SEM. *p<0.05, **p<0.01, determined by two-tailed Student's t-test.

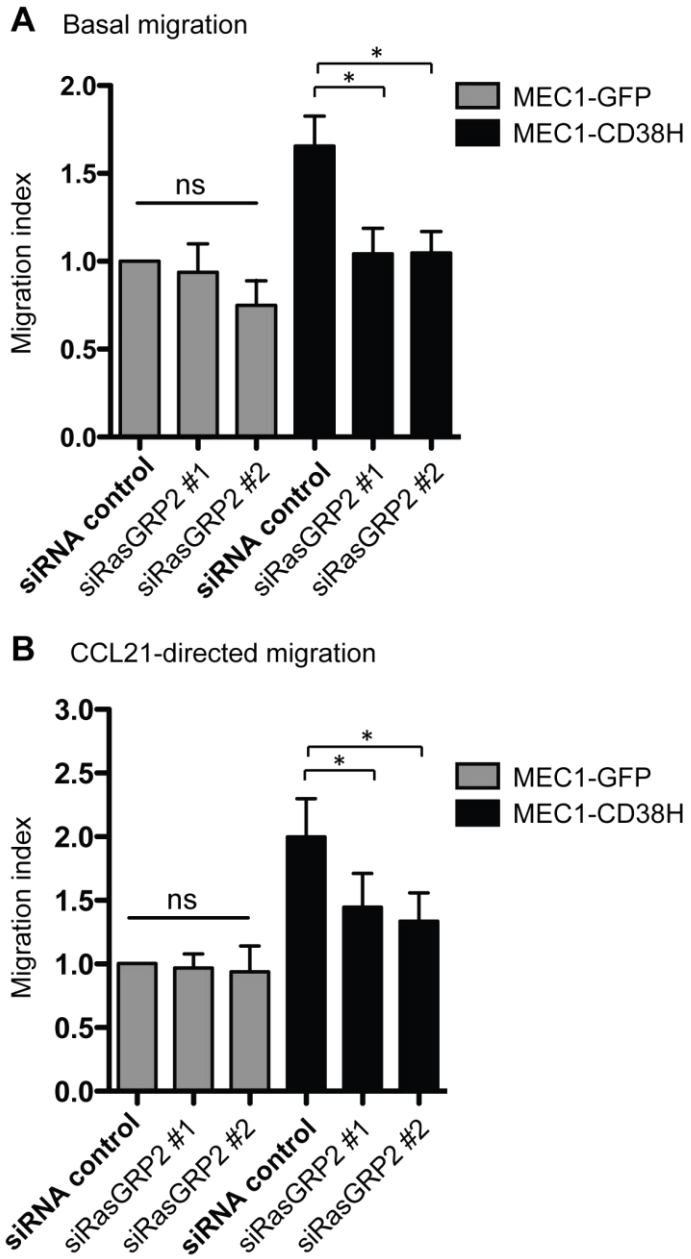

Figure 4.13 RasGRP2 down-regulation reduces cell migration in a CD38-dependent fashion.

MEC1-GFP and MEC1-CD38H cells were transfected with siRNAs targeting RasGRP2 (siRasGRP2#1 and siRasGRP2#2) or siRNA control. After 72 h, cells were added to Transwell™ filters previously coated with VCAM-1. After 2 hours cells that had transmigrated through the filters into the lower wells in the absence of chemoattractant (A) or in the presence of CCL21 (100 ng/ml) added to the bottom chamber (B) were counted using a Casy™ counter. A migration index was obtained by normalising the values against the MEC1-GFP siRNA control cells. Data shown are mean of three independent experiments ± SEM. *$p<0.05$, ns = not significant, determined by two-tailed Student's t-test.

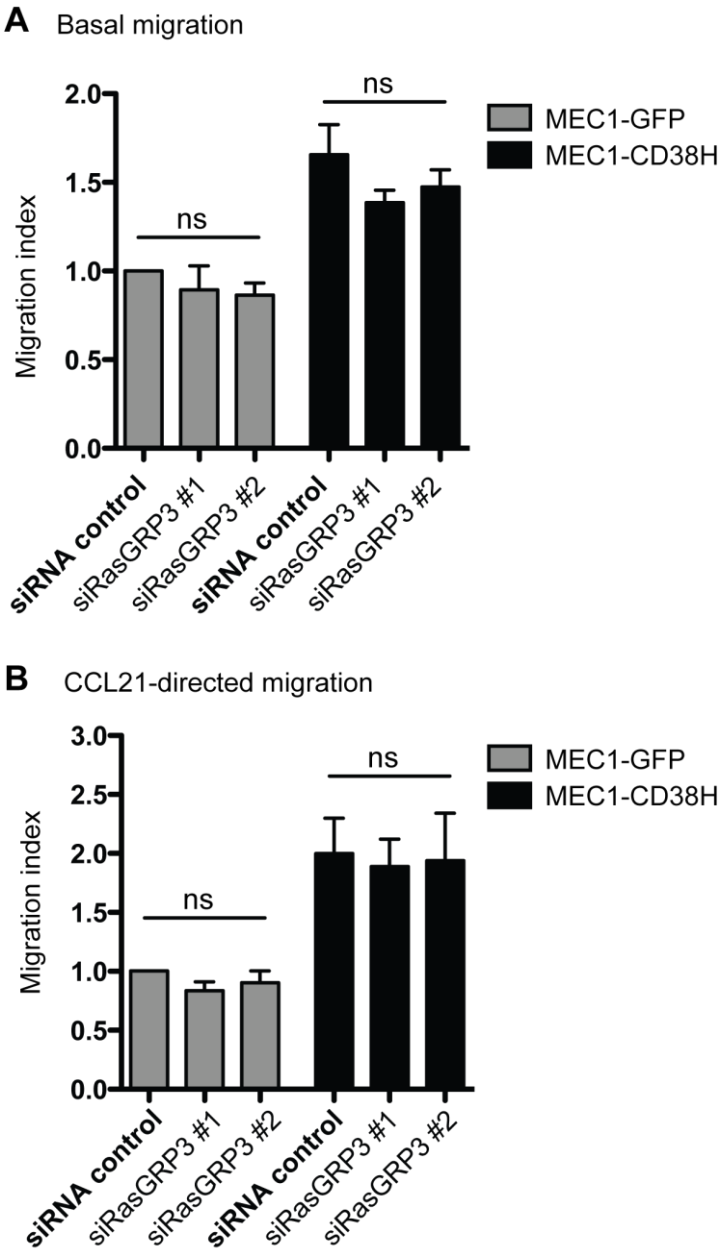

Figure 4.14 RasGRP3 down-regulation does not affect MEC1 cell migration.
MEC1-GFP and MEC1-CD38H cells were transfected with siRNAs targeting RasGRP3 (siRasGRP3#1 and siRasGRP3#2) or siRNA control and then added to Transwell™ filters previously coated with VCAM-1. After 2 hours cells that had transmigrated through the filters into the lower wells in the absence of chemoattractant (A) or in the presence of CCL21 (100ng/ml) added to the bottom chamber (B) were counted using a Casy™ counter. A migration index was obtained by normalising transmigrated MEC1-CD38H cells against the MEC1-GFP control cell. Data shown are mean of three independent experiments ± SEM. ns = not significant, determined by two-tailed Student's t-test.

4.4.2 RasGRP2 knockdown affects Rap1 activity in both MEC1-CD38H and MEC1-GFP cells.

As RasGRP2 depletion was observed to inhibit migration and chemotaxis specifically in MEC1-CD38H cells, the role of RasGRP2 in the basal Rap1 activity in both MEC1-GFP and MEC1-CD38H cells was investigated. RasGRP2 down-regulation induced a decrease in Rap1 activity in both MEC1-GFP and MEC1-CD38H populations (Fig 4.15). This suggests that the RasGRP2-Rap1 signalling axis is active in MEC1 cells either in the presence or in the absence of CD38, but its role in cell motility is limited to MEC1-CD38H cells.

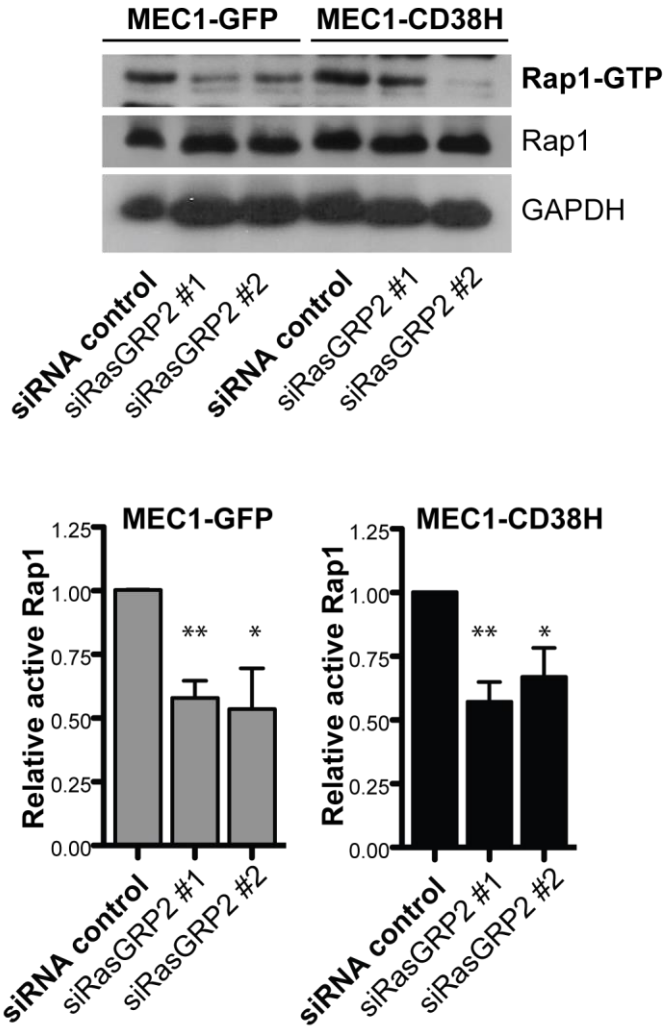

Figure 4.15 RasGRP2 down-regulation affects Rap1 activity.
MEC1-GFP and MEC1-CD38H cells were transfected with two individual siRNAs targeting RasGRP2 (siRasGRP2#1 and siRasGRP2#2) or with a siRNA control. Cell lysates were obtained 72 h after transfection and incubated with GST-RalGDS fusion protein and then analysed by western blotting with anti Rap1 antibody. Levels of active Rap1 were quantified by densitometry using ImageJ and normalised to total Rap1. Representative image is shown. Graphs show the mean of three independent experiments ± SEM. *p<0.5; **p<0.01; determined by two-tailed Student's t-test applied on data normalised to each respective siRNA control.

4.4.3 RasGRP2 shows a polarised localisation in MEC1-CD38H cells

To further investigate the role of RasGRP2, its subcellular localisation was analysed in MEC1-GFP and MEC1-CD38H cells by immunostaining performed on cells seeded on VCAM-1. RasGRP2 localisation was different between MEC1-GFP and MEC1-CD38H cells. Whereas in MEC1-GFP cells RasGRP2 showed a generally diffuse and only partly polarised localisation, in MEC1-CD38H cells RasGRP2 localisation was strongly polarised, and it mainly accumulated in the actin-rich subcellular regions (Fig. 4.16). The same localisation patterns were observed when cells were seeded on poly-L-lysin (data not shown) suggesting that VCAM-1 was not involved in the induction of RasGRP2 polarised localisation. Co-localisation of CD38 and RasGRP2 was also observed in the MEC1-CD38H cells (Fig. 4.17).

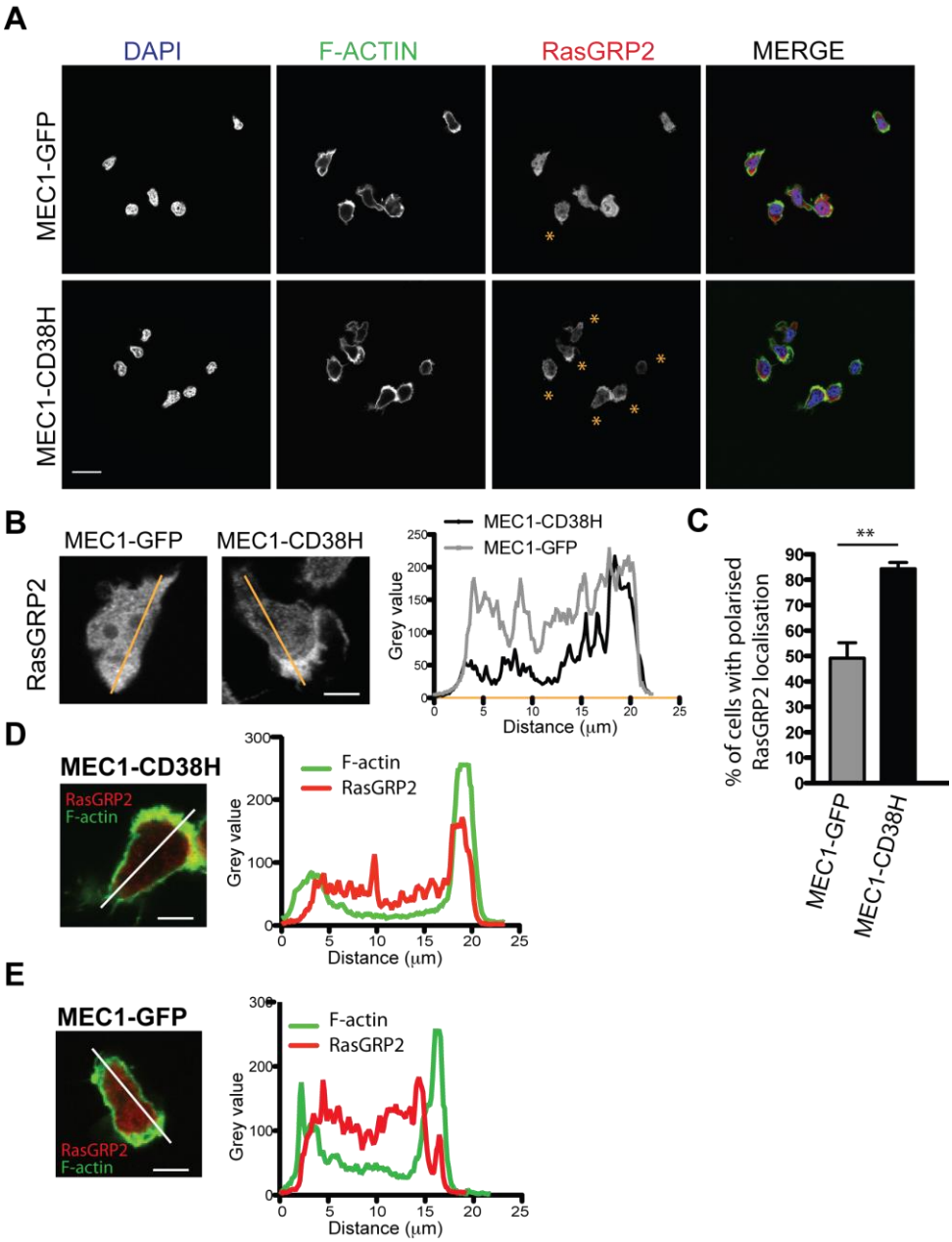

Figure 4.16 RasGRP2 shows a polarised localisation in MEC1-CD38H cells.
MEC1-GFP and MEC1-CD38H cells were seeded on VCAM-1 coated coverslips and allow to adhere for 15 min. Cells were then fixed and stained for RasGRP2. Nuclei were counterstained with DAPI and actin filaments (F-actin) was visualised by phalloidin staining. (A) Representative images of three independent experiments are shown. Stars indicate cells with polarised RasGRP2 localisation. Scale bar, 20 µm. (B) Fluorescence intensity profile of RasGRP2 obtained along the longitudinal axis of the representative MEC1-GFP and MEC1-CD38H cells shown; scale bar, 5 µm. (C) The percentage of cells with polarised RasGRP2 localisation was quantified by scoring n ≥ 100 cells in each of the 3 independent experiments. **p<0.01, determined by two-tailed Student's t-test. (D and E) Fluorescence intensity profile of RasGRP2 (red) and F-actin (green) obtained along the longitudinal axis of the representative MEC1-CD38H cell (D) and MEC1-GFP cell (E). Scale bar, 5 µm.

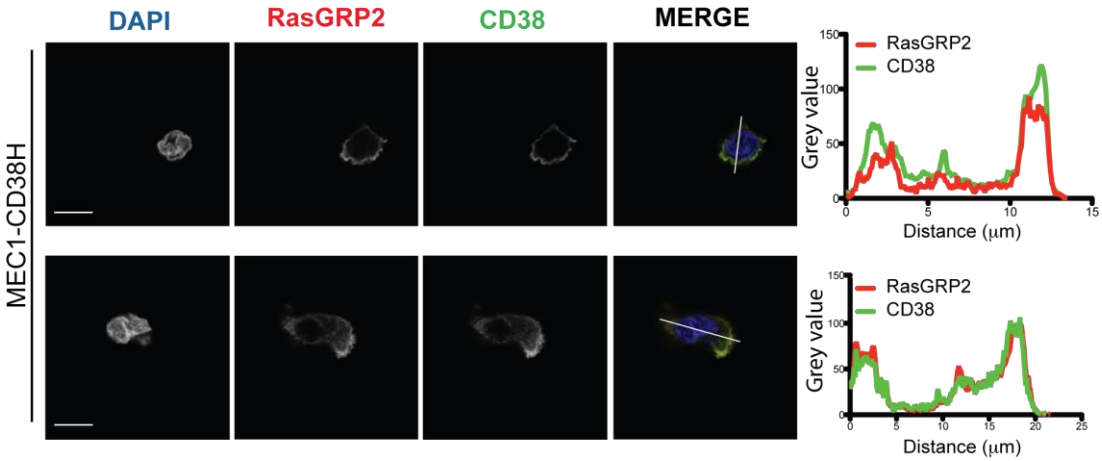

Figure 4.17 RasGRP2 and CD38 co-localise in MEC1-CD38H cells.
MEC1-CD38H cells were seeded on VCAM-1 coated coverslips and allow to adhere for 15 min. Cells were then fixed and stained for RasGRP2 and CD38. Nuclei were counterstained with DAPI. Scale bar, 10 μm. Two representative images from two independent experiments are shown. Plots show the fluorescence intensity profile of RasGRP2 (red) and CD38 (green) obtained along the longitudinal axis of the representative cells.

4.5 IQGAP1 knockdown affects the basal migration of MEC1-CD38H cells

IQGAP1 is a scaffolding protein that possesses several interaction motifs through which it associates with a wide range of proteins including F-actin, E-cadherin, ERK1/2, Cdc42, Rac1 and Rap1 (Jeong et al., 2007; Mataraza et al., 2003; Ren et al., 2008). These multiple interactions involve IQGAP1 in several cellular activities including actin polymerisation, microtubule organising centre formation, cell polarisation and regulation of calcium/calmodulin signalling.

In order to investigate if IQGAP1 was involved in the enhanced motility observed in MEC1-CD38H cells, the effect of IQGAP1 depletion on MEC1-GFP and MEC1-CD38H cell basal migration and chemotaxis was tested. The efficiency of IQGAP1 depletion was assessed by western blotting (Fig. 4.18). IQGAP1 down-regulation did not induce any change in the migration of MEC1-GFP cells, either in the presence or in the absence of CCL21 (Fig 4.19, A and B). Conversely, in MEC1-CD38H cells, IQGAP1 depletion induced a significant decrease in the cell basal migration (Fig. 4.19, A). A slight but not significant decrease in the chemotaxis towards CCL21 was observed in MEC1-CD38H cells when IQGAP1 was depleted (Fig 4.19, B), suggesting that IQGAP1 may have a role in the enhanced basal motility properties linked to CD38 expression but its role could be minimal in the CCL21-directed migration.

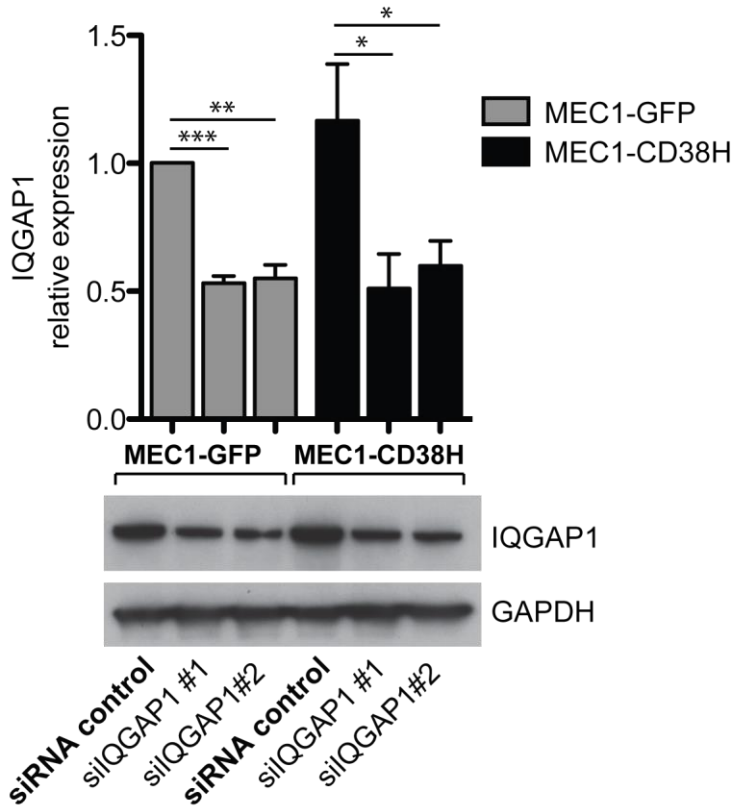

Figure 4.18 Efficiency of siRNA-mediated IQGAP1 knockdown.
MEC1-GFP and MEC1-CD38H cells were transfected with two individual siRNAs targeting IQGAP1 (IQGAP1#1 and IQGAP1#2) or with siRNA control. Cell lysates were obtained 72 h after transfection and protein levels were analysed by western blotting with antibodies against IQGAP1. GAPDH was used as a loading control. Representative image is shown. Graph shows the mean of three independent experiments ±SEM. *p<0.05, **p<0.01, ***p<0.001 determined by two-tailed Student's t-test.

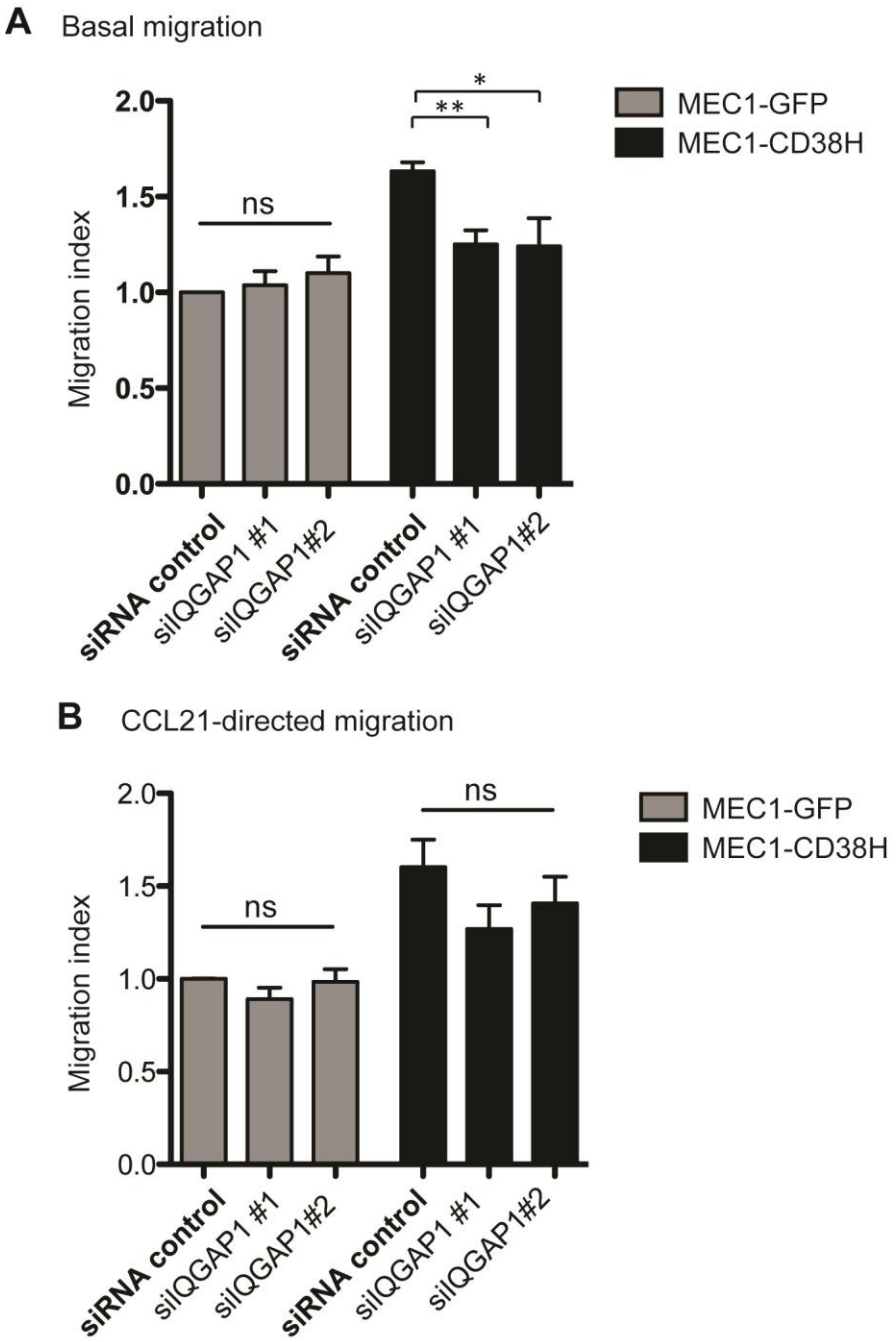

Figure 4.19 IQGAP1 knockdown affects MEC1-CD38H cell basal migration.
MEC1-GFP and MEC1-CD38H cells were transfected with siRNAs targeting IQGAP1 (IQGAP1#1 and IQGAP1#2) or siRNA control. After 72 hours cells were added to Transwell™ filters previously coated with VCAM-1. After 2 hours cells that had transmigrated through the filters into the lower wells in the absence of chemoattractant (A) or in the presence of CCL21 (100 ng/ml) added to the bottom chamber (B) were counted using a Casy™ counter. A migration index was obtained by normalising the values obtained to the MEC1-GFP cell transfected with siRNA control. Data shown are mean of three independent experiments ± SEM. *p<0.05, **p<0.01; ns, not significant; determined by two-tailed Student's t-test.

4.6 Effect of ibrutinib on MEC1 cell migration and Rap1 activity

Targeted therapy with kinase inhibitors is transforming the treatment of CLL. The properties of B-cell receptor (BCR) signalling in CLL have lead to target kinases of the BCR pathway, including Lyn, Syk, PI3K, and Btk (Wiestner, 2012). Treatment with these kinase inhibitors have been shown to cause, in the majority of patients, a rapid shrinkage of the lymph nodes and a transient increase in the number of lymphocytes in the blood, probably due to the egress of the malignant cells from the lymphatic tissues (Byrd et al., 2013; de Rooij et al., 2012; Friedberg et al., 2010; Furman et al., 2014b) (Chapter 1.5.4). These clinical results indicate that these inhibitors affect not only BCR-mediated pro-survival signalling but also the chemotactic and motility properties of CLL cells. Notably, studies performed *in vitro* on primary CLL cells have shown that ibrutinib, a Btk inhibitor, is able to impair integrin-mediated adhesion and chemotaxis (de Rooij et al., 2012).

Here, the effect of ibrutinib (PCI-32765) on the basal migration and chemotaxis of MEC1-GFP and MEC1-CD38H was tested. Cells were treated with ibrutinib (or with DMSO as a vehicle control) for 1 hour before performing a Transwell assay, both in the presence and absence of CCL21. Btk inhibition induced a small, but significant, decrease in the chemotaxis of both MEC1-GFP and MEC1-CD38H cells (Fig. 4.20). No statistical significance was observed in the basal migration of either MEC1-GFP or MEC1-CD38H after ibrutinib treatment (Fig 4.20).

The effect of Ibrutinib on the basal and chemokine-induced Rap1 activity was also investigated. MEC1-GFP and MEC1-CD38H were treated with Ibrutinib, or with DMSO as a control, for 1 hour before being stimulated for 1 minute with CCL21. Whereas the basal activity of Rap1 was not affected by the Btk inhibition, the Rap1 activation following CCL21 stimulation was inhibited by ibrutinib in both MEC1-GFP and MEC1-CD38H cells (fig 4.21). Hoewever, active Rap1 was still above each respective basal level in MEC1-GFP and MEC1-CD38H cells treated with Ibrutinib and a big variability was observed in the assay. To verify the activation of the CCL21-dependent pathway, ERK phosphorylation was tested, which was induced after 1 minute stimulation with

CCL21. As expected, ERK phosphorylation upon CCL21 stimulation was partly inhibited by Ibrutinib treatment (Fig. 4.21).

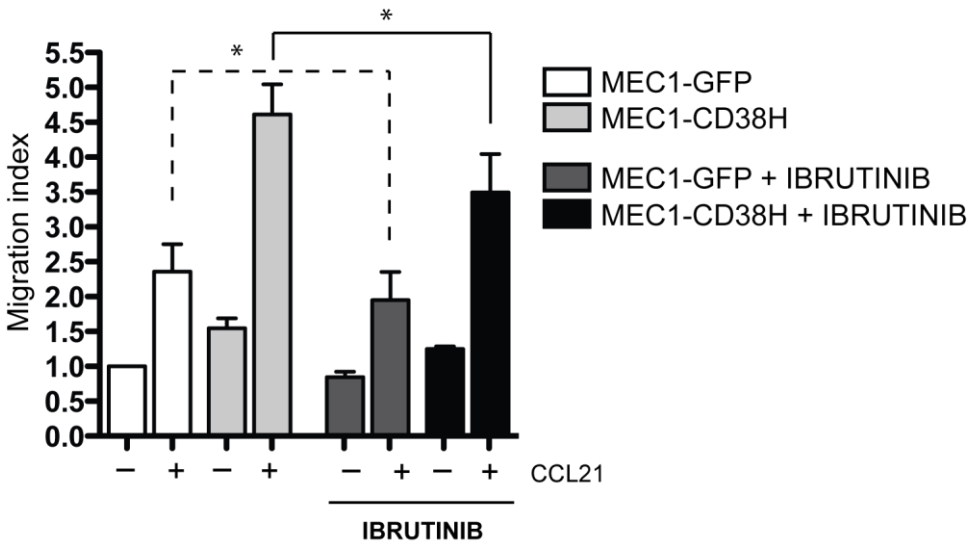

Figure 4.20 Effect of Ibrutinib on MEC1 cell migration.
MEC1-GFP and MEC1-CD38H cells treated with 1µM Ibrutinib (or with DMSO as a vehicle control) for 1 hour were added to Transwell™ filters previously coated with VCAM-1 and allowed to transmigrate for 2 hours. Cells that had transmigrated through the filters into the lower wells in the absence or in the presence of 100 ng/ml CCL21 were counted using a Casy™ counter. A migration index was obtained by normalising transmigrated cells for each condition to the MEC1-GFP control cells. Data shown are mean of three independent experiments ± SEM. *p<0.05 determined by two-tailed Student's t-test.

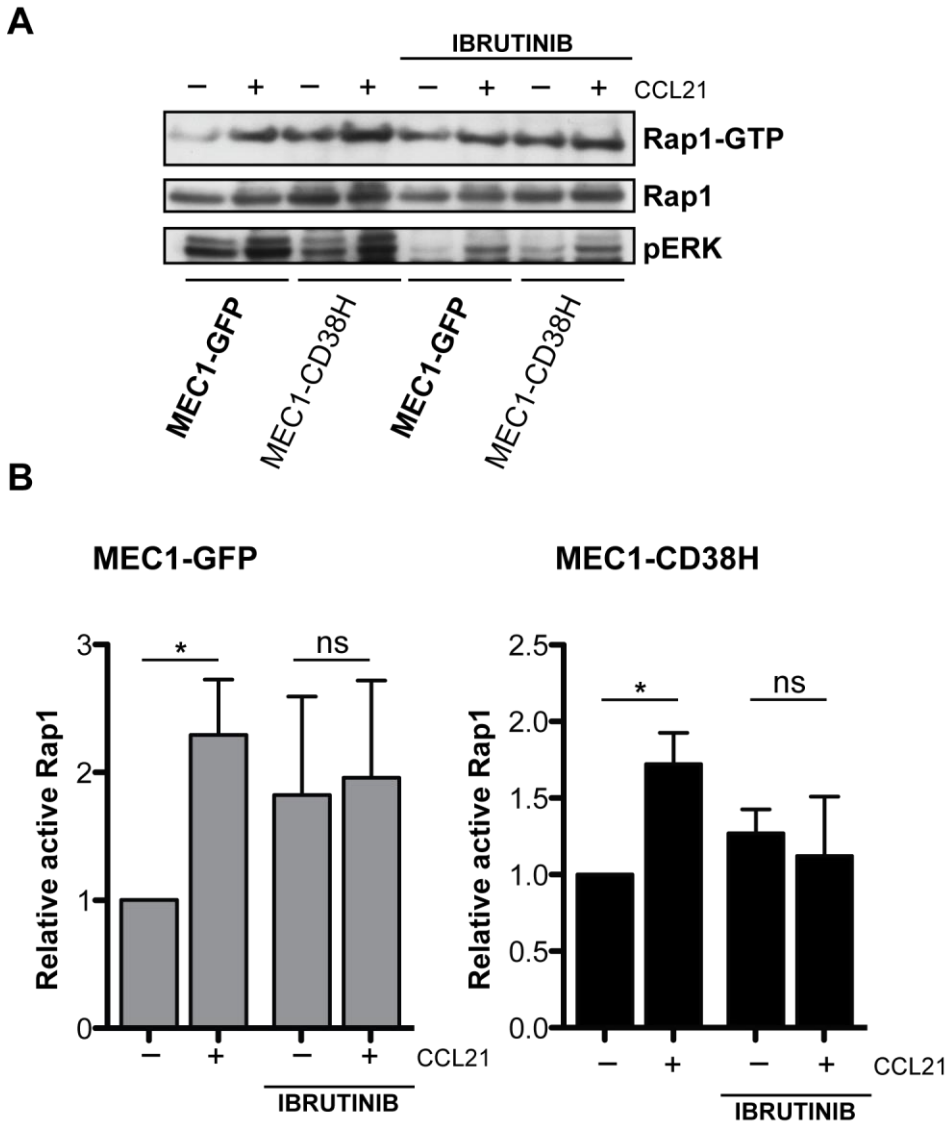

Figure 4.21 Effect of Ibrutinib on Rap1 activity.
MEC1-GFP and MEC1-CD38H cells were treated with 1µM Ibrutinib (or with DMSO as a vehicle control) for 1 hour before being stimulated with CCL21 (100 ng/ml) for 1 min. Cell lysates were obtained, incubated with GST-RalGDS fusion protein and then analysed by western blotting with anti Rap1 and anti-pERK antibodies. (A) Representative image of four independent experiments. (B) Levels of active Rap1 were quantified by densitometry using ImageJ and normalised to total Rap1. Relative Rap1 levels shown were obtained by normalisation against the untreated MEC1-GFP cell control. Data shown are the mean of four independent experiments ± SEM. *p<0.05; ns, not significant, determined by two-tailed Student's t-test.

4.7 Discussion

Despite the evidence that expression of CD38 in CLL correlates with increased migration of leukaemic cells (Deaglio et al., 2007a; Vaisitti et al., 2010; Zucchetto et al., 2012), the motility-related signalling pathways involved in this process have not being elucidated so far. The aim of the research described in this chapter was to investigate the motility-related signalling that could explain the difference in migration between MEC1-GFP and MEC1-CD38H cells described in Chapter 3. In order to do that, the role of selected small GTPases, well known key players of cell motility, together with some of their regulators was investigated.

Rap1 is well known for its role in stimulating integrin activation and mediating migration and trafficking of lymphocytes (Chen et al., 2008b; Dustin et al., 2004; Ebisuno et al., 2010; McLeod and Gold, 2001). Analysis of Rap1 activity in MEC1-GFP and MEC1-CD38H cells suggested that CD38 expression increases the basal level of active Rap1, with a consequently higher Rap1-GTP level in MEC1-CD38H cells compared to MEC1-GFP cells after CCL21-mediated activation. The CD38-associated increase in Rap1 activity could therefore explain the increased spreading observed in MEC1-CD38H cells described in the previous chapter. The higher Rap1 activity might also explain the increased migration and chemotaxis of MEC1-CD38H cells compared to MEC1-GFP cells, as Rap1a and Rap1b depletion experiments showed that both Rap1 proteins are involved in the basal migration and CCL21-directed chemotaxis of MEC1 cells. Interestingly, the decrease in migration observed in Rap1-depleted MEC1-CD38H cells was more pronounced compared to MEC1-GFP cells. CD38 expression could therefore lead to a stronger dependency on Rap1 in cell migration. Notably, up-regulation of Rap1 activity obtained in mice deficient for SPA-1, a Rap1 GTPase-activating protein, has been shown to lead to the development a B1-cell leukaemia resembling CLL (Ishida et al., 2006), suggesting that Rap1 could have a central role in the development of the disease.

Conversely, no difference between MEC1-GFP and MEC1-CD38H was detected in the activity of Rac1 and Cdc42. The activity of these two GTPases appeared to be the same in the two cell populations and stimulation with CCL21 did not show any

detectable difference at the time point tested. These data are in agreement with a recent report, which suggests that Rac1 is dispensable for CXCL12-mediated CLL cell migration, whereas it is required for CLL cell proliferation (Hofbauer et al., 2014). CXCL12 stimulation in fact does not appear to induce activation of Rac1 in CLL cells, in contrast with the CXCL12-induced Rac1 activation described in normal T and B cells (Fukui et al., 2001; Garcia-Bernal et al., 2005). It would be interesting to test the role of Rac2 in the migration of MEC1 cells. Rac1 and Rac2 were shown to have distinct roles in the motility of different immune cell types (Gu et al., 2003; Wheeler et al., 2006; Zhang et al., 2009). Although both Rac1 and Rac2 are required for B cell development and signalling upon BCR stimulation, only Rac2 appears to be involved in the BCR-induced B cell adhesion, with a mechanism that could involve Rap1 (Arana et al., 2008b).

CD38 is a multi-functional enzyme involved in the production of multiple Ca^{2+}-mobilising compounds (Malavasi et al., 2008). Here, the effect of CD38 expression on [Ca^{2+}] in resting and CCL21-/IgM-stimulated MEC1 cells was analysed. Whereas no significant difference between MEC1-GFP and MEC1-CD38H in the Ca^{2+}-mediated response to both stimuli was detected, a significantly higher basal [Ca^{2+}] was observed in the CD38-expressing cells. This is in agreement with recently published data, where expression of wild type CD38, and not the enzymatically inactive form, was shown to increase the basal [Ca^{2+}] of MEC1 cells (Vaisitti et al., 2014). Transient increases in intracellular [Ca^{2+}] have been linked to enhanced adhesive properties in different types of cells, including leukocytes (Schaff et al., 2010; Tsai et al., 2014). Moreover, it has long been known that chemokine-induced increase of intracellular [Ca^{2+}] is indispensable for the establishment of cell polarity and locomotion of leukocytes (Brundage et al., 1991; Evans and Falke, 2007; Gilbert et al., 1994). However, limited information is available about the role of basal intracellular [Ca^{2+}] in cell motility. In B-lymphocytes, elevated basal [Ca^{2+}] together with an impaired ability to respond to BCR stimulation is considered a typical feature of anergy (Yarkoni et al., 2010), but the molecular mechanism of this phenomenon has not been elucidated. Here, the increased basal [Ca^{2+}] observed in MEC1-CD38H compared to MEC1-GFP cells was not associated with a decrease in the cell responsiveness to BCR.

The intracellular Ca^{2+} rise detected upon IgM stimulation was the same in MEC1-CD38H and MEC1-GFP cells. This suggests that the enhanced basal [Ca^{2+}] in MEC1-CD38H cells does not reduce the BCR-mediated Ca^{2+} signalling, possibly because CD38-mediated Ca^{2+}-mobilisation involves different mechanisms to the PLC/IP_3 pathway activated upon BCR stimulation (Chapter 1.4.5 and 1.6.1). Data obtained by decreasing the intracellular Ca^{2+} level in MEC1 cells suggests that the CD38-associated elevated intracellular basal [Ca^{2+}] could be responsible for enhanced basal Rap1 activity. Calcium depletion in fact induced a decrease in the amount of Rap1-GTP in MEC1-CD38H cells, whereas no effect was observed in the MEC1-GFP population.

Two members of the RasGRP family, RasGRP2 and RasGRP3, have been characterised as Rap1 GEFs that could have the potential to integrate Ca^{2+} and DAG signalling with Rap1 activation (Stone, 2011). In support of this hypothesis, it has been shown that RasGRP2 is required for the CXCL12-induced Rap1 activation and LFA1-mediated adhesion in T-lymphocytes (Ghandour et al., 2007). The involvement of RasGRP3 in the BCR-mediated Ras activation in B-lymphocytes has been reported (Zheng et al., 2005). Here, RasGRP3 depletion did not significantly affect migration in MEC1-GFP or MEC1-CD38H cells. In contrast, RasGRP2 down-regulation specifically affected MEC1-CD38H cells. A significant decrease in both basal migration and CCL21-directed chemotaxis was observed in MEC1-CD38H cells upon RasGRP2 depletion, whereas no effect was detected in the migration and chemotaxis of MEC1-GFP cells. However, a decrease in the basal Rap1 activity was observed in both MEC1-GFP and MEC1-CD38H cells upon RasGRP2 depletion. A possible explanation for these results could be that RasGRP2 is targeting different pools of Rap1 in the two cell populations and could involve differential localisation of RasGRP2 and/or Rap1. In agreement with this hypothesis, RasGRP2 showed a different pattern of subcellular localisation in MEC1-CD38H compared to MEC1-GFP cells. Whereas in MEC1-GFP cells RasGRP2 showed mostly a diffuse localisation, in MEC1-CD38H cells its localisation was more polarised. Further studies would be necessary to investigate if RasGRP2 polarised localisation coincides with functional subcellular structures such as the lamellipodium or uropod.

Co-localisation studies suggested that CD38 and RasGRP2 co-localise in MEC1-CD38H cells. In primary CLL cells, CD38 has been shown to co-localise with the α4-integrin subunit (CD49d) (Buggins et al., 2011; Zucchetto et al., 2012). Experiments should be performed to assess the localisation of integrins and the potential co-localisation with CD38 in MEC1-CD38H cells. It would be tempting to speculate that CD38-mediated localised Ca^{2+} signalling could be involved in RasGRP2 subcellular compartmentalisation, which could in turn mediate differential Rap1 activation and consequent integrin activation.

IQGAP1 is a scaffolding protein with multiple recognition motifs, including a sequence homology to the Ras GTPase-activating protein (that led to the protein name); however this domain is non functional and IQGAP1 is not able to stimulate Ras-GTP or Rap-GTP hydrolysis (Malarkannan et al., 2012; Weissbach et al., 1994). IQGAP1 was shown to be involved in the establishment of cell polarity and cell motility, by co-localising with actin filaments at the leading edge of polarised fibroblasts in complex with activated Cdc42 and Rac (Mataraza et al., 2003; Watanabe et al., 2004). It has been reported that B-lymphocytes express IQGAP1 but its function has not been investigated (Malarkannan et al., 2012). Here, migration studies suggest that the enhanced basal migration of MEC1-CD38H cells could partly depend on IQGAP1, as its depletion significantly decreased the migration only in MEC1-CD38H cells whereas no effect was observed in MEC1-GFP cells. Interestingly, this dependency appeared to be overcome in the presence of CCL21, as no decrease in chemotaxis was observed in MEC1-CD38H cells when IQGAP1 was down-regulated.

Signalling pathways involved in CLL cell motility and trafficking *in vivo* have only partly been elucidated. *In vitro* experiments performed on primary human CLL cells have shown that treatment with inhibitors of protein kinases involved in BCR signalling, such as Syk, PI3K and Btk, impairs cell chemotaxis and integrin-mediated cell adhesion (de Rooij et al., 2012; Hoellenriegel et al., 2012; Hoellenriegel et al., 2011). These data are in agreement with the clinical observations of a transient and rapid increase of CLL cells in the blood and a concurrent shrinkage of the lymph nodes in patients treated with these inhibitors, suggesting that they are able to affect not only BCR-mediated pro-survival signalling but also the motility properties of CLL cells

(Wiestner, 2012) (Chapter 1.5.4). Here, data obtained indicate that inhibition of Btk with ibrutinib inhibits the CCL21-mediated Rap1 activation in MEC1-GFP and MEC1-CD38H cells and partly reduces the CCL21-directed chemotaxis of MEC1 cells.

Taken together, data presented in this chapter suggest that the enhanced migratory properties of CD38-expressing MEC1 cells could be linked to higher activity of the GTPase Rap1. Interestingly, a role of RasGRP2 in cell migration was observed. This Ca^{2+} regulated Rap1 GEF was involved in migration and chemotaxis exclusively in the MEC1-CD38H cells, where it showed a polarised localisation. The differences in the signalling between MEC1-GFP and MEC1-CD38H cells could be linked, at least partly, to the higher basal intracellular Ca^{2+} level observed in the CD38-expressing cells.

5. CD38-associated signalling in primary CLL cells

5.1 Introduction

A high degree of variability in the motility of CLL cells between patients has been reported, linked to differential expression of several molecules involved in cell migration such as CCR7 (Till, 2002), VLA-4 integrin (α4β1) (Till, 2002; Till et al., 2005) ZAP-70 (Calpe et al., 2011) and CD38 (Deaglio et al., 2007a).

Despite the fact that Rho and Ras GTPases, together with their regulators, play a central role in lymphocyte motility (Chapter 1.4), their role in CLL is largely unknown. As previously discussed (Chapter 4.2.2), CXCL12-mediated Rap1 activation in CLL cells has been reported to be variable between patients (Till et al., 2008), possibly due to defective Rap1 endosomal recycling (Pye et al., 2013).

Data described in Chapters 3 and 4 suggested that CD38 could enhance adhesion and motility of MEC1 cells by up-regulating Rap1 activity, which in turn could be mediated by intracellular Ca^{2+} content and the Ca^{2+}-regulated Rap1 GEF, RasGRP2. Interestingly, recent studies have shown that CLL cases with a cytogenetic trisomy 12 abnormality (described in Chapter 1.5.1.2) have increased gene expression of RasGRP2, Rap1B and its effector RAPL compared to other CLL cases (Riches et al., 2014). This correlated with an enhanced random motility and spreading mediated by the integrin VLA-4. Notably, surface expression levels of CD38 are also up-regulated in trisomy 12 CLL cases (D'Arena et al., 2001; Riches et al., 2014).

The molecular mechanism underlying the role of CD38 in the motility of CLL cells has not been elucidated so far. In this chapter, the effect of CD38 expression on the signalling pathway involving Rap1 and RasGRP2 in primary CLL cells is investigated for the first time. Intracellular Ca^{2+} levels, migration properties and Rap1 activation were investigated in a cohort of primary CLL samples expressing different CD38 surface levels. The intracellular distribution of RasGRP2 and its link with CD38 expression and localisation was also tested.

5.2 Analysis of Ca^{2+} signalling in CLL

5.2.1 Analysis and comparison of intracellular [Ca^{2+}] in primary CLL cells expressing different levels of CD38

Data obtained with the MEC1 cell line and described in Chapter 4 showed that MEC1-CD38H cells have a higher basal Ca^{2+} level compared to the control MEC1-GFP cells (Chapter 4.3.1). This suggested that CD38 expression could modify Ca^{2+} signalling in CLL cells by enhancing the intracellular [Ca^{2+}] in cells in resting conditions. In order to analyse if CD38 expression was linked to increased [Ca^{2+}] in primary CLL cells, CLL patient samples with different levels of CD38 expression were compared. Clinical details of samples used in this study are listed in Table 1. Based on a cut-off value set at 30% of CD38-expressing cells within each sample, CLL samples were categorised into $CD38^{low}$ and $CD38^{high}$. This cut-off was chosen as it was shown in previous studies to reflect CD38 prognostic impact (Damle et al., 2007; Malavasi et al., 2011) (Chapter 1.5.1.4). Cells loaded with Indo-1 were analysed by flow cytometry to determine basal intracellular [Ca^{2+}] and after stimulation with CCL21. A total of 18 CLL samples were analysed in three experiments. Each experiment was independently performed using 6 CLL samples. Because of the inherent inter-experimental variability and consequent difference in the range of Indo-1 fluorescence ratio values between the different experiments, intracellular Ca^{2+} levels were compared between the samples in the same experiment. Data obtained indicated that high CD38 expression was associated with an increased basal [Ca^{2+}]. With one exception (CLL 11, of the 18 CLL samples tested), $CD38^{low}$ samples showed a significantly lower level of basal intracellular [Ca^{2+}] compared to the $CD38^{high}$ samples (Fig. 5.1). No detectable changes in the intracellular [Ca^{2+}] after stimulation with CCL21 were observed in the samples analysed. However, CCR7 expression levels should be analysed to draw any conclusion regarding the effect of CCL21 stimulation on the intracellular [Ca^{2+}].

Table 5. 1 Clinical data of CLL samples used in this study

NA = not available

PATIENT ID	IGVH STATUS	CD38 EXPRESSION	Cytogenetics (FISH)	GENDER (M/F)/ AGE (YEAR)
CLL 1	UNMUTATED	97%	Normal karyotype	M/65
CLL 2	UNMUTATED	94%	13q14.3 del; 11q22.3 del; 17p13.1 del; Trisomy 12	F/75
CLL 3	UNMUTATED	84%	NA	M/73
CLL 4	UNMUTATED	18%	NA	NA/45
CLL 5	MUTATED	0%	Normal karyotype	F/74
CLL 6	MUTATED	3%	13q14.3 del	M/52
CLL 7	UNMUTATED	99%	Trisomy 12	F/51
CLL 8	MUTATED	91%	13q14.3 del	M/68
CLL 9	UNMUTATED	83%	Trisomy 12	M/74
CLL 10	MUTATED	4%	Normal karyotype	F/35
CLL 11	MUTATED	1%	NA	M/62
CLL 12	MUTATED	3%	NA	NA
CLL 13	MUTATED	65%	13q14.3 del	M/61
CLL 14	UNMUTATED	61%	13q14.3 del; 11q22.3 del	M/67
CLL 15	UNMUTATED	55%	NA	M/74
CLL 16	UNMUTATED	27%	13q14.3 del	M/46
CLL 17	UNMUTATED	23%	13q14.3 del; 17p13.1 del	M/52
CLL 18	NA	12%	Normal karyotype	M/55
CLL 19	MUTATED	98%	17p13.1 del	F/38
CLL 20	NA	0%	NA	M/51
CLL 21	NA	0%	NA	F/76
CLL 22	NA	92%	13q14.3 del; 11q22.3 del	M/72

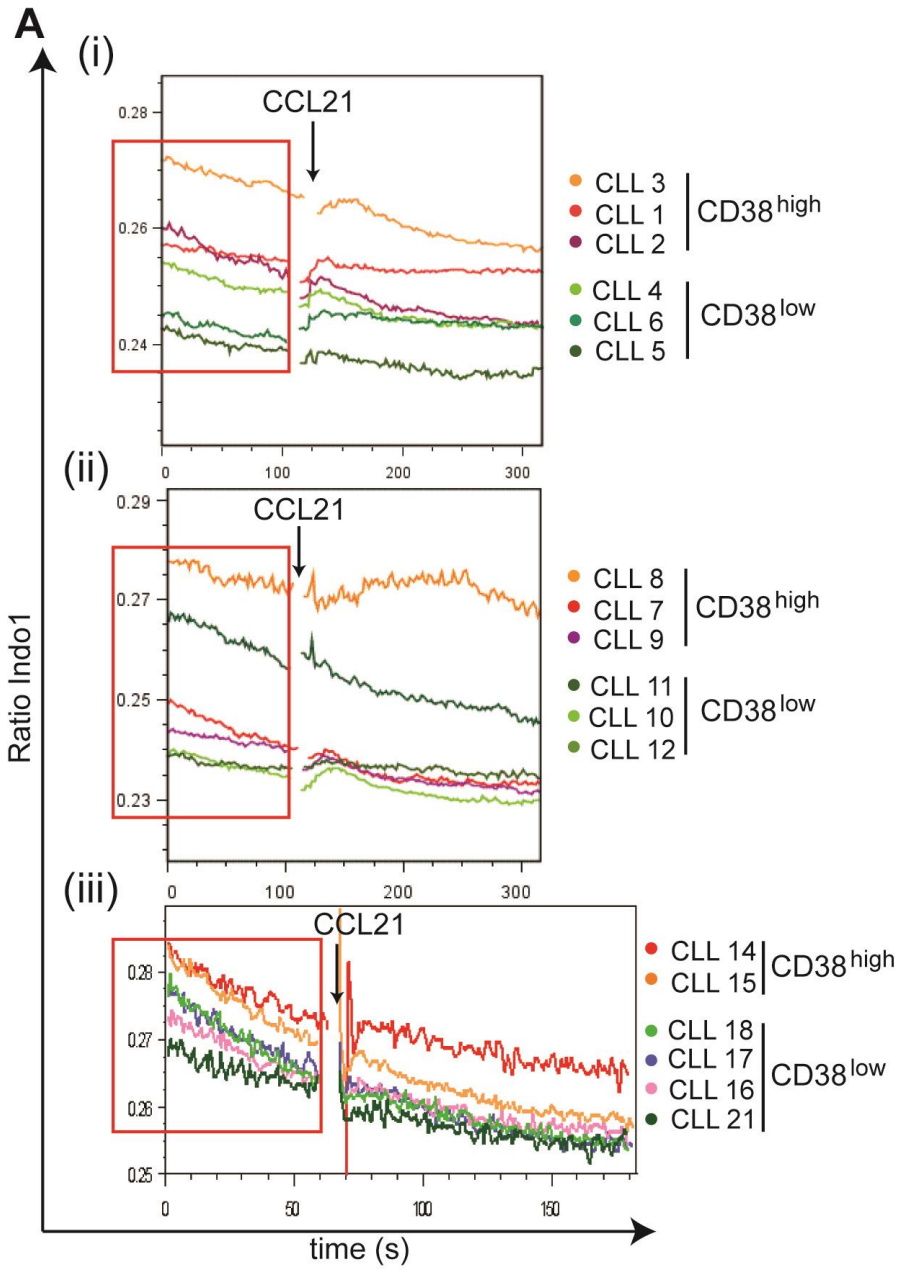
A
(i)
CCL21
0.28
0.26
0.24
0
100
200
300
CLL 3
CLL 1
CLL 2
CD38high
CLL 4
CLL 6
CLL 5
CD38low
(ii)
CCL21
0.29
0.27
0.25
0.23
CLL 8
CLL 7
CLL 9
CD38high
CLL 11
CLL 10
CLL 12
CD38low
(iii)
CCL21
0.28
0.27
0.26
0.25
0
50
100
150
CLL 14
CLL 15
CD38high
CLL 18
CLL 17
CLL 16
CLL 21
CD38low
Ratio Indo1
time (s)
B
n = 18
P = 0.031
0.30
0.28
0.26
0.24
0.22
A(i)
A(ii)
A(iii)
Ratio Indo1
1
2
3
Experiments
CD38high
CD38low

Figure 5.1 Analysis of the intracellular [Ca^{2+}] in $CD38^{low}$ and $CD38^{high}$ CLL samples. Primary CLL samples (n=18, 6 samples analysed in each independent experiment) with varying CD38 expression levels were incubated with the ratiometric Ca^{2+} detector Indo-1 (1 μM) and analysed by flow cytometry. The basal fluorescence level was recorded for 1 minute before stimulation with CCL21 (100 ng/ml) and then for a further 2 minutes. (A i, ii and iii) Bivariate plots of the median of fluorescent intensity ratio (Indo-1 emission wavelengths - 420/510 nm) against time obtained in each independent experiment. Red box indicates the basal fluorescence levels. (B) The peak of the fluorescence intensity of the basal fluorescence level was obtained for each CLL sample using FlowJo Kinetic statistic tool. Horizontal bars indicate mean values of the fluorescence intensity peak calculated in each experiment by grouping $CD38^{low}$ (green) and $CD38^{high}$ (red) samples. Significance of difference between $CD38^{low}$ and $CD38^{high}$ samples was obtained using two-tailed paired Student's t-test.

5.2.2 Ca^{2+} mobilisation in response to surface IgM stimulation

As previously discussed (Chapter 4.3.2), an overall association between CD38 expression and BCR responsiveness has been described in CLL, possibly linked to the fact that CD38 expression in CLL samples generally correlates with their unmutated IGVH status (Cutrona et al., 2008; Damle et al., 1999; Mockridge et al., 2007). Here, the ability of CLL cells to increase the intracellular free [Ca^{2+}] following IgM stimulation was analysed in 16 CLL patient samples. CLL cells were loaded with Indo-1 and analysed by flow cytometry; the percentage of responding cells was obtained as previously described (Chapter 2.2.14). In agreement with previous studies (Cutrona et al., 2008; Mockridge et al., 2007), the response to IgM stimulation was variable between CLL patient samples. A representative plot showing two CLL samples with different degrees of Ca^{2+} response upon IgM stimulation is shown (Fig. 5.2, A). Plotting CD38 expression (as a continuous variable) against the percentage of responding cells showed a modest positive correlation (Pearson's correlation coefficient = 0.57) (Fig. 5.2, B). However, as most of the CLL cases with a high CD38 expression were U-CLL (depicted in red), the responsiveness of these CLL samples is likely to depend on their IGHV mutational status. Importantly, as shown by previous studies (Coelho et al., 2013; Mockridge et al., 2007), CLL cell ability to respond to IgM stimulation is dependent on the surface levels of IgM. The expression levels of sIgM

should therefore be tested in order to provide a deeper understanding of the IgM responsiveness within the CLL samples here analysed.

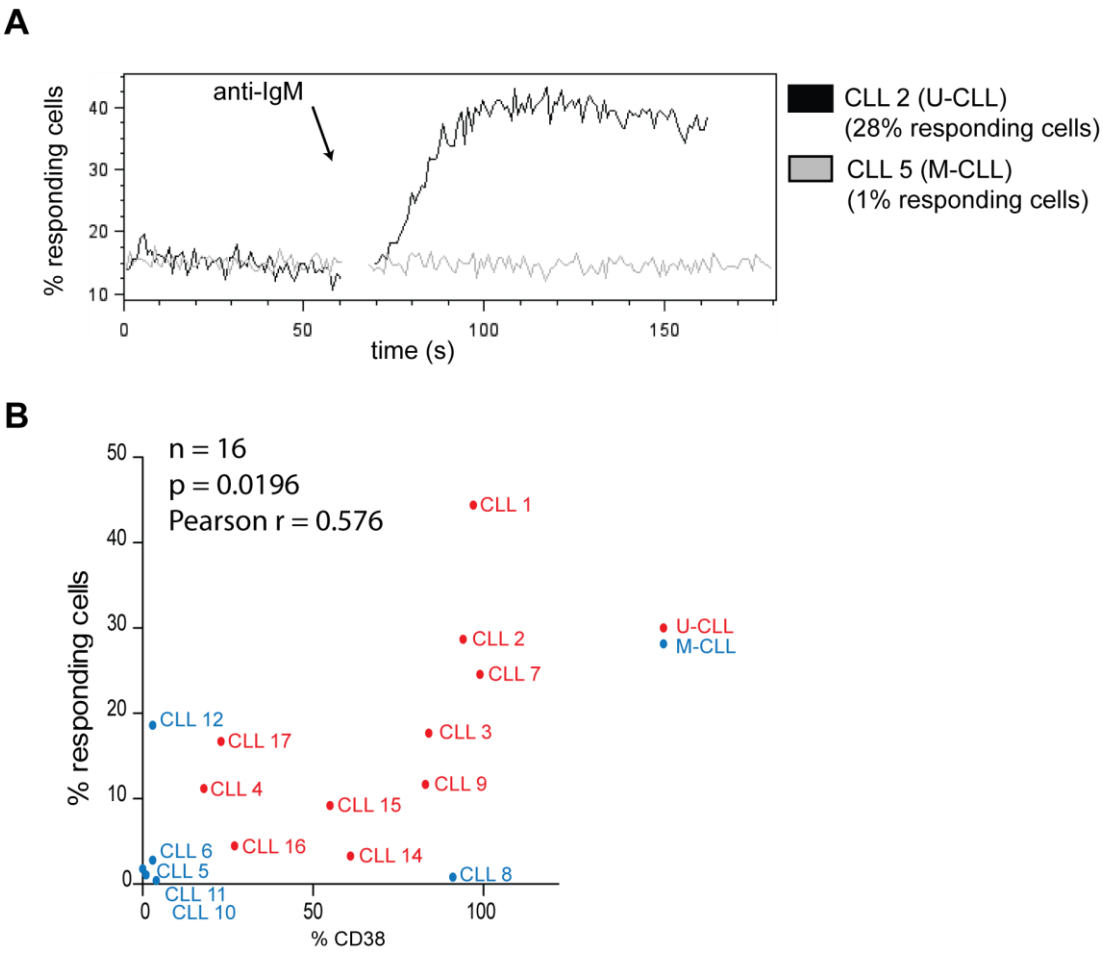

Figure 5.2 BCR-induced Ca^{2+} response in primary CLL cells.
Primary CLL cell samples, with different levels of CD38 expression and IGHV mutational status, were incubated with the ratiometric Ca^{2+} detector Indo-1 (1 µM) and analysed by flow cytometry. The basal fluorescence level was recorded for 1 minute before stimulation with anti-IgM (20 µg/ml) and then for a further 2 minutes. (A) Representative bivariate plot obtained by establishing the 85th percentile of basal fluorescence ratio intensity as a threshold. Overlay of flow cytometric data obtained from two CLL clones and respective % of responding cells are shown. (B) The % of cells with a fluorescence intensity ratio above the threshold (% responding cells) after stimulation with IgM was obtained with FlowJo Kinetic statistic tool. Values obtained for each CLL sample analysed (n=16) were plotted against the respective % CD38 expression. U-CLL and M-CLL samples are depicted in red and blue respectively.

5.3 Analysis of basal and CCL21-directed migration of primary CLL cells

As described in Chapter 3, CD38-expressing MEC1 showed an increased ability to migrate both in the absence and in the presence of a gradient of CCL21 compared to control cells. To investigate the migratory ability of primary CLL samples with differential expression levels of CD38, the Transwell™ assay was used. A total of 6 CLL patient samples were tested that had available cell numbers for both motility and Rap1 activity assays (described in Section 5.4): 3 $CD38^{high}$ and 3 $CD38^{low}$ CLL samples (patient ID and CD38 expression are listed in Table 5.2).

Table 5.2 Patient IDs and relative CD38 expression levels of CLL samples used for cell migration / Rap1 activity assay

PATIENT ID	CD38 EXPRESSION
CLL 1	97%
CLL 2	94%
CLL 8	91%
CLL 5	0%
CLL 6	3%
CLL 21	0%

In agreement with previous studies (Deaglio et al., 2007a; Vaisitti et al., 2010), $CD38^{high}$ CLL samples showed an enhanced migration towards CCL21 compared to $CD38^{low}$ CLL samples. Notably, an increased basal migration was also observed in the $CD38^{high}$ CLL samples (Fig. 5.3) compared to the $CD38^{low}$ ones.

The expression of the CCL21 receptor, CCR7, was analysed by flow cytometry analysis in 4 out of the 6 (2 $CD38^{high}$ and 2 $CD38^{low}$) CLL samples. All the 4 samples expressed CCR7, with a variable mean of fluorescence intensity (MFI) (Fig. 5.4). No correlation between CCR7 and CD38 expression was observed, in agreement with data obtained in the MEC1 cell line (Chapter 3.7), suggesting that the increased chemotaxis observed in the $CD38^{high}$ CLL samples is not likely to be due to an enhanced expression of CCR7.

Expression levels of the α4-integrin subunit (CD49d) were also investigated in the 2 $CD38^{high}$ and 2 $CD38^{low}$ CLL samples. The 4 samples showed a high variability in the level of α4 integrin expression (Fig. 5.5). A strong correlation between CD38 and α4 integrin expression in CLL cells has been previously reported (Buggins et al., 2011; Herens et al., 2006). Here, in agreement with this data, the 2 $CD38^{high}$ CLL samples (CLL 1 and CLL 8) showed a higher expression of α4-integrin compared to the 2 $CD38^{low}$ samples (CLL 6 and CLL 21). Therefore it can not be excluded that the increased α4 integrin expression could have a role in the increased migration observed in the $CD38^{high}$ primary CLL sample tested.

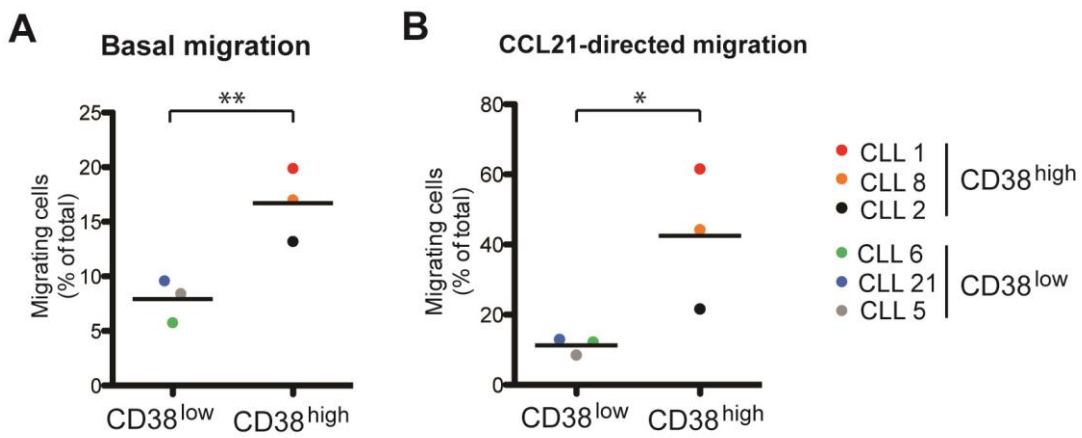

Figure 5.3 CD38 expression is linked to enhanced CLL cell migration and chemotaxis. Primary CLL cells were added to Transwell™ filters previously coated with VCAM-1 (n=6). After 4 hours cells that had transmigrated through the filters into the lower wells (A) in the absence of added chemoattractant or (B) in the presence of CCL21 (100 ng/ml) added to the bottom chamber were counted using a Casy™ counter. The % of migrating cells was obtained by dividing the number of transmigrated cells by the total number of cells added to the Transwell™ filters. Horizontal bars indicate mean values of migrating cells by grouping $CD38^{low}$ and $CD38^{high}$ samples. Data shown are from one experiment; *p<0.05, **p<0.01, determined by two-tailed Student's t-test.

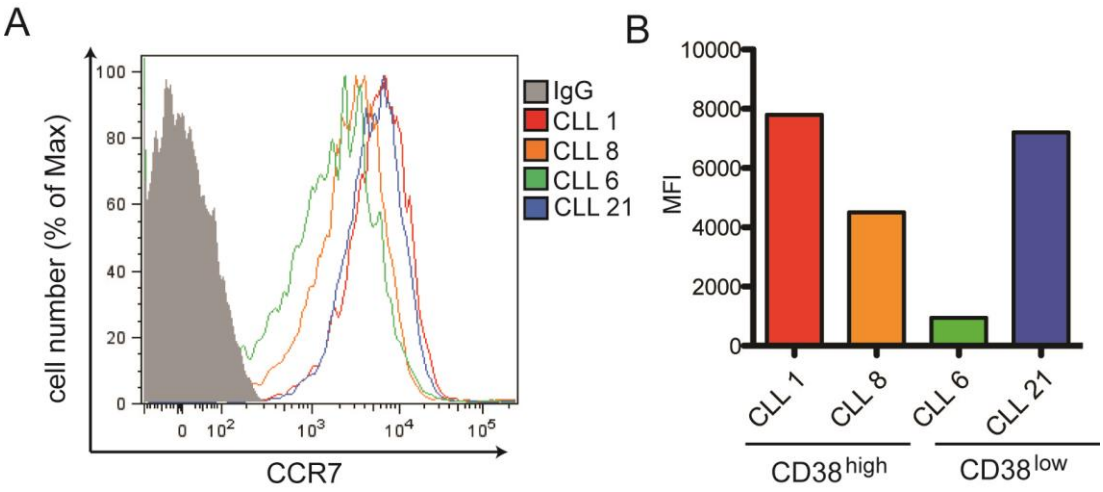

Figure 5.4 CCR7 surface expression in CD38^{low} and CD38^{high} CLL samples.
Surface levels of CCR7 were tested in 2 CD38^{low} and 2 CD38^{high} CLL samples. Cells were stained with a anti-CCR7 antibody and subsequently analysed by flow cytometry. IgG isotype control was used to detect non specific signal. (A) Overlay of flow cytometric data and (B) mean of the fluorescence intensity (MFI) obtained from the analysis of the 4 samples in one single experiment are shown.

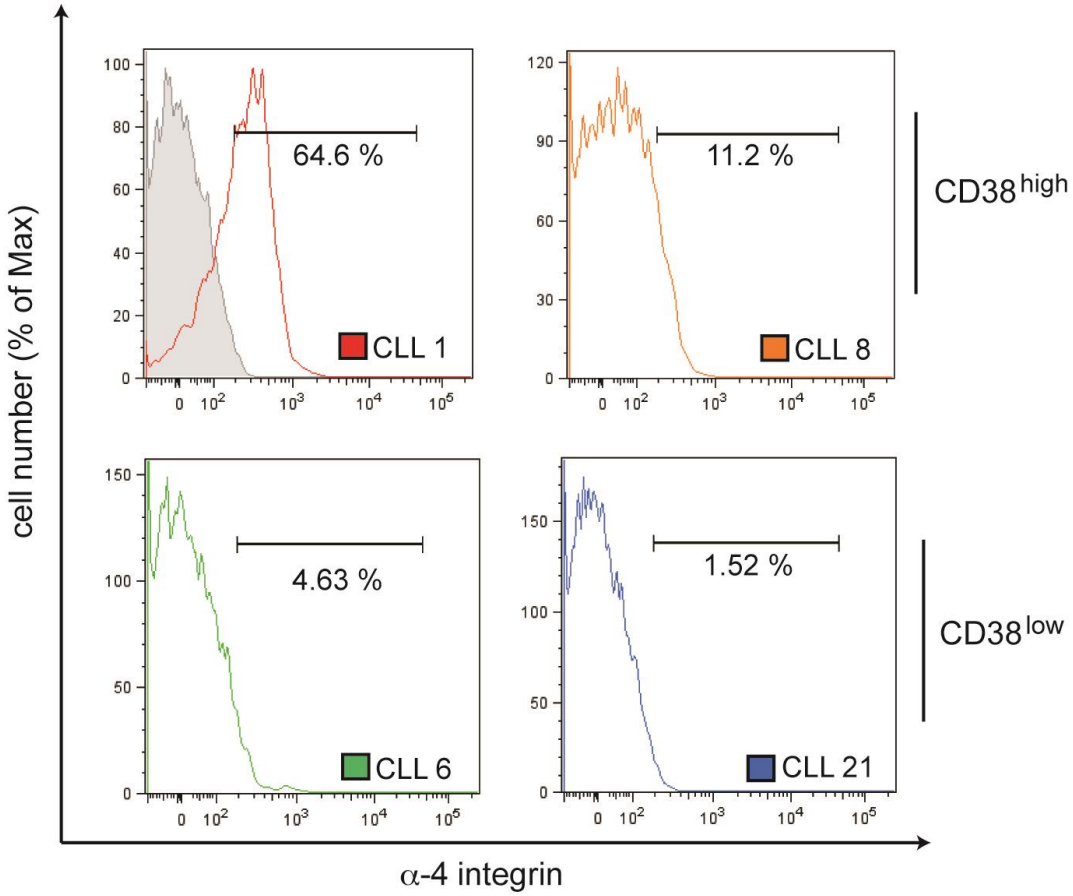

Figure 5.5 α4-integrin surface expression in CD38^{low} and CD38^{high} CLL samples. Surface levels of α4 integrin were tested in CLL patient samples with differential CD38 expression, 2 CD38^{high} (upper panels) and 2 CD38^{low} (lower panels). Cells were stained with a anti-α4-integrin antibody and subsequently analysed by flow cytometry. IgG isotype control was used to detect non-specific signal. Flow cytometric data obtained for each of the 4 CLL samples and percentage of cells expressing α4-integrin are shown. Data are from one single experiment.

5.4 Analysis of Rap1 activity in primary CLL cells

Data described in Chapter 4 indicated that CD38-expressing MEC1 cells have an enhanced basal level of active Rap1 (Chapter 4.2.2). MEC1-CD38H cells showed a stronger dependency on Rap1, both for random migration and chemotaxis, compared to MEC1-GFP cells (Chapter 4.2.3). Moreover, Ca^{2+} depletion reduced the enhanced basal Rap1 activity in MEC1-CD38H cells whereas no effect was observed in the MEC1-GFP population. To investigate if CD38 expression in primary CLL cells was associated with increased Rap1 activity, the basal level of active Rap1 was investigated and compared among the 6 primary CLL samples previously tested for migration. The Rap1-GTP was isolated in in the 6 CLL samples both in the presence of Ca^{2+} and after Ca^{2+} depletion (Chapter 2.2.5.3). Total and active Rap1 levels were assessed by western blotting, protein quantification was performed by densitometry analysis and comparison was performed between samples run on the same blot. Whereas a small level of variability was observed in the total Rap1 expression between the samples (Fig. 5.6, C), active Rap1 levels observed in the 3 $CD38^{high}$ samples in the presence of Ca^{2+} were highly enhanced when compared to the $CD38^{low}$ samples, with the latter showing barely detectable Rap1-GTP levels (Fig. 5.6, A and B). Of note, one of the 3 $CD38^{high}$ CLL patient samples was Trisomy 12 (CLL 2 - Table 5.1). Calcium depletion decreased the basal Rap1 activity in all the 3 $CD38^{high}$ samples analysed, suggesting that the basal calcium levels in the $CD38^{high}$ samples could be involved in the up-regulation of basal Rap1 activity. Due to the extremely low levels of Rap1-GTP detected in the $CD38^{low}$ samples, it was no possible to draw any conclusion regarding the effect of Ca^{2+} depletion in these samples.

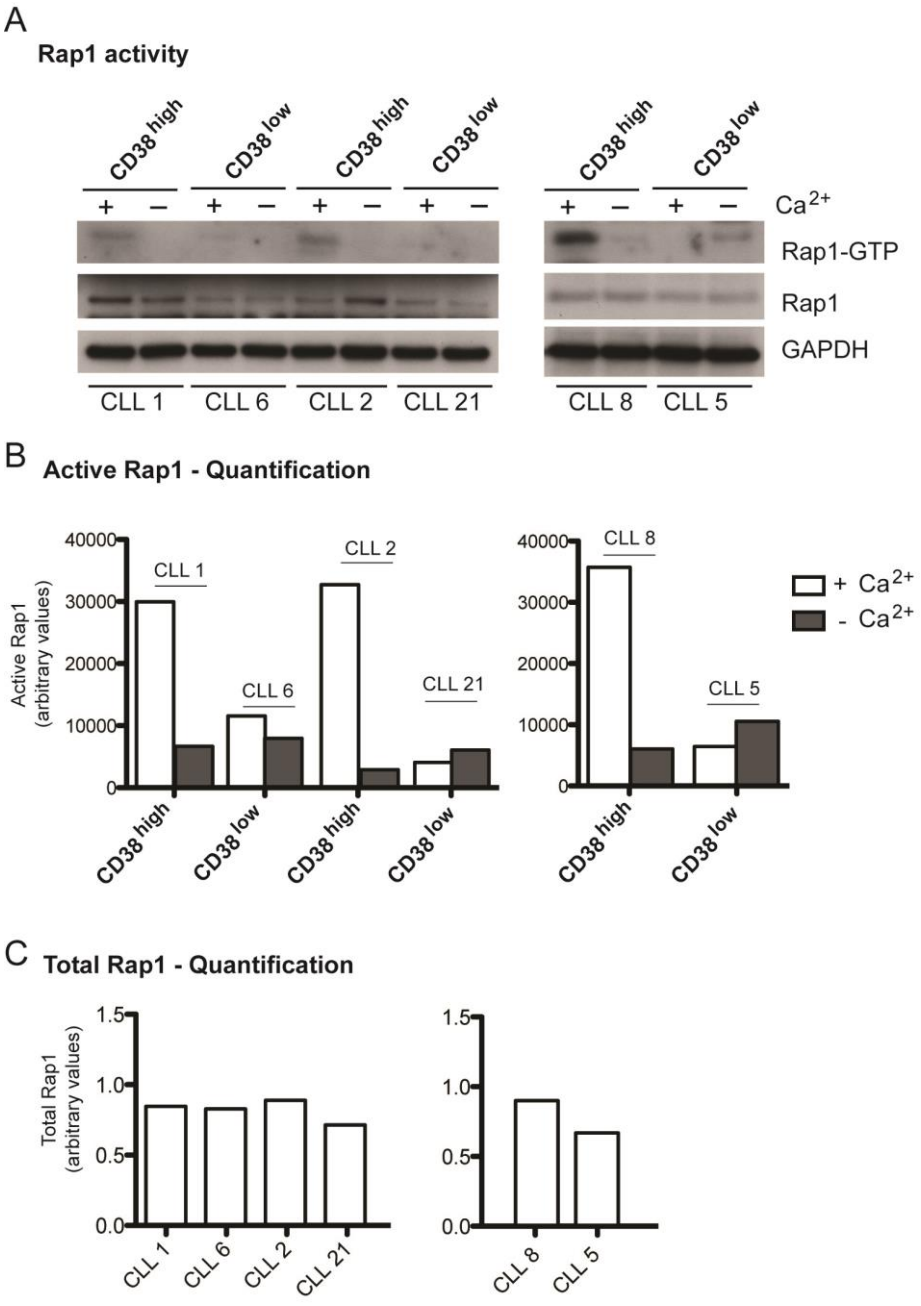

Figure 5.6 Calcium depletion reduces the enhanced basal Rap1 activity in CD38^{high} CLL cells.

CD38^{low} and CD38^{high} primary CLL samples were resuspended in calcium-free PBS and treated with the Ca^{2+} ionophore ionomycin (0.5 μM) for 5 minutes (Ca^{2+} -) or in PBS containing calcium and treated in parallel with the same amount of DMSO as a vehicle control (Ca^{2+} +). (A) Cells were lysed and lysates obtained were incubated with GST-RalGDS fusion protein and then analysed by western blotting with anti-Rap1 antibody. GAPDH was used as a loading control. (B) Levels of active Rap1 were quantified by densitometry using ImageJ and normalised on the respective total Rap1. (C) Levels of total Rap1 were quantified on samples in control condition (Ca^{2+} +) and normalised to the respective GAPDH level. Data shown are from one single experiment.

5.5 CD38 expression is linked to an increased RasGRP2 polarised localisation in primary CLL cells.

CD38 was originally identified as a cell surface protein in T-lymphocytes (Bhan et al., 1980). Whereas it has been reported that CD38 exclusively localises at the cell membrane of murine B-lymphocytes (Moreno-Garcia et al., 2005b), other studies have suggested that CD38 can localise in other intracellular compartments such as endosomes and on the nuclear membrane in human B and T cell line models (Muñoz et al., 2008; Orciani et al., 2008). Previous studies performed in primary CLL cells have shown that CD38 subcellular localisation patterns are variable between different CLL patient samples (Buggins et al., 2011).

Data described in Chapter 4 indicated that ectopic CD38 and endogenous RasGRP2 co-localise in MEC1-CD38H cells (Chapter 4.6.3). The subcellular localisation of CD38 and RasGRP2 was investigated in CLL cells from 3 $CD38^{high}$ patients. CD38 showed different patterns of localisation among cells of the same CLL sample, varying from a polarised localisation to a more diffuse one. Co-localisation of CD38 and RasGRP2 was observed in all the samples. However, due to the variability of the subcellular distribution of CD38, different levels of co-localisation were detected (Fig. 5.7, B and C).

Notably, RasGRP2 subcellular distribution was polarised in the 3 $CD38^{high}$ samples analysed (Fig. 5.7). As previously described (Chapter 4.6.3), RasGRP2 subcellular localisation was more polarised in the CD38-expressing MEC1 cells compared to MEC1-GFP cells. In order to investigate if CD38 expression was linked to a differential RasGRP2 subcellular localisation in primary CLL cells, CLL cells from 14 patients with different levels of CD38 expression were analysed. Immunofluorescence studies showed that RasGRP2 localisation was variable between the samples (Fig. 5.8). A RasGRP2 polarisation index was obtained for each individual cell analysed in the 14 CLL samples and the average polarisation index was then calculated per each CLL sample (strategy used for polarisation analysis is described in Chapter 2.2.16). Average RasGRP2 polarisation indexes obtained varied between ≈0.5 and ≈3.5. When treating CD38 expression as a continuous variable, a statistically significant positive

correlation between RasGRP2 polarisation and CD38 expression was observed (Pearson's correlation coefficient = 0.7307) (Fig. 5.9).

F-actin staining showed that primary CLL cells generally have a rounded cell morphology (Fig. 5.8). In order to understand if CD38 expression was linked to differential F-actin subcellular distribution, a F-actin polarisation index was calculated. The degree of variability observed between the CLL samples analysed was lower compared to the one of RasGRP2, as values obtained varied between ≈0.5 and ≈1.5. No correlation was observed between CD38 expression and F-actin polarisation (Pearson's correlation coefficient = 0.497) (Fig. 5.10).

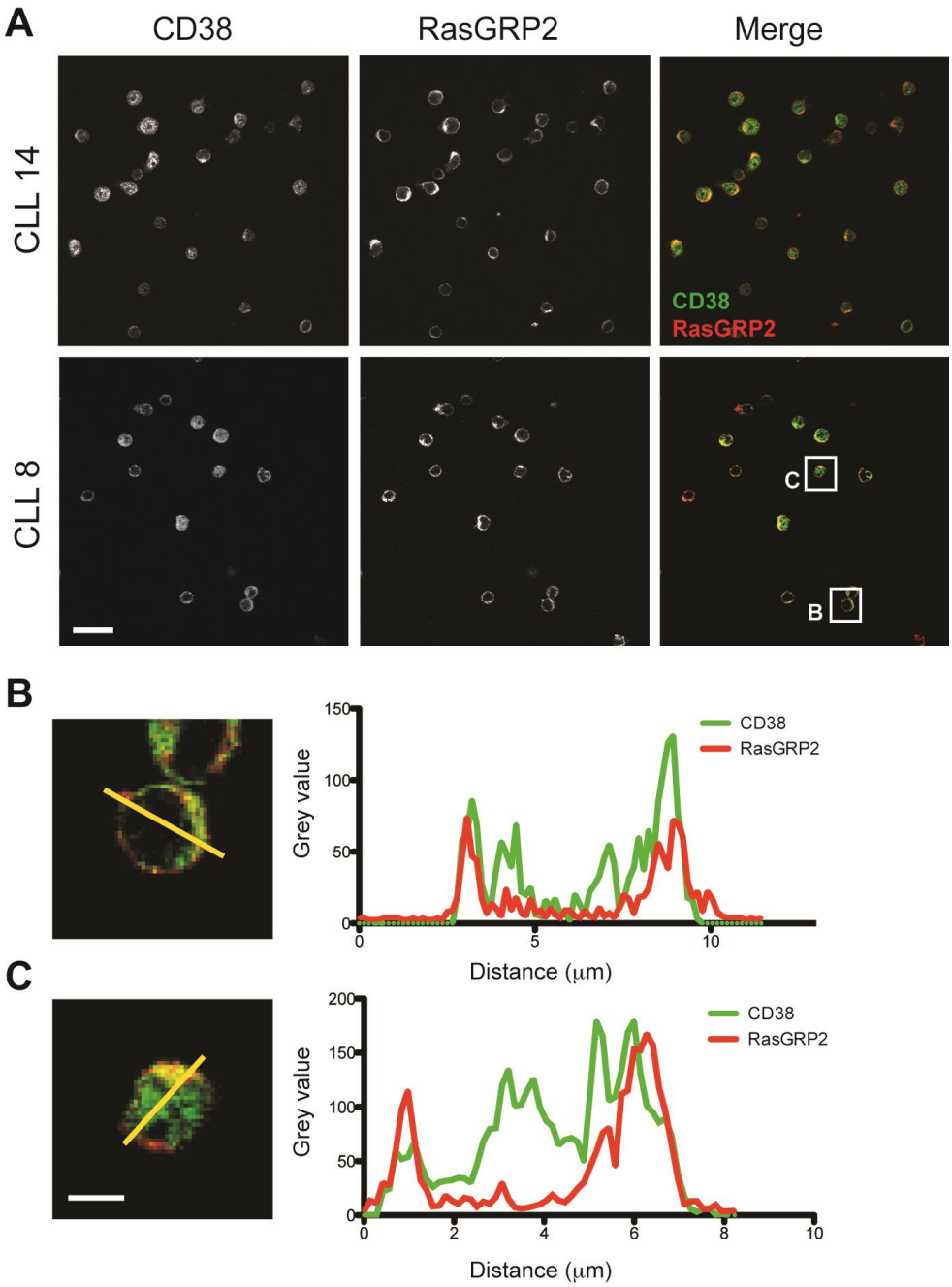

Figure 5.7 CD38 and RasGRP2 localisation in CLL cells.
CLL cells from CD38^{high} patients (n=3) were seeded on poly-L-lysine- coated coverslips and allow to adhere for 15 min. Cells were then fixed and stained for RasGRP2 and CD38. (A) Representative images from two patients are shown. Scale bar, 20 µm. (B and C) Representative fluorescence intensity profile of CD38 (green) and RasGRP2 (red) obtained along the axis indicated of one cell with a polarised CD38 localisation (B) and one cell with a more diffuse CD38 localisation (C). Scale bar, 5 µm. Images are representative of one single experiment.

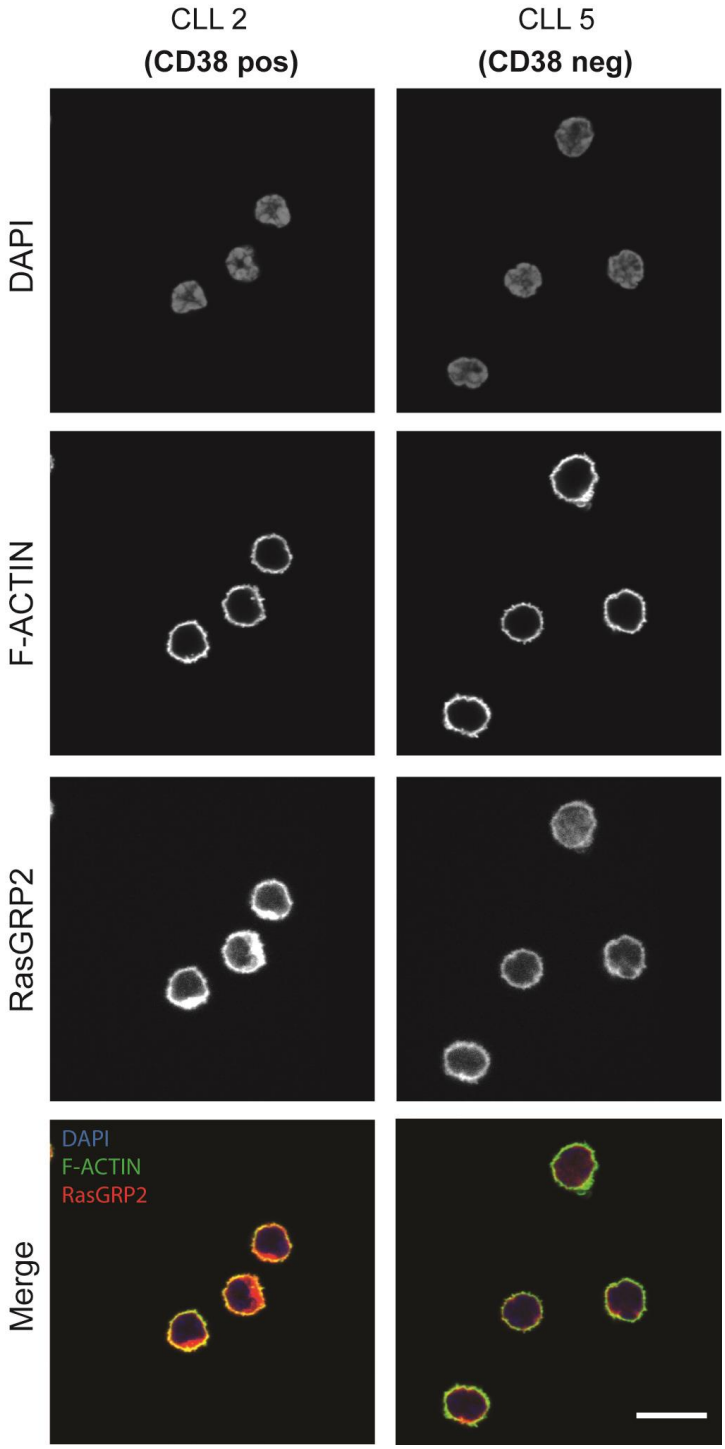

Figure 5.8 RasGRP2 localisation in CD38^{high} and CD38^{low} CLL samples.
Primary CLL cells were seeded on poly-L-lysin coated coverslips and allow to adhere for 15 min. Cells were then fixed and stained for RasGRP2. Actin filaments (F-actin) were visualised by phalloidin staining. Scale bar, 10 µm. Representative images of one CD38^{high} (CLL 2) and one CD38^{low} (CLL 5) CLL sample are shown.

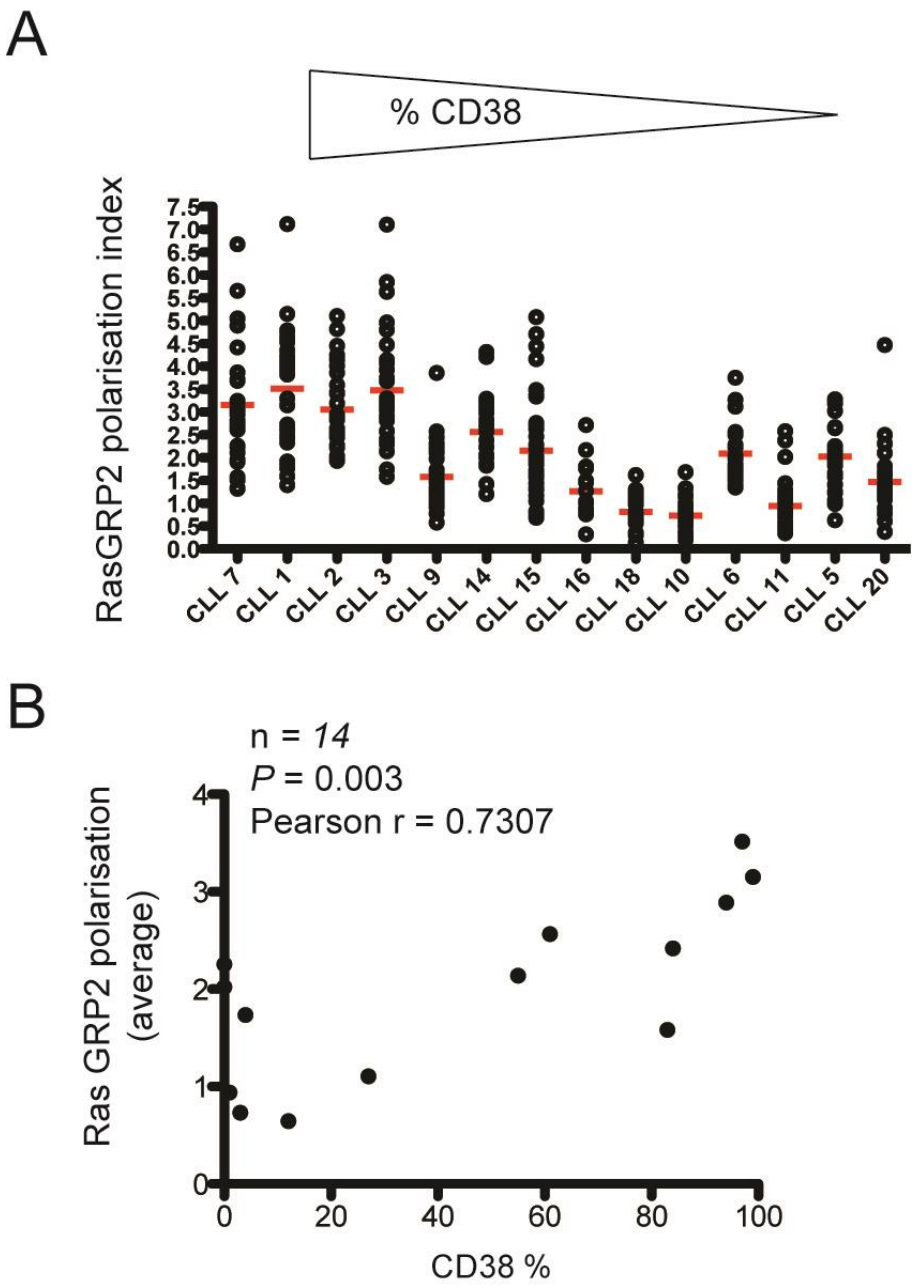

Figure 5.9 RasGRP2 polarisation correlates with CD38 expression in primary CLL cells.

CLL cells from 14 patients with different CD38 expression levels were seeded on poly-L-lysin coated coverslips and allow to adhere for 15 min. Cells were then fixed and stained for RasGRP2. The RasGRP2 polarisation index, indicating distribution of the fluorescence intensity, was obtained using ImageJ Oval Profile plug-in (method described in Chapter 2.2.16). (A) Graph shows the values obtained for each individual cell analysed (n ≥ 30 cells were analysed per sample). Horizontal red bars indicate mean values (A). (B) Mean values obtained per each patient sample were plotted against each respective CD38 expression.

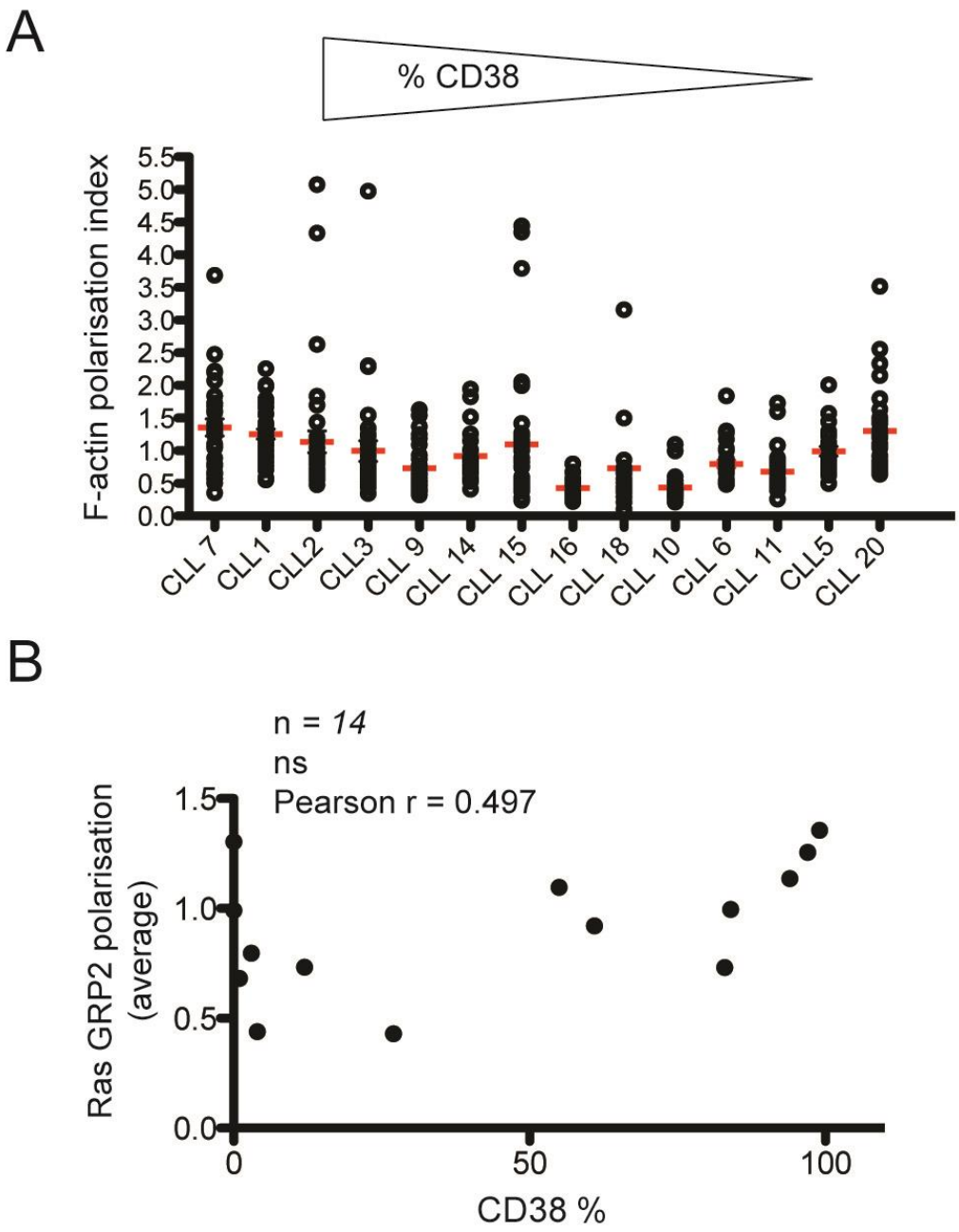

Figure 5.10 F-actin polarisation in primary CLL cells.
CLL cells from 14 patients with different CD38 expression levels were seeded on poly-L-lysin coated coverslips and allow to adhere for 15 min. Cells were then fixed and actin filaments (F-actin) were visualised by phalloidin staining. The F-actin polarisation index, indicating distribution of the fluorescence intensity, was obtained using ImageJ Oval Profile plug-in. At least 30 cells were analysed per sample and results are shown. Horizontal red bars indicate mean values (A). (B) Mean values obtained per each patient were plotted against each respective CD38 expression level.

5.6 Effect of Ibrutinib on Rap1 activity in primary CLL cells

Previous studies have shown that treatment with the BTK inhibitor Ibrutinib can reduce CLL cell migration and adhesion (de Rooij et al., 2012). Moreover, Ibrutinib inhibited the CCL21-induced activation of Rap1 in MEC1 cells (Chapter 4.8). In order to investigate if Ibrutinib could inhibit Rap1 activation in primary CLL cells, the effect of Ibrutinib on the basal and CCL21-induced Rap1 activity was tested in CLL cells from 3 patients. One $CD38^{high}$ (CLL 8) and two $CD38^{low}$ (CLL 18 and CLL 21) (Table 5.1) CLL samples were tested independently. Previous studies have shown that Rap1 activation upon CXCL12 stimulation is variable between patients (Pye et al., 2013; Till et al., 2008). In agreement with this data, only 2 of the 3 patients tested (1 $CD38^{high}$ and 1 $CD38^{low}$) showed Rap1 activation in response to CCL21 stimulation in the absence of Ibrutinib (Fig. 5.11). The effect of Ibrutinib treatment was also variable between the samples analysed. Of the 2 CCL21 responders, only the $CD38^{high}$ CLL sample was affected by Ibrutinb: Rap1 activation following CCL21 stimulation was completely abolished after treatment with the inhibitor in this sample. No effect was observed on CCL21-induced Rap1 activation in the $CD38^{low}$ CCL21 responder CLL sample (CLL 21). Ibrutinib treatment on the $CD38^{low}$ CCL21-unresponsive sample induced an overall decrease of Rap1-GTP levels. To test whether the cells were responding to CCL21 pathway, ERK phosphorylation (pERK) was assessed. In contrast with what was observed in MEC1 cells, no clear increase of ERK phosphorylation was detectable after 1 minute of CCL21 stimulation (Fig 5.11). Consequently, the effect of Ibrutinib on pERK could not be investigated. Due to the high cell number required to perform the assay, the number of patients analysed was limited by the sample availability. Screening more CLL samples would be required to draw any conclusion on the effect of Ibrutinib on Rap1 activity.

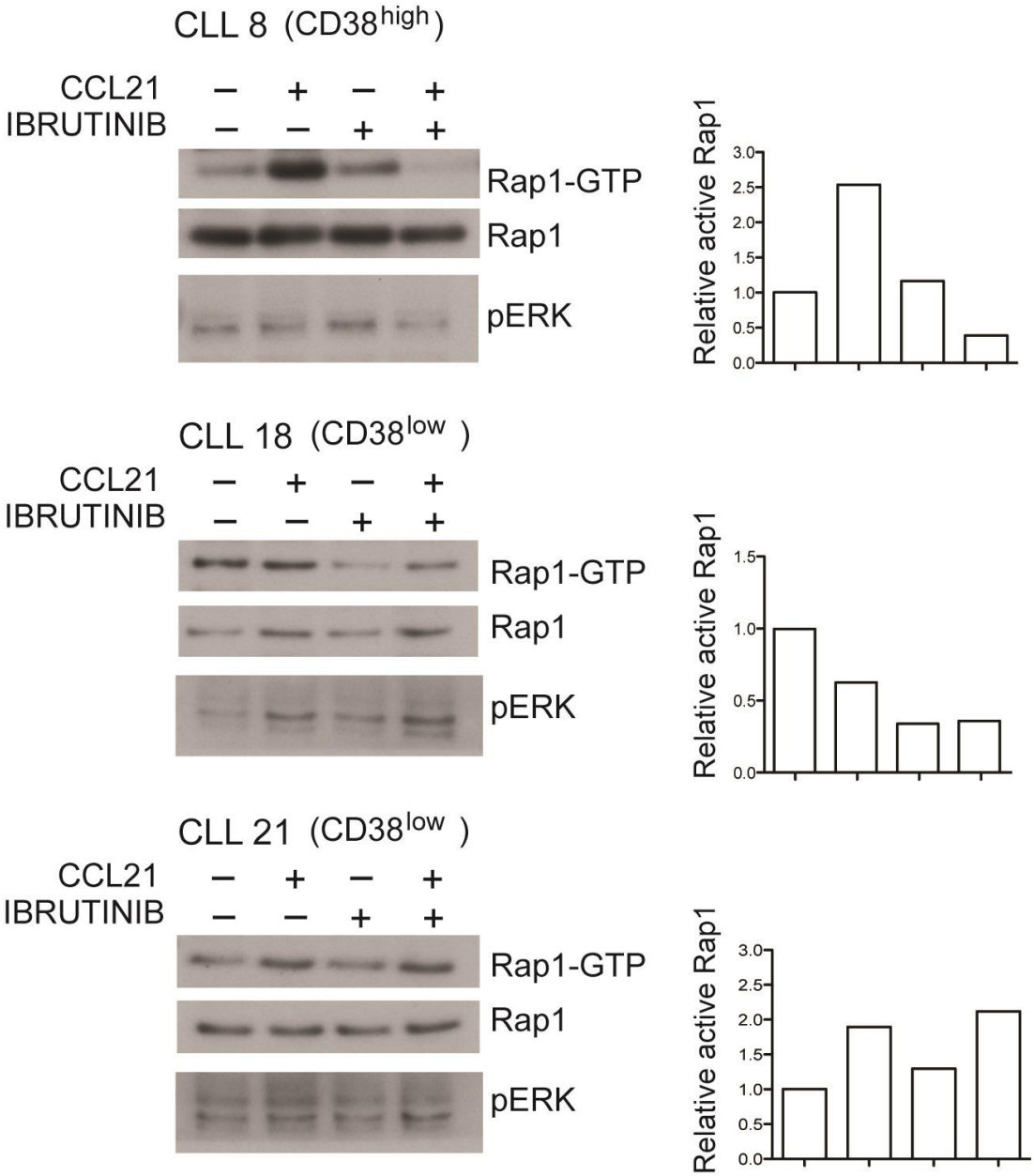

Figure 5.11 Effect of Ibrutinib on Rap1 activity in primary CLL cells.
CLL cells from 3 patients (one CD38^{high} and 2 CD38^{low}) were treated with 1µM Ibrutinib (or with DMSO as a vehicle control) for 1 hour before being stimulated with CCL21 (100 ng/ml) for 1 min. Cell lysates were obtained, incubated with GST-RalGDS fusion protein and then analysed by western blotting with anti-Rap1 and anti-pERK antibodies. Each sample was analysed independently. Relative levels of active Rap1 were quantified by densitometry using ImageJ and normalised to total Rap1. Data shown are from one single experiment.

5.7 Discussion

In Chapter 4 it was shown that MEC1-CD38H cells have an increased Rap1 activity compared to MEC1-GFP control cells, possibly induced by an enhanced intracellular Ca^{2+} level that could modulate RasGRP2 activity. This mechanism could explain the increased motility observed in the MEC1-CD38H cells compared to control cells (Chapter 3). The aim of the present chapter was to investigate this signalling pathway in primary CLL cells expressing differential levels of CD38.

Whereas a role for CD38 in the Ca^{2+} response to different stimuli was previously shown in formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated neutrophils (Partida-Sánchez et al., 2001) and dendritic cells stimulated with a subset of chemokines (Partida-Sánchez et al., 2004), the involvement of CD38 in regulating the basal intracellular [Ca^{2+}] is still undefined. A recent study performed using the MEC1 cell line showed that wild-type CD38-expressing MEC1 have increased intracellular [Ca^{2+}] (Vaisitti et al., 2014). In the same study, expression of the enzymatically inactive form of CD38 was not able to modulate the intracellular [Ca^{2+}], suggesting that the CD38 enzymatic products (cADPR, NAADP and ADPR) are involved in this effect. Here, data obtained by comparison of intracellular basal free [Ca^{2+}] levels between $CD38^{high}$ and $CD38^{low}$ CLL samples suggested that CD38 expression could be linked to an enhanced intracellular basal [Ca^{2+}] in primary CLL cells. However, whether the increased basal [Ca^{2+}] observed here can be directly ascribed to CD38 expression still needs to be determined. Moreover, it would be important to repeat this experiment with the same set of CLL samples, and screen a bigger cohort of CLL cases, in order to verify the results obtained. Antibodies or small inhibitor molecules able to block the enzymatic activity of CD38 (Ausiello et al., 2000) could be used in order to define the role of CD38 in regulating the basal intracellular [Ca^{2+}] in CLL. Notably, an association between the basal intracellular [Ca^{2+}] in CLL cells and their IGHV mutational status has recently been proposed (Muggen et al., 2014). Basal intracellular [Ca^{2+}] was reported to be enhanced in all the CLL patient samples examined compared to healthy B-cells with a further increase reported in mutated compared to unmutated CLL samples. Due to the known association between CD38 expression and IGVH unmutated status in CLL, an analysis based on discordant cases

(CD38^{high}/mutated IGVH and CD38^{low} /unmutated IGVH) would be required in order to understand the influence of both factors on CLL Ca^{2+} signalling. Moreover, it was postulated that increased basal [Ca^{2+}] observed in mutated CLL patient samples could reflect a distinct CLL anergic state. Data obtained here suggested that the enhanced intracellular basal [Ca^{2+}] observed in the CD38^{high} samples analysed was not linked with an anergic state, as Ca^{2+} response to IgM stimulation was associated with CD38 expression, and hence with IGHV unmutated status, in agreement with previous studies (Damle et al., 2007; Mockridge et al., 2007).

Analysis of primary CLL cell chemotaxis indicated that CD38^{high} CLL samples have an enhanced capacity to migrate towards CCL21, confirming previously published results (Deaglio et al., 2007a; Vaisitti et al., 2010). Interestingly, the migration observed in the CD38^{high} CLL samples tested in the absence of chemokine was also higher than the CD38^{low} samples, in agreement with what was observed in the MEC1-CD38H cells when compared to the MEC1-GFP cells (Chapter 3.6). The differences observed in chemotaxis in the CLL samples analysed did not correlate with their expression levels of CCR7. In contrast, the level of surface α4-integrin detected appeared to be linked to the enhanced migration observed in the CD38^{high} CLL cases. Several studies have shown that expression of CD38 and α4-integrin are highly correlated in CLL cells (Buggins et al., 2011; Herens et al., 2006; Zucchetto et al., 2012) and this needs to be taken into consideration when analysing the direct role of each single molecule. However, experiments performed in MEC1-CD38H cells suggested that their enhanced migration was not associated with an increase in α4-integrin expression level (Chapter 3.5), suggesting that high CD38 expression alone could be sufficient to enhance cell motility and that CD38 expression does not drive an increase in α4-integrin levels.

Analysis of Rap1 activity in the MEC1 cell line indicated that MEC1-CD38H cells have a significantly higher active Rap1 when compared to MEC1-GFP cells (Chapter 4.2.2). In this chapter, Rap1 activation was investigated in primary CLL cells. The amount of active Rap1 detected in the CD38^{high} CLL samples was dramatically higher than in the CD38^{high} cases in all the samples analysed. Despite the low number of CLL samples analysed, due to the lack of samples with the required number of cells to perform

this assay, data obtained indicate that CD38 expression in primary CLL cells could be linked to an increased basal level of Rap1-GTP. Screening a larger cohort of CLL patients would be useful to verify the hypothesis that elevated CD38 expression is linked to an increased Rap1 activity. Constitutive activation of Rap1 in the murine lymphoma B-cell line A20 has been shown to increase their *in vivo* dissemination (Lin et al., 2010). Moreover, deregulated Rap1 activity in mice was shown to induce the development of a leukaemia of B1-cell origin with characteristics similar to CLL (Ishida et al., 2006). An association between CD38 and Rap1 activity therefore could play an important role in the disease pathophysiology and trafficking of $CD38^{high}$ CLL cases. Gene expression studies recently published have reported that Rap1b, RasGRP2 and RAPL have variable expression between CLL patients and their expression levels are increased in cases with trisomy 12 cytogenic abnormality (Riches et al., 2014), which correlated with enhanced cell motility and adhesion. Here, total Rap1 protein levels (including both Rap1a and Rap1b) did not vary much between the CLL samples tested and did not correlate with the high differential levels of Rap1-GTP detected between the $CD38^{high}$ and $CD38^{low}$ subgroups. Remarkably, in agreement with the previous observation in MEC1-CD38H cells (Chapter 4.3.3), Ca^{2+} depletion reduced Rap1 activity to an undetectable level in all the $CD38^{high}$ CLL samples analysed. The low level of active Rap1 detected in the $CD38^{low}$ CLL samples did not allow any conclusion to be drawn on the effect of calcium depletion in these samples. Screening more CLL samples would be required to clarify the effect of Ca^{2+} depletion on the basal Rap1 activity in $CD38^{low}$ CLL cases.

The involvement of Rap1 in the intrinsic MEC1 cell migration was suggested by knockdown experiments (Chapter 4.2.3). The enhanced basal migration properties observed in the $CD38^{high}$ primary CLL samples analysed compared to the $CD38^{low}$ could therefore depend at least partly on their increased basal level of Rap1-GTP.

Data described in Chapter 4 led to the hypothesis that CD38-mediated Ca^{2+} signalling could regulate RasGRP2 subcellular distribution in MEC1 cells. Analysis of RasGRP2 subcellular localisation indicated that CD38 expression levels also positively correlated with RasGRP2 polarised distribution in primary CLL samples, indicating that CD38 expression could regulate the localisation of RasGRP2. The nature of this

distribution still needs to be defined. Particularly, it should be clarified if the polarised RasGRP2 accumulation observed is linked to any cellular compartment (e.g. plasma membrane, endocytic or exocytic vesicular compartments). The polarised distribution of RasGRP2 leads to the speculation that active Rap1 is also oriented in a cell pole of CLL cells with high CD38 expression. Notably, the polarisation observed was detected in cells under resting conditions, indicating that CD38 expression is linked to an enhanced basal activation status that could facilitate cell migration persistency during random migration as well as enhancing chemokine-directed cell chemotaxis. Detection of the subcellular spatial regulation of Rap1 activation, for example by using fluorescent resonance energy transfer (FRET)-based sensors (Mochizuki et al., 2001), would be one approach in order to verify this hypothesis. However, difficulties in transfecting expression vectors into primary CLL cells makes this strategy challenging.

It still needs to be investigated if CD38 and RasGRP2 co-localisation is required for the induction of a polarised RasGRP2 distribution. CD38 subcellular localisation observed in CLL cells varied between cells from the same CLL sample from a diffuse pattern to a more polarised one. Comparison of RasGRP2 polarisation levels among CLL cells with a different pattern of CD38 localisation could help to understand better if CD38 intracellular spatial localisation is involved in RasGRP2 redistribution. Interestingly, it was reported that ligation of CD38 with agonistic and non-agonistic anti-CD38 antibodies can induce the internalisation of a fraction of the surface CD38 pool in lymphoid cell lines (Funaro et al., 1998). Moreover, experiments performed in the human Namalwa B-cell line showed that incubation with the CD38 substrate, NAD+, can induce CD38 internalisation and that internalised CD38 is enzymatically active (Zocchi et al., 1996). The dynamics of CD38 distribution and internalisation in CLL cells have not been elucidated, but these observations lead to the hypothesis that CD38 catalytic activity could be regulated by its internalisation and possibly recycling in CLL cells. Moreover, the intracellular and extracellular availability of CD38 substrates and their modulation in the lymphatic CLL microenvironment could be particularly relevant in defining the role of CD38 enzymatic activity in CLL.

Taken together, data presented in this chapter suggest that CD38 expression in primary CLL cells is linked to an increased intracellular basal Ca^{2+} level and possibly correlates with enhanced constitutive Rap1 activation. This hypothesis is supported by the increased polarised subcellular distribution of RasGRP2 observed in the $CD38^{high}$ CLL samples, which could be involved in the differential migratory properties associated with CD38 expression.

6. Concluding remarks

High CD38 expression in CLL cells is a marker of poor prognosis that correlates with enhanced cell motility. However, the molecular mechanisms underlying this link have not been elucidated. The aim of this study was to investigate the effect of CD38 expression on the motility of CLL cells, focusing on the signalling pathways involved. Comparative analysis of the CLL-derived cell line MEC1 expressing CD38 or GFP (as a control) suggested that ectopic expression of CD38 alters cell motility by enhancing cell spreading and increasing CCL21-directed chemotaxis, in line with recently reported observations (Vaisitti et al., 2014; Vaisitti et al., 2010). Moreover, increased migration was also observed in the MEC1-CD38H cells compared to the MEC1-GFP cells in the absence of a chemokine gradient, suggesting that CD38 expression modifies the intrinsic cell motility properties. In agreement with this hypothesis, MEC1-CD38H cells showed a marked higher basal activity of Rap1, with a consequent increased CCL21-induced Rap1 activity, compared to control cells. These data indicate for the first time a possible link between CD38 and the activity of the GTPase Rap1 in CLL. Rap proteins have been shown to have a key role in several aspects of B-lymphocyte physiology and pathology (Kometani et al., 2004; McLeod and Gold, 2001; Minato and Hattori, 2009). Here, it was demonstrated that Rap1a and Rap1b depletion decreases MEC1 cell migration both in the presence and in the absence of a chemokine gradient. Moreover, knockdown of either Rap1 protein had a stronger effect on the migration of MEC1-CD38H cells compared to control cells. This leads to the assumption that the enhanced Rap1 activity observed in MEC1-CD38H is likely to be at least partly responsible for their enhanced basal migration and chemotaxis.

CD38 is an ectoenzyme that catalyses the synthesis of three different products (NAADP, cADPR and ADPR) involved in Ca^{2+}-mobilisation from intracellular stores and Ca^{2+} influx through the plasma membrane (Aarhus et al., 1995; Churchill et al., 2002; Liu et al., 2005). Analysis of the intracellular Ca^{2+} levels showed that MEC1-CD38H cells have a higher basal [Ca^{2+}] compared to the MEC1-GFP cells. The same observation was recently reported in another study performed in MEC1 cells (Vaisitti et al., 2014), where it was suggested that CD38 enzymatic activity, possibly through

the production of cADPR and ADPR, might mediate this effect. Here, analysis of the basal [Ca^{2+}] in a cohort of CLL samples suggests that CD38 expression also enhances the basal intracellular Ca^{2+} levels in primary CLL cells. However, as each CLL sample was tested only once and no normalisation method has been applied here to standardise the ratiometric values obtained, further studies are required to verify this results. The relevance of basal intracellular [Ca^{2+}] on CLL biology still needs to be determined. Considering the key role of Ca^{2+} as a second messenger, enhanced basal intracellular [Ca^{2+}] is likely to affect several cellular functions. It would be interesting to test if it could lead to an increase in the basal activity of Ca^{2+}-regulated signalling molecules such as calmodulin and calcineurin, which through NFAT and NFκB mediate the transcription of specific gene sets (Rickert, 2013). Elevated intracellular basal [Ca^{2+}] has been shown to characterise CLL cells when compared to other B cell malignancies (e.g. follicular lymphoma, multiple myeloma)(Duhren-von Minden et al., 2012) or normal B cells (Muggen et al., 2014). This effect is thought to be linked to constitutive BCR signalling, consistent with observations of features of anergy in CLL. Intracellular basal [Ca^{2+}] was reported to be particularly increased in CLL cases with mutated IGHV (Muggen et al., 2014), correlating with their reduced capability of a BCR response compared to unmutated CLL cases. If confirmed, data presented here would suggest that CD38 expression represents a further factor linked to increased basal Ca^{2+} level in CLL. It would be necessary to expand the analyses to a larger cohort of CLL cases to understand the relative contribution of CD38 levels and IGHV mutational status to this mechanism and its relevance to the CLL cell signalling and the disease physiology.

The enzymatic products of CD38 are known to act on molecular targets that differ from the IP_3-sensitive receptors (Malavasi et al., 2008), and thus CD38 could cooperate with the IP_3-mediated Ca^{2+}-signalling pathway and offer a further level of complexity to Ca^{2+} signalling. For example, studies performed in secretory cells, such as pancreatic acinar cells, have shown that polarised localisation of intracellular Ca^{2+} stores (IP_3-sensitive stores found in the luminal region of the cytoplasm and cAPDR- and NAADP-responsive stores restricted to the basolateral region) coordinate the Ca^{2+} signalling waves in response to several agonists and cooperate to establish cell

polarity (Kasai et al., 1993; Krause et al., 2002). It has been speculated that a similar mechanism could fine-tune T cell receptor-mediated signalling in Jurkat T cells, where IP_3, cADPR and NAADP have all been shown to be involved in the generation of Ca^{2+} signalling (da Silva and Guse, 2000).

CD38 has been reported to be enzymatically active in primary CLL cells and the ADPR-responsive Ca^{2+}-permeable TRPM2 channel was shown to be involved in the enhanced Ca^{2+} level observed in CD38-expressing MEC1 cells (Vaisitti et al., 2014). These observations together with the data presented here suggest that CD38 is likely to be involved in the regulation of Ca^{2+} signalling in primary CLL cells. Understanding the role of each CD38 enzymatic product will therefore be extremely useful in order to characterise their contribution to the Ca^{2+} signalling in CLL cells.

Data presented in Chapter 4 suggest that the intracellular basal [Ca^{2+}] in MEC1-CD38H cells is involved in maintaining the basal Rap1 activity. Studies performed in eosinophils have reported that basal Rap1 activity is independent of the homeostatic intracellular [Ca^{2+}] (Ulfman et al., 2008). This hypothesis is supported from the data obtained here in MEC1-GFP cells, in which Ca^{2+} depletion did not affect the basal level of active Rap1. However, Ca^{2+} depletion in MEC1-CD38H cells induced a decrease of Rap1-GTP, suggesting that the additional intracellular pool of Ca^{2+} in MEC1-CD38H cells could be specifically involved in the regulation of basal Rap1 activity. One hypothesis could be that the Ca^{2+} signalling mediated by CD38 enzymatic products could act in different subcellular regions and might act on discrete Rap1 pools.

Results presented in Chapter 5 showed that basal Rap1 activity was enhanced in the $CD38^{high}$ compared to $CD38^{low}$ primary CLL samples analysed, and Ca^{2+} depletion was able to reduce basal Rap1 activity in the former. These results are in keeping with the motility properties observed in the same cohort of CLL samples, where CD38 expression correlated with enhanced CCL21-directed migration, in agreement with other studies in which the chemokine CXCL12 was used as a chemoattractant (Deaglio et al., 2007a; Vaisitti et al., 2010). Moreover, the basal cell migration observed in the absence of CCL21 was also increased in the $CD38^{high}$ CLL samples analysed here compared to the $CD38^{low}$ CLL samples. These data, together with the

data obtained in the MEC1 cell line, suggest that the increased amount of basal active Rap1, possibly induced by CD38 expression and the consequent increase in basal [Ca^{2+}], is functionally active in contributing to CLL cell basal and chemokine-directed motility. Future studies will aim to screen a bigger cohort of CLL samples to further investigate this mechanism.

It was next investigated if the Ca^{2+}- and DAG-regulated Rap GEFs, RasGRP2 and RasGRP3, were involved in regulating Rap1 activity in MEC1 cells. RasGRP3 has been proposed to activate Rap and Ras proteins (Yamashita et al., 2000), and its role in B cells has been linked to the activation of Ras downstream of the BCR (Aiba et al., 2004). RasGRP2 was shown to act preferentially on Rap proteins and it is best characterised in platelets and T cells for its role in cell adhesion through Rap1 regulation (Bergmeier et al., 2007; Canault et al., 2014; Cifuni et al., 2008; Ghandour et al., 2007), whereas its function in B cells has not been investigated. Here, RasGRP3 depletion did not show any effect in the migration of MEC1-CD38H or MEC1-GFP cells. In contrast, RasGRP2 was demonstrated to be specifically involved in the migration of MEC1-CD38H cells, both basal and CCL21-directed, whereas it was dispensable for the migration of the MEC1-GFP cells. Surprisingly, pull-down experiments showed that RasGRP2 regulates Rap1 activity in both cell populations. The more polarised subcellular distribution of RasGRP2 in MEC1-CD38H cells compared to the MEC1-GFP cells could be the explanation for this discrepancy.

To date, the mechanisms regulating RasGRP2 subcellular localisation have not been elucidated. It has been reported that RasGRP2 expressed in COS7 cells localises in the cytoplasm, while co-expression of Vav proteins was shown to induce its translocation to the plasma membrane where it would mediate integrin activation through Rap1 (Caloca et al., 2004). Notably, RasGRP2 expression was shown to induce Rap1 activation in the same extent irrespective of RasGRP2 localisation when measured at the whole cell level, although localisation of RasGRP2 to the plasma membrane was shown to affect the compartmentalised activity of Rap1 (Caloca et al., 2004). This supports a hypothesis that here, in addition to an overall enhanced Rap1 activity, GTP-Rap1 subcellular localisation could also be different in MEC1-CD38H cells compared to the control MEC1-GFP cells. Interestingly, Vav1 phosphorylation was

reported to be increased in CD38-expressing MEC1 cells (Vaisitti et al., 2014), suggesting that this protein could have a role in the regulation of RasGRP2 localisation in MEC1 cells.

Data presented in Chapter 5 showed a correlation between CD38 expression and RasGRP2 polarised localisation in the cohort of primary CLL cells analysed. Based on the data obtained in the MEC1 cell line, this observation leads to the hypothesis that CD38 expression in primary CLL cells could up-regulate Rap1 activity through RasGRP2, in a spatially regulated fashion. Local accumulation of active GTPases (e.g. Rac and Cdc42) via positive feedback is known to be involved in the mechanism of symmetry-breaking during spontaneous cell polarisation. This mechanism, well characterised in fungal and plant cell models (Wu and Lew, 2013), has also been observed in neutrophils, which polarise in response to a uniform concentration of chemoattractants (Xu et al., 2003). Notably, constitutively active Rap1 expression also induces spontaneous cell polarisation in B cells (Shimonaka et al., 2003). Whether this mechanism occurs in CLL cells still needs to be defined. It would be interesting to investigate if localised enrichment of RasGRP2 in $CD38^{high}$ CLL samples is linked with PIP_3 accumulation, which is known to be involved in the spontaneous formation of the cell leading edge through the maintenance of a positive feedback loop involving Rac1 (Xu et al., 2003).

It could be speculated that the enhanced intracellular basal [Ca^{2+}] linked to CD38 expression could upregulate the basal Rap1 activity through RasGRP2. Further analyses are required to verify this hypothesis. RasGRP2 has shown weak binding affinity to DAG (Irie et al., 2004), and experiments performed in murine platelets have suggested that Ca^{2+} signalling could be the main regulator of RasGRP2 downstream of PLC (Stefanini et al., 2009). However, stimulation with DAG analogues was shown to induce RasGRP2-mediated Rap1 activation in T cells, suggesting that, even if not by direct binding, DAG could still have a role in RasGRP2 activation (Ghandour et al., 2007). B cell proliferation induced by CD38 cross-linking was shown to be PLCγ2 independent and to involve phosphatidylcholine-specific PLC and phospholipase D (Moreno-Garcia et al., 2005a), which are able to produce DAG without concomitant generation of IP_3 (Schutze et al., 1991). It is therefore possible that

alternative/additional signalling pathways apart from Ca^{2+}, such as DAG, could be involved in regulating activity of RasGRP2 in MEC1-CD38H cells.

Further mechanisms involved in the CD38-mediated signalling could involve the protein $G\alpha_q$. It has been shown that CD38 and its product cADPR are required for the CXCL12- and CCL19-mediated signalling and chemotaxis of murine dendritic cells (and not lymphocytes) via a mechanism involving the $G\alpha_q$ -subunit of the G-protein coupled to the chemokine receptors (Shi et al., 2007). This mechanism was proposed to be restricted to specific chemokine receptors as an alternative to the classical $G\alpha_i$ -mediated pathway that conversely appears to be CD38-independent. However, later studies suggest that both $G\alpha_q$ and $G\alpha_i$ proteins might be required for the CXCL12-directed chemotaxis of human T cells (Svensson et al., 2012). Interestingly, whereas $G\alpha_i$ depletion was shown to exclusively impair chemokine-directed migration, $G\alpha_q$ depletion reduced both basal migration and CXCL12-directed chemotaxis of T cells. $G\alpha_q$ was proposed to be involved in the basal cell motility by modulating LFA-1/αLβ2-integrin recycling. The hypothesis that CD38 signalling is mediated by $G\alpha_q$ would therefore be particularly relevant in light of the findings presented here. Given the increased spreading on VCAM-1 observed in the MEC1-CD38H cells compared to the MEC1-GFP cells, it would be interesting to analyse the role of $G\alpha_q$ in the modulation of α4β1-integrin. Investigating the role of $G\alpha_q$ proteins in CLL and the potential link with CD38-mediated signalling could reveal additional details of the molecular mechanisms involved. It was proposed that defective Rap1 recycling could be responsible for the impaired CXCL12-mediated Rap1 activation observed in a subset of CLL cases (Pye et al., 2013; Till et al., 2008). Future studies could examine if CD38, and RasGRP2, are involved in regulating Rap1 recycling, and hence motility, in CLL cells.

It is worth noting that one previous study performed in lymphokine-activated killer cells has shown Rap1 involvement in CD38-related signalling. CD38-mediated generation of cADPR upon stimulation with interleukin-8 was shown to induce Rap1 activation, and Rap1 activity was in turn required for the consequent CD38-mediated production of NAADP (Rah et al., 2010). These observations suggest that CD38 could

mediate Rap1 activation in different cell types and be involved in several cellular responses.

In summary, the data presented in this thesis suggest that high CD38 expression in CLL cells increases basal [Ca^{2+}], which then mediates an increase in Rap1 activity through RasGRP2 (Fig 6.1). The data are in agreement with the view that high CD38 expression contributes to the aggressiveness of CLL cells (Deaglio et al., 2008), as Rap1 is likely to be involved in increasing cell adhesion and migration, which are characteristics of $CD38^{high}$ CLL cells.

CD38 expression is higher in CLL cells resident in lymphoid tissues compared to peripheral blood and can be induced by microenvironmental signalling (Ghia et al., 2003; Jaksic et al., 2004; Patten et al., 2008). Moreover it has been proposed that CD38 expression in peripheral blood CLL cells decreases over time, while conversely CXCR4 expression increases in cells ready to re-enter the lymphoid tissues (Calissano et al., 2009; Calissano et al., 2011; Coelho et al., 2013). This dynamic pattern of expression leads to the hypothesis that the main role of CD38 in CLL cells could be restricted to the lymphoid compartments.

In vivo studies should be performed in the future to investigate the relevance of the RasGRP2/Rap1 signalling axis, together with the increase of intracellular basal [Ca^{2+}], in the trafficking of $CD38^{high}$ CLL cells. Different genetically modified mice and xenograft models of human CLL have been generated to reflect the heterogeneity of the disease. In one model, the *IgH-Eμ-Tcl-1* transgenic mice, the expression of the proto-oncogene T-cell leukaemia-1 (TCL-1) (expressed under the control of a V_H promoter and an IgH-Eμ enhancer) in immature and mature B cells drives the development of a leukaemia resembling the aggressive form of CLL (Bichi et al., 2002). This model has proved to be a useful tool to investigate *in vivo* the role of different proteins in CLL progression, including the Rho-family GTPase member RhoH, the expression of which is upregulated in CLL (Hofbauer et al., 2014; Sanchez-Aguilera et al., 2010), and the hematopoietic lineage cell-specific 1 (HS1) (Scielzo et al., 2010), an actin-binding protein involved in the BCR-mediated signalling cascade as a substrate of Lyn and Syk (Hao et al., 2004). Depletion of HS1 in a *IgH-Eμ-Tcl-1* background was

shown to affect the trafficking dynamics of the murine leukaemic cells (Scielzo et al., 2010). A similar approach could be adopted to investigate the role of CD38, RasGRP2 and Rap1 in CLL cell trafficking *in vivo*. Alternatively, the recently developed humanised mouse model, in which primary CLL cells co-transferred with autologous T cells proliferate in NOD/SCID/IL2Rγ-/- mice (Bagnara et al., 2011), could be used to investigate the migration properties *in vivo* of $CD38^{high}$ CLL samples with high basal $[Ca^{2+}]$ and RasGRP2/Rap1 activity.

The data presented here also represent an interesting basis to investigate the role of CD38 and its associated signalling in the motility properties of non-malignant B cells. Impaired neutrophil cell migration was reported in CD38-depleted mice (Partida-Sánchez et al., 2004), as well as RasGRP2-depleted mice (Bergmeier et al., 2007; Carbo et al., 2010). However, the motility properties of B cells in these models still needs to be characterised, and it could help to better understand the role of these proteins in CLL pathophysiology.

It can be speculated that the upregulation of the RasGRP2/Rap1 pathway could increase the retention of CLL cells in the lymphoid organs, thus favouring proliferative signalling. This effect might be mediated through several mechanisms. First, the role of Rap1 in controlling the lymphocyte interstitial migration (Ebisuno et al., 2010) might privilege high CD38-expressing cells to compartmentalise in close proximity to supporting cells. This hypothesis is in keeping with the observation that, within the lymphoid tissues of CLL patients, high CD38-expressing cells are more abundant in the tissue compartments containing pseudofollicles (Patten et al., 2008). Moreover, as Rap1 has been shown to be required for an optimal BCR signalling and cytoskeleton reorganisation during antigen-mediated B cell activation (Lin et al., 2010), its higher activity in $CD38^{high}$ CLL cells could strengthen antigen-triggered BCR signalling. Rap1 is best known for its role in integrin activation and its activity is necessary for the activation of α4β1- and αLβ2-integrin in lymphocytes (Shimonaka et al., 2003). Rap1 might therefore promote integrin binding to VCAM-1 and ICAM-1, which was shown to provide pro-survival signalling to CLL cells (Maffei et al., 2012) (Fig 6.2). The increased spreading on VCAM-1 observed in MEC1-C38H cells would support this hypothesis.

Understanding the signalling pathways mediated by CD38 is not only helpful in order to unravel the heterogeneity of CLL behaviour, but it could also be translationally beneficial. CD38 has recently gained attention as a potential therapeutic target in different types of blood cancers, and monoclonal antibodies against CD38 have been developed to target cancer cells through their Fc-dependent cytotoxic effect (Hersher, 2012). Delineating the signalling downstream of CD38 could help to identify alternative, and possibly more specific, therapeutic options for the treatment of CLL patients, such as small molecule inhibitors that can block CD38 enzymatic activity.

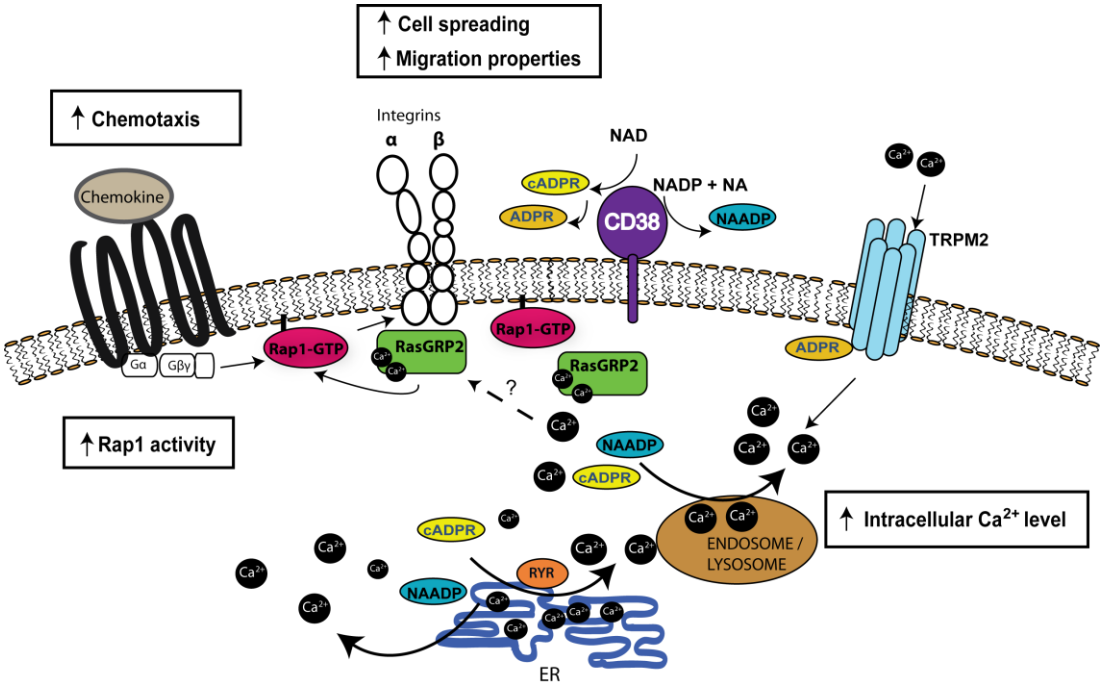

Figure 6.1 Hypothetical model of CD38 signalling in CLL cells.
CD38 expression in CLL cells is linked to an enhanced intracellular basal [Ca^{2+}], possibly mediated by the CD38 enzymatic products (such as cADPR, NAADP and ADPR) which mobilise Ca^{2+} from intracellular stores induce Ca^{2+} influx through the plasma membrane. The enhanced intracellular basal [Ca^{2+}] might increase RasGRP2 activity and polarise its subcellular localisation. RasGRP2 could regulate Rap1 activity both in resting conditions and after chemokine stimulation. Activation of RasGRP2/Rap1 signalling could explain the enhanced cell spreading, migration and chemotaxis linked to the expression of CD38 in CLL cells.

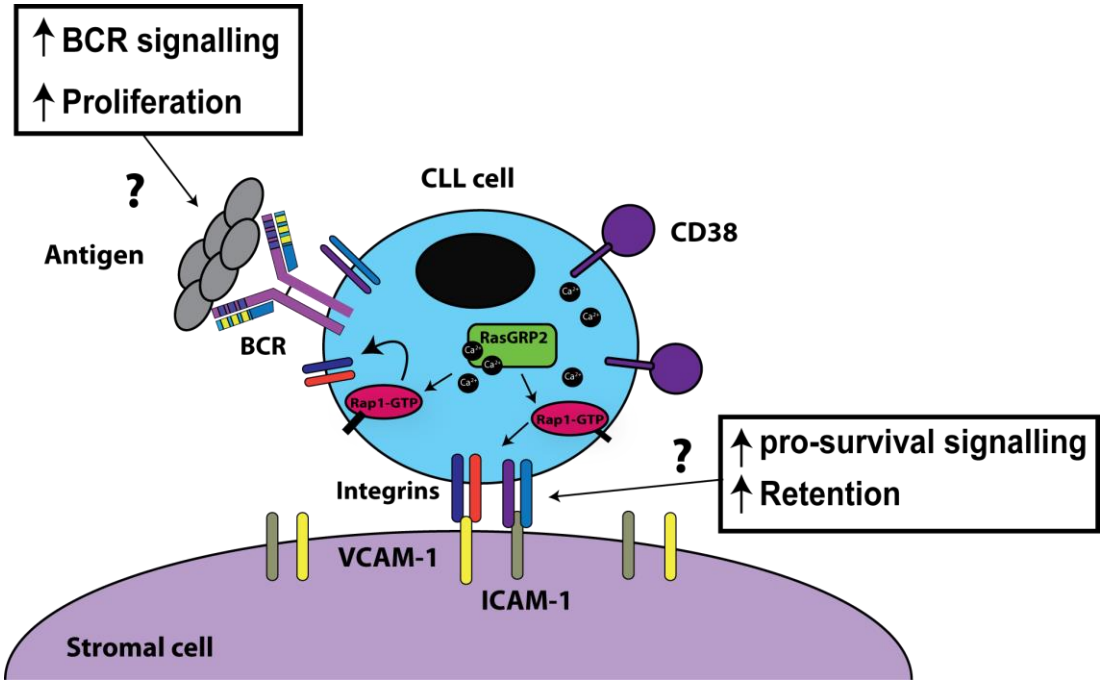

Figure 6.2 Hypothetical role of CD38-mediated signalling in the CLL microenvironment.
The proposed CD38-mediated Rap1 activation in CLL cells could enhance the integrin-mediated adhesive interactions between CLL cells and stromal cells (including NLCs, endothelial cells, MSCs). These interactions are involved in the pro-survival signalling that CLL cells receive in the microenvironment and could be involved in the retention of CLL cells in the lymphoid compartments. Higher Rap1 activity might also be involved in the antigen-driven BCR signalling and in the enhanced proliferation of high CD38-expressing CLL cells.

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