Chemokine Microenvironment in

Primary Central Nervous System Lymphoma

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Abbreviations

ABC-DLBCL	Activated B-cell-like Diffuse large B cell lymphoma
AID	Activation induced deaminase
ASHM	Aberrant somatic hypermutaion
BBB	Blood brain barrier
BCL2	B cell lymphoma 2
BCL6	B cell lymphoma 6
BCR	B cell receptor
BLIMP-1	B-lymphocyte-induced maturation protein 1
CARD11	Caspase recruitment domain member 11
CHOP regimen	Cyclophosphamide, Hydroxydaunorubicin, Oncovin, Prednison
CNS	Central nervous system
CpG	Cytosin and Guanin separated by a phosphate
CSF	Cerebrospinal fluid
CSR	Class switch recombination
CTL	Cytotoxic T lymphocyte
DNA	Desoxy-Ribonucleic acid
EBV	Epstein-Barr virus
ECOG-PS	Eastern Cooperative Oncology Group – Performance Score
FDC	Follicular dendritic cell
GC	Germinal center
GCL-DLBCL	Germinal center-like Diffuse larbe B cell lymphoma
HD-MTX	High-dose methatrexate
HEV	High endothelial vesses
HIV	Human immunodeficiency virus
HSC	Hematopoietic stem cell
IFN-γ	Interferon γ
IRF-4	Interferon regulatory factor 4
LDH	Lactate dehydrogenase
LFA-1	Lymphocyte function-associated antigen 1
LN	Lymph node
LPS	Lipopolysaccharide
NF-kB	Nuclear factor kappa B
NHL	Non-Hodgkin lymphoma
PAX-5	Paired box gene 5
PCNSL	Primary central nervous system lymphoma
pDC	Plasmocytoid dendritic cell
PNAd	Peripheral node adressin
PMBL	Primary mediastinal B cell lymphoma
PP	Peyer`s patches
RPVI	Reactive perivascular T cell infiltrate
SHM	Somatic hypermutation
SMA	Smooth muscle actin
TCR	T cell receptor
TFH	Follicular helper T cell
VEGF	Vascular endothelial growth factor
WBRT	Whole-brain radiation therapy
XBP-1	X-box binding protein 1

1. INTRODUCTION

1.1 The Chemokine System

Chemotactic signalling networks between resident cells within the tissue and migrating cells are essential in developmental biology and immunology where proper positioning of cells is crucial for lymphopoiesis, lymphoid organ development (1) or for mounting effective immune responses.(2-4)

The human immune system, consisting of an innate and an adaptive branch, is a complex and tightly regulated network of migrating and resident cells which is able to distinguish between non-dangerous self, non-dangerous-foreign and dangerous-foreign. The more ancient innate immunity is germ-line encoded and can bind bacterial and viral pathogens via pattern recognition receptors on their surface or within the cytoplasm. Macrophages, dendritic cells (DCs) and neutrophils represent innate immune effectors and serve as first line of defence against pathogens. The more recently emerged adaptive immune system is instructed during the lifespan of an individual. Exposure to different antigens leads to rearranged pathogen-specific surface receptors on B cells (B cell receptor) and T lymphocytes (T cell receptor) and the generation of memory B and T cells which elicit fast and powerful immune responses upon second encounter with the same pathogen. The ability of continuous rearrangement of surface receptors and the development of memory are hallmarks of the adaptive immunity.

Key players in migration, homing and retention of immune cells are chemotactic cytokines, so called CHEMOKINES**,** small secreted proteins ranging from 67 to 127 amino acids. The presence of four cysteine residues in conserved locations is crucial for the formation of disulfid bonds and therefore of their 3-dimensional shape.(5) Chemokines exert their biological effects by interacting with G protein-coupled seven transmembrane domain receptors that are selectively expressed on the surfaces of their target cells. Following interaction with their specific chemokine agonists, chemokine receptors undergo conformational changes and trigger a cascade of events, including flux of intracellular calcium (Ca^{2+}) ions (calcium signaling), phosphorylation of cytoplasmatic kinases, cytosceletal rearrangements, chemotaxis and activation of cell adhesion molecules such as intergrins. Some chemokine receptors can bind several chemokines with different affinities, such as CCR5,

indicating a certain redundancy or robstness of the sytem, other chemokine receptor, such as CXCR4, bind only one chemokine, the CXCL12.

Up to date, more than 40 chemokines and more than 20 chemokine receptors have been identified. *In vivo*, chemokines are thought to be released and bound to extracellular matrix proteins and glycosaminoglycans in the environment, creating a gradient indispensable for inducing cell migration. (6) The first protein to be identified as a chemoattractant in 1987 was CXCL8 (IL-8, NAP-1) that was shown to selectively recruit neutrophils.(7,8) Its receptor, CXCR1, was the first chemokine receptor to be cloned (9) followed by the identification of a second CXCL8 receptor, CXCR2.(10,11)

Migration is controlled by the expression of different chemokine receptors on the cell surface, which can be modulated upon maturation or differentiation. As an example, different types of immature DCs are localized at strategically important points of the body and serve as guardians against invading pathogens. They can also be recruited from blood at inflammatory sites, using chemokine receptors like CCR1 or CCR5. Upon antigen encounter, DCs get activated, downmodulate CCR1 and CCR5, and upregulate a specific chemokine receptor, CCR7, which allows them to migrate from the periphery into the T cell areas within draining secondary lymphoid organs where the appropriate ligand, CCL21, is expressed.(12-16) Here, activated DCs can activate naïve T and B cells which are specific for the antigen presented by the dendritic cell by a process known as antigen presentation. In this way an adaptive immune response can be initiated with the generation of antigen-specific T and B cell as well as antibody producing plasma cells.(17) Similar to activated dendritic cells, T and B lymphocytes also change their chemokine receptor profile upon activation, and become responsive to different chemokines which control the proper immune response.(18-20) All migratory processes of immature and mature dendritic cells, as well as naïve and activated B and T cells within and outside secondary lymphoid organs are orchestrated by a variety of chemokines, expressed at distinct time points, at distinct location, and by distinct cell types within the tissues. This crosstalk between chemokine expressing cells and their responding counterparts is essential to mount an adequat immune response.(21) Inappropriate activation of the chemokine network is associated with various pathological conditions such as autoimmunity, graft rejection or artheriosclerosis.(22)

Initially, newly discovered chemokines have been given a name according to their function (e.g.: MIG = Monokine Induced by Gamma Interferon; SDF-1 = Stromal-Derived Factor-1). Over the years, the proliferation of chemokine aliases that has accompanied the discovery of chemokines by multiple groups using bioinformatics, led to the development of a nomenclature system that parallels the receptor nomenclature. Chemokines were also classified accordingly to their functions in homeostatic and inflammatory. Even if this classification facilitates the understanding of the chemokine network, it does not correctly represent chemokine functions as several chemokines have been shown to exert a dual function activity.

1.1.1 Classification of chemokines by function

This classification distinguished chemokines which either function under **homeostatic** or **inflammatory** conditions.(23) Soon after, it became clear that some chemokines have a **dual-function:** they are expressed in homeostatsis as well as in inflammation.(24) Homeostatic chemokines, constitutively expressed, comprise molecules acting in primary and secondary lymphoid organs (25-27) as well as peripheral tissues.(28,29) In the bone marrow and in the thymus, for instance, homeostatic chemokines control the correct positioning and trafficking of committed lymphoid progenitor cells into “niches” to guarantee full maturation and functionality.(30-32) In addition, the development of secondary lymphoid organs (e.g. Peyer’s Patches and inguinal lymph nodes) is crucially dependent on the function of homeostatic chemokines.(33,34) Distinct T and B cell attractant chemokines in secondary lymphoid organs ensure the accurate segregation into T cell areas and B cell follicles. This segregation is essential for the initiation of adaptive immune responses. (33,35) In addition to directional guidance, homeostatic chemokines contribute to non-directional T cell migration (chemokinesis) within lymph nodes, thus promoting faster, more widespread movements of T cells to enhance the probability to encounter the specific antigen presenting dendritic cell.(36-38)

Inflammatory chemokines are induced upon inflammatory processes like bacterial and viral infections and control the recruitment of immune effectors to inflamed target tissues. Upon stimulation with pro-

inflammatory cytokines (e.g. IL-1, TNF) or bacterial and viral products (e.g. CpG, LPS) many cell types including leukocytes and stromal cells can upregulate the expression of a variety of inflammatory chemokines, which represents a “come-and-help-us” signal to other cell types. Under inflammatory conditions a variety of chemokines are upregulated and it is therefore likely that responding leukocytes are simultaneously exposed to a variety of inflammatory and homeostatic chemokines at the same time. The mechanism, how leukocytes integrate different simultaneous chemoktactic signals over their chemokine receptors and how they respond to them, is still poorly understood.

Dual-function chemokines play essential roles in guiding precursors and resting mature leukocytes to sites of leukocyte development and immune surveillance and in addition, are up-regulated during immune responses and target effector cells.(23) In addition, specific organs like the intestine are constantly exposed to an enormous number of microbes and may therefore express inflammatory chemokines even under homeostatic conditions.(39)

1.1.2 Classification of chemokines by structure

Over the years, the continous discovery of new chemokines led to confusion in the field since different groups concomitantly identified the same chemokine giving it a different name according to the function described. A new way of designating chemokines was introduced and summarized by Zlotnik and Yoshie in 2000.(5) The new classification is according to chemokine structural characteristics. Chemokines share four conserved cysteines which are essential for their tertiary structure, of which two are in their N-terminal domain and are adjacent or separated by one amino acid. Based on these first two cysteins, chemokines are classified into 2 major subfamilies, the CC chemokines and the CXC chemokines. The majority of chemokines fall into one or the other group. Most of the CC chemokines have been mapped on human chromosome 17q11-2 (40) while the majority of the CXC chemokine genes are clustered on human chromosome 4q12-21.(41) In addition to the 2 major subfamilies, there are two other classes of chemokines that have been described so far: the CX_3C chemokine, fractalkine, and the C chemokine, lymphotactin. Fractalkine, one of the two membrane-

bound chemokines with a mucin-like stalk, has three amino acids between the first two cysteines (42) whereas lymphotactin misses the first and the third cysteine.(43)

Chemokines from the CC chemokine family act in a broad range on different cell types including monocytes, T lymphocytes, DCs, basophils and eosinophils. An important feature of the chemokine system is illustrated in Table1: redundancy versus robustness. One chemokine can bind different receptors and in reciprocal, one receptor can bind different chemokines. This adds an additional step in the complexity of cellular migration.

Table 1. CC Chemokine family

CC chemokines	Former known as	Agonistic receptor	Subfamily
CCL1	Intercrine-β-glycoprotein 309 (I-309)	CCR8	D
CCL2	Monocyte Chemotactic Protein-1 (MCP-1)	CCR2	I
CCL3	Macrophage Inflammatory Protein-1α (MIP1α), LD78α, SISα	CCR1, CCR5	I
CCL4	Macrophage Inflammatory Protein-1β (MIP1β), LAG-1	CCR5	I
CCL5	Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES)	CCR3, CCR5	I
CCL7	MCP-3	CCR1, CCR2, CCR3	I
CCL8	MCP-2	CCR2, CCR3, CCR5	I
CCL11	Eotaxin	CCR3, CCR5	I
CCL13	MCP-4, NCC-1	CCR1, CCR2.CCR3	I
CCL14	Hemofiltrate CC chemokine-1 (HCC-1), HCC-3, NCC-2	CCR1, CCR5	I
CCL15	HCC-2, NCC-3	CCR1, CCR3	I
CCL16	HCC-4, NCC-4	CCR1, CCR2, CCR3, CCR5	I
CCL17	Thymus- and Activation-Regulated Chemokine (TARC)	CCR4	D
CCL18	Dendritic cell- chemokine1 (DC-CK1), PARC, AMAC-1, MIP-4,	unknown	D
CCL19	ELC, MIP-3β, Exodus-3	CCR7	H
CCL20	LARC, MIP-3α, Exodus-1	CCR6	D
CCL21	SLC, Exodus-2, TCA4, 6Ckine	CCR7	D
CCL22	MDC, STCP-1, ABCD-1, DC/B-CK	CCR4	D
CCL23	MPIF-1, MIP-3	CCR1	I
CCL24	MPIF-2, Eotaxin-2	CCR3	I
CCL25	Thymus- Expressed Chemokine (TECK)	CCR9	D
CCL26	MIP-4α, Eotaxin-3, IMAC	CCR3	I
CCL27	Cutaneous T cell-attracting chemokine (CTACK)	CCR10	I
CCL28	Mucosae-associated Epithelial Chemokine (MEC),	CCR10	I

CC Chemokines and their receptors. The first column indicates the chemokine name according to the structure. The second column indicates the historical chemokine name according to its function. The third column indicates the chemokine receptors which can bind the chemokine. The last column groups the chemokines according to their mode of action into homeostatic (H), inflammatory (I) or dual-function (D) chemokines.

The second major subfamily of chemokines is the group of CXC chemokines (Table 2) which can be further subcategorized based on the presence of the tripeptide motif Glu(E)-Leu(L)-Arg(R), into ELR+

or ELR- chemokines. ELR+ chemokines (CXCL1, CXCL2, CXCL3, CXCL5 to CXCL8) have been reported to act on neutrophils and are also angiogenic (44,45), whereas ELR- chemokines (CXCL4, CXCL9 to CXCL14) activate different leukocytes (T and B lymphocytes) and are angiostatic.(46)

Table 2. CXC, C and CX_3C chemokine families

CXC chemokines	Former known as	Agonistic receptor	Subfamily
CXCL1	Growth-Related Oncogene-α (GRO-α), MGSA-α, NAP-3,	CXCR1, CXCR2	I
CXCL2	GRO-β, MGSA-β, MIP-2α,	CXCR2	I
CXCL3	GRO-γ, MGSA-γ, MIP-2β	CXCR2	I
CXCL4	Platelet Factor-4 (PF-4),	CXCR3B	I
CXCL5	ENA-78	CXCR2	I
CXCL6	Granulocyte Chemotactic Protein-2 (GCP-2), CKA-3	CXCR1, CXCR2	I
CXCL7	Neutrophil-Activating Peptide-2 (NAP2)	CXCR2	I
CXCL8	Interleukin-8 (IL-8), NAP-1	CXCR1, CXCR2	I
CXCL9	Monokine Induced by Gamma interferon (MIG),	CXCR3	D
CXCL10	10 kDa Interferon-gamma-induced Protein (γ-IP10 or IP-10)	CXCR3	D
CXCL11	Interferon-inducible T-cell Alpha Chemoattractant (I-TAC)	CXCR3	D
CXCL12	Stromal cell -Derived Factor-1α/β (SDF1α/β)	CXCR4, CXCR7	H
CXCL13	B Cell Activating chemokine-1 (BCA-1)	CXCR5	D
CXCL14	Breast and Kidney-expressed chemokine (BRAK), bolekine, Kec, MIP-2γ	not known	H
CXCL15	Lungkine		I
CXCL16	SR-PSOX, CXCLG16	CXCR6	D

CxC chemokines	Former known as	Agonistic receptor	Subfamily
CX3CL1	Fractalkine	CX3CR1	I

XC chemokine	Former known as	Agonistic receptor	Subfamily
XCL1	Lymphotactin	XCR1	I

CXC, CX_3C and C chemokines and their receptors. The first column indicates the chemokine name according to the structure. The second column indicates the historical chemokine name according to its function. The third column indicates the chemokine receptors which can bind the chemokine. The last column groups the chemokines according to their mode of action into homeostatic (H), inflammatory (I) or dual-function (D) chemokines.

1.1.3 Lymphocyte trafficking under homeostatic conditions

Interaction between chemokine and chemokine receptor alone are not sufficient to induce cell migration into the tissue. Transmigration of a leukocyte into the tissue is rather a multi-step process which can be divided in 4 different steps: 1) tethering and rolling, 2) activation, 3) firm adhesion and spreading, 4) intravascular crawling and transcellular transmigration into the tissue (Figure 1).

Thethering and rolling is mainly dependent on selectins, whereas activation of integrins depends on chemokines. Upon chemokine interactions, cells adhere firmly to the endothelium via integrin activation and start to transmigrate into the tissue.

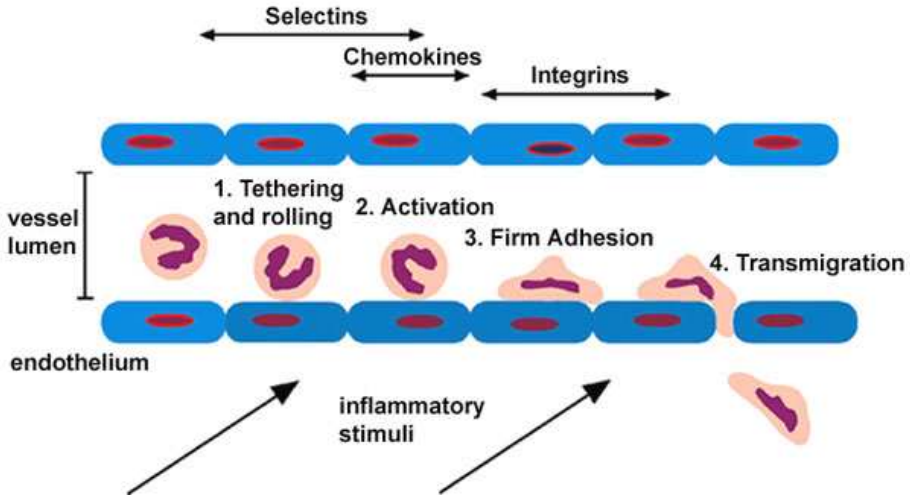

Figure 1: Multistep transmigration of leukocytes from vessels into the tissue

Transmigration of cells strongly depends on chemokine interactions. Various chemokines can be transported or bound onto the luminal site of endothelial cells and in this way influence migratory behaviour of circulating leukocytes under homeostatic or inflammatory conditions.(47) On the luminal site of the endothelium, chemokines play an important role in the transmigration of cells by inducing integrin activation, which leads to tight binding between transmigrating cell and endothelial cell.(48)

At birth, our lymphocyte compartment consists mainly of naïve, antigen-unexperienced cells. During lifetime, our memory pool of B and T lymphocytes is constantly increasing so that adult individuals have a mixture between naïve lymphocytes, which have not seen their specific antigen yet and memory lymphocytes, which have already encountered their specific antigen during a previous infection and are now patrolling through the body. Naïve as well as memory lymphocytes have the ability to circulate through the blood and transmigrate into peripheral lymphoid organs or peripheral tissue, screening for specific antigens or antigen presenting cell, which would activate them via the B or T cell receptor on their cell surface. The route of homing/patrolling through secondary lymphoid

organs and peripheral tissues is regulated by the chemokine receptor expression on the surface and the expression pattern of the cognate agonists in the tissue.

Under homeostatic conditions, lymphocytes enter the lymph node from the blood via a specialized type of postcapillary venules, the high endothelial venules (HEV). On the luminal site, HEVs specifically express the CD62L-ligand peripheral node addressin (PNAd) as well as the CCR7-ligand CCL21. Transmigration is initiated by a tethering step that allows leukocytes to loosely bind to endothelial cells. This step is mediated by L-selectin (CD62L) expressed on lymphocytes that interacts with its endothelial ligand peripheral node addressin (PNAd). Subsequently, CD62L-dependent lymphocytes rolling on the endothelium is followed by chemokines recognition via the appropriate chemokine receptor.(21,49) Chemokine receptor triggering induces intracellular signals that activate integrins from a low affinity to a high affinity state (inside-out-signal). In this case, the homeostatic chemokine CCL21, ligand of CCR7, mediates the activation of the integrin Leukocyte Function-associated Antigen 1 (LFA-1) on rolling T cells which in turn interacts with its ligand, the InterCellular Adhesion Molecule-1 (ICAM-1) on HEVs.(50) Upon activation of integrins, lymphocytes firmly stick to the endothelium and transmigrate into the tissue (Figure 1). After transmigration into the lymph node, naïve $CD4^+$ T cells migrate towards the T cell areas (paracortex) where the ligands for CCR7, CCL19 and CCL21, are expressed by stromal cells.(51) In this area, they actively move by chemokinesis and scan for antigen-bearing DC.(36-38). Not only chemokinesis is important in cell-cell contact in the T area, but also release of selective chemokines has been shown to guide naïve $CD8^+$ T cells to APCs in a CCR5 dependent manner.(52) In contrast to naïve T cells, naïve B cells express lower levels of CD62L.(53) Rolling B cells can be induced to arrest on HEVs by either CCR7 or CXCR4 agonists. After transmigration, B cells migrate in a CXCR5-dependent manner towards the B cell areas where its cognate agonist CXCL13 is produced by follicular dendritic cells (FDC) and T_{FH} cells.

B and T cells that have entered a lymph node exit through the medulla via efferent lymphatics.(54-57) This active process of re-circulation ensures that lymphocytes can be exposed to their cognate antigen independently on where the pathogen has been sampled.

A key player among chemokines in lymphopoiesis and immune homeostasis is the house-keeping chemokine CXCL12, together with its cognate receptor CXCR4.(58,59) CXCL12 as well as CXCR4 are the only chemokines/chemokine receptors identified so far, which has been shown to be pivotal for life, since gene targeted disruption of CXCR4 or CXCL12 leads to perinatal death in mice.(60,61) Various physiological as well as pathological processes have been shown to depend on the CXCL12/CXCR4 axis. The chemokine receptor CXCR4 is the coreceptor for HIV entry in human $CD4^+$ T cells.(58,59,62) Stromal cell-derived CXCL12 has a crucial role within the bone marrow (BM). B cell maturation occurs in complex BM microenvironments known as bone marrow niches.(63) Reports in gene-targeted mice where CXCR4 was selectively deleted in B cells, have documented its requirement for retention of B cell precursors in the BM.(64) CXCL12 is therefore not only important for B cell migration, but also for trapping of B cells within the BM environment. CXCR4- and CXCL12-deficient animals exhibit deficient B cell development and a lack of bone marrow myelopoiesis.(60) During their maturation in the bone marrow, B cells highly express CXCR4 yet gradually loose their responsiveness to CXCL12 which could account for the release into circulation. (65,66) Hematopoietic stem cells (HSC) in the bone marrow also depend on stromal-derived CXCL12 and blocking CXCL12/CXCR4 interactions in the bone marrow leads to a release of different progenitors into the circulation.(67-71) Only recently, the CXCR4-inhibitor (AMD3100, Plerixafor) has been introducded in clinical protocols to increase circulating hematopoietic progenitors before bone marrow transplantation.(72,73) In addition to its niche-like signal for HSCs in the bone marrow, CXCL12 is an important survival factor for B cells and plasma cells and is essential for plasma cell homing to the bone marrow.(74,75) Another important function of CXCL12 in B cell biology is during an adaptive immune response where CXCL12 together with CXCL13 seem to regulate germinal center B cell trafficking between dark and light zone.(76)

More recently CXCR7 has been described as a second receptor for CXCL12 (77) Mice with gene targeted deletion of CXCR7 succumb perinatally due to ventricular septal defects in the heart while hematopoiesis including B cell development was not affected.(61) In zebrafish development, CXCR7 seems to function as a non-signaling scavenging receptor, which internalizes CXCL12 from the environment and regulates in this way its extracellular concentration.(78)

Another B cell attractant chemokine is CXCL13 which acts on the chemokine receptor CXCR5, expressed on B cells and a subset of activated T cells. It is produced and released by germinal center T cells and FDCs in the germinal center. This germinal center T cell subset is called follicular B helper T cells (T_{FH}) and is localized in the light zone of the germinal center, where they provide help to germinal center B cells to differentiate towards memory B or plasma cells.(79) CXCL13 is specifically expressed by follicular dendritic cells (FDC) in the light zone of the germinal center and is essential for B cell migration into B cell follicles in lymph nodes and in the spleen.(27,33,76) Knock out mice deficient in CXCR5 show a disrupted T/B cell segregation and fail to develop inguinal lymph nodes and Peyer's Patches demonstrating a chemokine-driven positive feedback loop that is essential to develop some secondary lymphoid organs.(33,34)

1.1.4 Lymphocyte trafficking under inflammatory conditions

Depending on the type of pathogen, cell-to-cell signals received from the antigen presenting cells (APC) as well as environmental influences, T cells can differentiate into different T cell subsets. Up to date 5 such T helper subsets have been described, Th1, Th2, Th17, natural and induced regulatory T cells (T_{regs}) and follicular B helper T cells (T_{FH}) cells. The differentiation into each subset depends on distinct cytokine signals in the environment and the induction of specific transcription factors (Figure 2).

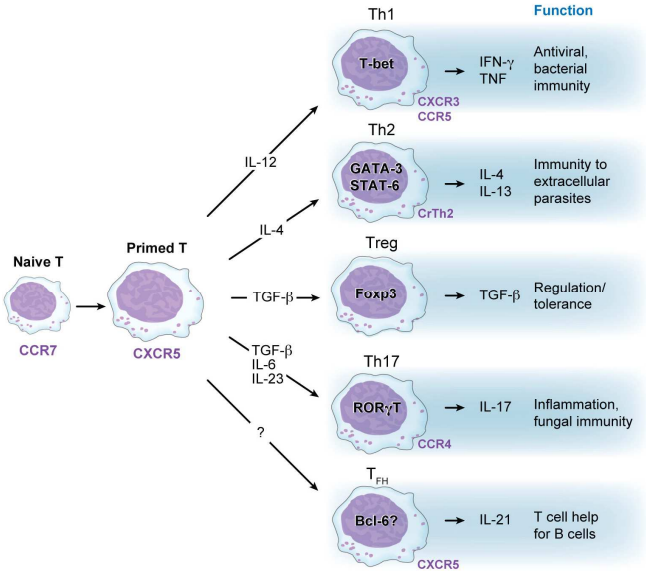

Figure 2: Effector T cell differentiation and the expression of transcription factors, effector cytokines, and chemokine receptors. Transcription factors for each subset have been placed in the nucleus. The list of chemokine receptors, or cytokines, for each of the subsets is not complete. (*from King et al. Ann. Rev Imm 2008, 26:741-66*)

Each T cell subset releases specific cytokines and expresses a different repertoire of chemokine receptors. Viruses and intracellular bacteria induce mainly Th1 responses. Th1 lymphocytes release IFN-γ and IL-2. A Th1 response activates mononuclear phagocytes and promotes isotype switching. Th2 cells, in contrast, are induced during parasite and helminth infection, produce IL-4 and IL-5 and are involved in responses mediated by IgE, eosinophils and basophils. Fungi tend to induce a Th17 differentiation, where T cells mainly express IL-17 and IL-22. While Th1, Th2 and Th17 are favoring an inflammatory environment by releasing a variety of proinflammatory cytokines, a fourth subset of T_{reg} can dampen an immune response by releasing anti-inflammatory cytokines such as IL-10 and TGF-β. A fifth group comprises T_{FH}: this subset is found within the light zone of the germinal centers where they provide costimulatory help to late germinal center B cells. T_{FH} cells are characterized by their expression of the B cell attractant chemokine CXCL13, CD57 and depend on the transcriptioin factor BCL6.(80-82) Whether T cell differentiation is a one way road, or whether plasticity between T cell subsets occur *in vivo* is subject of intense research. Past studies emphasized terminal commitment of effector T cells. (83-86) More recently it has become clear that flexibility between T cell subsets is probably not an exception. (87) Beside their different cytokine expression, T cell subsets also differ in their chemokine receptor profiles and show therefore different migratory capacities.(88-90) The upregulation of CXCR5 for instance enables T_{FH} cells to migrate towards the T/B cell border where they can interact with the appropriate B cell in a cognate fashion. In contrast to T cells, follicular B cells can upregulate CCR7 upon engagement of their antigen-receptor, which induces B cell migration towards the T/B border.(91) Interestingly, activated follicular B cells do not seem to downregulate CXCR5 expression but the upregulation of CCR7 seems to be sufficient to induce B cell migration to the B/T cell- zone boundary to seek for costimulatory help by follicular helper T cells (T_{FH}). During plasma cell differentiation, activated B cells upregulate CXCR3 and CXCR4, which allows them to home into the bone marrow or into inflamed peripheral tissues (Figure 3).(92)

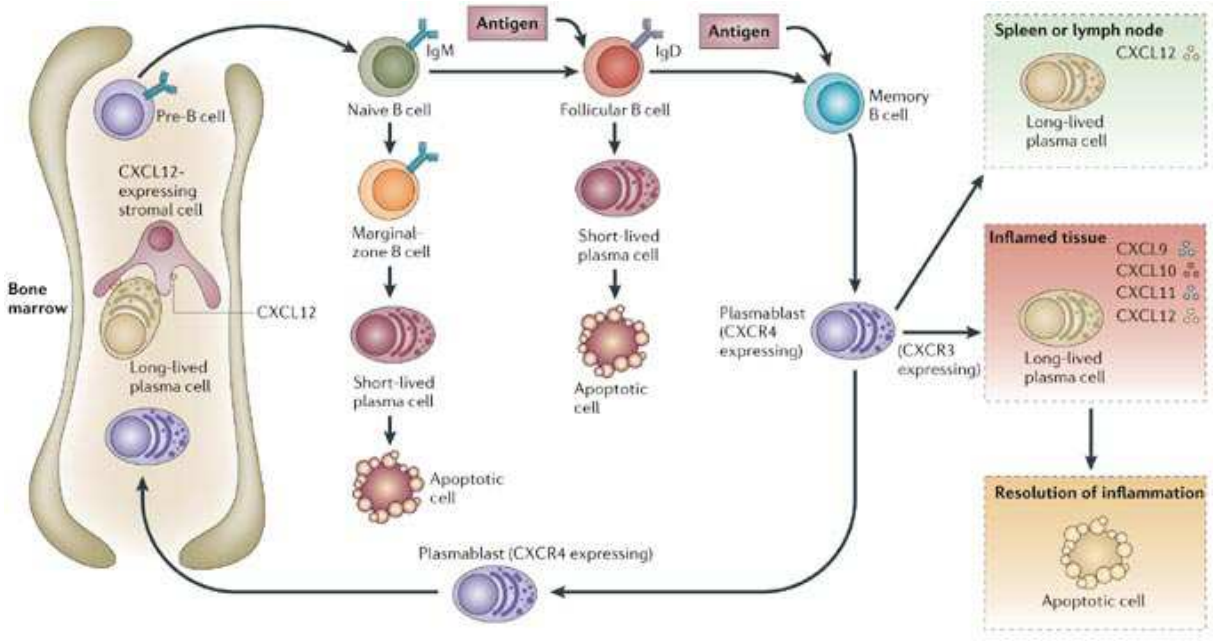

Figure 3. CXCR4- and CXCR3- depedent migration during plasma cell differentiation. B cells are generated in the bone marrow and exit as precursor B cells (pre-B cells), which are immature and express IgM. These cells further mature into naive B cells and then into either marginal-zone B cells or follicular B cells. When activated, these marginal-zone and follicular B cells can differentiate into plasmablasts and short-lived plasma cells, both of which can secrete antibody. Alternatively, with the help T_{FH} cells, follicular B cells can also differentiate into memory B cells, which are long-lived, and express antibodies of switched class and high affinity for antigen. When reactivated by antigen, memory B cells can differentiate into plasmablasts, which are competent to become long-lived plasma cells. A small proportion of these plasmablasts stay in the secondary lymphoid organ (the spleen or the lymph node) where they were generated. Most of the plasmablasts migrate either to inflamed tissue, under the control of the CXC-chemokine receptor 3 (CXCR3; which binds CXC-chemokine ligand 9 (CXCL9), CXCL10 and CXCL11), or to the bone marrow, under the control of chemotaxis towards CXCL12 (which binds CXCR4). All three tissues have finite numbers of plasma-cell survival niches. Plasmablasts that succeed in the acquisition of such a niche differentiate into plasma cells and become immobile. Resolution of inflamed tissue after a successful immune response terminates the survival niches in the tissue and therefore eliminates the resident plasma cells, and this is the peak of the immune response. In the bone marrow, and to a lesser degree in secondary lymphoid organs, long-lived plasma cells survive and provide humoral memory. (*from Radbruch et al. Nat. Rev. Imm. 2006, 6:741-50*)

Beside activated B cells, also activated $CD4^+$ and $CD8^+$ T cells, plasmacytoid DCs and NK cells express CXCR3 and CXCR4 and can respond to inflammation-induced chemokines CXCL9, CXCL10 and CXCL11.(93,94) Under inflammatory conditions, cell migration within draining lymph nodes undergoes rapid changes. Different cell types can be recruited to inflamed lymph nodes also in a CXCR3-dependent manner.(95-97). Of note, there is strong evidence that the place of chemokine expression does not always correlates with the mRNA expression and that transcytosis of chemokines is an important functional mechanism for cell recruitment.(98-101)

1.1.5 Chemokines and chemokine receptors in haematological malignancies

After malignant transformation, tumor cells can retain their chemokine receptor profile or can produce chemokines.(102) Malignant cells, in general, express distinct and non-random patterns of chemokine receptors, which render the tumor able to respond to chemokines in the environment, and favor its infiltratation and dissemination into different organs following a chemokine gradient. The first evidence that chemokine receptors are used by tumor cells for metastatic dissemination came from the work of Müller and colleagues that showed the involvement of CXCR4 in breast cancer metastasis.(103) More recently a role for chemokine receptors has also been found in a variety of haematological malignancies. Lymphocyte leukemias and B cell lymphomas express several chemokine receptors, including CCR6, CCR7, CXCR3, CXCR4, and CXCR5.(24,104-108) In addition, expression of various chemokines has been detected in a variety of human B cell lymphomas (24,109,110), follicular dendritic cell sarcomas (111), and in T-cell lymphomas, such as Angioimmunoblastic T-cell Lymphoma, which is thought to derive from germinal center T cells.(112) Of note, most of the studies assessed mRNA expression by rt-PCR or micro-array analyis and therefore neglect, that tumors include a variety of bystander cells, such as stromal, endothelial and immune effector cells which may contribute to the upregulation of gene products. Detailed histopathological examination of chemokine expression within the tumor and its environement is required to improve our understanding on the complex relationship between tumor cells resident their bystander cells in the microenvironment.

1.1.6 Synergistic activities among chemokines

Up to date, *in vitro* and *in vivo* studies have evaluated cell migration induced by a single chemokine. However, under homeostatic and inflammatory conditions, leukocytes are exposed to variety of chemoattractans at the same time. Moreover, leukocytes often express several chemokine receptors on their surface and can therefore receive different chemoattractant stimuli at the same time. The mechanisms, how leukocytes integrate signals from the environments and respond to simultaneous

stimulation with different chemokines are not well understood. For instance, immature B cells express CCR2 and CXCR4 and while CXCL12 induces a strong response when added as first chemokine, the pre-treatment with CCL2 diminishes CXCL12-induced activation.(113) In other words, the duration, the concentration and the timing of a first stimulation can make a cell refractory to further engagements by other ligands. The concomitant presence of two ligands, in contrast, can induce a response that exceeds additive effects. Such synergistic activity represents on amplification mechanism that is only active once a cell encounters two ligands at the same time. This mode of action could represent a "security code" that ensures that powerful effects are only generated in response to the right combination of ligands. Moreover, the first ligand could sensitize the cells to respond to a second stimulus.(114,115) These cells have an advantage compared with unstimulated cells that can lead to better activation or even survival. Synergistic activites between chemokines include two separate mechanisms: in the first scenario a leukocyte is exposed to different chemokines, for which the cell expresses the cognate chemokine receptors. In a second scenario a leukocyte is exposed to a variety of chemokines but expresses only the chemokine receptors selective for a single chemokine. One of the first reports on synergistic activities between chemokines demonstrated that regakine-1, a CC chemokine isolated from bovine serum, could synergise with CXCL8 and CCL7 in the recruitment of neutrophils and lymphocytes, respectively.(116) Alternatively, plasmacytoid DC (pDCs) responded synergistically to suboptimal concentrations of CXCL12 and CXCL10.(117) Even more striking, the presence of CXCL10 in the upper well of the chemotaxis chamber seemed to prime the cells to migrate to low CXCL12 concentrations and excluded the involvement of a CXCL10 chemokine gradient. Since pDCs express the specific receptors, the synergistic effect was believed to be mediated by both CXCR3 and CXCR4.(114) These observations were followed by several studies *in vitro* and *in vivo*.(118,119) A different mode of synergism has been identified by various groups whereby the responses of cells can be influenced by the presence of non-ligand chemokines. In this case, cells bearing receptors for one chemokine, strongly respond to a suboptimal agonist concentration in the presence of a non-ligand chemokine.(120-122) Different cellular responses, such as adhesion assays, chemotaxis, receptor internalization and the activation of intracellular signalling cascades, were addressed and all showed increased responses when a non-ligand chemokine was present. The

depicted chemokines were chosen also because of their relative distribution *in vivo*. CXCL13 and CCL21 are both expressed in secondary lymphoid organs and the migration of antigen-experienced T cells to the B/T- cell boundary zone could potentially be enhanced by CXCL13.(121) CCL22, the agonist of CCR4, is concomitantly expressed with the synergy-inducing chemokine CXCL10 in atopic and allergic dermatitis, and skin-homing $CCR4^+$ T cells could benefit from the co-presence of both chemokines.(122) CXCL4 and CCL5 are two chemokines stored in platelets and are released upon activation. The arrest of monocytes under flow conditions, mediated by CCL5, could be enhanced when CXCL4 was applied at the same time.(120) The receptor for CXCL4, recently identified being CXCR3B (123) is not expressed on monocytes (124) and therefore does not contribute to the synergistic effect. These studies could show that the agonist and the synergy-inducing chemokine can form heteromeric complexes, suggesting that the synergy-inducing chemokine could fix the agonist in the best conformation for triggering. The structural requirements for the formation of heterodimers were elucidated and showed for the CCL22/CCL7 and CCL5/CXCL4 complexes.(111,122,125) Moreover, disrupting heterocomplexes formation between CCL5 and CXCL4 *in vivo* inhibits monocyte recruitment into artheriosclerotic plaques.(125)

1.2 B Cell Physiology and Lymphomagenesis

1.2.1 B cell trafficking

Trafficking of B cells to LNs and Peyer's patches (PPs) through HEV involves, similar to T cells, a combination of chemokine receptors, such as CCR7 and CXCR4, and adhesion molecules. (33,126,127) In contrast to T cells, B cell homing to LNs is mainly directed by CXCR4. B cell-HEV interactions are largely unaffected by the absence of functional CCR7.(128) However, simultaneous absence of CXCR4 and CCR7 eliminates most B-cell homing to sencondary lymphoid organs.(126) PPs have an additional level of control for B cell homing. In addition to CCR7 and CXCR4 signaling, CXCR5 contributes to B cell homing to PPs and to the positioning of B cells in the B area of the LNs. (33,126). Activated B cells within B cell follicles upregulate the chemokine receptor CCR7 which is guiding the cells to the T/B border where the CCR7 agonists are present, to allow T/B cell interactions.(129) B cell trafficking to non-lymphoid organs is less understood. B cell homing to selective tissues is controlled by the expression of distinct receptors. For example, a subset of differentiated IgA^+ B cells expresses the chemokine receptor CCR10, which is only rarely expressed by B cells that secrete other immunoglobulin isotypes. This B cell subtype trafficks to mucosal tissues where CCL28, the agonist of CCR10 is preferentially expressed.(130-132) During their final differentiation to plasmablasts, activated B cells upregulate CXCR3 and become responsive to the CXCR3-ligands CXCL9, CXCL10 and CXCL11.(92) This pattern of receptor expression seems to be important for early plasma cell migration to site of inflammation.

1.2.2 B cells during germinal center formation

The hallmark of adaptive immune responses is specificity and memory towards encountered antigens. Specificity and memory require gene rearrangement and recombination events within lymphocytes. These events have to be strictly regulated because they harbour an increased risk for malignant recombination events and therefore malignant transformation of normal lymphocytes. The majority of malignant lymphomas in humans derive from B cells, which undergo malignant transformation at a certain timepoint during their development. The germinal center microenvironment is the main source

of memory B cell and plasma cells that produce high-affinity antibodies, which are necessary to protect against invading microorganisms.(133) The beneficial role of germinal center B cells in immunity is somewhat counterbalanced by their detrimental role in lymphomagenesis, as the majority of B cell lymphomas originate from germinal center B cells (Table 4).(134,135) In fact, with the exception of the rare lymphblastic and mantle-cell lymphoma subtypes, most B cell non-Hodgkin lymphomas (B-NHL) display somatically rearranged IgV genes, indicating that they are derived from B cells that are blocked within or have passed through the germinal center.(134,135) The genomes of these B-NHL subtypes display two main types of genetic lesions – chromosomal translocations and aberrant somatic hypermutation, (ASHM) – which represent mistakes in the immunoglobulin gene remodelling mechanisms. (136) Of note, as SHM can also occur outside of the GC, extrafollicular B cells may represent the targets of malignant transformation in certain subtypes of lymphoma.(137-139) The initiation of the GC response requires the interaction of co-stimulatory B-cell-surface receptors with ligands expressed by T cells and/or antigen-presenting cells, of which the most important is that between the tumour-necrosis factor (TNF)-receptor family member CD40, which is expressed by all B cells, and its ligand CD154 expressed by helper T cells. Activated B cells can then either develop directly into antibody-secreting cells in specialized extrafollicular sites of plasmablast growth and differentiation, such as the medullary cords of lymph nodes, or mature into GC-precursor B cells and move to the primary follicle, a structure made of recirculating IgM^+IgD^+ B cells within a network of follicular dendritic cells (FDCs) (Figure 4). Here, B cells start to proliferate rapidly and push the IgM^+IgD^+ B cells aside to form the mantle zone around the GC, yielding a structure known as the secondary follicle. After a few days of vigorous proliferation, the characteristic structure of the GC becomes apparent (Figure 4): a dark zone consisting almost exclusively of densely packed proliferating B cells known as centroblasts, and a light zone comprised of smaller, non-dividing centrocytes situated within a mesh of FDCs, T cells and macrophages (Figure 4). Centroblasts diversify their IgV genes by SHM, and those cells that express newly generated modified antibodies are selected for improved antigen binding in the light zone. Some centrocytes eventually differentiate into memory B cells or plasma cells.

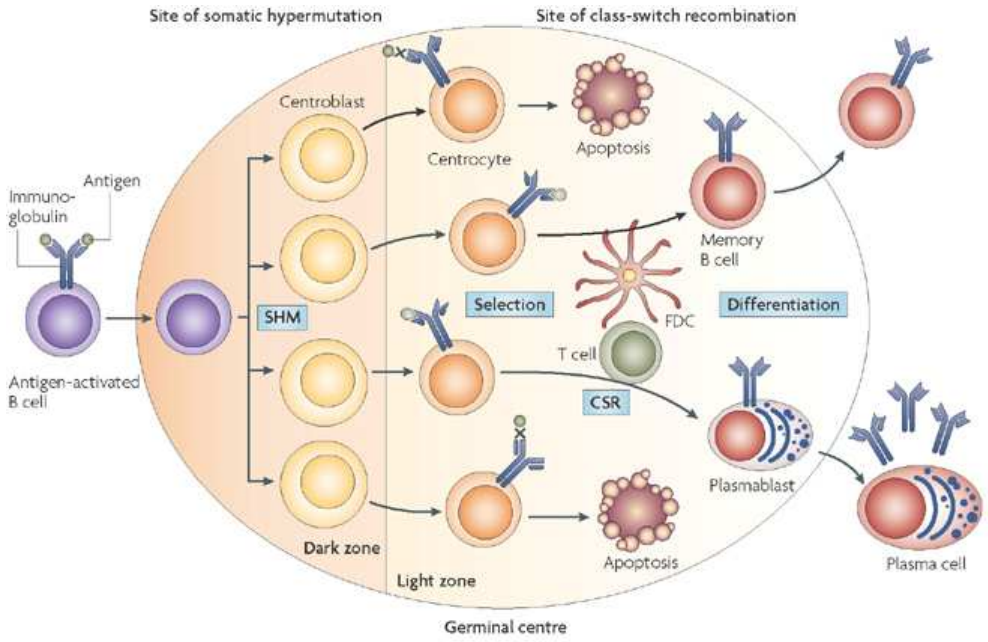

Figure 4: The germinal center microenvironment: Antigen-activated B cells differentiate into centroblasts that undergo clonal expansion in the dark zone of the germinal centre. During proliferation, somatic hypermutation (SHM) introduces base-pair changes into the V(D)J region of the rearranged genes encoding the immunoglobulin variable region (IgV) of the heavy chain and light chain; some of these base-pair mutations lead to a change in the amino-acid sequence. Centroblasts then differentiate into centrocytes and move to the light zone, where the modified antigen receptor, with help from immune helper cells including T_{FH} cells and follicular dendritic cells (FDCs), is selected for improved binding to the immunizing antigen. Newly generated centrocytes that produce an unfavourable antibody undergo apoptosis and are removed. A subset of centrocytes undergoes immunoglobulin class-switch recombination (CSR). Cycling of centroblasts and centrocytes between dark and light zones seems to be mediated by a chemokine gradient of CXCL12 and CXCL13, presumably established by stromal cells in the respective zones (not shown). Antigen-selected centrocytes eventually differentiate into memory B cells or plasma cells. (*from Klein et al. Nat. Rev Imm. 2008, 8:22-23*)

The GC reaches its maximal size within approximately two weeks, after which the structure slowly involutes, and it disappears within several weeks. The formation of germinal centers is a complex process, which includes the interaction of several cell types within the germinal center environment as FDCs and T_{FH} as well as a variety of transcription factors in activated B cells. Centroblasts express CXCR4 and migrate towards a CXCL12 gradient that originates in the light zone, whereas centrocytes express CXCR5, which is responsive to a CXCL13 gradient produced in the dark zone. Alternating upregulation and downregulation of the expression of these two chemokine receptors on GC B cells may promote the cycling of cells back and forth between the dark and light zones.(76) Nevertheless, a more recent study reveals that B cells tend to migrate along the dark-light zone border rather than to cross it.(140) A characteristic of centroblasts is their dramatically fast proliferation rate in the dark

zone of the germinal center.(141) The fast proliferation rate is required for the generation, within a short time frame, of large numbers of modified immunoglobulins, from which the few B cells that display antibodies with high antigen-binding affinity will be selected. Centroblasts upregulate genes associated with cell proliferation and downregulate genes encoding negative regulators of clonal expansion.(142,143) The physiology of dark zone centroblasts differs markedly from that of other proliferating cells in the body. Notably, the sensing of a response to DNA damage are specifically suppressed in centroblasts.(144) Furthermore, centroblasts express the enzyme telomerase (145), thereby ensuring that the vigorous clonal expansion of GC B cells does not result in a loss of their replicative potential, which would be a direct consequence of telomere shortening. GC B cells have long been known to be especially prone to apoptosis. Isolated GC B cells rapidly undergo apoptosis *in vitro* if they are not rescued by anti-apoptotic signals.(146) These findings indicate that the GC microenvironment provides crucial signals to GC B cells and decides their life and death. Centroblasts lack the expression of anti-apoptotic factors, including B cell lymphoma 2 (BCL-2) and its family members, whereas they express several pro-apoptotic molecules (133,142,147) (148), which allow the rapid execution of cell death by default or in response to exogenous signals. The major benefit of this pro-apoptotic nature of GC B cells is thought to be the rapid elimination of B cells with newly generated immunoglobulin mutations that produce a non-functional or non-binding antibody. This feature and the spatial concentration of proliferation, mutation and selection of antigen-activated B cells may have been the driving force for the evolution of the highly specialized GC structure. The transcription factor BCL6 is the master transcriptional regulator for centroblasts. BCL6 may allow GC B cells to sustain the physiological genotoxic stress that is associated with high proliferation, and sustain the DNA breaks that are induced by SHM and Class Switch Recombination (CSR). In addtion, BCL-6 inhibits the expression of genes involved in B cell activation during T cell-dependent immune responses, including CD69, CD80 and STAT1.(149) Interaction between CD80 on B cells and CD28 on T_{FH} cells in the light zone of the GC is essential for further differentiation towards memory B and plasma cells.(150) BCL6 further inhibits differentiation of B cells into memory and plasma cells by suppressing transcription factors, like Blimp-1 which are required for final B cell differentiation. (149,151,152) Signalling through the B-cell receptor (BCR) leads to BCL6 ubiquitin-mediated

proteasomal degradation.(150) Signalling through CD40, which physiologically can be induced by T helper cells, results in the transcriptional silencing of BCL6 (153) through NF-κB-mediated activation of IRF4.(154) Together, these mechanisms are likely to be crucial for the transition of GC B cells from centroblasts to centrocytes. Compared with centroblasts, centrocytes are very heterogeneous, perhaps consistent with the fact that they undergo diverse developmental fates, including the differentiation back into centroblasts and into post-GC cells: memory B cells or plasma cells. BCR stimulation coupled with co-stimulatory signals that are transmitted to the B cell by GC T cells provides survival signals to the cell. By contrast, failure of the BCR to bind antigen causes cell death. Recent evidence suggests that the differentiation of a GC B cell into a plasma cell is driven by the acquisition of a high-affinity BCR.(155) For memory B cells, the differentiation process instead seems to be stochastic, as throughout GC formation GC B cells are constantly selected to enter the memory pool.(156) During GC development, it is thought that CSR-inducing signals are transmitted to B cells by GC T cells that are present in the light zone. Recent studies demonstrated an important role for IRF4 in CSR. In *Irf4*$^{-/-}$ B cells, *Aid* mRNA and AID protein expression are not optimally induced, although IRF4 seems to regulate AID expression through intermediate molecules rather than through a direct mechanism.(157,158) In a subset of centrocytes induced to undergo CSR, IRF4 may be upregulated by the CD40–NF-κB signalling pathway that is activated in these cells (159,160) The fact that, in T-cell-dependent responses, class-switched cells are present among centroblasts as well as post-GC memory B cells and plasma cells suggests that the mechanism of CSR is activated in a distinct developmental window in the GC, and therefore not directly linked with the differentiation into a specific cell type.(161)

Besides the requirement of signalling through the BCR in affinity-driven plasma-cell development (155), the signals that induce a GC B cell to differentiate into a plasma cell or a memory B cell are largely unknown. However, several transcription factors have been identified, of which the regulation is crucial for plasma-cell commitment.(162-164)

IRF4 is one essential regulator of plasma-cell differentiation.(157,158) IRF4 has been suggested to act upstream of or in parallel to BLIMP1 for the generation of plasma cells. IRF4 also represses BCL6, thereby terminating the GC transcriptional programme.(154,158) Recent work suggest that the

expression of BLIMP1, IRF4 and XBP1 is independently regulated, but that the three transcription factors are jointly required for the establishment of the terminally differentiated plasma cells (165), as reflected by the dramatic effects of their individual deficiencies on plasma-cell development. (152,157,158) (166,167) In contrast to the steps that lead to plasma cell differentiation, those that lead to the differentiation of a centrocyte into a memory B cell are less well characterized. Recent findings shed new light into our understanding of B cell memory formation and maintenance.(168)

1.2.3 The role of germinal centers in B cell lymphomagenesis

The GC response, which develops specific memory and plasma cells during the immune response against invading microorganisms, also comes with a risk. In fact, with the exception of the relatively rare lymphoblastic and mantle-cell lymphoma subtypes, most B-cell non-Hodgkin lymphomas (B-NHL) display somatically mutated IgV genes, indicating that they are derived from B cells that are blocked within or have passed through the GC.(134,135) Gene-expression profiling has been used to define 3 molecular subtypes of diffuse large B cell lymphoma (DLBCL): i) germinal center B-cell-like (GCB) DLBCL; ii) activated B cell-like (ABC) DLBCL; iii) primary mediastinal B-cell lymphoma (PMBL). While GCL-DLBCL resembles germinal center B cells in the dark zone, ABC-DLBC resembles activated B cells during late germinal center reaction or postgerminal center B cells and is characterized by a constitutive activity of the NF-kB signaling pathway.(169,170) The mechansism underlying constitutive NF-kB activation in ABC-DLBCL are not completely understood yet, but recent findings have indicated that mutations in genes regulating NF-kB activity may induce its constitutive activation.(171,172) Nevertheless, in a large number of cases no mutations could be found and it remains to be determined whether the activation of the NF-kB signaling pathway, normally quiescent in centroblasts (143,159), may provide an ectopic survival signal to B cells or may reflect a particular developmental stage of the tumor-cell precursor (a centrocyte) that is subjected to signals that induce NF-kB activation. Among all DLBCL,

the ABC-DLBCL subtype represents the least curable. The third subypte, PMBL-DLBCL may derive from thymic B cells.(173,174)

1.2.4 B cell lymphomas at nodal and extranodal site

While the majority of DLBCL originate in lymph nodes, about 40% of all DLBCL present as extranodal tumors outside of secondary lymphoid organs.(175) Primary extranodal lymphoma is a disease confined to a single extranodal site, with or without regional lymph node involvement The major sites of involment can vary but in general there is agreement that the gastrointestinal tract, Waldeyer`s ring, and skin make up the majority of primary extranodal lymphomas (176) Extranodal low-grade lymphomas of the marginal B-cell origine within mucosa-associated lymphoid tissue (MALT) in the stomach are associated with the presence of *Helicobacter pylori*.(177) In these cases, antibiotic treatment can lead to resolution of the lymphma in many but not all patients.(178,179) A similar relationship has been reported between ocular adnexal lymphoma and the presence of Chlamydia psittaci.(180,181) These findings highlight the importance of environmental stimuli for the generation and progression of some malignant lymphomas. In the last 20 years, a distinct increase of extranodal large cell lymphoma has been recognized in association with acquired immunodeficiency syndrome (AIDS) and intense immunosuppression following organ transplantation. In both cicumstances, extranodal presentation such as Primary Cenrtral Nervous System Lymphoma (PCNSL) was common and associated with Epstein-Barr virus (EBV).(182) It is therefore important to distinguish extranodal large cell lymphoma development in immunocompetent or immunocompromised patients (AIDS, post-transplantation). The site at which lymphoma cells proliferate is probably a reflection of an interaction between tumor cell characteristics (e.g. homing and adhesion molecules, growth factor receptors, etc.) and growth promoting microenvironmental features (presence of corresponding ligands and growth factors). Nevertheless, the crosstalk between malignant lymphocytes and its tumor microenvironment in extranodal lymphomas are poorly understood. A number of autoimmune diseases, e.g. rheumatoid arthritis and Wegener`s granulomatosis have been linked to an increased risk of NHL in general, an of primary extranodal

NHL in particular.(183,184) Primary lymphoma of the thyroid has been often reported in patients with Hashimoto`s thyroiditis.(185) Of note, the primary organ of origin can influence the outcome. Two of the most unfavorable localizations of primary extranodal lymphomas are the CNS (PCNSL) and the testis (primary testicular NHL), which are both almost always from DLBCL subtype. The question, if malignant B cells are recruited to extranodal sites where they proliferate, or if malignant transformation takes place at extranodal site, remains to be resolved. The survial of patients with PCNSL only slightly increased over the last decades and a better understanding of the biology of this tumor is a major goal to improve patient`s outcome.

1.3 Primary Central Nervous System Lymphoma

1.3.1 Incidence and epidemiology

Primary central nervous system lymphoma (PCNSL) is a rare from of extranodal lymphoma which occurs in the brain, leptomeninges, spinal cord or eyes. Typically, it remains confined to the CNS and accounts for about 3% of primary brain tumors.(186,187) Its incidence increased nearly 3-fold between 1973 and 1984 (188), but more recent data suggest that it may be stabilizing or declining slightlgy.(189) The only established risk factor is congenital (ataxia telangiectasia, Wiskott-Aldrich sydrome and severe common and combined variable immunodeficiencies) or acquired immunodeficiency (HIV infection, iatrogenic immune suppression). HIV-infected individuals with a $CD4^+$ T cell count < 50cell / µl and a high peripheral viral load have a 3600-fold increased risk of developing PCNSL compared with healthy individuals (190) AIDS-related PCNSL are usually associated with EBV infection.(189) Since highly active antiretroviral therapy (HAART) has been introduced, the incidence of AIDS-related PCNSL has significantly declined.(191) Most cases of non-AIDS related PCNSL are diagnosed in patients between 45 and 70 years of age, with a median age at diagnosis in the fifth decade.(192-195) Men and women are equally affected.

1.3.2 Clinical presentation and diagnosis

PCNSL can manifest in the brain, its coverings, the eye or the spinal cord. Most cases of PCNSL present as symptoms related to the corresponding brain lesion. Symptoms at presentation include focal neurological deficits, neuropsychiatric symptoms, signs of raised intracranial pressure, seizures or ocular symptomes. In addition headaches, blurred vision, motor difficulties and personality changes (depression, apathy, psychosis, confusion, visual hallucinations) can occur depending on the infiltration of the tumor. In addition to a detailed history and physical examination, the evaluation of patients suspected of having PCNSL should include imaging of the CNS with contrast-enhancing magnetic resonance imaging (MRI), cerebrospinal fluid (CSF) analysis and slit lamp examination of both eyes. Fifty to seventy percent of immunocompetent patients with PCNSL develop solitary lesions, the remaining 25 percent developing multifocal brain lesions. Periventricular lesions

(thalamus, basal ganglia and corpus callsoum) are most common followed by lesions in the frontal, parietal, temporal and occipital lobes. Although mild surrounding edema is present in the majority of cases, it is usually less profound than accompanying metastatic foci of carcinoma. Evaluation of the cerebrospinal fluid (CSF) may reveal the presence of malignant lymphoid cells in up to 30 percent of patients with PCNSL. The CSF often reveals an elevated protein concentration and a lymphocytic predominant pleocytosis. The demonstration of neoplastic lymphocytes in CSF is sufficient to confirm the diagnosis of PCNSL and obviates the need for a brain biopsy. However, while a positive CSF analysis can provide valuable diagnostic informations, negative results do not exclude PCNSL. The diagnostic procedure of choice to confirm PCNSL is a stereotactic needle biopsy, followed by immunohistochemical analysis.(196) The pathologic evaluation and classification of PCNSL is similar to that of tumors of systemic non-Hodgkin lymphoma. Tumors are evaluatied based upon their morphology and immunophenotype. The vast majority of PCNSL are of the aggressive or highly aggressive, diffuse large B cell subtypes from an activated B-cell-like phenotype (ABC-DLBCL).(197) A histopathological feature of PCNSL is the angiocentric behaviour of malignant B cells. Malignant B cells tend to infiltrate along the perivascular spaces building up a concentric circle of densely packed blasts around small and intermediate size vessels. PCNSL often contain mixtures of tumor cells, reactive astrocytes and infiltrating T lymphocytes. Reactive perivascular T cell infiltrates (RPVI) are found in about half of PCNSL cases and their presence is associated with better overall survival compared to RPVI-negative PCNSL cases.(198) It remains elusive, whether the immune system can recognize PCNSL and elicit antitumor effector function, or whether infiltrating lymphocytes are tolerogenic. T cell subsets in PCNSL have not been analyzed up to date and the role of tumor infiltrating lymphocytes in the progression or suppression of PCNSL remains to be determined. In addition, crosstalk mechanisms between bystander cells like activated microglia or infiltrating lymphocytes and malignant B cells in PCNSL have not been studied. Signals from the tumor environment may provide tumor promoting or tumor suppressing signals to malignant B cells in PCNSL. Several attempts to isolate and culture PCNSL-derived malignant B cells have failed. Similar to B cells isolated from germinal centers, PCNSL B cells rapidly undergo apoptosis *in vitro*, indicating that PCNSL cells may depend on signals provided by the tumor environment. Blocking signals

between malignant cells and its environment may therefore have important clinical implications and may improve therapeutic intervention and patients' outcome.

1.3.3 Pathogenesis

1.3.3.1 Cellular origin of PCNSL

The cellular and molecular events leading to neoplastic lymphocytic infiltration of the CNS seen in PCNSL are not well understood.(199) It has been suggested that clonal proliferation may occur among normal B lymphocytes drawn to the CNS, a theory that is supported by the occurrence of white matter brain lesions that precede brain lymphoma.(200) The observation that systemic dissemination of PCNSL only occurs rarely suggests that the cell of origin may be derived from neoplastic lymphocytes that are eradicated from the periphery by an intact immune system, but which are able to traffic and survive in an immunological privileged site, such as the CNS.(193,201-203) In several PCNSL patients, B cell clones have been identified in the bone marrrow which share BCR rearrangement with the malignant B cell clone in the CNS (204), indicating, that lymphoma cells in the CNS may have a growth advantage, while circulating lymphoma cells lack specific survival signals in the microenvironment or may be recognized and deleted by the immune system. Several adhesion molecules including CD44 have been implicated in the interaction between lymphoma cells and tumor endothelial cells.(205,206) CNS tropism may be facilitated through the expression of specific cell-surface adhesion molecules or chemokine receptors on malignant B cells. Malignant B cells in PCNSL have been reported to express the chemokine receptors CXCR4, CXCR5 and CCR7 (108) showing a phenotype resembling activated B cells during late germinal center reaction. In addition several homeostatic chemokines have been described in PCNSL (203,207) and in CSF from PCNSL patients.(208)

1.3.3.2. Molecular features

The increased incidence of PCNSL in immunodefiecient individuals strongly implicates the immune system in the pathogensis of PCNSL. In fact, in immunocompromised patients, EBV may have a

causal link to the development of PCNSL (209,210) In contrast, the majority of immunocompetent patients do not appear to have EBV genomic DNA within their tumors. PCNSLs show clonal rearrangement in their IgHV genes and high levles of somatic hypermutation (SHM) of immunoglobulin genes and intraclonal heterogeneity (211-214), pointing towards their derivation from late germinal center B cells. Complementary DNA expression profiling experiments support the existence of similar molecular subtypes for PCNSL and systemic DLBCL, including germinal center B-cell-type (GCB) and activated B-cell-type (ABC) expression profiles.(170,215) The vast majority of PCNSL have an ABC-DLBCL phenotype (197) sharing molecular characteristics of late germinal center B cells such as the expression of the transcription factor IRF4 but not the plasma cell marker CD138.(197) However, Rubenstein at al. reported an overlapping state of differentiation in malignant B cells in PCNSL, characterized by expression of both GCB and ABC genes.(216) Aberrant SHM targeting the regulatory or coding regions of proto-oncogenes such as c-MYC, PIM1, RhoH/TTF and PAX5 has been described in both systemic lymphoma and PCNSL.(217,218) It is therefore tempting to think of PCNSL and systemic DLBCL as disorders reflecting faulty class-switch recombination or SHM.(219)

1.3.3.3. The role of infectious agents

Infectious agents promote lymphomagenesis through direct transforming properties or sustained antigenic stimulation. EBV genomic material is identified in over 90% of PCNSL tissue from immunocompromised patients.(220) EBV episomes are not found in PCNSL occurring in immunocompetent patients, and the notion that EBV might still be involved in B cell oncogenesis in this population remains speculative. Intriguingly, it has been shown that EBV infection of B cells results in expression of AID and polymerase η, both of which are crucial enzymes for SHM. Aberrant SHM involving growth-regulatory genes might provide B cells with a survival advantage independent from EBV infection.(221) Another important mechanism of pathogenesis in systemic lymphoma is chronic antigenic stimulation. Relatively strong evidence for this mechanism exists for *Borrelia Burgdorferi*-associated cutaneous marginal zone lymphoma and *Helicobacter Pylori*-related gastric

mucosa-associated lymphoid tissue lymphoma (MALT) as well as ocular adnexal lymphoma and the presence of Chlamydia psittaci.(180,181)

1.3.4 Therapeutic intervention

Treatment options for immunocompetent patients with newly diagnosed PCNSL include corticosteroids, radiation therapy, and chemotherapy. A stereotactic biopsy is indicated for all patients with suspected PCNSL. The administration of corticosteroids should be withheld prior to biopsy to avoid diagnostic inaccuracy. PCNSL patients relapse quickly when treated exclusively with corticosteroids and always require additional therapy. Whole brain radiation therapy (WBRT) is often deferred in PCNSL patients older than 60 years in complete remission after primary chemotherapy. The administration of chemotherapy regimens that are effective for extracranial aggressive lymphomas (i. e. CHOP regimen) showed poor results in PCNSL patients, (222,223) probably due to limited penetration of the chemotherapeutic drug through the blood-brain barrier (BBB). The most effective drug against PCNSL is high-dose Methotrexate (HD-MTX).(224-227) Several drugs with potential BBB penetrating properties have been added to HD-MTX and are currently tested in clinics. Preliminary results from a few trials in relapsed patients are available with temoxolomide, topotecan, rituximab and the PCV regimen (228-231) Chemotherapy alone has been emphasized to minimize radiation-related neurotoxicity, especially in elderly patients. With chemotherapy alone, durable responses are possible, although most patients experience relapse. Additional intrathecal MTX administration to the HD-MTX treatment demonstrated no survival benefit.(232) The vast majority of PCNSL patients will experience tumor relapse or progression and will requrie salvage therapy. The precise mechanism that leads to treatment resistance in PCNSL is not well understood. It has been hypothesized that promoter methylation of the reduced folate carrier (RFC) gene is responsible for MTX resistance.(233) The five year overall survival remains poor and is between 40–70 %.(226,227,234) To improve the prognosis of patients with PCNSL a better understanding of the biological mechanisms of this tumor is therefore necessary.

1.4 The Role of the Microenvironment in Malignancies

1.4.1 The inflammatory environment in malignancies

Oncogenic processes are typically viewed independently of the immune response against the tumor. The hallmarks of cancer include tumour-cell proliferation and survival, tumour angiogenesis and metastasis.(235) The activation of proto-oncogenes and oncogenic signalling pathways together with inactivation of tumour-suppressor genes in cancer cells are crucial processes in malignant transformation and progression.(235-237) At the same time, tumor immunologists have uncovered many cellular and molecular mechanisms that mediate tumor escape from natural immune surveillance, which functions as an extrinsic tumor suppressor.(238-240) The paucity of immunological danger signals necessary for immune activation, the increased concentration of immunosuppressive factors, and the accumulation of immunosuppressive cells in the tumor microenvironment indicate that immune regulation has an active role in cancer progression.

An inflammatory component is present in the microenvironment of most neoplastic tissues, including those not causally related to an obvious inflammatory process. Hallmarks of cancer-associated inflammation include the infiltration of white blood cells, the presence cytokines and chemokines and the occurrence of tissue remodeling and angiogenesis.

Strong evidence suggests that cancer-associated inflammation promotes tumor growth and progression.(239,241,242) Previous studies have shown that a high frequency of tumor-associated macrophages (TAMs) was linked with poor prognosis.(243,244) Interestingly, this pathological finding has reemerged in the postgenomic era: genes associated with leukocyte or macrophage infiltration (e.g. CD68) are part of the molecular signatures associated with poor prognosis in different malignancies.(243,245) In recent years, TAMs have been divided into M1 and M2 type according to their antitumoral or protumoral cytokine profiles. Classically M1 macrophages can exert cytotoxic activity on tumor cells and elicit tumor-destructive reactions. In contrast M2 macrophages express several protumoral functions, including promotion of angiogenesis, matrix remodelling and suppression of adaptive immunity.(246-248) TAMs can exert dual functions on malignant cells (the macrophage balance hypothesis) (246), and can negatively affect the activation of antigen-specific

$CD4^+$ T cells.(249) Signal transducer and activator of transcription 3 (STAT3) is a transcriptional factor activated by a number of cytokines that seem to be situated at the crossroads of multiple oncogenic signalling pathways. It is constititutively activated in tumor and immune cells of the tumor microenvironment, thus contributing to the regulation of the cell composition and role of the immune infiltrate.(250) In fact, macrophages with STAT3 activity are thought to play an important role in the crosstalk between tumor cells and their immunological microenvironment, leading to tumor-induced immunosuppression. Consequently, they have been proposed as being a potential target for cancer immunotherapy.(251)

Tumor infiltrating $CD4^+$ and $CD8^+$ T cells are likely to be important in achieving immunological control of tumors.(252-256) While $CD8^+$ T cells are believed to kill malignant cells, $CD4^+$ T cells have a role in the activation of $CD8^+$ cytotoxic T cells. $CD8^+$ T cells are potent effectors of the adaptive antitumour immune response. The target antigens that are recognized by tumour-reactive $CD8^+$ T cells have been shown to be mostly non-mutated self-antigens that are also expressed by tumour cells.(257,258) Tumour-specific $CD4^+$ T cells have been also identified, but their functionality can be manifold because $CD4^+$ T cells can help or hinder anti-tumour immune responses.(259,260) Regulatory T cells (T regs) are crucial for the maintenance of peripheral self-tolerance and for the suppression of antitumor responses.(261) T reg cells represent a unique T cell lineage that is characterized by expression of the transcription factor forkhead box P3 (FOXP3) and high levels of expression of cell-surface molecules associated with T cell activation. By releasing immunosuppressive cytokines such as IL-10 and TGF β, regulatory T cells, which are over-represented in tumor lesions from patients with melanoma and lung cancer, can inhibit the function of infiltrating T cells.(262,263) The presence and the suppressinve effects of T reg cells might therefore contribute to the poor clinical outcome of certain types of malignancies.

1.4.2 The tumor microenvironment in B cell malignancies

Development and progression of B cell malignancies are driven by a combination of cell intrinsic events and cell extrinsic events. Cell intrinsic events, such as oncogene activation or tumor-suppressor

gene inactivation lead to increased proliferative activity or decreased apoptosis.(264) Lymphoid neoplasms are characterized by well-defined chromosomal translocations and by the accumulation of subsequent molecular alterations involving mainly the cell cycle and/or apoptotic pathways. However, *ex vivo* culture of isolated malignant B cells is limited and is an indicator, that their survival *in vivo* is also dependent on cell extrinsic signals from accompanying cells in the microenvironment.(265)

Signals in the tumor environment can reach malignant B cells via cell-cell interactions or via soluble factors. In chronic lymphocyte leukemia, cell-cell interactions via CD40/CD40L between CLL and T cells cooperate with BCR signalling to trigger survival of tumor B cells.(266,267) In addition, survival of CLL cells *in vivo* (268) and *in vitro.*(269) have been associated to increased B cell survival factors, such as BAFF and APRIL BCR transmits survival and death signals throughout B cell development and abnormal BCR stimlulation provides constitutive signalling for the survival of B lymphoma cells.(270,271) These findings indicate that environmental signals strongly influence the behaviour and survival of malignant B cells. Uncontrolled tumor growth leads to mass effects and elicit inflammatory responses. Immune responses in malignant lymphomas can be manifold depending on the recruitment of different immune effectors.

The presence of T_{regs} for instance has been shown to be associated with different clinical oucomes, depending on their location, number and tumor context.(272-275) In epithelial derived cancers, the presence of T_{regs} generally correlates with poor clinical prognosis.(262,263) In contrast, high numbers of T_{regs} in follicular lymphomas are associated with improved overall survival and small numbers of T_{regs} is associated with transformation to DLBCL.(273) Tumor-infiltrating lymphocytes in Hodgkin lymphoma are enriched in T_{reg} cells, which create an immunosuppressive environment that explains the lack of an effective anti-tumor response.(276) Differences in clinical outcome between lymphoid malignancies and epithelial malignancies have also been described in terms of $CD8^+$ T cells. While in various tumors, the presence of CD8+ T lymphocytes correlate with better prognosis, conflicting results have been reported in B cell malignancies. In Hodgkin lymphoma $CD8^+$ T cells represent a small proportion of the T cell infiltrate and their presence in the tissue is a marker for unfavourable prognosis associated with shorter progression-free survival.(277,278) Beside different adaptive immune effectors, innate immune effectors also seem to play an important role in the clinical outcome

of B cell malignancies. Tumor-associated macrophages with increased STAT1 activity and immunosuppressive capacity have been found in some lymphomas, especially surrounding tumor cells in Hodgkin lymphomas and follicular lymphomas, where they have been associated with an unfavourable prognosis.(277,279,280) A classical example of tumor-cell survival dependent on the presence of environmental factors are B cell malignancies associated with microbial or viral pathogens. Several lines of evidence support the hypothesis that chronic antigenic stimulus can promote and sustain the growth of MALT-type marginal zone B cells in the stomach, fist causing acute and chronic gastritis, and subsequently gastic lymphoma.(281) Detailed analysis of the crosstalk between malignant B cells and their microenvironment will provide the basis for the development of alternative treatments by interfering with survival signals derived from antigens and inflammatory cells.

2. THE STUDY

2.1 Aim of the study

The germinal center is the main source of memory B cells and plasma cell generation. They produce high affinity antibodies, which are necessary to protect us against invading microorganisms. However, the beneficial role of GC B cells in immunity is somewhat counterbalanced by their detrimental role in lymphomagenesis, as the majority of B-cell lymphomas originate from GC B cells. Germinal center B cells express a distinct set of chemokine receptors such as CXCR4, CXCR5 and CCR7, which regulate their migration and positioning during and after germinal center reaction. Similar to centrocytes and centroblasts, malignant B cells derived from germinal centers can retain a particular set of chemokine receptors which allow them to respond to their cognate ligands expressed in the microenvironment. Investigation of B cell attracting chemokines within and outside germinal centers may not only improve our understanding of B cell trafficking within secondary lymphoid organs, but also help understanding tumor cell distribution and dissemination of malignant B cells derived from germinal centers.

The germinal center microenvironment is essential for survival of B cells. It is therefore tempting to hypothesize, that GC-derived B cell malignancies may at least partly depend on survival signals from their microenvironment. This hypothesis is corroborated by the fact, that malignant B cells from DLBCL quickly undergo apoptosis *in vitro*. Special emphasis should therefore be given to bystander cells in DLBCL and their effects on malignant B cells.

PCNSL remains a poorly understood and often fatal DLBCL confined to the CNS. Malignant B cells in PCNSL resemble late germinal center B cells and express classical B cell chemokine receptors, such as CXCR4, CXCR5 and CCR7. The present work was focusing on B cell attracting chemokines in the microenvironment of PCNSL, with the aim of improving our understanding on PCNSL microenvironment, including type, density and localization of tumor infiltrating lymphocytes and the potential role of specific chemokines in their recruitment. Detailed analysis of the crosstalk between normal and malignant B cells with their chemokine environment may provide insights on the strict organ tropism shown by malignant B cells.

The presented project includes four major sections:

I. Analysis of the expression of B cell attracting chemokines under normal and inflammatory conditions in human secondary lymphoid organs.

II. Analysis of T and B cell attracting chemokines in extranodal CNS lymphomas

III. Analysis of type, density and localization of tumor infiltrating lymphocytes in PCNSL

IV. Analysis of the effect of coexpressed chemokines in PCNSL on the migratory responses of tumor-infiltrating lymphocytes and malignant B cells.

2.2 Strenght of the study

PCNSL is a rare and often fatal CNS tumor. Thanks to the collaboration with one of the most important European center for the study and management of patients with PCNSL, we had at our disposal samples from 22 patients. Due to the fact that animal models for PCNSL are not jet available, these samples represent a unique collection for assessing both tumour cell characteristics and tumour microenvironment.

In the majority of malignancies, tumor cells are surrounded by a heterogenous population of bystander cells that contribute to tumor progression. To better understand the tumour characteristics is therefore important to strictly distinguish between tumor-derived factors and microenvironment-derived factors. Gene expression studies on malignant B cell lymphomas are often performed without prior separation of malignant B cells and bystander cells, resulting in the analysis of genes, which are upregulated in the microenvironment rather than in the tumor cells. A way of bypassing this issue is detailed histomorphological analysis or cell separation prior to analysis. Cell separation methods generally lead to a significant loss of cells during processing and require large patient samples. We therefore focused on detailed histomorphologic analysis of various proteins and their mRNA expression. Our work provides new findings in the crosstalk between malignant B cells and its microenvironment and improves our understanding of cellular dynamics within PCSNL. The strict organ tropism of PCNSL may at least partly be explained by our findings, supporting a model, where the inflammatory CNS response creates a niche-like environment, in which malignant B cells find optimal conditions for

further proliferation and progression. The data provided here may also be valid for a variety of other malignancies or chronic inflammatory conditions, such as multiple sclerosis. It emphasizes the importance of inflammatory bystander cells in releasing homing/trapping or survival signals to normal and malignant cells.

2.3 Limitations of the study

Biomedical research on human tissue samples is indispensable for improving our understanding of physiology and pathology in human diseases. Nevertheless, scientific investigation on human samples often remains restricted due to well defined ethical constraints and limited accessibility of human tissue. In immunology and tumor biology, murine models have developed as powerful tools to investigate health and model diseases. PCNSL are rare extranodal lymphomas restricted to the CNS and our understanging of the pathophysiology of this B cell lymphoma remains very limited. The difficult accessibility, the lack of appropriate animal models and the inability to maintain malignant B cells from PCNSL in culture challenges our scientific investigation. Clinical and radiological investigation, stereotactic biopsy, immunohistopathological analysis and chemotherapeutic treatment are standard procedures for patients with PCNSL. To perform functional assays, such as migration and killing assays isolation of malignant B as well as tumor-infiltrating T cells from patient`s samples would be required. Unfortunately, we did not have access to fresh tissue samples, which would have allowed us to perform these experiments. Therefore this study remains mainly based on description, while proof of principle experiments are missing. While recent gene expression profiles of PCNSL added important information, they analyzed total tumor mRNA and therefore neglect the cellular heterogeneity within PCNSL consisting of malignant B cells, infiltrating immune effectors and resident bystander cells such as activated microglia. Despite being partially descriptive, this work adds important value to our understanding on the interaction between the tumor microenvironment and tumor cells, and encourages future studies on the crosstalk between organ specific resident cells and proliferating malignant B cells.

3. MATERIAL AND METHODS

Material and methods regarding the manuscripts are listed there. This section includes only material and methods not listed in the manuscript.

Tissue specimens

Normal resting human lymph nodes from different localizations (inguinal, iliac, mesenteric, pulmonary) were received from human organ donors. Samples were fixed in 10% buffered formalin and embedded in paraffin. Sections from reactive lymph nodes were from patients that underwent lymphadenectomy due to chronic lymphadenopathy. Histopathological analysis showed absence of hematologic malignancies and samples were considered as reactive, inflammatory lymph nodes. Sections were received from the Department of Pathology, Scientific Institute of San Raffaele, Milan, Italy. Human tonsils were from patients undergoing tonsillectomy due to chronic tonsillitis. Samples were fixed in 10% buffered formalin and embedded in paraffin. Paraffin-embedded tissue was cut in sections of 4-5µm thickness and analyzed by immunohistochemistry, *in situ* hybridization or immunofluorescence microscopy.

Antibodies

Rabbit anti-human CD3 (RM-9107) was purchased from Thermo Scientific (Cheshire, UK) and used for immunostaining at 1:50. Mouse anti-human CD8 (M7103), CD20cy (M0755), CD68 (M0876), Smooth muscle actin (SMA) (M0851) and Cytokeratin (M0821) were purchased from DAKO (Glostrup, Denmark) and used at concentrations following manufacturer`s recommendations. Mouse anti-human CXCL12 (MAB350) was purchased from R&D Systems (Abingdon, UK) and used for immunostaining at 5 µg/ml. Goat anti-human CXCL9 (AF392), CCL21 (AF366) and CXCL13 (AF801) were purchased from R&D Systems, and used at 1 µg/ml, 1 µg/ml and 2 µg/ml. Rat anti-mouse PNAd (550564) was purchased from PharMingen and used at 2.5 µg/ml.

Immunostaining

Immunostaining was performed by initial deparaffinization and rehydration of tissue sections. Slides were boiled at 95°C for 30 minutes in target retrieval solution (S1699, DAKO Cytomation). Tissue

slides were treated with Peroxidase Blocking Reagent (S2001, DAKO) and Protein Block Serum-Free (X0909, DAKO). As negative controls for immunostainings, nonrelated isotype-matched immunoglobulins were used. Primary antibodies were diluted in Antibody Diluent (S3022, DAKO). Sections were then incubated overnight at room temperature in a humid chamber, followed by incubation with the appropriate secondary antibodies: rabbit anti-mouse Ig/biotinylated/F(ab`)2 (E0413, DAKO) or rabbit anti-goat Ig/biotinylated (0466 DAKO) diluted 1:200 in Antibody Diluent (S3022, DAKO) for 30 minutes at room temperature. Slides were washed and incubated with StreptABComplex/ Horseradish peroxidase (HRP) (K0377, DAKO). After washing, sections were developed with 3-amino-9-ethylcarbazole (A-6926, Sigma-Aldrich, Buchs, CH), washed in water and counterstained with Hematoxylin. Immunofluorescence stainings were performed following the same protocol used for immunohistochemistry. Fluorescence-labelled secondary antibodies purchased form Molecular Probes (Invitrogen, Carlsbad, CA, USA) were as follows: goat anti-mouse IgG1 Alexa Fluor 488 (A-21121) and 594 (A-21125), goat anti-rat IgG Alexa 594 (A-11007), goat anti-mouse IgG2a Alexa Fluor 594 (A-21135), goat anti-mouse IgG3 Alexa Fluor 488 (A-21151), goat anti-rabbit IgG Alexa Fluor 488 (A11070), rabbit anti-goat IgG Alexa Fluor 594 (A-11080). Appropriate secondary antibodies were diluted 1:400. After washes, slides were mounted in 4,6 diamidino-2-phenylindole (DAPI)-containing mounting medium (H-1200, Vectashield, Vector Laboratories, Burlingame, CA, USA).

In situ hybridization

^{35}S-labeled sense and antisense for CCL21mRNA probes, 310 bp in length and corresponding to position -59 to -368 bp of CCL21 sequence (NM002989), CXCL13mRNA probes, 355 bp in length and corresponding to position -35 to -364 bp of CXCL13 sequence (NM006419) CXCL12mRNA probes, 236 bp in length and corresponding to position -111 to -346 bp of CXCL12 sequence (NM199168) and CXCL9 mRNA probes, 372 bp in length and corresponding to position -46 to -417bp of the CXCL9 sequence (NM002416), probes were generated by *in vitro* transcription (10 999 644 001, Roche, Rotkreuz, CH). Tissue sections were kept under RNAse free conditions during the whole procedure. After dewaxing and rehydrating the slides in graded ethanol solutions, *in situ*

hybridization was performed, according to a previously described method.(24) Finally, the sections were dipped in Kodak photo emulsion NTB-2 (Rochester, NY) and exposed in complete darkness for 14 days at 4°C. Development and fixation were performed according to the instructions provided by Kodak, and counterstaining was performed with hematoxylin.

Image analysis

Immunohistochemical and *in situ* hybridization staining were analyzed with Nikon Eclipse E800 light microscope. Digital pictures were acquired with a Nikon Digital Camera DXM1200, and Act-1 software (Nikon AG, Egg, CH). Digital image analysis for CXCL9 mRNA expression was performed with Nikon NIS-Elements BR 2,10. Immunofluorescence stainings were analyzed with a Nikon Eclipse E800. Image acquisitions were performed with Hamamatsu EM-CCD Digital camera C9100 and Openlab 5,5 software (Improvision, Coventry, UK). Lymphocyte distribution was assessed by quantitative analysis of cell per high power field (HPF), counting 10-20 different areas within each sample at 400x magnification.

Statistical analysis

Statistical analsysis was performed with GraphPad Prism 4 using Mann Whitney test.

4. RESULTS

4.1 B cell attracting chemokines in human secondary lymphoid organs

4.1.1 B cell attracting chemokines in normal human secondary lymphoid organs

During their lifetime, B cells express various chemokine receptors. The two classical chemokine receptors of B cells are CXCR4 and CXCR5. Depending on their developmental and activation state, they can also express CCR7 and CXCR3. While ligands for these receptors have been extensively studied in murine secondary lymphoid organs under homeostatic and inflammatory conditions, their expression under homeostatic and inflammatory conditions in humans is less well studied. To improve our understanding of lymphocyte trafficking in human secondary lymphoid organs under normal and inflammatory conditions, we focused on the expression of their appropriate ligands CXCL12 (CXCR4), CXCL13 (CXCR5), CCL21 (CCR7), and the three CXCR3-ligands CXCL9, CXCL10 and CXCL11. We performed immunohistochemical analysis for the abovementioned chemokines in resting and inflamed secondary lymphoid organs received from organ donors, patients undergoing tonsillectomy or lymphadenectomy. Sense and antisense probes have been generated for CXCL12, CXCL13 and CCL21 and *in situ* hybridization has been performed on resting lymph nodes from organ donors. The analysis showed three distinct expression patterns for CCL21, CXCL13 and CXCL12 in normal resting lymph nodes (Figure 5).

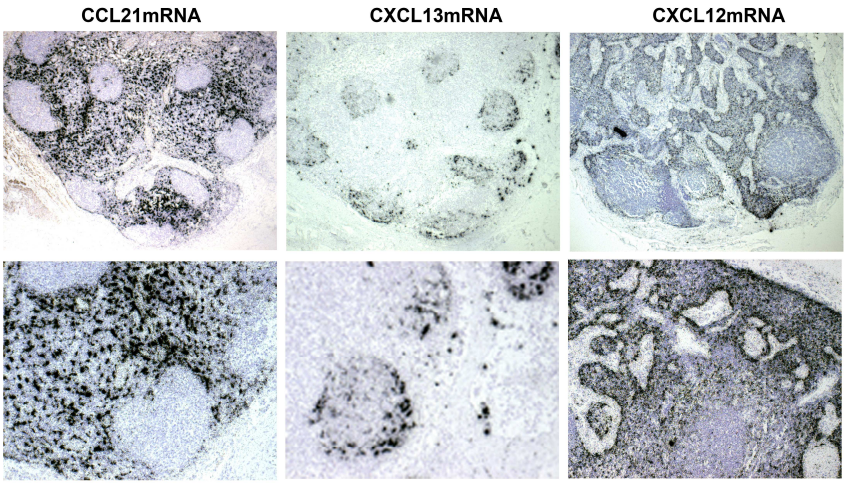

Figure 5: mRNA expression of chemokines in normal resting human lymph nodes. *In situ* hybridization for CCL21, CXCL13 and CXCL12 in normal resting inguinal lymph node. CCL21 expression is restricted to parafollicular T cell areas and is absent in B cell follicles (left panels). CXCL13 expression is mainly restricted to B cell follicles underlying the subcapsular sinus (intermediate panels). CXCL12 expression is most prominent along subcapsular, intermediate and medullary sinuses (right panels). Representative results are shown. Images show signal intensity with antisense probes, sense probes showed no signal. (Magnification 60x in top panels, and 125x in bottom panels).

Consistent with previous reports in mouse (282), CXCL13 was mainly confined to the B cell follicles underlying subcapsular sinuses in normal lymph nodes, while CCL21 was completely absent in B cell follicles, but was present in high concentration in the parafollicular T cell area.(26) Under homeostatic conditions, CXCL12 expression was most prominent along subcapsular, intermediate and medullary sinuses within resting lymph nodes. Similar to CCL21mRNA, CXCL12mRNA was also present in the T area, but could not been detected in B cell follicles (Figure 5). Immunohistochemical stainings for the CXCR3-ligands CXCL9, CXCL10 and CXCL11 showed no positive staining in normal resting lymph nodes.

To confirm translation of chemokine mRNA into proteins, we analyzed expression of CCL21, CXCL13 and CXCL12 by immunohistochemistry. We found similar reticular expression pattern of CCL21 and CXCL12 in the T area, consistent with stromal compartments (51) CCL21, CXCL13 and CXCL12 protein expression showed complementary pattern compared to mRNA expression (Figure 6) indicating that immunohistochemical stainings with the antibodies detect specifically the appropriate chemokines. CCL21 showed expression within the T area and around HEVs (Figure 6A). CXCL13 stained follicular dendritic cells in primary B cell follicles (Figure 6B). Strong expression of CXCL12mRNA in the subcapsular, indermediate and medullary sinuses could also be confirmed by its protein expression (Figure 6C).

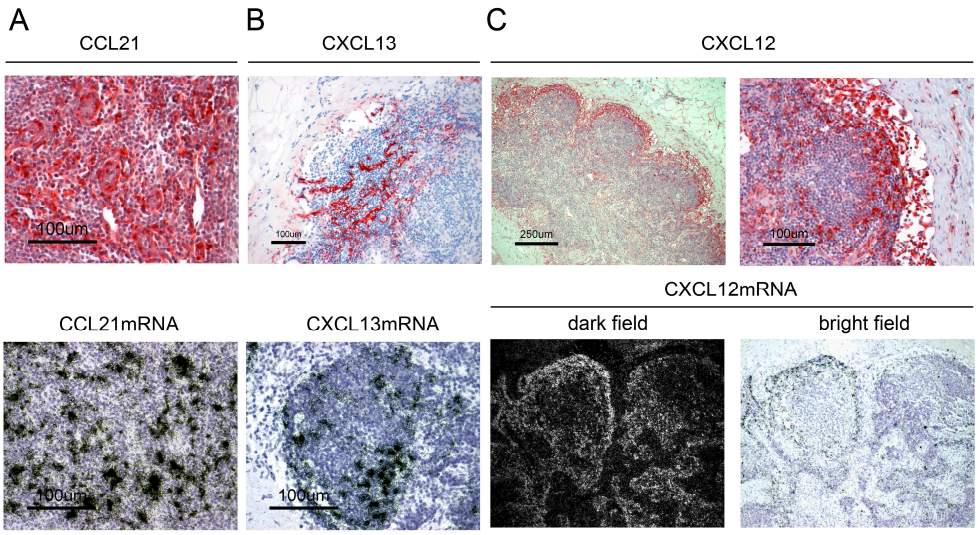

Figure 6: chemokine protein expression reflects mRNA expression in normal resting lymph nodes (A) Images show CCL21 protein expression (top panel) and CCL21mRNA expression (bottom panel) in the T cell area. The image shows a reticular expression pattern with close association to HEV. (B) CXCL13 protein (upper panel) and mRNA (lower panel) expression in primary B cell follicles. (C) CXCL12 protein (upper panels) and mRNA (lower panels) expression in the subcapsular, intermediate and medullary sinuses (Magnification 100x).

Slight differences between protein and mRNA expression were detected on HEVs. While HEVs showed positivity for CXCL12 expression, no mRNA was detected, indicating a chemokine transport from the abluminal T cell compartment towards the luminal side of HEVs (Figure 7B). CCL21mRNA and protein was detected in close proximity to HEVs. Nevertheless, no direct overlay with CXCL12 was found on HEVs (Figure 7A/C). In contrast, CCL21 and CXCL12 colocalized in the parafollicular T area (Figure 7C).

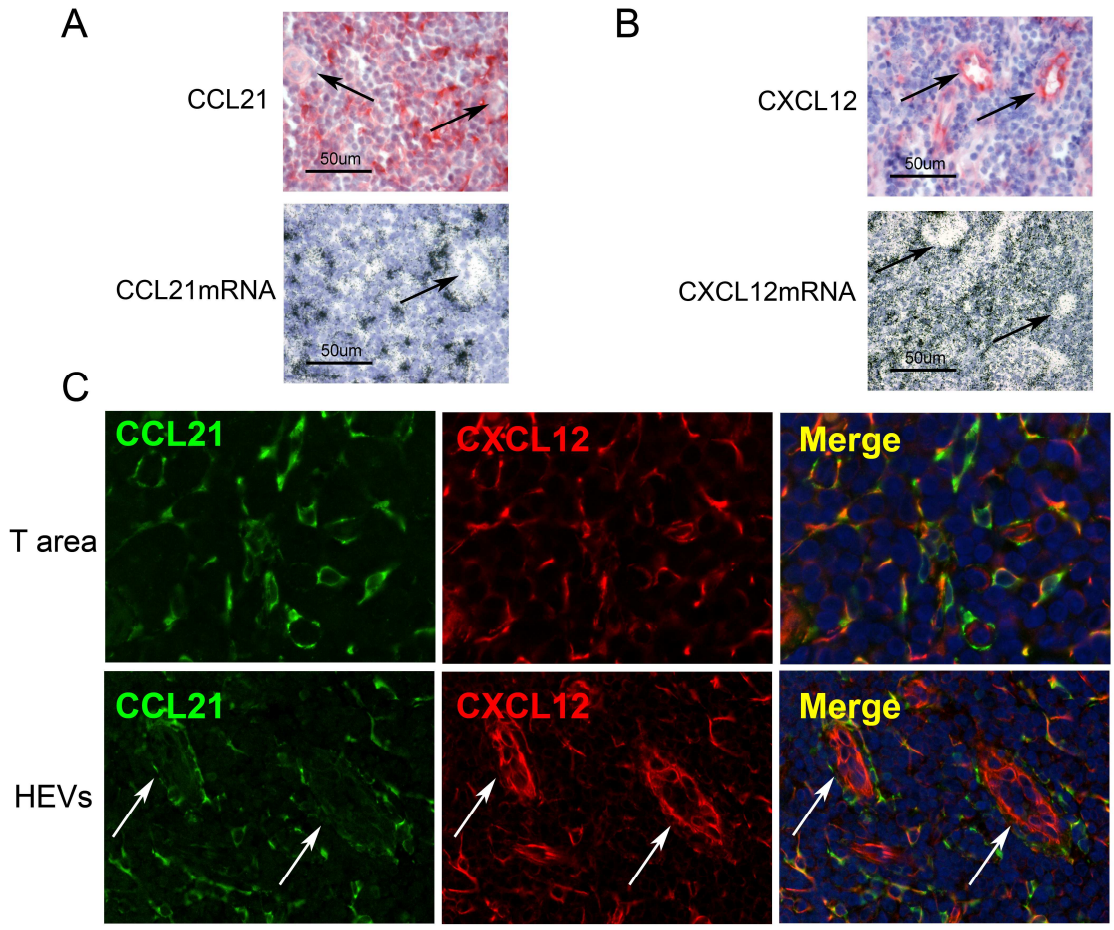

Figure 7: Expression of CCL21 and CXCL12 in T cell areas and on HEVs in normal resting lymph nodes (A) Immunhistochemical (top panel) and *in situ* hybridization (bottom panel) for CCL21. Images show T cell areas including HEVs (B) Immunhistochemical (top panel) and *in situ* hybridization (bottom panel) for CXCL12. Images show T cell areas including HEVs (C) Double immunofluorescence stainings for CCL21 and CXCL12 in T cell areas (upper panels, Magnification 800x) and HEVs (lower panels, Magnification 400x) arrows indicate HEVs. Representative images are shown.

In resting human lymph nodes, the B cell attractant chemokine CXCL13 mainly localizes in B cell follicles underlying the subcapsular sinus (Figure 6B). Cells with long dendrites can also be found in close proximity the subcapsular sinuses (Figure 6B, upper panel).

4.1.2 Inflammation-induced changes of chemokine expression

We further investigated inflammation-induced changes of homeostatic B cell attracting chemokines in secondary lymphoid organs. Chemokine expression in inflamed human tonsils and reactive lymph nodes was analyzed and the expression pattern was compared to normal lymph nodes. In contrast to normal resting lymph nodes, inflamed secondary lymphoid tissue classically presents with germinal center formation. As summarized in the introduction, germinal center formation is a complex process of subsequent events which requires B cell and plasma cell migration at several time points. In humans, these migration steps are poorly understood. To improve our understanding of B cell trafficking within and outside of germinal centers, we analyzed the expression pattern of CCL21, CXCL12, CXCL13 and CXCL9.

Under inflammatory conditions, immunostainings for the CCR7-ligand CCL21, showed a similar expression pattern compared to resting secondary lymphoid organs. CCL21 protein and mRNA was mainly confined to the parafollicular T area and in close proximity to HEVs (Figure 8). The germinal centers as well as the mantle zone were negative for CCL21 (Figure 8). While downregulation of CCL21mRNA expression has been described under several acute inflammatory conditions in the mouse (283,284), we have not detected major differences in CCL21mRNA expression between normal and chronically inflamed secondary lymphoid organs.

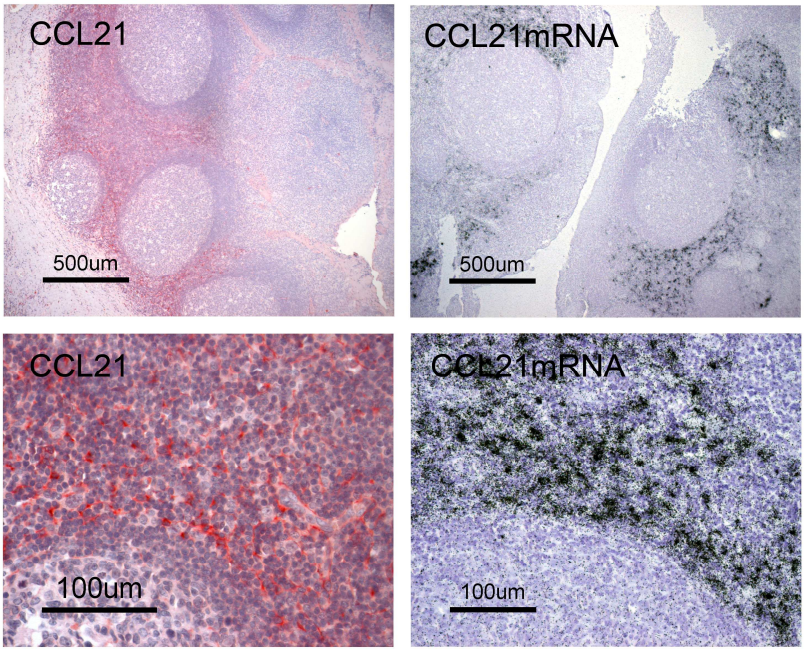

Figure 8: CCL21 expression in chronically inflamed secondary lymphoid organs.
Immunhistochemical and *in situ* hybridization for CCL21 in chronically inflamed human tonsils. Germinal centers are polarized towards the tonsillar crypt. GC and tonsillar crypt is free from CCL21.

We further investigated the expression of CXCL12 in inflamed secondary lymphoid organs. Inflamed tonsils showed positive staining in three distinct areas: 1) in the tonsillar crypt 2) within germinal centers and 3) in T cell areas (Figure 9).

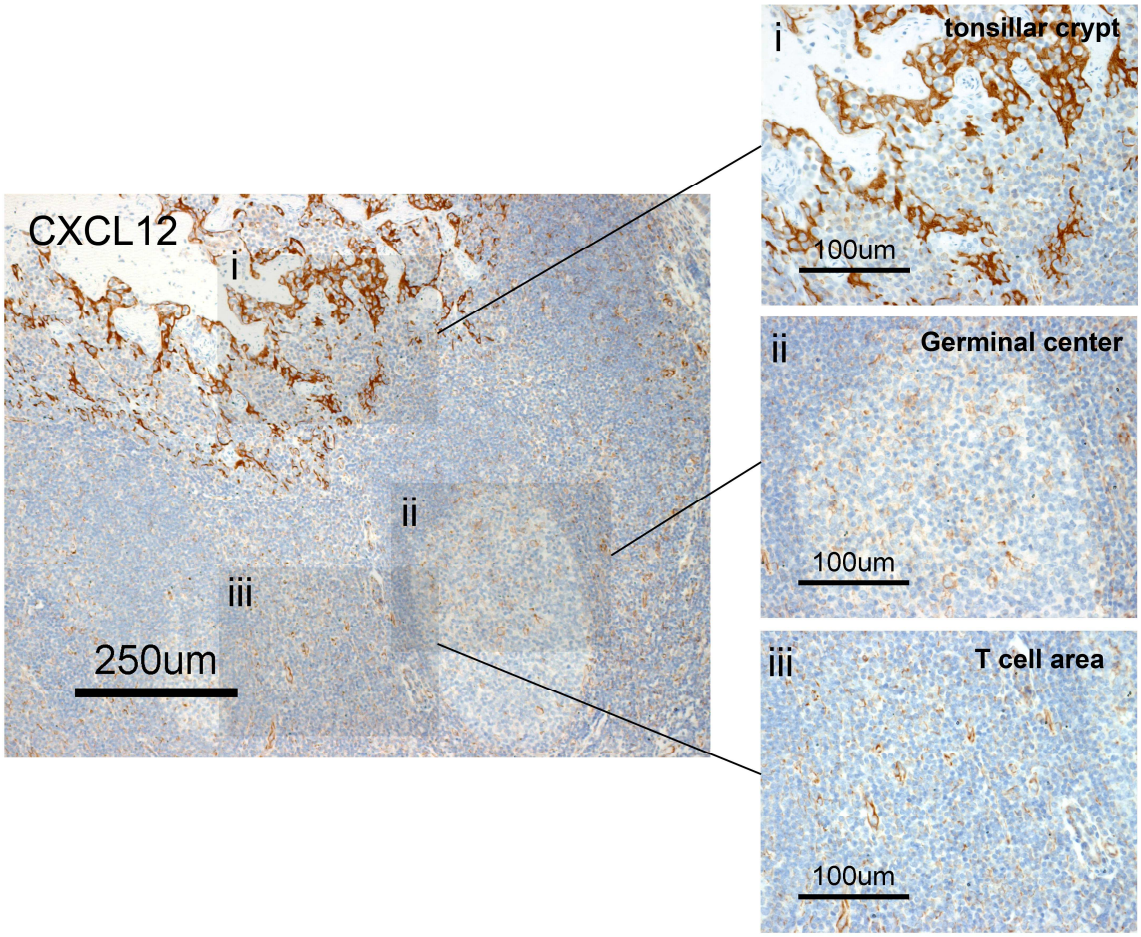

Figure 9: CXCL12 expression in tonsillar crypt, germinal centers and T cell areas of inflamed secondary lymphoid organs. Immunohistochemical staining for CXCL12 in inflamed human tonsils show expression in the tonsillar crypt (i), within the germinal center (ii) and in the T cell area (iii).

Similar as in resting lymph nodes, CXCL12 is found on $PNAd^+$ HEVs in parafollicular areas of human inflamed tonsils and reactive lymph nodes and did not colocalized on smooth muscle actin positive (SMA^+) pericytes (Figure 10D). Interestingly perivascular accumulation of $CD20^+$ B cells around HEVs in the T cell area is commonly found in human secondary lymphoid organs (Figure 10B). This phenomenon has also been reported in murine lymph nodes.(126)

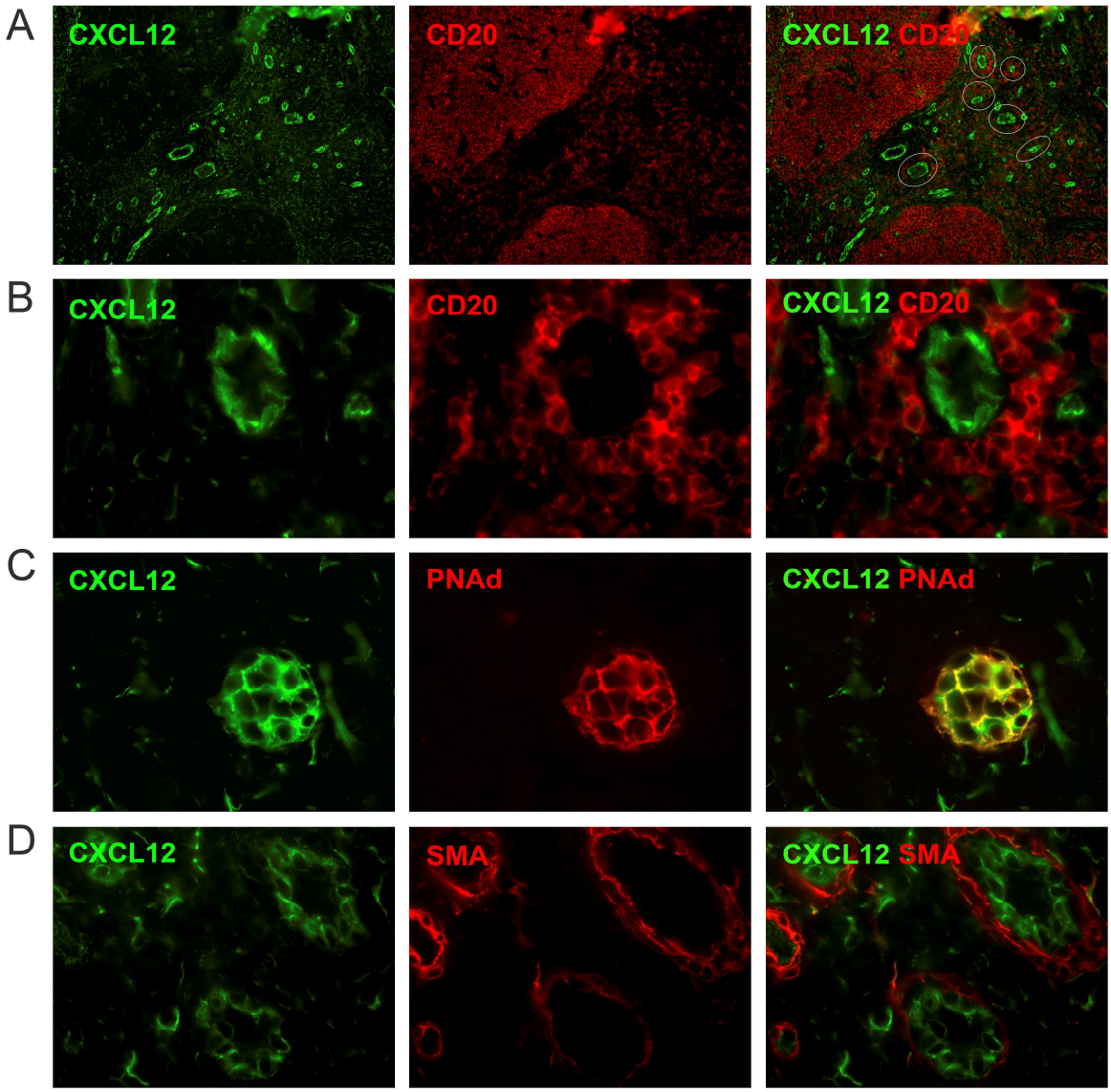

Figure 10: Perivascular accumulation of B cells around CXCL12$^+$ HEVs in parafollicular T areas. Double immunofluorescence microscopy of inflamed human tonsil for CXCL12 and CD20, PNAd and SMA. (A) T area with CXCL12$^+$ HEVs between 2 germinal centers. White circles indicate B cell distribution around HEVs in the T area (Magnification 100x). (B) High magnification of a CXCL12$^+$ HEV in the T area with perivascular B cell distribution (Magnification 800x) Double staining with PNAd (C) (Magnification 800x) and SMA (D) (Magnification 600x) confirm its expression on HEVs SMA: smooth muscle actin, PNAd: peripheral node addressin.

CXCL12 expression within the B cell follicles changed under inflammatory conditions. While in B cell follicles of normal lymph nodes, follicular CD68$^+$ marcophages do not show CXCL12 expression, highly phagocytic tingible body macrophages (TBM) in germinal centers showed strong CXCL12 staining in inflamed tonsils as well as reactive lymph nodes (Figure 11). In contrast to FDCs, TBMs are equally distributed within the germinal center and are positive for the macrophage marker macrosialin (CD68) (Figure 11). TBMs engulf apoptotic germinal center B cells which fail to develop

further towards memory B or plasma cells. They can regulate germinal center reactions.(133,285) and decreased phagocytic activity of TBMs in the germinal center can lead to autoimmunity.(286) In contrast to previous reports in the mouse (76), we did not detect an obvious gradient of CXCL12 between the light and dark zone of the germinal center.

Interestingly, strongest expression of CXCL12 in normal and inflamed secondary lymphoid organs was found at the sites, where antigen encounter occurs. This includes the subcapsular sinuses in lymph nodes (Figure 5 and 6) and the crypt epithelium of inflamed tonsils (Figure 9).

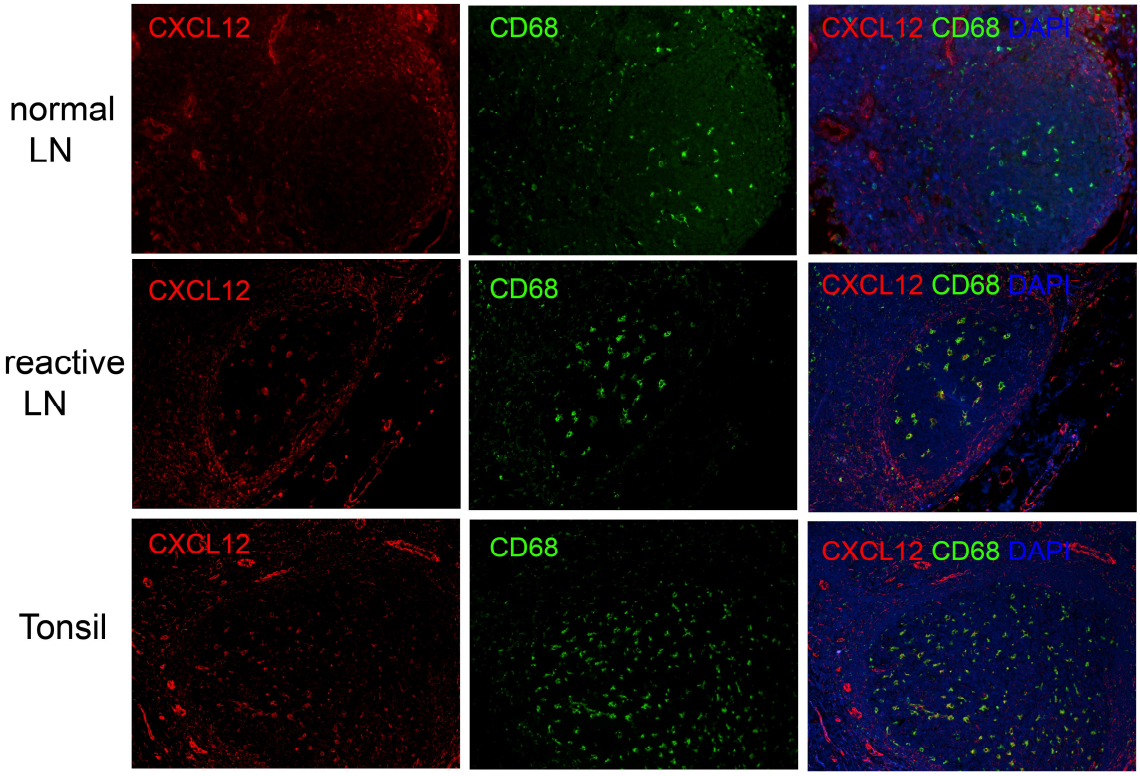

Figure 11: CXCL12 expression in the germinal center Double immunofluorescence microscopy on normal (Mag 200x), reactive (Mag 100x) and tonsillar (Mag 100x) secondary lymphoid tissues for CXCL12 and the macrophage marker macrosialin (CD68).

Crypt-derived CXCL12 in the tonsil is produced by cytokeratin positive epithelial cells. This network of CXCL12-expressing epithelial mainly harbors $CD20^{+}$ B cells (Figure 12). A considerable amount of plasma cells is also present within this meshwork, while only a few T cells are present in the crypt (data not shown).

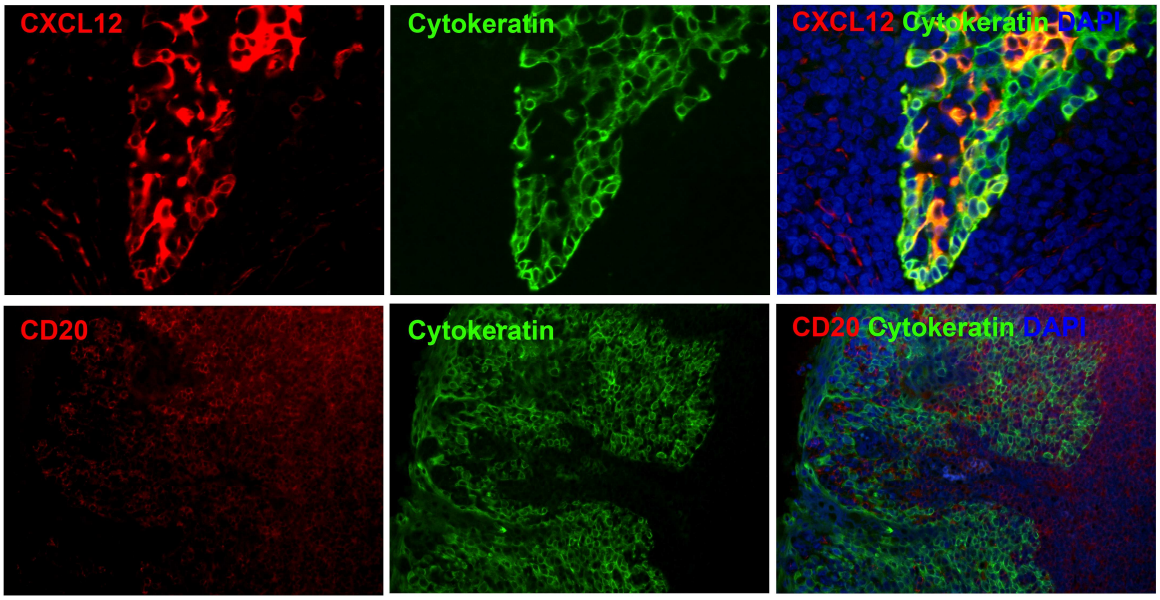

Figure 12: Tonsillar crypt cells build a CXCL12-expressing reticular network filled with B cells.
Double immunofluoresence microscopy of inflamed human tonsils stained with CXC12, the epithelial marker Cytokeratin (upper panels, Magnification 400x) or the B cell marker CD20 (bottom panels, Magnification 100x). Images show network of tonsillar crypt epithelial cells interspersed by CD20+ B cells. Crypt epithelium stains strongly positive for CXCL12.

In inflamed secondary lymphoid tissue, CXCL13 expression is mainly confined to areas within the germinal center. In germinal centers, CXCL13 shows a polarized expression patter and is found exclusively in the light zone of the germinal center and the adjacent mantle zone (Figure 13).The highest density of $CXCL13^{+}$ cells occurs at the border between the germinal center light zone and the adjacent mantle zone (Figure 13).

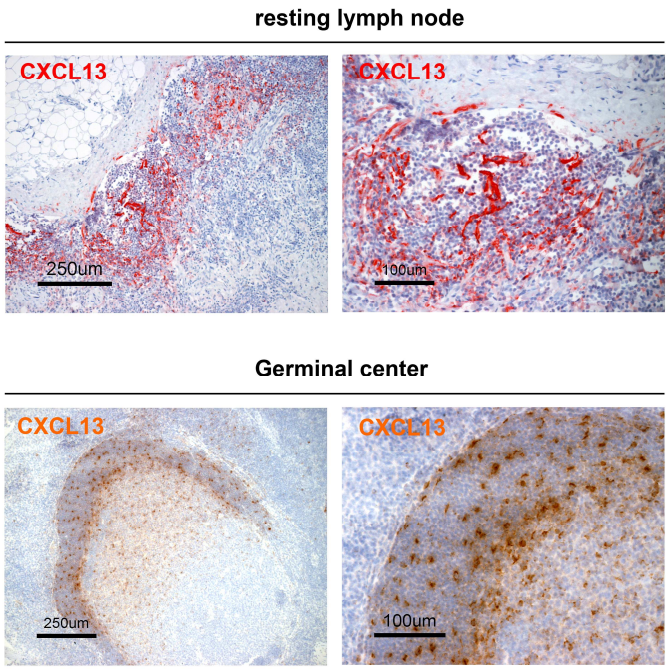

Figure 13: CXCL13 expression in primary and secondary B cell follicles in secondary lymphoid organs Immunohistochemical staining for CXCL13 in resting (LN) and inflamed (tonsil) secondary lymphoid organs. Upper panels show CXCL13 expression in resting lymph node in B cell areas underlying the subcapsular sinus. The expression pattern is mainly reticular and cells show long dedrites which can reach the subcasular sinus. Bottom panels show CXCL13 expression in the germinal center light zone and the mantle zone of inflamed tonsils. The strongest staining occurs on the edges between light zone and mantle zone.

$CXCL13^+$ cells in the light zone of GCs represent a fraction of $CD3^+$ T_{FH} cells as well as $CD3^-$ FDCs (Figure14). Interestingly the majority of $CD3^+$ follicular T cells are negative for CXCL13, indicating either functional heterogeneity between T_{FH} cells or upregulation of CXCL13 at a special time point within the germinal center reaction (Figure14). $CXCL13^+$ T_{FH} cells are enriched in close proximity to the germinal center border between light zone and mantle zone (Figure14). $CD3^+$ T cells outside the germinal center and mantle zone are negative for CXCL13 (Figure 14).

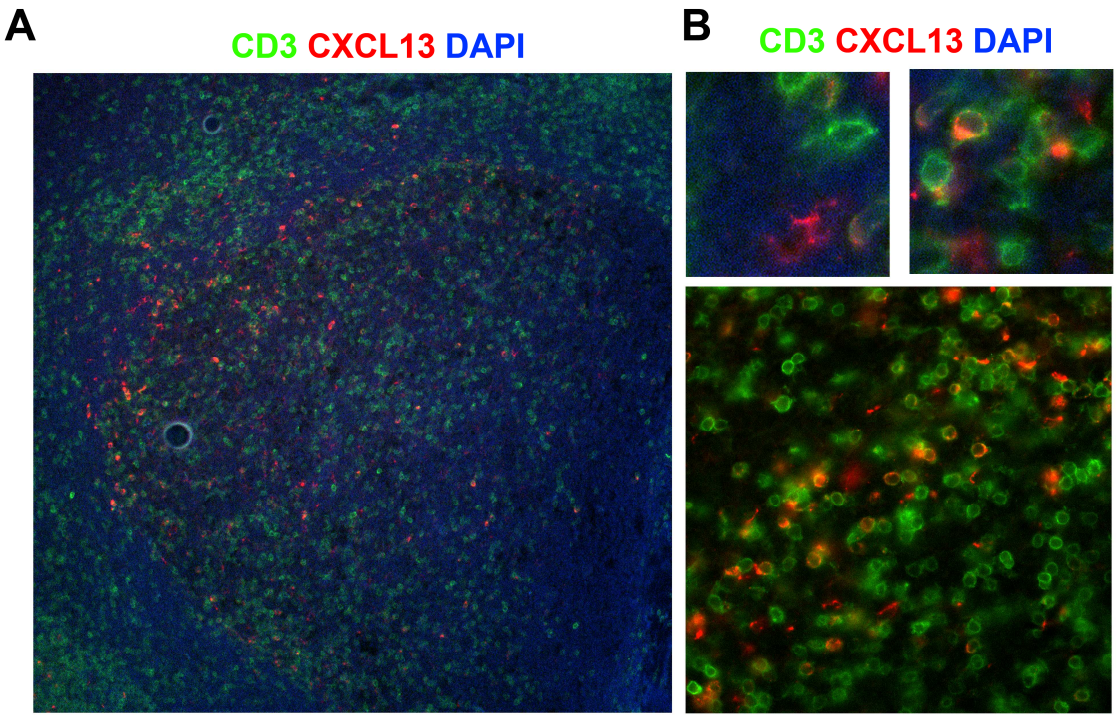

Figure 14: CXCL13 expression in the germinal center (A) Double immunofluorescence microscopy of representative germinal center in inflamed tonsil for CXCL13 and CD3. Yellow color indicates $CXCL13^+CD3^+$ TFH cells. Red color only stains FDCs (Magnification 100x) (B) $CXCL13^+$ $CD3^-$ FDC (left panel) and $CXCL13^+$ $CD3^+$ TFH cells (right panel) in the germinal center light zone (upper panels, magnification 800x). Inset shows accumulation of $CXCL13^+$ T_{FH} cells on the edge of the germinal center between light zone and mantle zone (bottom panel) (Magnification 400x).

CXCL9, CXCL10 and CXCL11, ligands for the chemokine receptor CXCR3, could only been detected in secondary lymphoid organs under inflammatory conditions. While CXCL10 and CXCL11 expression was restricted to a few cells (data not shown), CXCL9 expression showed a T cell area-restricted expression pattern. Protein and mRNA for CXCL9 were found in dinstinct interfollicular clusters and showed a reticular expression pattern, whereas germinal centers showed no CXCL9 expression (Figure 15). $CXCL9^+$ cells show a dendritic-cell-like morphology (Figure 15).

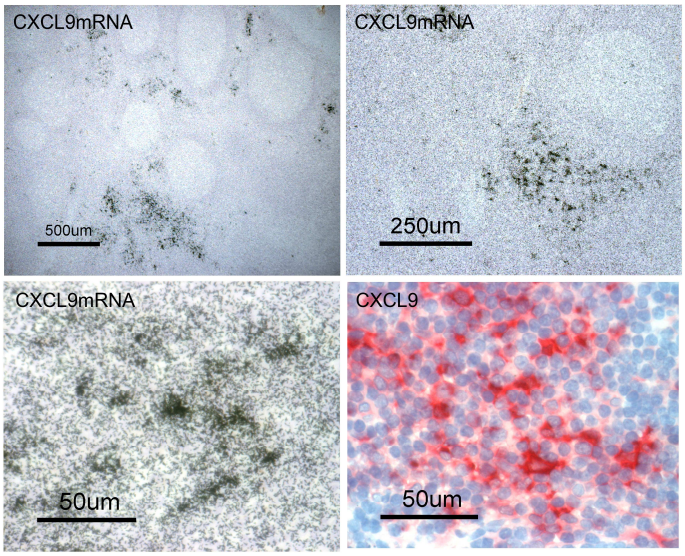

Figure 15. *In situ* hybridization and immuno-histochemistry for CXCL9 in inflamed secondary lymphoid organs. T area-restricted mRNA expression of CXCL9 in the parafollicular areas of inflamed tonsils (upper panels). Insets show CXCL9-expressing cells in the T area with dendritic cell-like morphology.

CXCR3, the receptor for CXCL9 is typically expressed on $CD8^+$ T cells and may therefore play and important role in the tissue distribution of $CD8^+$ T cells in the T area of inflamed secondary lymphoid organs. Indeed, tonsillar $CD8^+$ T cells accumulate in areas with high CXCL9 expression (Figure 16). While the vast majority of $CD8^+$ T cells are found in the T area outside germinal centers, a few $CD8^+$ T cells are also present within germinal centers (Figure 16A). In contrast to $CD4^+$ follicular T cells, which accumulate only in the germinal center light zone, $CD8^+$ follicular T cells seem to be equally distributed in the germinal centers or accumulate in small $CD8^+$ T cells clusters.

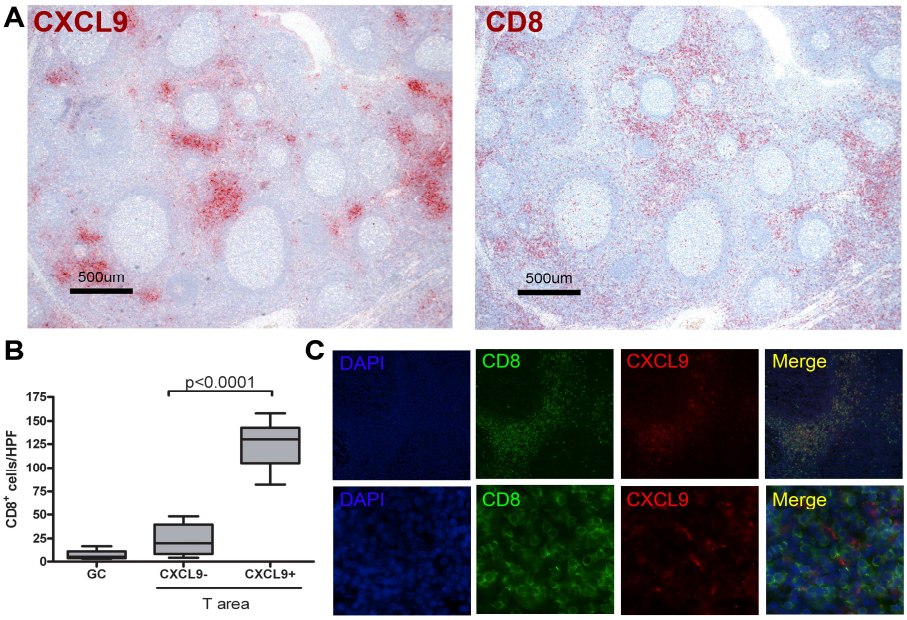

Figure 16. $CD8^+$ T cells accumulate in parafollicular areas with CXCL9 expression. (A) Immunhistochemical staining for CXCL9 (left panel) and CD8 (right panel) on consecutive slides in inflamed human tonsil show complementary images. (B) Graph showing $CD8^+$ T cell accumulation in T cell areas with CXCL9 expression. Cell were counted in high power fields in at least 10 different GC or areas with or without CXCL9 expression (C) Double immunofluorescence microscopy for CXCL9 and CD8 shows accumulation of $CD8^+$ T cells in $CXCL9^+$ area (Magnification 125x upper panels, 800x bottom panels).

4.2 T and B cell attracting chemokines in PCNSL

4.2.1 Patients

To investigate whether there are parallels between the B cell attractant chemokine environment in secondary lymphoid organs and extranodal DLBCL confined to the CNS, we performed a retrospective study on samples from 22 patients with PCNSL. Table 3 summerizes patients`s characteristics and the extension of disease. Brain specimens were taken for diagnosis of B cell PCNSL between 2001 and 2005, from 11 men and 11 women, aged 35 to 75 years (median 65 years). All patients were immunocompetent, HIV negative, and Epstein-Barr virus encoded RNAs (EBER) negative. Diagnosis of PCNSL by immunophenotyping and routine hematoxylin and eosin staining was used to characterize the tumors according to the current World Health Organization (WHO) classification. No patient selected for this study received an antitumor treatment (neither chemotherapy nor radiotherapy) before surgical biopsy. All patients received corticosteroids before biopsy to control symptoms by reducing brain edema and tumor mass effect. Steroids were interrupted 10-15 days before biopsy to reduce the interfering effect of these drugs on histopathological features.

Table 3. Patients' characteristics and extension of disease.

		Patients(n= 22)
Age	median 65	
	range 35-75	
	age >70 ys.	7/22 (32%)
Males		11/22 (50%)
ECOG-PS	0 - 1	12/22 (55%)
	2 - 3	10/22 (45%)
Histotype [α]	Diffuse large B-cell lymphoma	21/22 (95%)
	Burkitt-like lymphoma	1/22 (5%)
Systemic symptoms		0/22 (0%)
LDH ratio >1		3/17 (18%)
Ocular disease[β]		0/11 (0%)
Positive CSF cytology examination [β]		1/10 (10%)
High CSF protein level [β]		3/11 (27%)
Multiple lesions		10/22 (45%)
Involvement of deep structures [χ]		10/22 (45%)

[α] Histotype was defined according to the WHO Classification.
[β] Relationship between the number of positive cases and the total number of assessed patients.
[χ] Involvement of deep structures of the brain (basal ganglia and/or corpus callosum and/or brain stem and/or cerebellum).

4.2.2 CXCL12 expression in PCNSL

In most of the PCNSL cases, malignant B cells remain confined to the CNS. It is therefore tempting to hypothesize that the CNS microenvironment releases factors, which favor CNS restriction. CXCL12 is well known as a key player for the retention of HSC, B cell precursors and plasma cells in the bone marrow.(63,64,67-71,74,75) Previous reports have shown, that malignant B cells in PCNSL resemble late germinal center B cells and uniformly express CXCR4.(108,197) We therefore investigated in detail the expression pattern of its ligands CXCL12 in samples from PCNSL patients. In line with a previous report (207), we detected strong expression of CXCL12 in all 22 PCNSL cases (22/22). Yet, our findings indicated, that the source of CXCL12 in all analyzed cases was the inflammatory tumor microenvironment, rather than the malignant B cells themselves. Similar to our findings in the germinal center (Figure 11), CXCL12 in PCNSL was mainly restricted to $CD68^+$ microglia/macrophages with large cytoplasm or dendritic cell-morphology as well as endothelial cells (Figure 18A/B). Tumor associated macrophages in PCNSL show large phagocytic morphology, similar to tingible body macrophages within germinal centers (Figure 17A). High numbers of $CD68^+$ macrophages were found in all investigated PCNSL cases. Their density of TAMs in PCNSL was comparable with the density of TBM within germinal centers (Figure 17B).

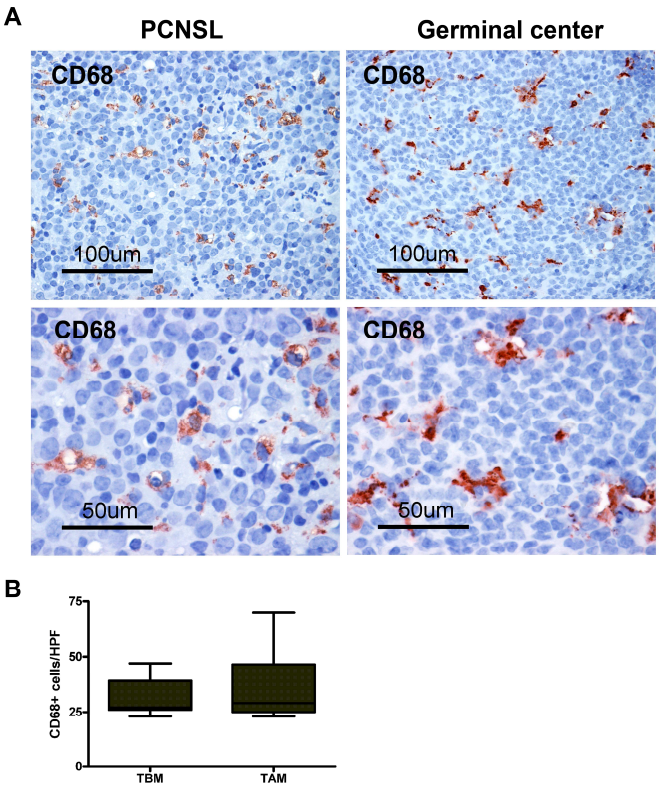

Figure 17. Morphology and distribution of macrophages in PCNSL and germinal centers Immunohistochemical stainings for CD68 in PCNSL cases and germinal centers. (A) Representative images for tumor associated macrophage (TAM) distribution in PCNSL (left panels) and tingible body macrophages (TBM) in germinal centers (B) Graph showing average numbers of TBMs and TAMs in germinal centers and PCNSL cases respectively. $CD68^+$ macrophages were counted in high power fields (Magnification 800x) in 10 cases of PCNSL and 20 germinal centers.

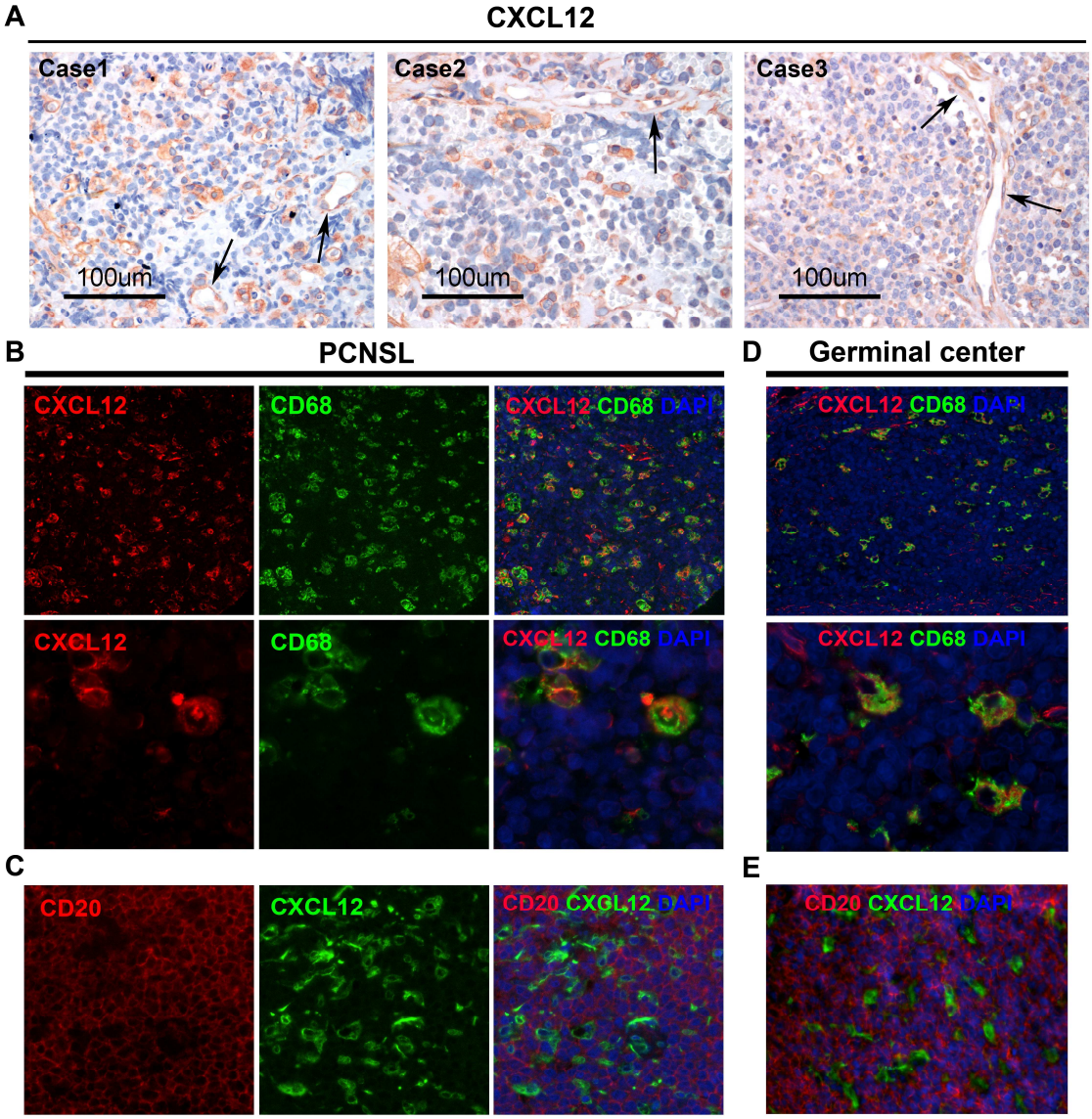

Figure 18: CXCL12 expression in PCNSL microenvironment. (A) Immunohistochemical staining for CXCL12 in 3 representative cases of PCNSL show CXCL12 positivity in large cells with big cytoplasm and small nuclei as well as on small and intermediate size vessels (black arrows). Double immunofluorescence microscopy for CXCL12 and $CD68^+$ microglia/macrophages in the tumor microenvironment (B) (Magnification 200x) and $CD20^+$ malignant B cells (C) (Magagnification 400x). (D) and (E) show stainings as in (B) and (C) in the germinal center of inflamed human tonsils (Magnification 800 and 300x, respectively).

To improve our understanding of tumor infiltration and distribution within the CNS, we analyzed in more detail CXCL12 distribution and the presence of $CD20^+$ malignant B cells at the tumor border from 3 out of 22 cases, where peritumoral CNS tissue was present. As expected, we found that in peritumoral areas, elongated cells show CXCL12 expression in the absence of $CD20^+$ malignant B cells (Figure 19A ii). CXCL12 expressing cells decreased with increasing distance to the tumor border (Figure 18). These findings indicate that peritumoral microglial cells may sense alterations in

their environment, such as edema and upregulate lymphocyte attractants to which malignat B cells can respond.

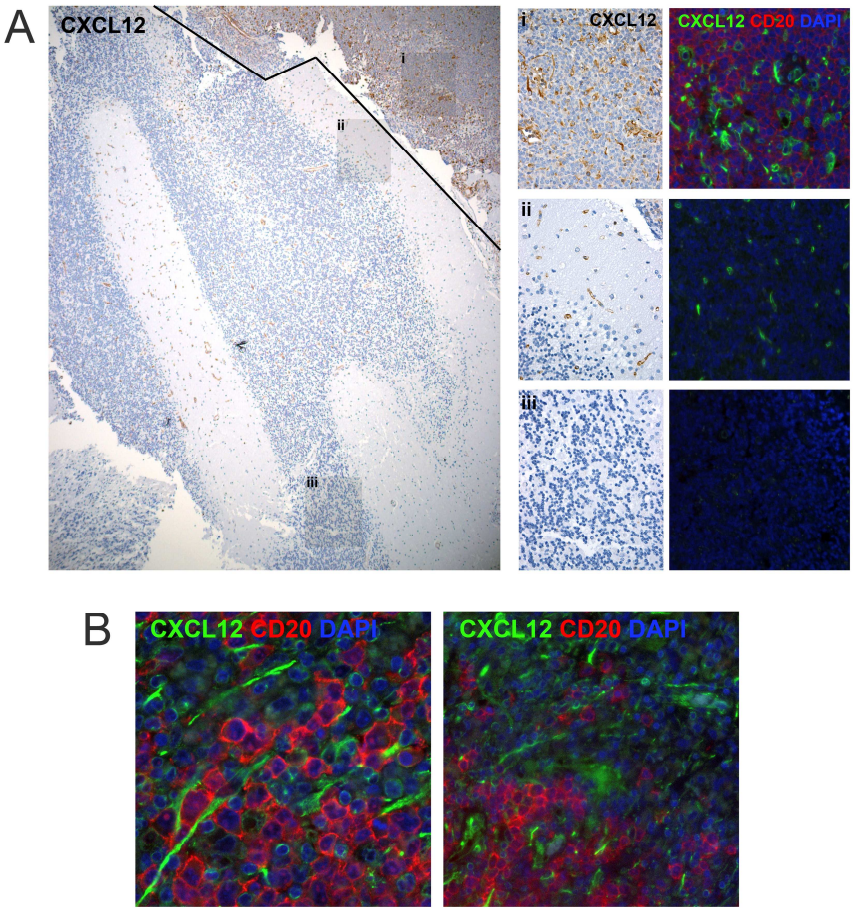

Figure 19: CXCL12 expression at the tumor border of PCNSL.
(A) Immuno-histochemical analysis of CXCL12 expression (brown) at the tumor border of PCNSL cases. Black line in the large image indicates tumor border. PCNSL (upper right corner) and adjacent cerebellar tissue. Insets are marked and represent i) intratumoral, ii) peritumoral and iii) extra-tumoral areas. Larger magnifications of insets are shown on the right. Immunofluorescense stainings for CD20 and CXCL12 indicates malignant B cell distribution on the tumor edge with elongated $CXCL12^+$ cells (green) in the peritumoral area in the absence of $CD20^+$ malignant B cells (Magnification 300x)
(B) Images show tumor border in two additional PCNSL cases where malignant B cells are infiltrating CNS tissue. CXCL12 is found in the tumor microenvironment. Tumor mass is on the bottom left, CNS tissue on the upper right (Magagnification 600x and 300x, respectively).

4.2.3 CXCL13 expression in PCNSL

Beside CXCR4, malignant B cells in PCNSL express CXCR5.(108) Its ligand CXCL13 has been reported to be upregulated in PCNSL.(203,287) By morphological analysis part of these cells producing CXCL13 has been ascribed to B cell blasts, whereas the analysis of gene expression profile, performed later, could not specifically focus on the CXCL13 producing cells. We performed additional immunhistochemical and immunofluorescence stainings for CXCL13 alone and in combination with CD20 and CD68. In line with previous reports, we found upregulation of CXCL13 in all PCNSL analyzed. In addition to the previus data, we have observed CXCL13 staining in $CD68^+$

macrophages/microglia, as well as on a fraction of tumor infiltrating $CD3^+$ T cells (Figure 20). These findings indicate that malignant B cells in PCNSL are embedded in an inflammatory environment of activated microglia/macrophages, which in addition release B cell attractant chemokines.

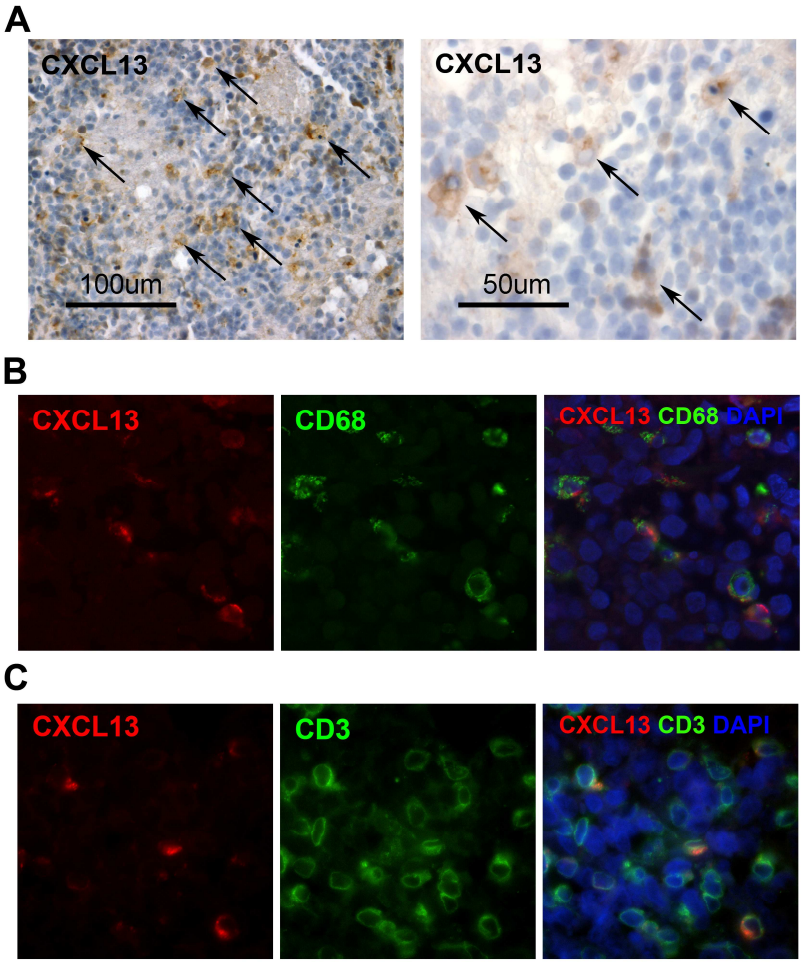

Figure 20: CXCL13 expression in the PCNSL microenvironment .
(A) Immuno-histochemical stainings for two representative cases of PCNSL stained with CXCL13. Positive staining is found on a fraction of cells (black arrows).
Double immunofluorescence microscopy for CXCL13, CD68 and CD3 in PCNSL shows overlay with CD68+ microglia/ macrophages (B) and CD3+ T cells (C).

4.2.4 Manuscript: International Journal of Cancer 2010

Perivascular Expression of CXCL9 and CXCL12 in Primary Central Nervous System Lymphoma: T Cell Infiltration and Positioning of Malignant B Cells

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Short title: Inflammatory microenvironment in PCNSL

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Key words: Chemokines; PCNSL; DLBCL; perivascular microenvironment; cell migration.

Journal category: Cancer Cell Biology.

Novelty and impact of the paper

This study addresses the hierarchy of the distinct co-expression of B and T cell attractant chemokines in the tumor microenvironment which control cell trafficking of both tumor infiltrating lymphocytes and malignant B cells and improves our understanding of cellular dynamics within PCNSL.

Therapies targeting chemokine interactions between malignant cells and its microenvironment might disrupt tumor-supporting interactions and improve anti-tumor immunity.

Abstract

Primary central nervous system lymphomas (PCNSL) are aggressive malignancies confined to the CNS, mostly of diffuse large B cell histotype. Despite improved understanding of the malignant B cells, little is known on the tumor microenvironment and on the response of the adaptive immunity against PCNSL. We investigated the phenotype of tumor infiltrating lymphocytes (TILs), and the expression of chemokines that could affect malignant B cells and trafficking of TILs.

TILs and chemokine expression were evaluated by immunohistochemistry and *in situ* hybridization. Furthermore, we performed *in vitro* migration assays to analyze the migratory capacity of lymphocytes and malignant B cells towards chemokines and chemokine heterocomplexes.

We show in 22 cases of PCNSL from immunocompetent patients that $CD8^+$ T cells represent the majority of TILs in the tumor mass. They tend to accumulate in perivascular areas, show Granzyme B expression, and proliferate *in situ*. Their localization and density correlates with the expression of the inflammatory chemokine CXCL9, which is transcribed and translated by perivascular macrophages and pericytes in the perivascular microenvironment. Moreover, CXCL9 and CXCL12 are coexpressed on the tumor vasculature and form heterocomplexes. In the presence of CXCL9, CXCL12-induced migration is enhanced not only on $CXCR4^+/CXCR3^+/CD8^+$ T cells but also on $CXCR4^+/CXCR3^-$ malignant B cells.

These findings indicate the presence of a strong chemoattractant stimulus in the perivascular microenvironment, which might serve as regulator for the recruitment of TILs and for the angiocentric positioning of malignant B cells in the perivascular cuff.

Introduction

Primary central nervous system lymphomas (PCNSL) are aggressive malignancies confined to the central nervous system (CNS), mostly of diffuse large B cell histotype. It counts for about 5% of primary CNS tumors and its incidence augmented significantly over the last three decades.[1,2] Despite improved therapeutic intervention, the prognosis of PCNSL remains poor. The majority of previous studies on PCNSL focused on the phenotype of malignant B cells, and only recent findings indicate a potential role of the microenvironment.[3] Moreover, recent studies in various tumors highlight the

importance of the environment for neoplastic development and progression.[4] Type, density, and location of different tumor infiltrating lymphocytes (TILs) influence clinical outcome due to the recruitment of T cell subsets that provide tumor-suppressive or tumor-promoting stimuli.[5] To improve our knowledge on the tumor pathophysiology it is therefore essential to investigate the T cell subsets that are attracted to the tumor, and the chemotactic stimuli which regulate their recruitment.[6-8]

Unlike most other organs, the CNS is an immunoprivileged site with restricted access and unique microenvironment that profoundly affects the capacity of T cells to enter and exert their functions.[9] Lymphocyte recruitment is regulated by the chemokine receptors expressed on their surface and by selective chemokines expressed in tissues. In addition, the chemokine receptor profile of malignant B cells allows their migration towards chemotactic cues in the tumor microenvironment. Perivascular T cells and accumulation of malignant B cell around vessels are commonly found in PCNSL.[10] This histological characteristic may be initiated and sustained by distinct molecules, including T and B cell attracting chemokines. We show that the CXC chemokine ligand (CXCL) 9, agonist of the CXC chemokine receptor (CXCR) 3, is expressed in the perivascular area and that TILs accumulate in areas with high CXCL9 expression. In addition, CXCL9 forms heterocomplexes with the B cell chemoattractant CXCL12, expressed on the tumor vasculature, and enhances CXCL12-induced migration on malignant B cells.

The perivascular microenvironment in PCNSL can therefore regulate the recruitment of specific T cell subsets to the tumor and modulate the migratory behavior of both TILs and malignant B cells within the tissue.

Materials and Methods

Patients and tissues

Brain specimens were taken for diagnosis of B-cell PCNSL (2001-2005), from 11 men and 11 women, aged 35 to 75 years (median 65 years). All patients were immunocompetent, HIV negative, and Epstein-Barr virus encoded RNAs (EBER) negative. Diagnosis of PCNSL by immunophenotyping and routine hematoxylin and eosin staining was used to characterize the tumors according to the current World Health Organization (WHO) classification. Corticosteroid regimen was

stopped 10-15 days prior to biopsy. Initial screening for CXCR3, CXCR3-ligands and the CCL21 was performed on micro-tissue arrays on 20 cases.

Immunostaining procedures

Tissue sections from formalin-fixed, paraffin-embedded blocks were used for immunohistochemical and immunofluorescence stainings as previously described [11] using antibodies against cellular markers as described below.

Rabbit anti-human CD3 (RM-9107) and mouse-anti human CD57 (MS-136-P0) were from Thermo Scientific (Cheshire, UK) and used at 1:50 and 2 μg/ml, respectively. Mouse anti-human CD4 (Clone 4B12) was from Novocastra (Newcastle, UK) and used at 1:100. CD8 (M7103), CD20cy (M0755), CD68 (M0876) Granzyme B (M7235), Interferon regulatory factor 4 (IRF4) (M7259), Smooth muscle actin (SMA) (M0851) and rabbit anti-human Ki-67 (A0047) were from DAKO (Glostrup, Denmark) and used for immunostaining at 4.6 μg/ml, 7 μg/ml, 0.6 μg/ml, 1.1 μg/ml, 4.8 μg/ml, 6 μg/ml, and 5.5 μg/ml, respectively. Mouse anti-human Forkhead box P3 (Foxp3) (CM299A) was from BioCare Medical (Concord, USA) and used at 1:100. Mouse anti-human CXCL12 (MAB350) and CXCR3 (MAB160) were from R&D Systems (Abingdon, UK) and used for immunostaining at 5 μg/ml and 2 μg/ml, respectively. Anti-human CXCR3-PE (557185), CXCR4-APC (555976) were from BD Biosciences (Belgium), and used for cytofluorimetric analysis at 1:50. Anti-human CC chemokine receptor (CCR) 1 (MAB145), CCR5 (MAB182), CCR7 (MAB197), CXCR3 (MAB160), CXCR4 (MAB173) and CXCR5 (MAB190), CXCL9 (AF392), CXCL13 (AF801) were from R&D Systems used at 1 μg/ml.

Secondary antibodies were used according to manufacture instructions: rabbit anti-mouse Ig/biotinylated/F(ab`)2 (E0413, DAKO), rabbit anti-goat Ig/biotinylated (0466 DAKO), goat anti-mouse Ig/RPE (R0480, DAKO), goat anti-mouse IgG1/ Alexa Fluor 488 (A-21121, Invitrogen, Carlsbad, CA, USA), goat anti-mouse IgG2a Alexa Fluor 594 (A-21135, Invitrogen), goat anti-rabbit IgG Alexa Fluor 488 (A11070, Invitrogen), rabbit anti-goat IgG Alexa Fluor 594 (A-11080, Invitrogen).

In situ hybridization

^{35}S-labeled sense and antisense CXCL9 mRNA probes, 372 bp in length and corresponding to position -46 to -417bp of the CXCL9 sequence (NM002416), were generated by *in vitro* transcription (10 999 644 001, Roche, Rotkreuz, CH). *In situ* hybridization was performed as previously described.[12,13]

Image analysis

Immunohistochemical, immunofluorescence, and *in situ* hybridization stainings were analyzed with Nikon Eclipse E800 microscope. Digital pictures were acquired with a Nikon Digital Camera DXM1200, and Act-1 software (Nikon AG, Egg, CH), or with Hamamatsu EM-CCD Digital camera C9100 and Openlab 5,5 software (Improvision, Coventry, UK). Lymphocyte infiltration was assessed by quantitative analysis of cells in all PCNSL cases, counting 3 different areas within each tumor sample at 400x magnification. CXCL9 mRNA expression was quantified by digital image analysis (NIS-Elements BR 2.10) on the whole tumor area and expressed as percentage.

Chemokine synthesis

All chemokines were chemically synthesized using tBoc solid-phase chemistry.[14] All chemokines were tested for full length and homogeneity by mass spectrometry.

Co-Immunoprecipitation

Immunoprecipitation of CXCL12 and CXCL9 at 200nM was performed using chemically synthesized chemokines. CXCL9 alone or in combination with CXCL12 was incubated for 30 min at 37°C in RPMI containing 0.5% BSA and protease inhibitors (Sigma Aldrich P8340, 1:1000). Chemokine solutions were then incubated at 4°C overnight with 2.5 µg/ml anti-CXCL9 (R&D, MAB392) and for additional 2h with 5 µg/ml biotinylated rabbit anti-mouse antibody (DAKO, E0413). Immune complexes were isolated and analyzed by Western blotting as previously described.[15]

Cells

PBMC were isolated from buffy coats of donor blood (Central Laboratory of the Swiss Red Cross, Basel, Switzerland) by Ficoll-Paque density centrifugation. $CD8^+$ T lymphocytes were isolated by a positive immunoselection procedure (CD8 MicroBeads, Miltenyi Biotec, Bergisch Gladbach, Germany), and cultured for 24 h before chemotaxis assays as previously described.[15] DLBCL cell lines (OCI-Ly7, OCI-Ly10, OCI-Ly19, SU-HDL6) from different DLBCL subtypes (Activated B-Cell-like and Germinal Center B-Cell-like) [16] were kindly provided by Dr. Eric Davis, National Cancer Institute, NIH, Bethesda, MD, USA, analyzed for different surface molecules (Supplementary Fig. S2), and used for migration assays.

Chemotaxis assays

Chemotaxis was assayed in 48-well Boyden microchambers (Neuro Probe, Cabin John, MD) as previously described.[17] Cells migrated to the lower sides of the filters were counted at 1000x magnification in 5 high-power fields.

Statistical analysis

Statistical analysis was performed by GraphPad Prism 4 using Mann Whitney test.

Results

Lymphocyte infiltration, distribution, and activation state in PCNSL

Immunohistochemical analysis for CD8, CD4, and Foxp3 expression in 22 PCNSL cases revealed that $CD8^+$ and $CD4^+$ lymphocytes are selectively recruited to the tumor mass (Fig. 1A). This observation was corroborated by the analysis of five cases with enough peritumoral tissue to allow quantification of $CD8^+$ T cells both within the tumor and in the surrounding tissue (Fig. 1C). In most cases, intratumoral $CD8^+$ T cells were the prominent subset, followed by $CD4^+$ cells (Fig. 1B). In contrast, $Foxp3^+$ lymphocytes were rarely observed (Fig. 1A-B). TILs, and in particular $CD8^+$ T cells, showed a predominant accumulation in perivascular areas within the tumor mass (Fig. 1A and 1D).

To further characterize the $CD8^+$ T cell subset, we analyzed the presence of Granzyme B, an indicator of cytotoxic activity, and Ki67, an indicator of cell proliferation. Approximately half of the intratumoral $CD8^+$ T cells were positive for Granzyme B with a typical polarized pattern directed towards the target cells (Fig. 2A, left panels). Up to 15% of intratumoral $CD8^+$ T cells were $Ki67^+$ (Fig. 2A, right panels).

$CXCL13^+$ follicular dendritic cells (FDCs) [12,13] and follicular B helper T cells (T_{FH}) are present in normal germinal centers (Supplementary Fig. S1). T_{FH} cells consist of an heterogeneous population that have been shown to provide help to the B cells, by inducing activation-induced cytidine deaminase (AID) and promoting survival of germinal center B cells [18-20]. Of note, the tumor cells in PCNSL resemble late germinal center B cells [21] and produce CXCL13 [11] that might also be responsible for the recruitment of T_{FH} lymphocytes. Indeed, we found that a fraction of $CD3^+$ TILs produces CXCL13 and expresses CD57 (Fig. 2B), comparable to T_{FH} cells present in the germinal center of human tonsil (Supplementary Fig. S1).

The transcription factor interferon regulatory factor 4 (IRF4) is upregulated in T lymphocytes after T cell receptor stimulation, and plays a critical role for the differentiation of naïve to Th2 or Th17 effector lymphocytes [22,23]. IRF4 is also upregulated during late germinal center B cell differentiation and its expression has been shown in PCNSL [24,25]. In the human tonsil, $CD3^+IRF4^+$ lymphocytes are mainly restricted to the light zone of the germinal center and are in close proximity to $IRF4^{high}$ light zone germinal center B cells (Supplementary Fig. S1). In PCNSL, malignant B cells as well as around 10% of the TILs show IRF4 expression (Fig. 2B) suggesting TCR-mediated activation.

These findings indicate that different subsets of adaptive immune effectors are recruited to PCNSL. A fraction of intratumoral $CD8^+$ T cells show an activated phenotype with cytotoxic and proliferative activity. The recruitment or induction of T_{FH}-like T cells may provide tumor promoting signals to malignant B cells, while T cells with regulatory function ($FoxP3^+$) seem to play a minor role.

Perivascular CXCL9 expression in the PCNSL microenvironment

The abundance of TILs led us to investigate the expression of chemotactic cytokines which may regulate $CD8^+$ and $CD4^+$ T lymphocyte recruitment. Beside CCR7, which is expressed on naïve and central memory $CD4^+$ and $CD8^+$ T cells [26], CXCR3 and CXCR4 are expressed on all $CD8^+$ T lymphocyte subsets and on activated $CD4^+$ lymphocytes.[27,28] Immunohistochemical analysis for the agonists of CCR7 (CCL21), CXCR3 (CXCL9, CXCL10, and CXCL11), and CXCR4 (CXCL12) showed high expression of CXCL9 (Fig. 3) and CXCL12 in all the investigated cases, whereas CXCL10, CXCL11 and CCL21 were undetectable (data not shown). While CXCL9 and CXCL12 showed similar expression pattern in the vascular and perivascular area, CXCL12 showed in addition a broad distribution within the tumor microenvironment, as previously shown by other groups.[29,30] To further investigate the expression profile of CXCL9, we generated antisense and sense probes for the detection of CXCL9 mRNA and performed *in situ* hybridization on all 22 cases. CXCL9 mRNA colocalized with the protein detected by immunohistochemistry, confirming its production *in situ* (Fig. 3A). This chemokine, produced in the perivascular environment, can be additionally transported to the luminal surface of endothelium, as it has been shown for inflammatory chemokines [31]. Double immunofluorescence stainings reveal the perivascular microenvironment (SMA^+ and $CD68^+$) as the major source of CXCL9 (Fig. 3C). In contrast, malignant B cells, which have been shown to produce CXCL13 [11], do not seem to contribute to CXCL9 production. The percentage of $CXCL9^+$ area within the tumor mass was calculated in all cases by digital image analysis and ranged from 0.5% to 55%. Tumor samples were divided according to their CXCL9 mRNA expression into 3 subgroups: i) low (0.5-5 %), ii) intermediate (>5-20 %), or iii) high (> 20%) CXCL9 expression (Fig. 4A), and further analyzed.

T cell infiltration correlates with CXCL9 expression in PCNS

The amount of CXCL9 in each PCNSL sample was compared to the number of TILs (as from Fig. 1B). Samples with intermediate and high CXCL9 expression showed significantly higher $CD8^+$ T cell infiltration than the ones with low CXCL9 expression (Fig. 4B, top panel). Similar differences,

although less prominent, were found between expression of CXCL9 and the presence of $CD4^+$ or $Foxp3^+$ T cells (Fig. 4B, middle and bottom panels).

The distinct perivascular expression of CXCL9 and the distribution of $CD8^+$ TILs prompted us to quantify, on consecutive slides from five different patients, $CD8^+$ lymphocytes accumulating in areas with high CXCL9 expression (Fig. 4C). The analysis showed that $CD8^+$ lymphocytes preferentially accumulate in areas with CXCL9 expression (Fig. 4D). These findings strengthen the role of CXCL9 as important factor for the recruitment of adaptive immune effectors into the perivascular area.

CXCL9 forms heterocomplexes with CXCL12 and enhances CXCL12-induced migration of $CD8^+$ T cells and malignant B cells

Reactive perivascular T cell infiltrates and angiocentric behavior of malignant B cells represent one of the histological characteristics of PCNSL. T lymphocytes form an inner circle, and malignant B cells an outer concentric circle of densely packed cells in the perivascular sheet [10] (Fig. 5A-B). In line with previous reports we found that CXCL12 is expressed in PCNSL microenvironment (Fig. 5B, right panels).[29,30] Here we show that, both CXCL9 and CXCL12, are present on endothelial cells (Fig. 5B-C),

Our group and others reported that inflammatory chemokines can synergize, by forming heterocomplexes, to induce stronger responses to homeostatic chemokine receptor agonists [15,32,33]. Indeed, we show that also CXCL9 and CXCL12 form heterocomplexes in solution (Fig. 5D). We therefore investigated the potential role of CXCL9 as enhancer of CXCL12-induced migration of $CD8^+$ T cells and malignant B cells.

To investigate the effect of these chemokines on circulating $CD8^+$ T cells, we performed *in vitro* migration assays on cells from healthy individuals. Circulating $CD8^+$ T cells express CXCR4, as well as CXCR3 and respond to both CXCL12 and CXCL9 (Fig. 6A). When $CXCR3^+/CXCR4^+/CD8^+$ T cells are incubated simultaneously with CXCL9 and CXCL12 at any chemokine concentration, the effect on cell migration is additive, indicating increased capacity to migrate towards areas where these chemokines are coexpressed (Fig. 6B).

To investigate the effects of CXCL9 and CXCL12 on malignant B cells, we used four different DLBCL cell lines, tested for phenotypic markers including chemokine receptors (Supplementary Fig. S2). Their chemokine receptor profiles were in line with previous studies in PCNSL.[29,34] None of the investigated DLBCL cell lines expressed CXCR3, the selective receptor for CXCL9, or responded to CXCL9 (Supplementary Fig. S2, and Fig 6C), whereas they efficiently migrated at 100 nM CXCL12 (migrated cells/5 high power fields: OCI-Ly10 249.8±27.1, OCI-Ly7 293.7±29.4, OCI-Ly19 107.1±6.3, SU-HDL6 277.7±23.1 and Fig.6C). Interestingly, only in the presence of CXCL9 all four cell lines efficiently migrated at sub-optimal concentration of CXCL12 (10 nM) (Fig. 6D), indicating synergistic activity between CXCL9 and CXCL12 on CXCR4. This synergistic activity was observed in all DLBCL cell lines regardless their subtype (Fig. 6D).

To further investigate the chemokine requirements for inducing synergism with CXCL12, we performed the same migration assays in the presence of CCL4. Synergistically acting chemokines have been shown to feature polar and /or basic residues in the first β-strand [32], which results in patch of low electronegativity in the respective location of the chemokine surface. While CXCL9 fulfils these criteria, CCL4 has two acidic residues in the first β-strand, and consequently high electronegativity in the respective surface area. Moreover, it is an agonist for CCR1 and CCR5, both of them absent on DLBCL cell lines (Supplementary Fig. S2). In contrast to CXCL9, CCL4 lacked synergistic activity on CXCL12-induced migration (data not shown) suggesting specific structure requirements for CXCL12 synergy-inducing chemokines.

Our findings show that CXCL9 expressed in perivascular areas can form heterocomplexes with CXCL12 and in this way enhances CXCR4-dependent migration of malignant B cells towards the vessel wall. Furthermore, CXCL9 and CXCL12 cooperate acting on their selective receptors to recruit $CXCR3^+/CXCR4^+/CD8^+$ T cells. The data presented support a model in which the pattern of chemokine expression in the perivascular area regulates the recruitment of adaptive immune effectors and might explain the angiocentric positioning of malignant B cells in PCNSL.

Discussion

This study focuses for the first time on T cell subsets present in the microenvironment of PCNSL, and analyzes the co-expression of chemokines that might regulate TILs infiltration and malignant B cell positioning. The finding that the majority of TILs are $CD8^+$ T cells which express Granzyme B and proliferate *in situ* indicates TCR-mediated recognition, activation-induced proliferation, and cytotoxic activity. Nevertheless, PCNSL have a poor prognosis and the immune response might not be sufficient to appropriately counterbalance tumor growth. Inefficient migration of cytotoxic TILs within the tumor may be one of the mechanisms leading to tumor evasion. In addition other factors, including *in situ* released cytokines such as Transforming Growth Factor β (TGFβ) [35] may profoundly decrease the cytotoxic activity of $CD8^+$ TILs. A fraction of TILs share phenotypic markers of T_{FH} cells present in the light zone of germinal centers, such as CXCL13 and CD57, and show IRF4 expression indicating TCR-mediated activation and differentiation towards Th2 or Th17.[22,23,36] Interestingly, a recent report highlighted the importance of Th2 cell help for the development of lymphomas in a mouse model.[37] Those large B cell lymphomas are also $IRF4^+$, as PCNSL [24], and developed consistently at extranodal sites.[37] The fraction of TILs with a T_{FH} phenotype, which are present in PCNSL, might provide costimulatory and antiapoptotic signals to malignant B cells, similar to the activity of T_{FH} cells on the B cells present in the light zone of the germinal centers [38]. It remains to be determined, whether malignant proliferating B cells educate their environment to produce soluble factors or upregulate costimulatory signals on infiltrating T cells, to build up a germinal center-like environment. The $CXCL13^+$ T cells could also correspond to a T cell subset recently described in rheumatoid arthritis[39] or to Th17 lymphocytes.[40]

Functional studies on TILs and malignant B cells derived from PCNSL are impractical due to the fact that accessible tissue samples are taken for diagnosis or post-mortem. In the recent years several laboratories have shown gene expression profiles of extranodal B cell lymphomas including the PCNSL [3], allowing their phenotypical characterization. The availability of diffuse large B cell lymphomas cell lines that resemble phenotypically the malignant B cells in PCNSL, has allowed us to analyze their migratory capacity in response to the chemokines that we show are produced in the tumor.

Adaptive immune effectors as well as malignant B cells express particular sets of chemokine receptors and respond to chemotactic cues. Our data strongly suggests that the vascular and perivascular microenvironment in PCNSL may be responsible for $CD8^+$ T cell recruitment, as well as for the accumulation of malignant B cells by releasing specific chemokines such as CXCL9 and CXCL12. The chemokine coexpression provides synergistic signals to T and malignant B lymphocytes. This synergistic activity between CXCL9 and CXCL12 may explain reactive perivascular T cell infiltrates and the angiocentric behavior of PCNSL cells. Recent studies confirmed the *in vivo* relevance of chemokine heterocomplexes and show the potential of disrupting their functional interactions to attenuate cell recruitment [41]. CXCR3-agonists also affect CXCL12-induced migration on plasmacytoid dendritic cells, but most likely with a mechanism that does not imply the presence of heterocomplexes due to their additional expression of CXCR3.[42]

It remains matter of debate whether malignant B cells in PCNSL are recruited from the periphery and/or trapped within the CNS. CXCL12 is a potent factor for homing and survival of long lived plasma cells in the bone marrow.[43,44] It is therefore possible, that the peculiar expression of B cell attractant chemokines like CXCL12 and other soluble factors provided by the tumor microenvironment may provide a niche-like environment for malignant B cells in the CNS.

Our findings highlight the importance of distinct regional expression and cellular sources of chemokines for the recruitment of TILs, and improve our understanding of both cellular dynamics and histological features in PCNSL.

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References

1. Montesinos-Rongen M, Siebert R, Deckert M. Primary lymphoma of the central nervous system: just DLBCL or not? Blood 2009;113:7-10.
2. Hochberg FH, Baehring JM, Hochberg EP. Primary CNS lymphoma. Nat Clin Pract Neurol 2007;3:24-35.
3. Tun HW, Personett D, Baskerville KA, Menke DM, Jaeckle KA, Kreinest P, Edenfield B, Zubair AC, O'Neill BP, Lai WR, Park PJ, McKinney M. Pathway analysis of primary central nervous system lymphoma. Blood 2008;111:3200-3210.
4. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature 2008;454:436-444.
5. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, Tosolini M, Camus M, Berger A, Wind P, Zinzindohoue F, Bruneval P, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 2006;313:1960-1964.
6. Zou W. Regulatory T cells, tumour immunity and immunotherapy. Nat Rev Immunol 2006;6:295-307.
7. Trapani JA, Sutton VR. Granzyme B: pro-apoptotic, antiviral and antitumor functions. Curr Opin Immunol 2003;15:533-543.
8. Gattinoni L, Powell DJ, Jr., Rosenberg SA, Restifo NP. Adoptive immunotherapy for cancer: building on success. Nat Rev Immunol 2006;6:383-393.
9. Engelhardt B, Ransohoff RM. The ins and outs of T-lymphocyte trafficking to the CNS: anatomical sites and molecular mechanisms. Trends Immunol 2005;26:485-495.
10. Ponzoni M, Berger F, Chassagne-Clement C, Tinguely M, Jouvet A, Ferreri AJ, Dell'Oro S, Terreni MR, Doglioni C, Weis J, Cerati M, Milani M, et al. Reactive perivascular T-cell infiltrate predicts survival in primary central nervous system B-cell lymphomas. Br J Haematol 2007;138:316-323.
11. Smith JR, Braziel RM, Paoletti S, Lipp M, Uguccioni M, Rosenbaum JT. Expression of B-cell-attracting chemokine 1 (CXCL13) by malignant lymphocytes and vascular endothelium in primary central nervous system lymphoma. Blood 2003;101:815-21.
12. Mazzucchelli L, Blaser A, Kappeler A, Scharli P, Laissue JA, Baggiolini M, Uguccioni M. BCA-1 is highly expressed in Helicobacter pylori-induced mucosa-associated lymphoid tissue and gastric lymphoma. J Clin Invest 1999;104:R49-R54.
13. Vermi W, Lonardi S, Bosisio D, Uguccioni M, Danelon G, Pileri S, Fletcher C, Sozzani S, Zorzi F, Arrigoni G, Doglioni C, Ponzoni M, et al. Identification of CXCL13 as a new marker for follicular dendritic cell sarcoma. J Pathol 2008;216:356-364.
14. Clark-Lewis I, Vo L, Owen P, Anderson J. Chemical synthesis, purification, and folding of C-X-C and C-C chemokines. Methods Enzymol 1997;287:233-250.
15. Paoletti S, Petkovic V, Sebastiani S, Danelon MG, Uguccioni M, Gerber BO. A rich chemokine environment strongly enhances leukocyte migration and activities. Blood 2005;105:3405-3412.
16. Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, Boldrick JC, Sabet H, Tran T, Yu X, Powell JI, Yang L, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature 2000;403:503-511.
17. Uguccioni M, D'Apuzzo M, Loetscher M, Dewald B, Baggiolini M. Actions of the chemotactic cytokines MCP-1, MCP-2, MCP-3, RANTES, MIP-1a and MIP-1b on human monocytes. Eur J Immunol 1995;25:64-68.
18. Schaerli P, Willimann K, Lang AB, Lipp M, Loetscher P, Moser B. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. J Exp Med 2000;192:1553-1562.
19. Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, Forster R. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. J Exp Med 2000;192:1545-1552.
20. Vinuesa CG, Tangye SG, Moser B, Mackay CR. Follicular B helper T cells in antibody responses and autoimmunity. Nat Rev Immunol 2005;5:853-865.

21. Montesinos-Rongen M, Brunn A, Bentink S, Basso K, Lim WK, Klapper W, Schaller C, Reifenberger G, Rubenstein J, Wiestler OD, Spang R, la-Favera R, et al. Gene expression profiling suggests primary central nervous system lymphomas to be derived from a late germinal center B cell. Leukemia 2008;22:400-405.
22. Mittrucker HW, Matsuyama T, Grossman A, Kundig TM, Potter J, Shahinian A, Wakeham A, Patterson B, Ohashi PS, Mak TW. Requirement for the transcription factor LSIRF/IRF4 for mature B and T lymphocyte function. Science 1997;275:540-543.
23. Brustle A, Heink S, Huber M, Rosenplanter C, Stadelmann C, Yu P, Arpaia E, Mak TW, Kamradt T, Lohoff M. The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. Nat Immunol 2007;8:958-966.
24. Camilleri-Broet S, Criniere E, Broet P, Delwail V, Mokhtari K, Moreau A, Kujas M, Raphael M, Iraqi W, Sautes-Fridman C, Colombat P, Hoang-Xuan K, et al. A uniform activated B-cell-like immunophenotype might explain the poor prognosis of primary central nervous system lymphomas: analysis of 83 cases. Blood 2006;107:190-196.
25. Lu R. Interferon regulatory factor 4 and 8 in B-cell development. Trends Immunol 2008;29:487-492.
26. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature 1999;401:708-712.
27. Loetscher P, Seitz M, Baggiolini M, Moser B. Interleukin-2 regulates CC chemokine receptor expression and chemotactic responsiveness in T lymphocytes. J Exp Med 1996;184:569-77.
28. Hess C, Means TK, Autissier P, Woodberry T, Altfeld M, Addo MM, Frahm N, Brander C, Walker BD, Luster AD. IL-8 responsiveness defines a subset of CD8 T cells poised to kill. Blood 2004;104:3463-3471.
29. Brunn A, Montesinos-Rongen M, Strack A, Reifenberger G, Mawrin C, Schaller C, Deckert M. Expression pattern and cellular sources of chemokines in primary central nervous system lymphoma. Acta Neuropathol 2007;114:271-276.
30. Smith JR, Falkenhagen KM, Coupland SE, Chipps TJ, Rosenbaum JT, Braziel RM. Malignant B cells from patients with primary central nervous system lymphoma express stromal cell-derived factor-1. Am J Clin Pathol 2007;127:633-641.
31. Middleton J, Patterson AM, Gardner L, Schmutz C, Ashton BA. Leukocyte extravasation: chemokine transport and presentation by the endothelium. Blood 2002;100:3853-3860.
32. Sebastiani S, Danelon G, Gerber B, Uguccioni M. CCL22-induced responses are powerfully enhanced by synergy inducing chemokines via CCR4: evidence for the involvement of first beta-strand of chemokine. Eur J Immunol 2005;35:746-756.
33. Weber C, Koenen RR. Fine-tuning leukocyte responses: towards a chemokine 'interactome'. Trends Immunol 2006;27:268-273.
34. Jahnke K, Coupland SE, Na IK, Loddenkemper C, Keilholz U, Korfel A, Stein H, Thiel E, Scheibenbogen C. Expression of the chemokine receptors CXCR4, CXCR5, and CCR7 in primary central nervous system lymphoma. Blood 2005;106:384-385.
35. Willimsky G, Czeh M, Loddenkemper C, Gellermann J, Schmidt K, Wust P, Stein H, Blankenstein T. Immunogenicity of premalignant lesions is the primary cause of general cytotoxic T lymphocyte unresponsiveness. J Exp Med 2008;205:1687-1700.
36. Huber M, Brustle A, Reinhard K, Guralnik A, Walter G, Mahiny A, von LE, Lohoff M. IRF4 is essential for IL-21-mediated induction, amplification, and stabilization of the Th17 phenotype. Proc Natl Acad Sci U S A 2008;105:20846-20851.
37. Zangani MM, Froyland M, Qiu GY, Meza-Zepeda LA, Kutok JL, Thompson KM, Munthe LA, Bogen B. Lymphomas can develop from B cells chronically helped by idiotype-specific T cells. J Exp Med 2007;204:1181-1191.
38. Kim CH, Lim HW, Kim JR, Rott L, Hillsamer P, Butcher EC. Unique gene expression program of human germinal center T helper cells. Blood 2004;104:1952-1960.
39. Manzo A, Vitolo B, Humby F, Caporali R, Jarrossay D, Dell'accio F, Ciardelli L, Uguccioni M, Montecucco C, Pitzalis C. Mature antigen-experienced T helper cells synthesize and secrete the B cell chemoattractant CXCL13 in the inflammatory environment of the rheumatoid joint. Arthritis Rheum 2008;58:3377-3387.

40. Takagi R, Higashi T, Hashimoto K, Nakano K, Mizuno Y, Okazaki Y, Matsushita S. B cell chemoattractant CXCL13 is preferentially expressed by human Th17 cell clones. J Immunol 2008;181:186-189.
41. Koenen RR, von HP, Nesmelova IV, Zernecke A, Liehn EA, Sarabi A, Kramp BK, Piccinini AM, Paludan SR, Kowalska MA, Kungl AJ, Hackeng TM, et al. Disrupting functional interactions between platelet chemokines inhibits atherosclerosis in hyperlipidemic mice. Nat Med 2009;15:97-103.
42. Vanbervliet B, Bendriss-Vermare N, Massacrier C, Homey B, de Bouteiller O, Briere F, Trinchieri G, Caux C. The inducible CXCR3 ligands control plasmacytoid dendritic cell responsiveness to the constitutive chemokine stromal cell-derived factor 1 (SDF-1)/CXCL12. J Exp Med 2003;198:823-830.
43. Cassese G, Arce S, Hauser AE, Lehnert K, Moewes B, Mostarac M, Muehlinghaus G, Szyska M, Radbruch A, Manz RA. Plasma cell survival is mediated by synergistic effects of cytokines and adhesion-dependent signals. J Immunol 2003;171:1684-1690.
44. Radbruch A, Muehlinghaus G, Luger EO, Inamine A, Smith KG, Dorner T, Hiepe F. Competence and competition: the challenge of becoming a long-lived plasma cell. Nat Rev Immunol 2006;6:741-750.

Figure Legends

FIGURE 1. Infiltration and distribution of TILs in 22 cases of PCNSL.

(A) Immunohistochemical analysis of $CD8^+$, $CD4^+$ and $Foxp3^+$ lymphocytes. TILs are limited to the tumor mass (*) (black lines indicate tumor border, positive cells are shown in brown). Scale bar: 250 µm. Bottom panels show, for each staining, typical accumulation of TILs mixed with large malignant B cells (arrows) in the perivascular areas. Scale bar: 100 µm. Representative pictures are shown. (*B*) Quantitative analysis of tumor-infiltrating $CD8^+$, $CD4^+$ and $Foxp3^+$ TILs. Data are shown as lymphocyte number/0.3 mm^2 analyzed in three lymphocyte rich areas. (*C*) Quantitative analysis of $CD8^+$ TILs in tumor area compared to peritumoral $CD8^+$ TILs in 5 cases of PCNSL with ample peritumoral tissue. $CD8^+$ TILs selectively accumulate in the tumor areas ($p<0.01$). (*D*) Quantitative analysis of $CD8^+$ TILs in perivascular versus diffuse areas within the tumor mass. $CD8^+$ TILs tend to accumulate in the perivascular area within the tumor mass ($p<0.05$).

FIGURE 2. Phenotypic characterization of TILs in PCNSL. **(***A***)** $CD8^+$ TILs express Granzyme B and proliferate *in situ*. Expression of Granzyme B (GrzB, left panel) and Ki67 (right panel) (scale bars: 20 µm). Two higher magnifications of positive cells are shown for each staining in the lower panels (scale bars: 10 µm). Representative pictures are shown. Approximately half of $CD8^+$ TILs show a polarized expression of Granzyme B, whereas about 15% are $Ki67^+$. Pie charts on the right indicate the percentage of positive and negative cells in all samples analyzed. (*B*) A fraction of TILs shares phenotypic markers of T_{FH}. Representative pictures of $CD3^+$ TILs expressing CXCL13 (left panel, white arrows), CD57 (middle panel, white arrows) and IRF4 (right panel, white arrows). $IRF4^+$ malignant B cells are indicated in the right panel with yellow arrows. Scale bars: 20 µm. Two higher magnifications of $CD3^+/CXCL13^+$, $CD3^+/CD57^+$, and $CD3^+/IRF4^+$ cells are shown in the lower panels (scale bars: 10 µm).

FIGURE 3. CXCL9 is transcribed and translated by the perivascular microenvironment. (*A*) Analysis of CXCL9 mRNA performed by *in situ* hybridization (left panel) show colocalization with CXCL9 protein detected by immunohistochemistry (right panel). Scale bars: 100 µm. (*B*) CXCL9 expression is

associated to vascular/perivascular areas (scale bars: 100 µm). (*C*) Double immunofluorescence microscopy shows CXCL9 expression in SMA^+ pericytes (top panels), and in $CD68^+$ perivascular macrophages/microglia (bottom panels). Scale bars: 40 µm.

FIGURE 4. TILs accumulate in areas with high CXCL9 expression. (*A*) The CXCL9 positive area was calculated as percentage of the total tumor area. The 22 samples were divided in three groups according to CXCL9 expression: low (<5%, white), intermediate (>5-20%, light grey) and high (>20%, black). (*B*) Analysis of $CD8^+$ (top panel), $CD4^+$ (middle panel) and $Foxp3^+$ (bottom panel) TILs according to CXCL9 expression. TILs accumulate in $CXCL9^+$ tissue. (*C*) The analysis of consecutive slides shows that perivascular CXCL9 mRNA (i,iii), detected by *in situ* hybridization, is associated with $CD8^+$ lymphocytes (ii,iv). Scale bars: 250 µm (i, ii), and 100 µm (iii, iv). (*D*) $CD8^+$ (left panel) and $CD4^+$ (right panel) TILs were counted in positive and negative CXCL9 area in 5 cases. $CD8^+$ TILs selectively accumulate in CXCL9 positive area ($p<0.01$).

FIGURE 5. CXCL9 and CXCL12 are coexpressed in the perivascular microenvironment and can form heterocomplexes. (*A*) PCNSL reactive perivascular T cell infiltrates. Top panel shows a typical accumulation of $CD3^+$ TILs in the inner rim and of $CD20^+$ malignant B cells in the outer rim. Lower panel shows a vessel on the tumor border with typical accumulation of $CD3^+$ TILs and $CD20^+$ malignant B cells in the perivascular cuff. Scale bars: 100 µm. (*B*) CXCL9 (left panels) and CXCL12 (right panels) protein expression in the perivascular microenvironment (top panels) and on the tumor vasculature (bottom panels). (*C*) Double immunofluorescence analysis shows colocalization of CXCL12 and CXCL9 on PCNSL vessel. (*D*) CXCL9 forms heterocomplexes with CXCL12. Western blot analysis of immunecomplexes identified with anti-CXCL9 and CXCL12 antibodies.

FIGURE 6. CXCL9 enhances CXCL12-induced migration of $CD8^+$ T cells and malignant B cells

(A) Flow cytometric analysis of CXCR3 and CXCR4 expression, and migratory responses towards CXCL9 and CXCL12 of $CD8^+$ T cells. Migrated cells were counted in 5 high power fields and are shown as mean±SEM of 3 independent experiments performed with cells from different donors.

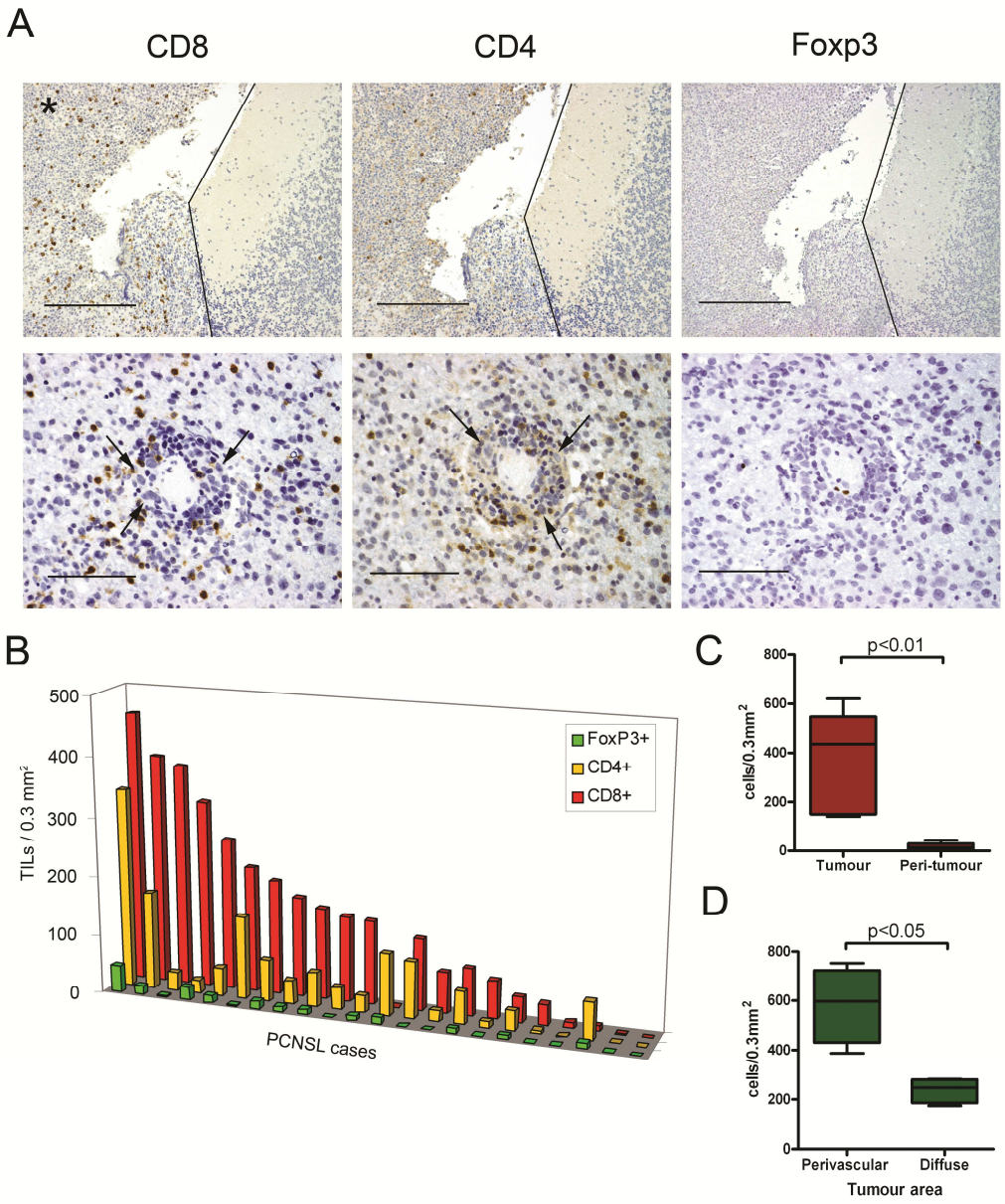
(*B*) Migratory responses of $CXCR3^+CXCR4^+CD8^+$ T cells towards 300 nM CXCL9 (checker bar), different concentration of CXCL12 alone (white bars) or in the presence of 300 nM CXCL9 (grey bars). Migrated cells were counted in 5 high power fields and are shown as mean±SEM of 3 independent experiments performed with cells from different donors. (*C***)** Flow cytometric analysis of CXCR3 and CXCR4 expression, and migratory responses towards CXCL9 and CXCL12 of OCI-Ly10. (*D***)** Chemotaxis assays performed on 4 DLBCL cell lines at sub-optimal CXCL12 concentration (10 nM) in the absence and presence of 300nM CXCL9. Migrated cells were counted in 5 high power fields and are shown as mean±SEM of three independent experiments.

Supplementary Figure S1. Expression of CXCL13, CD57 and IRF4 by T_{FH} cells in the light zone of the germinal center from human tonsil. Representative pictures of CXCL13, CD57 and IRF4 at three different magnifications. (*A)* CXCL13 is restricted to FDCs, and T_{FH} cells of the light zone of the germinal center (left panel). Higher magnification for $CD3^+CXCL13^+$ lymphocytes (middle panel, white arrows). Two higher magnifications of $CD3^+CXCL13^+$ lymphocytes are shown on the right panels. (*B)* CD57 is restricted to T_{FH} in the light zone of the germinal center (left and middle panels, white arrows). Two higher magnifications of $CD3^+CD57^+$ cells are shown on the right panels. (*C)* IRF4 is expressed by $CD3^+$ T cells (white arrows), as well as B cells ($IRF4^{high}$) mostly in the light zone of the germinal center (left and middle panels). Two higher magnifications of $CD3^+IRF4^+$ lymphocytes are shown on the right panels. Scale bars: white 40µm, green 10µm.

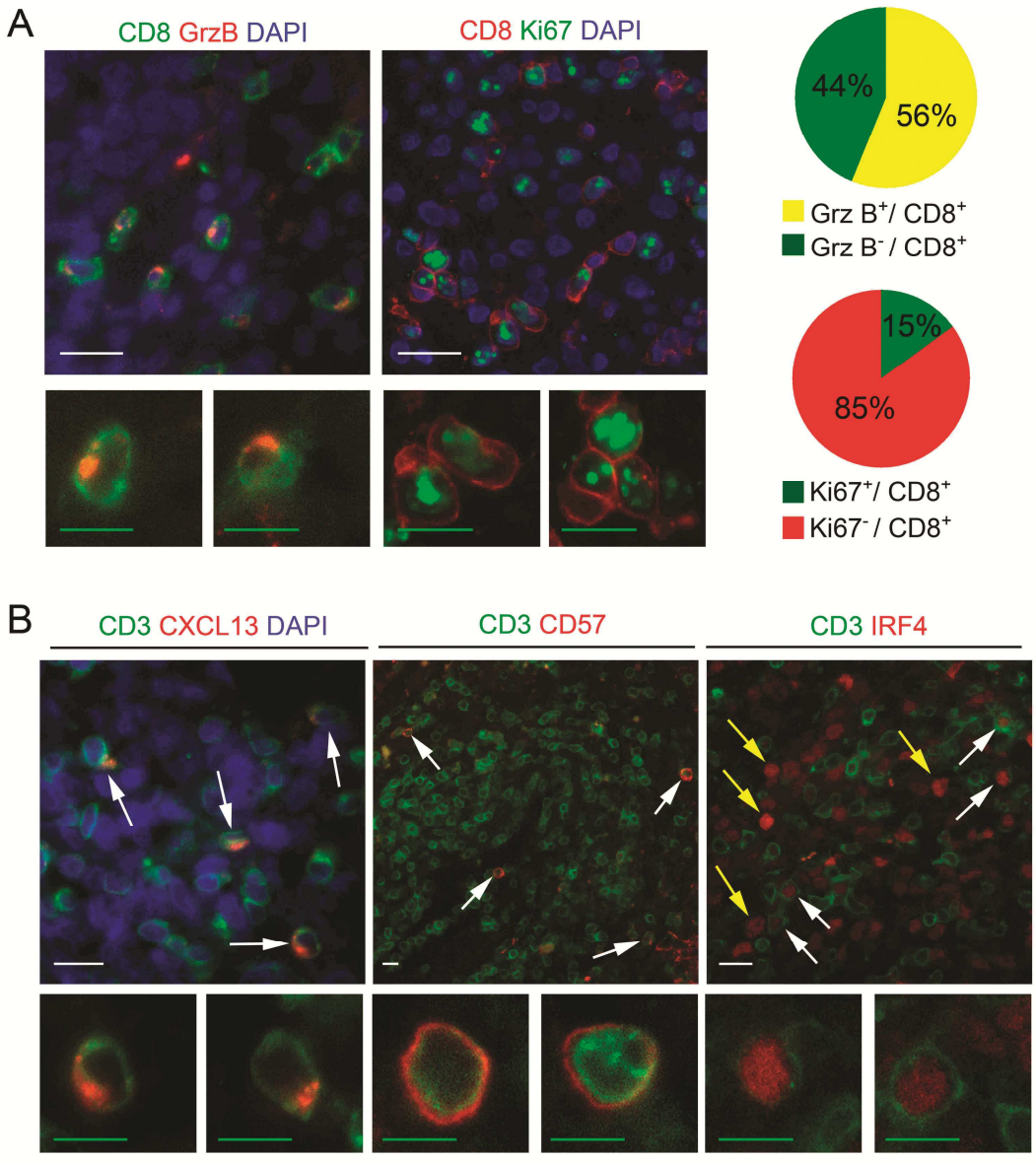
Supplementary Figure S2. Chemokine receptor profiles and surface markers for different DLBCL cell lines.

Cytofluorimetric analysis of the 4 different DLBCL cell lines used (OCI-Ly7, OCI-Ly10, OCI-Ly19, SU-HDL6) for phenotypic markers. Color code shows absence (green), low (yellow) or high (red) expression of the appropriate molecule.

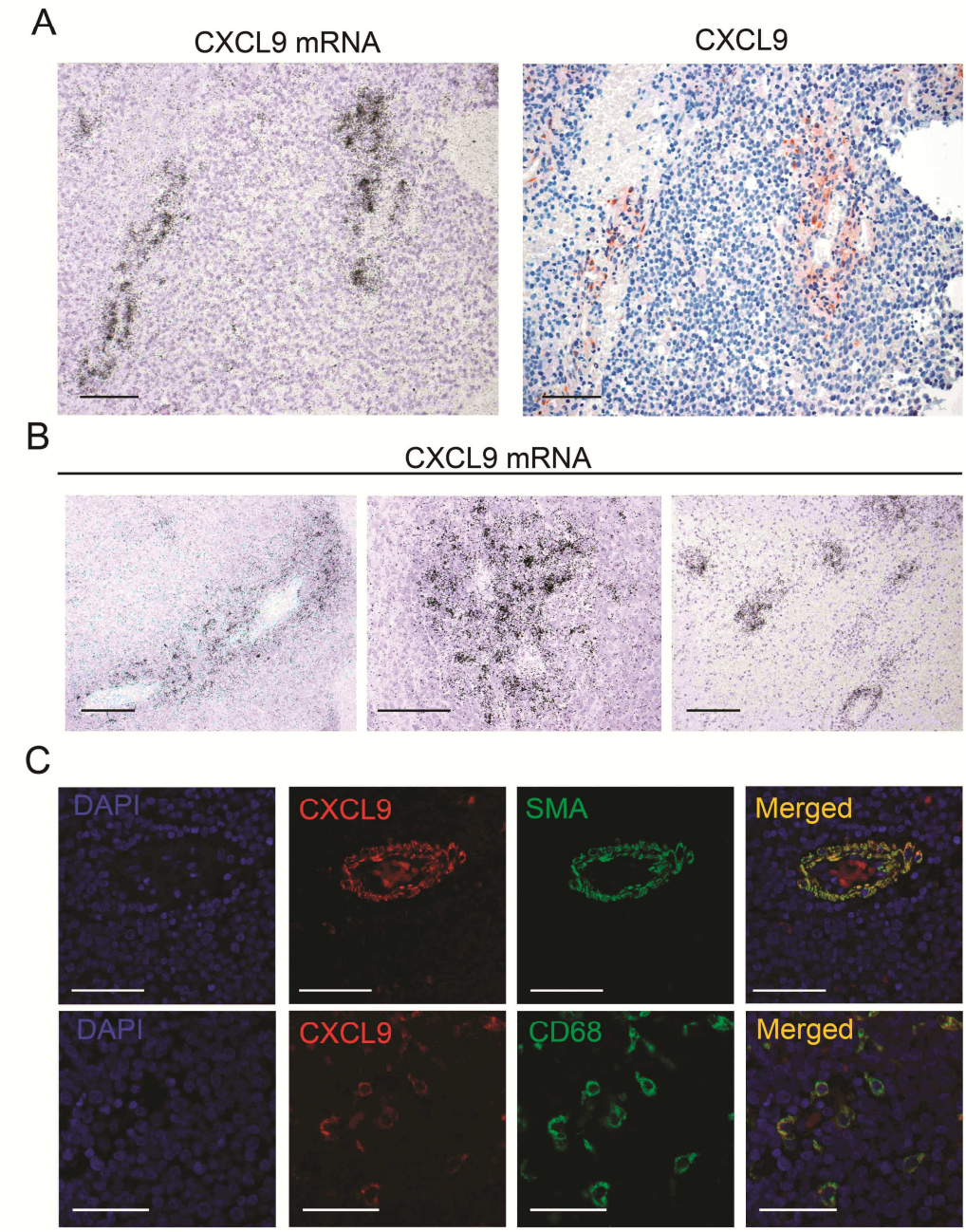
Venetz et al. Figure 1



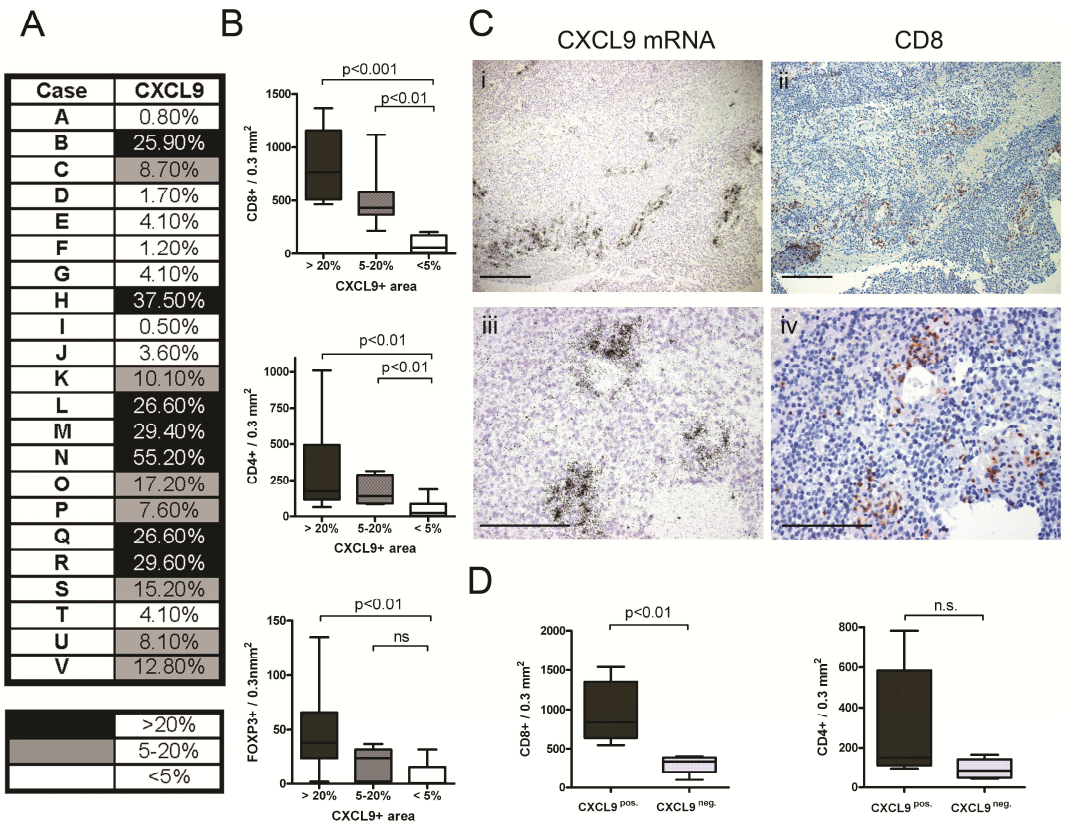
Venetz et al - FIGURE 2



Venetz et al - FIGURE 3



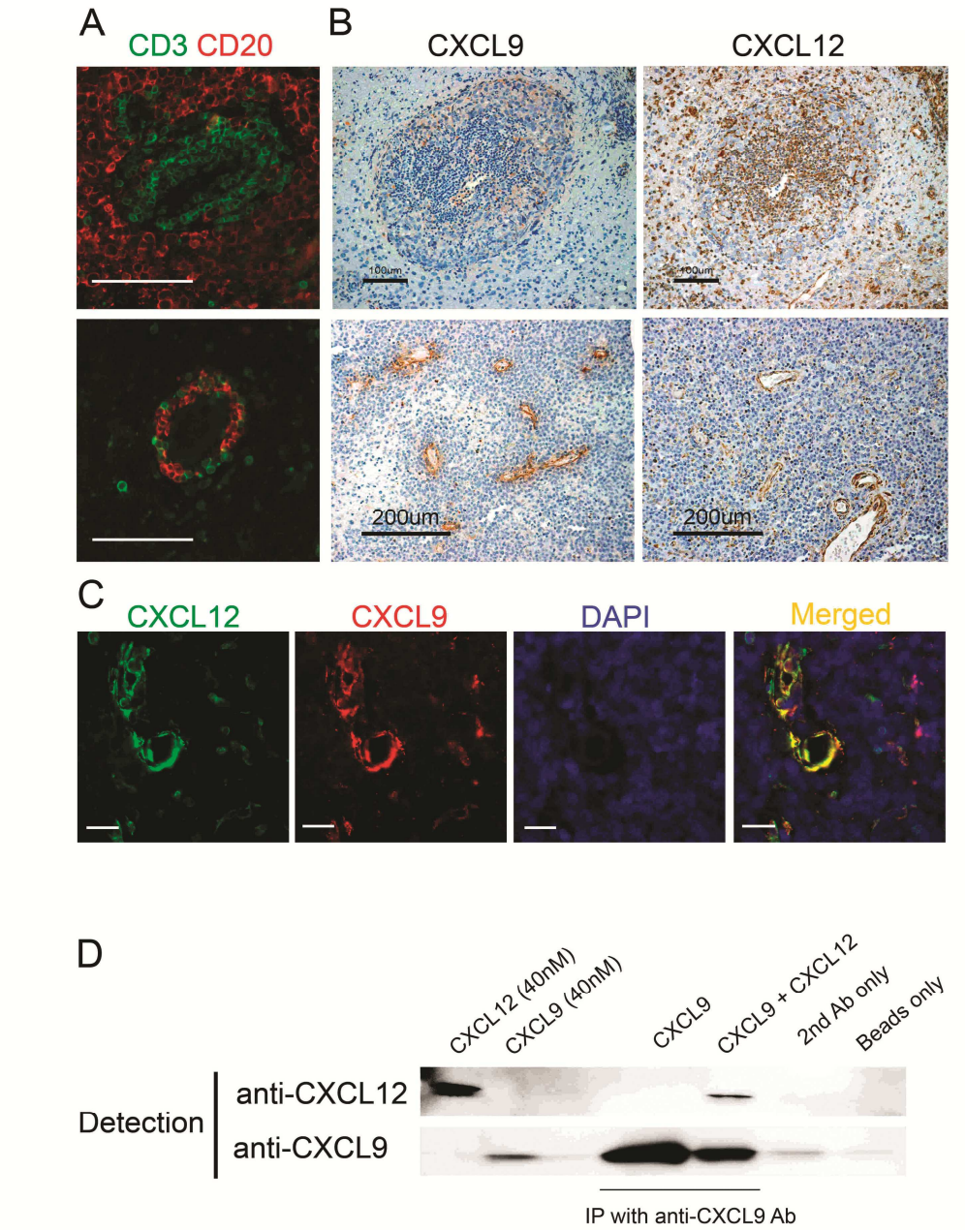
Venetz et al. Figure 4



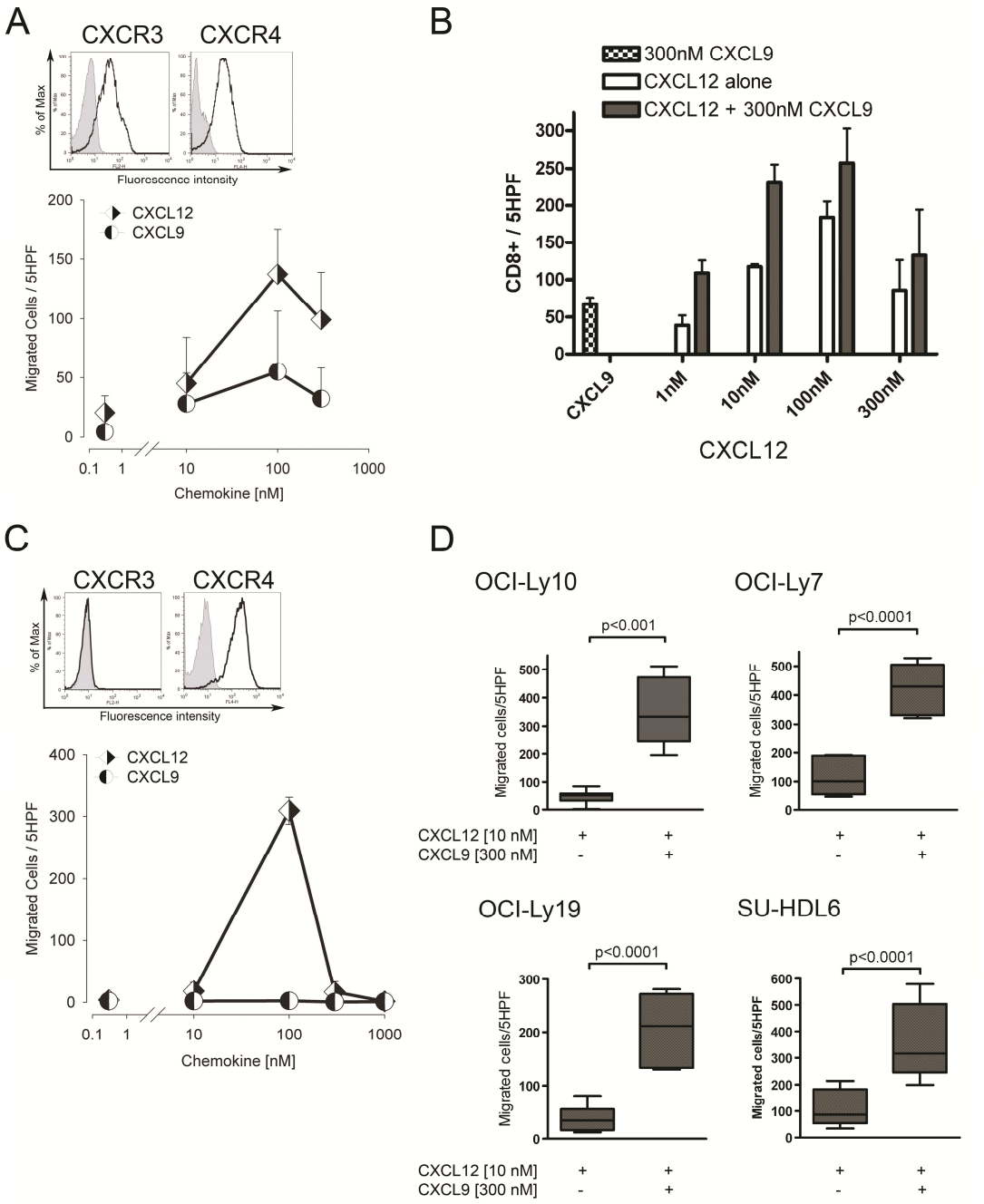
Case	CXCL9
A	0.80%
B	25.90%
C	8.70%
D	1.70%
E	4.10%
F	1.20%
G	4.10%
H	37.50%
I	0.50%
J	3.60%
K	10.10%
L	26.60%
M	29.40%
N	55.20%
O	17.20%
P	7.60%
Q	26.60%
R	29.60%
S	15.20%
T	4.10%
U	8.10%
V	12.80%

	>20%
	5-20%
	<5%

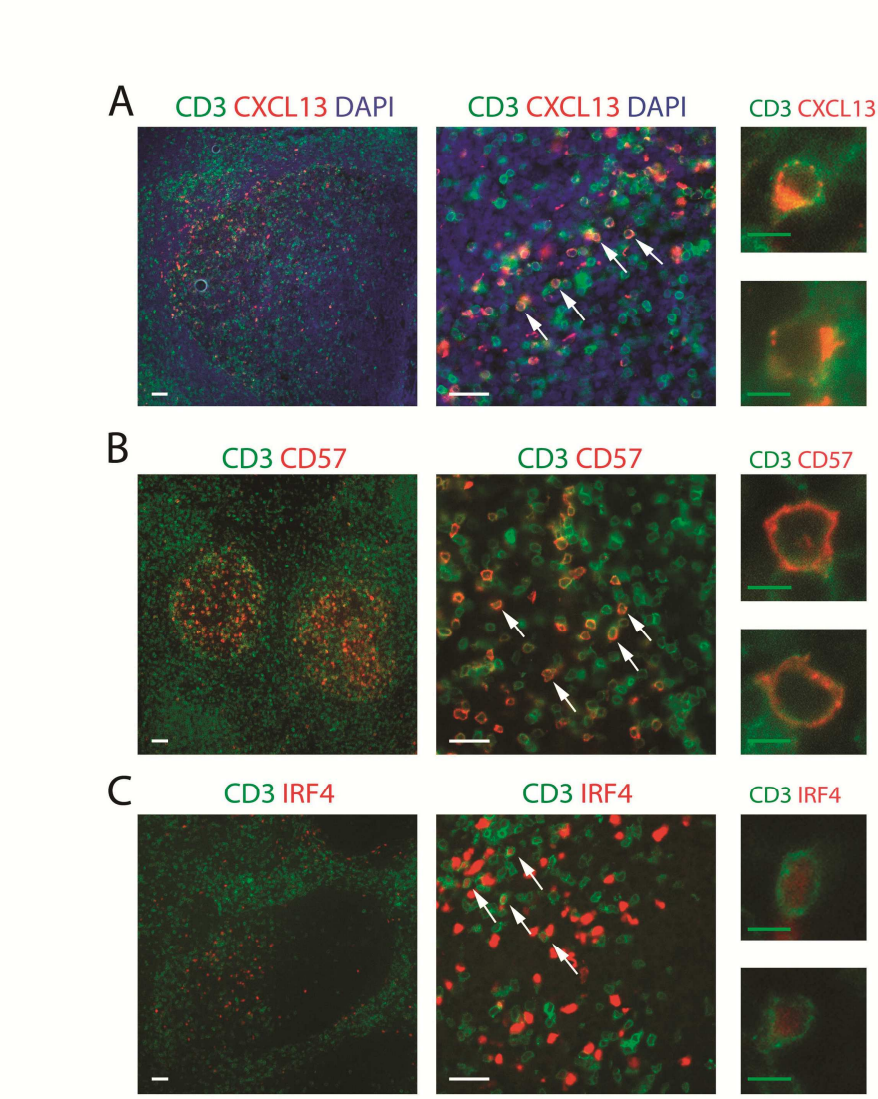
Venetz et al - FIGURE 5



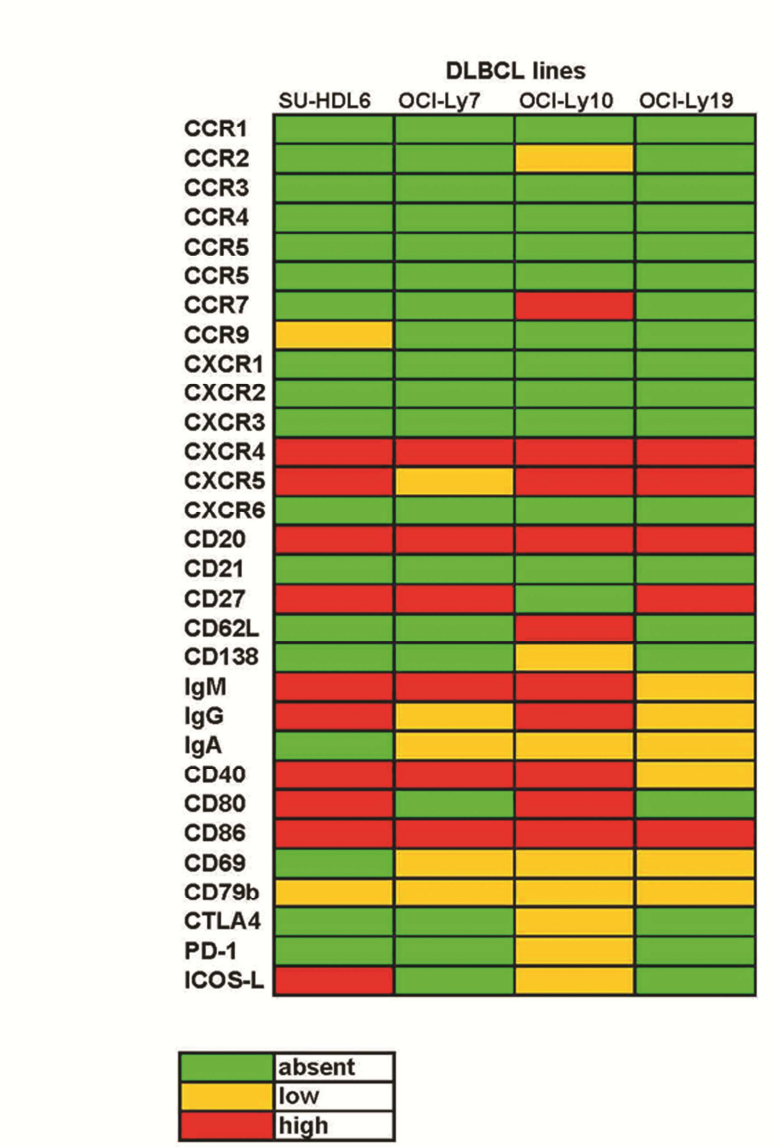
Venetz et al. Figure 6



Venetz et al. Supplementary Figure S1



Venetz et al. Supplementary Figure S2



5. DISCUSSION

5.1 B cell attractant chemokines in secondary lymphoid organs

Understanding chemokine-guided immune responses is subject of intense research and many questions remain to be resolved. Previous work on human samples and in murine models has investigated the role of T and B cell attractant chemokines and has greatly improved our understanding of lymphocyte trafficking within secondary lymphoid organs. Studies on lymphocyte trafficking in secondary lymphoid organs have been mainly performed in murine models. The work presented here has been entirely performed on human tissue samples and describes in more detail the expression of B cell attractant chemokines, CXCL12 and CXCL13, and of the inflammatory chemokine CXCL9 in secondary lymphoid organs under homeostatic and inflammatory conditions, as well as in B cell maligancies at extranodal site.

Recent work in murine infection models have revealed downregulation of homeostatic CCL21 and CXCL13 under acute inflammatory conditons, which leads to disruption of lymph node architecture.(283,284) Our date show a clear gradient of CXCL13 between the dark and the light zone of the germinal center. Interestingly, the majority of the T_{FH} cells expressing CXCL13 are located at the border between the ligh zone of the germinal center and the mantle zone. Yet, they represented only a minority of follicular T cells, indicating the presence of different T cell subtypes or upregulation of CXCL13 during a distinct time point within the germinal center. It is well known, that T_{FH} cells provide help to B cells in the light zone of the germinal center, but whether germinal center B cells also play a role in the differentiation of follicular T cells remains to be clarified.

Beside CXCR5, the chemokine receptor for CXCL13, CXCR4 is a classical B cell chemokine receptor and is constitutively expressed on normal and malignant B cells. We analyzed the expression of its ligand, CXCL12, in human secondary lymphoid organs.

The reticular network of epithelial cells in the tonsillar crypts, creates a mesh in which B cells and plasma cells are in close proximity to incoming antigens.(288) We have observed a high expression of CXCL12 by tonsillar crypt epithelial cells that may account for a significant CXCL12 gradient between underlying B cell follicles and the crypt epithelium. Plasma cells and memory B cells which

have been generated in the light zone of germinal centers underlying the crypt epithelium, upregulate CXCR4 during late B cell differentiation.(76,92) They may sense a CXCL12 gradient outside the germinal center and migrate towards the epithelium of the tonsillar crypts. This hypothesis is also supported by the finding that $CD138^+$ plasma cells generated within the germinal center align at the germinal center border underlying the crypts. It is tempting to hypothesize, that plasma cells and memory B cells generated in the germinal centers traffick towards the tonsillar crypt, where they encounter a niche like-environment build up by CXCL12 expressing epithelial cells. CXCL12 expression in normal lymph nodes was mainly found in the subcapsular sinuses. In line with the crypt epithelium in the tonsil, the subcapsular sinus is exposed to incoming antigens. Shuttling of follicular B cells towards the sinus where they take up antigen from subcapsular macrophages has been recently reported by several groups.(289,290) Our findings in human lymph nodes indicate, that CXCL12 expression in the subcapsular sinuses may play an important role in a B cell shuttling towards the subcapsular sinus. Of interest, we have found that CXCL12 mRNA expression is downmodulated upon inflammation, whereas protein levels remain confind to vessels, subcapsular sinuses, and in the GC. The role of CXCL12 inside the germinal center needs further investigation. Our findings reveal, that the CXCL12 expression in the T area is generally more abundant than in GC. GC-derived CXCL12 was mainly found on highly phagocytic tingible body macrophages. Whether the CXCR4-CXCL12 axis between tingible body macrophages and GC B cells is important in phagocytic activity is currently unknown. The finding that $CD20^+$ B cells in the T area accumulate around CXCL12-expressing HEVs is in line with a previous report in the mouse.(126) Yet, the phenotype and the role of perivascular B cells around CXCL12-expressing HEVs in secondary lymphoid organs need further characterization. Interestingly, malignant B cells in PCNSL also tend to accumulate around CXCL12-expressing vessels and it is tempting to hypothesize that perivascular B cells around HEVs and malignant perivascular B cells in PCNSL share angiocentric behaviour due to common genetic programs. It remains to be clarified, whether perivascular B cells around HEVs are pre- or post-GC B cells. Of special interest is the coexpression of CCL21 and CXCL12 in the T area. This finding highlights the fact, that T and B cells in the T area of secondary lymphoid organs are simultaneously exposed to several chemokines and adds a new level of complexity to lymphocyte trafficking.

Lymphocyte movement within the T area may therefore be regulated by minor changes in CCL21 and CXCL12 chemokine expression between stromal cells. Coexpression of chemokines at low concentrations may overcome higher concentrations of a single chemokine. In addition, coexpression of homeostatic chemokines by stromal cells may regulate trafficking of different immune effectors to the same area.

CXCL9 as a classical inflammatory chemoattractant for T cells and plasma cells was found in distinct areas within the T cell area of inflamed tonsils. $CD8^+$ T cell accumulation could be observed in these distinct areas. This is a strong indicator that $CD8^+$ T cell distribution in the T area is at least partly regulated by CXCL9 expression in the environment. While previous work in the mouse has described CXCL9 expression on HEV, which induces $CD8^+$ T cell recruitmen into secondary lymphoid organs (97), we could not detect CXCL9 on HEVs. Its expression was restricted to cells in the parafollicular area with dendritic cell-like morphology. The receptor for CXCL9, CXCR3 is classically expressed on activated $CD4^+$ T cells and the majority of $CD8^+$ T cells. $CD4^+$ T helper cells are well known for their role in providing critical signals during priming of cytotoxic $CD8^+$ T lymphocytes. These interactions take place in the T cell area of secondary lymphoid organs. CXCL9 expression by dendritic cells in the T area may therefore be a good chemokine candidate to bring recently activated $CD4^+$ T cells in close contact with $CD8^+$ T cells.

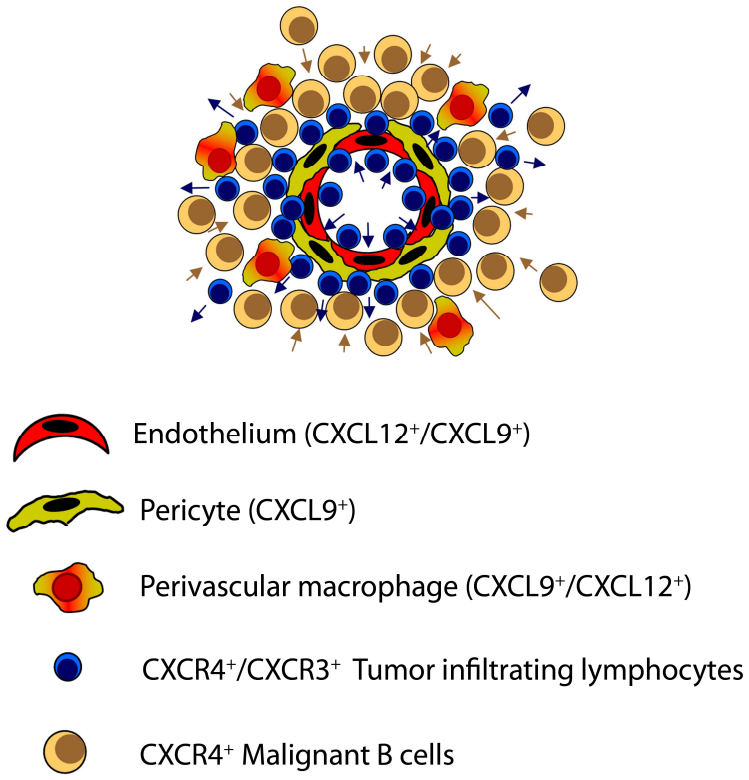
5.2 B and T cell attractant chemokine environment in PCNSL

PCNSL is a poorly understood extranodal CNS lymphoma mainly due to limited access to tissue and the lack of animal models. This study focused for the first time primarily on the tumor microenvironment, with special emphasis on the the chemokine environment and the potential impact on the recruitment of adaptive immune effectors into PCNSL.

In this work, we analyzed in detail the lymphocyte subsets in PCNSL, and the role that different T and B cell attractant chemokines might have in regulating their recruitment. We have found that TILs consist of a heterogeneous population of T cells, including $CD8^+$, Granzyme B^+ T cells showing an activated phenotype and T cells which share phenotypic marker of T_{FH} in the germinal center, indicating that a fraction of TILs may provide help to malignant B cells.

The chemokine environment showed upregulation of different B and T cell attractant chemokines, such as CXCL9, CXCL12 and CXCL13 in PCNSL microenvironment. These chemoattractans modulate migratory behaviour of lymphocytes expressing their cognate receptors CXCR3, CXCR4 and CXCR5. Previous reports have shown that malignant B cells in PCNSL are typically positive for CCR7, CXCR4 and CXCR5.(108) This chemokine receptor profile is also found on antigen experienced B cells, which upon activation upregulate CCR7 but do not downregulate CXCR4 and CXCR5.(129) While we could detect CCR7 expression in half of the cases, CCL21 could not be detected within PCNSL. In contrast, the finding that the two B cell attractant chemokines CXCL12 and CXCL13 are expressed by activated macrophages/microglia in PCNSL indicates that there is a crosstalk between macrophage/microglia-derived chemokines and malignant B cells. CXCL12 is known to play a central role in trapping of hematopoietic stem cells, B cell precursor and plasma cells in the bone marrow.(67-71) (74,75) In addition, CXCL13 regulates B cell trafficking of germinal center B cells.(33,76) The release of these two B cell chemoattractant in the PCNSL microenvironment may strongly influence the behavior of malignant B cells in PCNSL. It is tempting to hypothesize, that the inflammatory responses in the CNS during PCNSL may create a niche-like environment for malignant B cells. Of note, the presence of chemokines induces activation of integrins such as LFA-1 and VLA-4 in normal B cells. It will therefore be important to investigate whether the inflammatory CNS environment in PCNSL expresses the cognate ligands for B cell integrins, such as

ICAM-1 and VCAM1. ICAM-1 expression by activated microglia may enhance B cell adhesion. Previous reports have shown that activated microglia express ICAM-1.(291,292) FDCs in germinal center light zone also express ICAM-1 and VCAM-1 and the interaction of LFA-1 and VLA-4 on germinal center B cells have been shown *in vitro* to prevents them from undergoing apoptosis.(293) Upregulation of CXCL12 and CXCL13 has also been described in other CNS pathologies (294-297), such as multiple sclerosis, indicating their potential role in inflammatory CNS conditions. While upregulation of CXCL12 and CXCL13 in the CNS microenvironment during the course of an infection may be important to attract and trap normal B cells in the CNS, the presence of a malignant proliferating B cell clone may induce a positive feedback mechanism, which drives tumor expansion by inducing peritumoral edema which leads to the activation of peritumoral microglia, upregulation of chemoattractants and subsequent migration of malignant B cells towards activated microglia in the peritumoral space.



Model for perivascular accumulation of infiltrating CD8$^+$ T cells and angiogenctric behaviour of malignant B cells in PCNSL. Vascular and perivascular CXCL9 and CXCL12 found on endothelium and pericytes act in an additive and synergistic way on infiltrating T cells and malignant B cells.

This scenario is supported by the finding, that peritumoral microglia stains positive for CXCL12. In addition to a previous report from the laboratory, that has found CXCL13 expression by malignant B cells in PCNSL (203), we found here that also activated microglia/macrophages as well as infiltrating T cells express CXCL13. Microglial cells share morphological and functional features of dendritic cells and are able to present antigen to lymphocytes. This raises the question whether there are parallels between CXCL13-expressing follicular dendritic cells during germinal center reactions and CXCL13-expressing activated microglia in PCNSL. In addition, we found TILs expressing CXCL13, a typical feature for T_{FH} cells in the light zone of germinal centers. FDCs and T_{FH} cells play a key role during late germinal center reaction and provide costimulatory signals to late germinal center B cells which support their survival. (79) Whether proliferating malignant B cells are able to influence their CNS microenvironment is currently unknown and requires further investigation.

While CXCL12 and CXCL13 show a scattered expression pattern within PCNSL, CXCL9 is specifically expressed in perivascular areas around small and intermediate size vessels by perivascular macrophages/microglia. Its distinct expression pattern within the tumor could therefore regulate recruitment and trapping of TILs and malignant B cells in the perivascular cuff. The finding that CXCL9 expression correlates with $CD8^+$ T cell recruitment to PCNSL indicates its role in the attraction of adaptive immune effectors to the CNS. Although $CD8^+$ TILs represent the major T cell subset in PCNSL, $CD8^+$ T cell recruitment into PCNSL shows variations among different patients. This might be due to a reduced CXCL9 expression in the perivascular area. Our findings support the idea, that perivascular macrophages/microglia plays an important role in the recruitment of adaptive immune effectors to PCNSL. It remains to be determined, whether perivascular macrophages/microglia are activated in an antigen-specific or cytokine-driven manner. In contrast to CXCL12 expression, we could not find CXCL9 upregulation and $CD8^+$ T cells outside the tumor mass, indicating that TILs access the tumor via inflamed vessels inside the tumor. Interestingly we found that CXCL12 and CXCL9 can be coexpressed on vessels within PCNSL and could therefore simultaneously act on circulating T lymphocytes as well as on resident malignant B cells via CXCR3 and CXCR4. A histopathological peculiarity of PCNSL is the angiocentric behaviour of malignant B cells and reactive perivasular T cell infiltrates. Our *in vitro* findings reveal that coexpressed CXCL9

and CXCL12 on tumor vessels synergize on malignant B cells to induce stronger migratory responses and lead to perivascular accumulation of malignant B cells. Circulating $CD8^+$ T cells did not show synergistic, but rather additive migration capacity towards simultaneous exposure to CXCL9 and CXCL12. These findings support a model, in which vascular and perivascular expressed CXCL9 and CXCL12 act in a synergistic way in malignant B cells and on an additive way on infiltrating $CD8^+$ T cells and build up an inner rim of T cells and an outher rim of malignant B cells and therefore angiocentric behaviour. Our findings highlight the importance of distinct regional expression of chemokines in PCNSL, and improve our understanding of cellular dynamics and histological features in this tumor.

It remains matter of debate whether perivascular malignant B cells are recruited from the periphery or are relocated in areas due to a particular chemokine environment in the perivascular cuff.

5.3 Adaptive immune responses in PCNSL

Our results reveal that adaptive immune effectors from different T cell subsets are recruited to PCNSL and show different phenotypes. Tumor-infilatrating $CD8^+$ T cells represent the major T cell subset in PCNSL. Proliferating $CD8^+$ T cells are numerous and are a strong indication for TCR-mediated recognition and activation-induced proliferation. In certain cases, the number of proliferating $Ki67^+CD8^+$ was comparable to the number of proliferating tumor cells. Yet, we can not formerly prove that $CD8^+$ TILs specifically kill malignant B cells in an antigen-specific manner in PCNSL, because their activation may be cytokine-driven but antigen independent. The isolation of $CD8^+$ TILs from tissue to analyze their killing activity will be a difficult and challenging task due to the lack of sufficient tissue. Nevertheless, the large number of $CD8^+$ T cells in many PCNSL cases remains rather surprising, taking into account the poor prognosis of this tumor. Our results reveal that the adaptive immunity might be able to mount a specific cytotoxic immune response against PCNSL, indicated also by the high numbers of apoptotic cells *in situ* within the tumor mass (data not shown). Nevertheless, the adaptive immune response is not sufficient to appropriately counterbalance tumor growth. In addition, CD8+ T cell recruitment is typically seen in various viral infections. Despite the fact that all investigated PCNSL cases were negative for EBV, other viral pathogens cannot be excluded. Analysis

of clonality and TCR specificity of tumor infiltrating $CD8^+$ T cells may shed new light into their specific antitumoral function in PCNSL. Despite being the most prominent T cell subset in PCNSL, tumor-infiltrating $CD8^+$ T cells varied significantly between patients. Of note, patients with low $CD8^+$ T cell infiltration showed low or no CXCL9 expression, indicating that activated perivascular macrophages/microglia and pericytes may have a central role in $CD8^+$ TIL recruitment. A recent report in a mouse model describes $CD8^+$ T cells recruited to the lymph node via CXCL9. These $CD8^+$ T cells kill antigen presenting cells and in this way limit the ability of dendritic cells to activate additional T cells.(97) It is therefore also possible that a higher ratio of $CD8^+$ TILs may dampen the antitumor immune response. Further investigation is needed to verify the effect of tumor-infiltrating $CD8^+$ T cells in counterbalancing PCNSL.

Compared to $CD8^+$ T cells, intratumoral $CD4^+$ lymphocytes are less abundant and their distribution within the tumor mass does not reflect $CD8^+$ T cell infiltration and CXCL9 expression. This finding indicates that alternative chemotactic cues might be responsible for $CD4^+$ T cell recruitment to PCNSL. Interestingly, numerous TILs show IRF4 expression indicating TCR-mediated activation and differentiation towards Th2 or Th17.(167,298,299) A recent report highlighted the importance of Th2 cell help for the development of lymphomas in a mouse model.(300) Those large B cell lymphomas are also $IRF4^+$, as PCNSL (197), and developed consistently at extranodal sites.(300) It is tempting to hypothesize that a subset of TILs represent T_{FH}-like phenotypes and can provide costimulatory and antiapoptotic signals, or modulate the activity of AID, similar to T_{FH} cells in the light zone of the germinal center. Future studies are needed to investigate the clonality and TCR specificity of tumor infiltrating $CD4^+$ T cells to assess their potential tumor-promoting activities.

It has been previously shown, that the B cell attracting chemokine-1, CXCL13 is constitutively expressed in secondary lymphoid organs, in the light zone of the germinal center by FDCs and T_{FH} cells(24,301), as well as under several pathological conditions.(24,110,203,296) We show that CXCL13 is also expressed by activated microglia/macrophages as well as by a fraction of TIL. Further characterization of T cell subsets and their specific function will be important to distinguish between tumor-suppressive and tumor-promoting T cell activities.

A recent gene expression study reported a strong upregulation of the extracellular matrix molecule Osteopontin in PCNSL cases.(287) Interestingly, Osteopontin similar to CXCL13, is expressed in the light zone of germinal centers. It remains to be determined, whether malignant proliferating B cells can educate their environment to produce soluble factors or upregulate costimulatory signals on infiltrating T cells, to build up a germinal center-like environment.

5.4 Outlook

The data presented here has been performed entirely on human tissue samples. This underlies its relevance for human immunology and human immunopathology. The availability of PCNSL murine models would help verifying our hypothesis and perform proof of principle studies. Distinct expression of CXCL12 by subcapsular macrophages reveals a role as chemoattractant towards the subcapsular sinus. This gradient may be responsible for B cell shuttling to the subcapsular sinus, where B cells can pick up antigens from subcapsular macrophages.(289,290) B cell shuttling towards the subcapsular sinus may be reduced by specific inhibition of CXCR4.

A better understanding of germinal center reactions as the cradle for plasma cell and memory cell development requires special attention. On one hand to improve our knowledge on the generation and maintenance of immunological memory and on the other hand to improve our understanding on germinal center derived B cell malignancies. We showed here that there are paralles between the light zone of the germinal center and the tumor microenvironment in PCNSL, such as the presence of CXCL12 and CXCL13 expressing myeloid cells and the presence of CXCL13 expressing T cells, The mutual interactions between germinal center B cells and the germinal center environment is not fully understood yet and it remains to be determined whether germinal center B cells are able to instruct the germinal center environment, such as FDCs and TFH cells. GC-derived B cell malignacies may also have the capacity to induce GC-like properties in their environment, which support their survival in a niche-like environment.

The role of germinal center $CD8^+$ T cells remains unknown. They are less numerous compared to $CD4^+$ T cells in the germinal center, but present in almost all germinal center reactions. Why are some $CD8^+$ T cells able to migrate into the GC, whereas the majority is not and what is their role within the

germinal center? Our data in inflamed secondary lymphoid organs reveals that CXCL9 plays an important role in $CD8^+$ T cell distribution in the T area. Future studies on lymphocyte trafficking may have to distinguish between lymphocyte recruitment to the tissue and distribution of lymphcytes within a given tissue. As presented here, simultaneous coexpression of different chemokines is not an exception, but rather common, particularly under inflammatory conditions. The mechanisms how lymphocytes integrate simultaneous signals from different chemokines needs to be investigate, as well as the ability of chemokines to form heterocomplexes and induce stronger migratory responses *in vivo.* Further studies in PCNSL may focus on role of the inflammatory CNS microenvironment as niche for malignant B cells. Chemokines such as CXCL12 are known to induce inside-out signals to induce activation of integrins by conformational changes, which leads to tight binding of lymphocytes to cells expressing the appropriate integrin ligands. Special focus should be given to the crosstalk between malignant B cells and activated microglia/macrophages.

5.5 Concluding remarks

This study adds valuable and important data on the crosstalk between the tumor microenvironment and malignant B cells in the CNS. The results presented here describe the chemokine environment in PCNSL and its potential implication on intracerebral tumor expansion, as well as on the recruitment of adaptive immune effectors to the tumor. B cell chemoattractants such as CXCL12 and CXCL13 are expressed by the inflammatory microenvironment, while malignant B cells in PCNSL express the cognate receptors CXCR4 and CXCR5 and are therefore capable of responding to the ligands in the microenvironment. These findings reveal a direct crosstalk between activated microglia/macrophages and malignant B cells in PCNSL and indicate a potential niche-like environment for malignant B cells in the inflamed CNS. Blocking these interactions by specific inhibitorsn such as a CXCR4 antagonist (AMD3100), may disrupt CXCL12-induced effects on malignant B cells and inhibit CXCL12-dependent expansion. Our results refer to PCNSL but may also be valid for other CNS diseases where inflammatory cells accumulate in the perivascular area. The results presented here highlight the importance of distinct analysis of bystander cell types present in a given malignancies to better

understand its seeding behavior. In this regard, gene expression studies on whole tissue samples may be misleading in terms of interpretation.

The immune system is likely to play an important role in PCNSL, given the fact that immunosuppressed patient have a higher risk for PCNSL. Adaptive immune responses in PCNSL seem to be elicited in the majority of the cases. We described various T cells subsets in PCNSL with potential tumor suppressive as well as tumor promoting properties. A difficult but important task will be to discriminate different T cell subsets in the tumor, and to address their tumor promoting or tumor suppressive effects. Regulatory T cells do not seem to play a major role for tumor escape in PCNSL, nevertheless, the presence of T_{FH}-like T cells within the tumor may provide tumor supporting signals. The simultaneous coexpression of chemokines in the tumor microenvironment and their synergistic activites on malignant B cells adds an additional level of complexity.

REFERENCES

1. Ansel, K. M., and J. G. Cyster. 2001. Chemokines in lymphopoiesis and lymphoid organ development. *Curr. Opin. Immunol.* 13: 172-179.
2. Rot, A., and U. H. Von Andrian. 2004. Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells. *Annu. Rev. Immunol.* 22: 891-928.
3. Baggiolini, M., B. Dewald, and B. Moser. 1997. Human chemokines: an update. *Annu Rev Immunol* 15: 675-705.
4. Baggiolini, M., B. Dewald, and B. Moser. 1994. Interleukin-8 and related chemotactic cytokines - CXC and CC chemokines. *Adv. Immunol.* 55: 97-179.
5. Zlotnik, A., and O. Yoshie. 2000. Chemokines: A new classification system and their role in immunity. *Immunity* 12: 121-127.
6. Allen, S. J., S. E. Crown, and T. M. Handel. 2007. Chemokine: receptor structure, interactions, and antagonism. *Annu. Rev. Immunol.* 25: 787-820.
7. Walz, A., P. Peveri, H. Aschauer, and M. Baggiolini. 1987. Purification and amino acid sequencing of NAF, a novel neutrophil-activating factor produced by monocytes. *Biochem. Biophys. Res. Commun.* 149: 755-761.
8. Yoshimura, T., K. Matsushima, S. Tanaka, E. A. Robinson, E. Appella, J. J. Oppenheim, and E. J. Leonard. 1987. Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines. *Proc. Natl. Acad. Sci. USA* 84: 9233-9237.
9. Holmes, W. E., J. Lee, W.-J. Kuang, G. C. Rice, and W. I. Wood. 1991. Structure and functional expression of a human interleukin- 8 receptor. *Science* 253: 1278-1280.
10. Murphy, P. M., and H. L. Tiffany. 1991. Cloning of complementary DNA encoding a functional human interleukin-8 receptor. *Science* 253: 1280-1283.
11. Lee, J., R. Horuk, G. C. Rice, G. L. Bennett, T. Camerato, and W. I. Wood. 1992. Characterization of two high affinity human interleukin- 8 receptors. *J. Biol. Chem.* 267: 16283-16287.
12. Sallusto, F., B. Palermo, D. Lenig, M. Miettinen, S. Matikainen, I. Julkunen, R. Forster, R. Burgstahler, M. Lipp, and A. Lanzavecchia. 1999. Distinct patterns and kinetics of chemokine production regulate dendritic cell function. *Eur. J. Immunol.* 29: 1617-1625.
13. Yanagihara, S., E. Komura, J. Nagafune, H. Watarai, and Y. Yamaguchi. 1998. EBI1/CCR7 is a new member of dendritic cell chemokine receptor that is up-regulated upon maturation. *J. Immunol.* 161: 3096-3102.
14. Dieu, M. C., B. Vanbervliet, A. Vicari, J. M. Bridon, E. Oldham, S. Ait-Yahia, F. Briere, A. Zlotnik, S. Lebecque, and C. Caux. 1998. Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J. Exp. Med.* 188: 373-386.
15. Lin, C. L., R. M. Suri, R. A. Rahdon, J. M. Austyn, and J. A. Roake. 1998. Dendritic cell chemotaxis and transendothelial migration are induced by distinct chemokines and are regulated on maturation. *Eur. J. Immunol.* 28: 4114-4122.
16. Sozzani, S., P. Allavena, G. D'Amico, W. Luini, G. Bianchi, M. Kataura, T. Imai, O. Yoshie, R. Bonecchi, and A. Mantovani. 1998. Differential regulation of chemokine receptors during dendritic cell maturation: A model for their trafficking properties. *J. Immunol.* 161: 1083-1086.
17. Cyster, J. G. 1999. Chemokines and the homing of dendritic cells to the T cell areas of lymphoid organs. *J. Exp. Med.* 189: 447-450.
18. Sallusto, F., J. Geginat, and A. Lanzavecchia. 2004. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu. Rev. Immunol.* 22: 745-763.

19. Hess, C., T. K. Means, P. Autissier, T. Woodberry, M. Altfeld, M. M. Addo, N. Frahm, C. Brander, B. D. Walker, and A. D. Luster. 2004. IL-8 responsiveness defines a subset of CD8 T cells poised to kill. *Blood* 104: 3463-3471.
20. Wong, S., and D. Fulcher. 2004. Chemokine receptor expression in B-cell lymphoproliferative disorders. *Leuk. Lymphoma* 45: 2491-2496.
21. Von Andrian, U. H., and T. R. Mempel. 2003. Homing and cellular traffic in lymph nodes. *Nat. Rev. Immunol.* 3: 867-878.
22. Gerard, C., and B. J. Rollins. 2001. Chemokines and disease. *Nat Immunol* 2: 108-15.
23. Moser, B., M. Wolf, A. Walz, and P. Loetscher. 2004. Chemokines: multiple levels of leukocyte migration control. *Trends Immunol.* 25: 75-84.
24. Mazzucchelli, L., A. Blaser, A. Kappeler, P. Scharli, J. A. Laissue, M. Baggiolini, and M. Uguccioni. 1999. BCA-1 is highly expressed in Helicobacter pylori-induced mucosa-associated lymphoid tissue and gastric lymphoma. *J. Clin. Invest* 104: R49-R54.
25. Willimann, K., D. F. Legler, M. Loetscher, R. S. Roos, M. B. Delgado, I. Clark-Lewis, M. Baggiolini, and B. Moser. 1998. The chemokine SLC is expressed in T cell areas of lymph nodes and mucosal lymphoid tissues and attracts activated T cells via CCR7. *Eur. J. Immunol.* 28: 2025-2034.
26. Gunn, M. D., K. Tangemann, C. Tam, J. G. Cyster, S. D. Rosen, and L. T. Williams. 1998. A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. *Proc. Natl. Acad. Sci. USA* 95: 258-263.
27. Legler, D. F., M. Loetscher, R. S. Roos, I. Clark-Lewis, M. Baggiolini, and B. Moser. 1998. B cell-attracting chemokine 1, a human CXC chemokine expressed in lymphoid tissues, selectively attracts B lymphocytes via BLR1/CXCR5. *J Exp Med* 187: 655-60.
28. Morales, J., B. Homey, A. P. Vicari, S. Hudak, E. Oldham, J. Hedrick, R. Orozco, N. G. Copeland, N. A. Jenkins, L. M. McEvoy, and A. Zlotnik. 1999. CTACK, a skin-associated chemokine that preferentially attracts skin-homing memory T cells. *Proc. Natl. Acad. Sci. U. S. A* 96: 14470-14475.
29. Zabel, B. A., W. W. Agace, J. J. Campbell, H. M. Heath, D. Parent, A. I. Roberts, E. C. Ebert, N. Kassam, S. X. Qin, M. Zovko, G. J. LaRosa, L. L. Yang, D. Soler, E. C. Butcher, P. D. Ponath, C. M. Parker, and D. P. Andrew. 1999. Human G protein-coupled receptor GPR-9-G/CC chemokine receptor 9 is selectively expressed on intestinal homing T lymphocytes, mucosal lymphocytes, and thymocytes and is required for thymus-expressed chemokine-mediated chemotaxis. *J. Exp. Med.* 190: 1241-1255.
30. Ma, Q., D. Jones, and T. A. Springer. 1999. The chemokine receptor CXCR4 is required for the retention of B lineage and granulocytic precursors within the bone marrow microenvironment. *Immunity* 10: 463-471.
31. Cyster, J. G. 2003. Lymphoid organ development and cell migration. *Immunol. Rev.* 195: 5-14.
32. Hirose, J., T. Kouro, H. Igarashi, T. Yokota, N. Sakaguchi, and P. W. Kincade. 2002. A developing picture of lymphopoiesis in bone marrow. *Immunol. Rev.* 189: 28-40.
33. Forster, R., A. E. Mattis, E. Kremmer, E. Wolf, G. Brem, and M. Lipp. 1996. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell* 87: 1037-1047.
34. Ansel, K. M., V. N. Ngo, P. L. Hyman, S. A. Luther, R. Forster, J. D. Sedgwick, J. L. Browning, M. Lipp, and J. G. Cyster. 2000. A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature* 406: 309-314.
35. Förster, R., A. Schubel, D. Breitfeld, E. Kremmer, I. Renner-Müller, E. Wolf, and M. Lipp. 1999. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* 99: 23-33.
36. Okada, T., and J. G. Cyster. 2007. CC chemokine receptor 7 contributes to Gi-dependent T cell motility in the lymph node. *J. Immunol.* 178: 2973-2978.

37. Woolf, E., I. Grigorova, A. Sagiv, V. Grabovsky, S. W. Feigelson, Z. Shulman, T. Hartmann, M. Sixt, J. G. Cyster, and R. Alon. 2007. Lymph node chemokines promote sustained T lymphocyte motility without triggering stable integrin adhesiveness in the absence of shear forces. *Nat. Immunol.* 8: 1076-1085.
38. Worbs, T., T. R. Mempel, J. Bolter, U. H. Von Andrian, and R. Forster. 2007. CCR7 ligands stimulate the intranodal motility of T lymphocytes in vivo. *J. Exp. Med.* 204: 489-495.
39. Sansonetti, P. J., and R. Medzhitov. 2009. Learning tolerance while fighting ignorance. *Cell* 138: 416-420.
40. Naruse, K., M. Ueno, T. Satoh, H. Nomiyama, H. Tei, M. Takeda, D. H. Ledbetter, E. Van Coillie, G. Opdenakker, N. Gunge, Y. Sakaki, M. Iio, and R. Miura. 1996. A YAC contig of the human CC chemokine genes clustered on chromosome 17q11.2. *Genomics* 34: 236-240.
41. Modi, W. S., and Z. Q. Chen. 1998. Localization of the human CXC chemokine subfamily on the long arm of chromosome 4 using radiation hybrids. *Genomics* 47: 136-139.
42. Bazan, J. F., K. B. Bacon, G. Hardiman, W. Wang, K. Soo, D. Rossi, D. R. Greaves, A. Zlotnik, and T. J. Schall. 1997. A new class of membrane-bound chemokine with a CX_3C motif. *Nature* 385: 640-644.
43. Kelner, G. S., J. Kennedy, K. B. Bacon, S. Kleyensteuber, D. A. Largaespada, N. A. Jenkins, N. G. Copeland, J. F. Bazan, K. W. Moore, T. J. Schall, and A. Zlotnik. 1994. Lymphotactin: A cytokine that represents a new class of chemokine. *Science* 266: 1395-1399.
44. Strieter, R. M., P. J. Polverini, D. A. Arenberg, and S. L. Kunkel. 1995. The role of CXC chemokines as regulators of angiogenesis. *Shock* 4: 155-160.
45. Arenberg, D. A., P. J. Polverini, S. L. Kunkel, A. Shanafelt, J. Hesselgesser, R. Horuk, and R. M. Strieter. 1997. The role of CXC chemokines in the regulation of angiogenesis in non-small cell lung cancer. *J. Leukocyte Biol.* 62: 554-562.
46. Arenberg, D. A., S. L. Kunkel, P. J. Polverini, S. B. Morris, M. D. Burdick, M. C. Glass, D. T. Taub, M. D. Iannettoni, T. I. Whyte, and R. M. Strieter. 1996. Interferon-gamma-inducible protein 10 (IP-10) is an angiostatic factor that inhibits human non-small cell lung cancer (NSCLC) tumorigenesis and spontaneous metastases. *J. Exp. Med.* 184: 981-992.
47. Middleton, J., A. M. Patterson, L. Gardner, C. Schmutz, and B. A. Ashton. 2002. Leukocyte extravasation: chemokine transport and presentation by the endothelium. *Blood* 100: 3853-3860.
48. Ley, K., C. Laudanna, M. I. Cybulsky, and S. Nourshargh. 2007. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat. Rev. Immunol.* 7: 678-689.
49. Gunn, M. D., S. Kyuwa, C. Tam, T. Kakiuchi, A. Matsuzawa, L. T. Williams, and H. Nakano. 1999. Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. *J. Exp. Med.* 189: 451-460.
50. Warnock, R. A., S. Askari, E. C. Butcher, and U. H. Von Andrian. 1998. Molecular mechanisms of lymphocyte homing to peripheral lymph nodes., 187 ed.
51. Luther, S. A., H. L. Tang, P. L. Hyman, A. G. Farr, and J. G. Cyster. 2000. Coexpression of the chemokines ELC and SLC by T zone stromal cells and deletion of the ELC gene in the plt/plt mouse. *Proc. Natl. Acad. Sci. U. S. A* 97: 12694-12699.
52. Castellino, F., A. Y. Huang, G. Altan-Bonnet, S. Stoll, C. Scheinecker, and R. N. Germain. 2006. Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell-dendritic cell interaction. *Nature* 440: 890-895.
53. Tang, M. L., D. A. Steeber, X. Q. Zhang, and T. F. Tedder. 1998. Intrinsic differences in L-selectin expression levels affect T and B lymphocyte subset-specific recirculation pathways. *J. Immunol.* 160: 5113-5121.
54. Matloubian, M., C. G. Lo, G. Cinamon, M. J. Lesneski, Y. Xu, V. Brinkmann, M. L. Allende, R. L. Proia, and J. G. Cyster. 2004. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature* 427: 355-360.

55. Schwab, S. R., J. P. Pereira, M. Matloubian, Y. Xu, Y. Huang, and J. G. Cyster. 2005. Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients. *Science* 309: 1735-1739.
56. Pappu, R., S. R. Schwab, I. Cornelissen, J. P. Pereira, J. B. Regard, Y. Xu, E. Camerer, Y. W. Zheng, Y. Huang, J. G. Cyster, and S. R. Coughlin. 2007. Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. *Science* 316: 295-298.
57. Lira, S. A. 2005. A passport into the lymph node. *Nat. Immunol.* 6: 866-868.
58. Oberlin, E., A. Amara, F. Bachelerie, C. Bessia, J. L. Virelizier, F. Arenzana-Seisdedos, O. Schwartz, J. M. Heard, I. Clark-Lewis, D. F. Legler, M. Loetscher, M. Baggiolini, and B. Moser. 1996. The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. *Nature* 382: 833-835.
59. Bleul, C. C., M. Farzan, H. Choe, C. Parolin, I. Clark-Lewis, J. Sodroski, and T. A. Springer. 1996. The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature* 382: 829-833.
60. Ma, Q., D. Jones, P. R. Borghesani, R. A. Segal, T. Nagasawa, T. Kishimoto, R. T. Bronson, and T. A. Springer. 1998. Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc. Natl. Acad. Sci. USA* 95: 9448-9453.
61. Sierro, F., C. Biben, L. Martinez-Munoz, M. Mellado, R. M. Ransohoff, M. Li, B. Woehl, H. Leung, J. Groom, M. Batten, R. P. Harvey, A. Martinez, C. R. Mackay, and F. Mackay. 2007. Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. *Proc. Natl. Acad. Sci. U. S. A* 104: 14759-14764.
62. Feng, Y., C. C. Broder, P. E. Kennedy, and E. A. Berger. 1996. HIV-1 entry cofactor: Functional cDNA cloning of a seven- transmembrane, G protein-coupled receptor. *Science* 272: 872-877.
63. Nagasawa, T. 2006. Microenvironmental niches in the bone marrow required for B-cell development. *Nat. Rev. Immunol.* 6: 107-116.
64. Nie, Y., J. Waite, F. Brewer, M. J. Sunshine, D. R. Littman, and Y. R. Zou. 2004. The role of CXCR4 in maintaining peripheral B cell compartments and humoral immunity. *J. Exp. Med.* 200: 1145-1156.
65. Honczarenko, M., R. S. Douglas, C. Mathias, B. Lee, M. Z. Ratajczak, and L. E. Silberstein. 1999. SDF-1 responsiveness does not correlate with CXCR4 expression levels of developing human bone marrow B cells. *Blood* 94: 2990-2998.
66. Fedyk, E. R., D. H. Ryan, I. Ritterman, and T. A. Springer. 1999. Maturation decreases responsiveness of human bone marrow B lineage cells to stromal-derived factor 1 (SDF-1). *J. Leukocyte Biol.* 66: 667-673.
67. Dar, A., P. Goichberg, V. Shinder, A. Kalinkovich, O. Kollet, N. Netzer, R. Margalit, M. Zsak, A. Nagler, I. Hardan, I. Resnick, A. Rot, and T. Lapidot. 2005. Chemokine receptor CXCR4-dependent internalization and resecretion of functional chemokine SDF-1 by bone marrow endothelial and stromal cells. *Nat. Immunol.* 6: 1038-1046.
68. Petit, I., M. Szyper-Kravitz, A. Nagler, M. Lahav, A. Peled, L. Habler, T. Ponomaryov, R. S. Taichman, F. Arenzana-Seisdedos, N. Fujii, J. Sandbank, D. Zipori, and T. Lapidot. 2002. G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. *Nat. Immunol.* 3: 687-694.
69. Broxmeyer, H. E., C. M. Orschell, D. W. Clapp, G. Hangoc, S. Cooper, P. A. Plett, W. C. Liles, X. Li, B. Graham-Evans, T. B. Campbell, G. Calandra, G. Bridger, D. C. Dale, and E. F. Srour. 2005. Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. *J. Exp. Med.* 201: 1307-1318.
70. Rettig, M. P., P. Ramirez, B. Nervi, and J. F. DiPersio. 2009. CXCR4 and mobilization of hematopoietic precursors. *Methods Enzymol.* 460: 57-90.

71. Pitchford, S. C., R. C. Furze, C. P. Jones, A. M. Wengner, and S. M. Rankin. 2009. Differential mobilization of subsets of progenitor cells from the bone marrow. *Cell Stem Cell* 4: 62-72.
72. Uy, G. L., M. P. Rettig, and A. F. Cashen. 2008. Plerixafor, a CXCR4 antagonist for the mobilization of hematopoietic stem cells. *Expert. Opin. Biol. Ther.* 8: 1797-1804.
73. DiPersio, J. F., E. A. Stadtmauer, A. Nademanee, I. N. Micallef, P. J. Stiff, J. L. Kaufman, R. T. Maziarz, C. Hosing, S. Fruehauf, M. Horwitz, D. Cooper, G. Bridger, and G. Calandra. 2009. Plerixafor and G-CSF versus placebo and G-CSF to mobilize hematopoietic stem cells for autologous stem cell transplantation in patients with multiple myeloma. *Blood* 113: 5720-5726.
74. Tokoyoda, K., T. Egawa, T. Sugiyama, B. I. Choi, and T. Nagasawa. 2004. Cellular niches controlling B lymphocyte behavior within bone marrow during development. *Immunity.* 20: 707-718.
75. Cassese, G., S. Arce, A. E. Hauser, K. Lehnert, B. Moewes, M. Mostarac, G. Muehlinghaus, M. Szyska, A. Radbruch, and R. A. Manz. 2003. Plasma cell survival is mediated by synergistic effects of cytokines and adhesion-dependent signals. *J. Immunol.* 171: 1684-1690.
76. Allen, C. D., K. M. Ansel, C. Low, R. Lesley, H. Tamamura, N. Fujii, and J. G. Cyster. 2004. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. *Nat. Immunol.* 5: 943-952.
77. Balabanian, K., B. Lagane, S. Infantino, K. Y. Chow, J. Harriague, B. Moepps, F. renzana-Seisdedos, M. Thelen, and F. Bachelerie. 2005. The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. *J. Biol. Chem.* 280: 35760-35766.
78. Boldajipour, B., H. Mahabaleshwar, E. Kardash, M. Reichman-Fried, H. Blaser, S. Minina, D. Wilson, Q. Xu, and E. Raz. 2008. Control of chemokine-guided cell migration by ligand sequestration. *Cell* 132: 463-473.
79. King, C., S. G. Tangye, and C. R. Mackay. 2008. T follicular helper (TFH) cells in normal and dysregulated immune responses. *Annu. Rev. Immunol.* 26: 741-766.
80. Chtanova, T., S. G. Tangye, R. Newton, N. Frank, M. R. Hodge, M. S. Rolph, and C. R. Mackay. 2004. T follicular helper cells express a distinctive transcriptional profile, reflecting their role as non-Th1/Th2 effector cells that provide help for B cells. *J. Immunol.* 173: 68-78.
81. Nurieva, R. I., Y. Chung, G. J. Martinez, X. O. Yang, S. Tanaka, T. D. Matskevitch, Y. H. Wang, and C. Dong. 2009. Bcl6 mediates the development of T follicular helper cells. *Science* 325: 1001-1005.
82. Johnston, R. J., A. C. Poholek, D. DiToro, I. Yusuf, D. Eto, B. Barnett, A. L. Dent, J. Craft, and S. Crotty. 2009. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* 325: 1006-1010.
83. Messi, M., I. Giacchetto, K. Nagata, A. Lanzavecchia, G. Natoli, and F. Sallusto. 2003. Memory and flexibility of cytokine gene expression as separable properties of human T(H)1 and T(H)2 lymphocytes. *Nat. Immunol.* 4: 78-86.
84. Ansel, K. M., D. U. Lee, and A. Rao. 2003. An epigenetic view of helper T cell differentiation. *Nat. Immunol.* 4: 616-623.
85. Afkarian, M., J. R. Sedy, J. Yang, N. G. Jacobson, N. Cereb, S. Y. Yang, T. L. Murphy, and K. M. Murphy. 2002. T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nat. Immunol.* 3: 549-557.
86. Zheng, W., and R. A. Flavell. 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 89: 587-596.
87. Bluestone, J. A., C. R. Mackay, J. J. O'Shea, and B. Stockinger. 2009. The functional plasticity of T cell subsets. *Nat. Rev. Immunol.* 9: 811-816.

88. Sallusto, F., D. Lenig, C. R. Mackay, and A. Lanzavecchia. 1998. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J. Exp. Med.* 187: 875-883.

89. Acosta-Rodriguez, E. V., L. Rivino, J. Geginat, D. Jarrossay, M. Gattorno, A. Lanzavecchia, F. Sallusto, and G. Napolitani. 2007. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat. Immunol.* 8: 639-646.

90. Duhen, T., R. Geiger, D. Jarrossay, A. Lanzavecchia, and F. Sallusto. 2009. Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. *Nat. Immunol.* 10: 857-863.

91. Okada, T., M. J. Miller, I. Parker, M. F. Krummel, M. Neighbors, S. B. Hartley, A. O'Garra, M. D. Cahalan, and J. G. Cyster. 2005. Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells. *PLoS. Biol.* 3: e150.

92. Muehlinghaus, G., L. Cigliano, S. Huehn, A. Peddinghaus, H. Leyendeckers, A. E. Hauser, F. Hiepe, A. Radbruch, S. Arce, and R. A. Manz. 2005. Regulation of CXCR3 and CXCR4 expression during terminal differentiation of memory B cells into plasma cells. *Blood* 105: 3965-3971.

93. Loetscher, M., B. Gerber, P. Loetscher, S. A. Jones, L. Piali, I. Clark-Lewis, M. Baggiolini, and B. Moser. 1996. Chemokine receptor specific for IP10 and Mig: Structure, function, and expression in activated T-lymphocytes. *J. Exp. Med.* 184: 963-969.

94. Cole, K. E., C. A. Strick, T. J. Paradis, K. T. Ogborne, M. Loetscher, R. P. Gladue, W. Lin, J. G. Boyd, B. Moser, D. E. Wood, B. G. Sahagan, and K. Neote. 1998. Interferon-inducible T cell alpha chemoattractant (I-TAC): a novel non- ELR CXC chemokine with potent activity on activated T cells through selective high affinity binding to CXCR3. *J Exp Med* 187: 2009-21.

95. MartIn-Fontecha, A., L. L. Thomsen, S. Brett, C. Gerard, M. Lipp, A. Lanzavecchia, and F. Sallusto. 2004. Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming. *Nat. Immunol.* 5: 1260-1265.

96. Janatpour, M. J., S. Hudak, M. Sathe, J. D. Sedgwick, and L. M. McEvoy. 2001. Tumor necrosis factor-dependent segmental control of MIG expression by high endothelial venules in inflamed lymph nodes regulates monocyte recruitment. *J. Exp. Med.* 194: 1375-1384.

97. Guarda, G., M. Hons, S. F. Soriano, A. Y. Huang, R. Polley, A. MartIn-Fontecha, J. V. Stein, R. N. Germain, A. Lanzavecchia, and F. Sallusto. 2007. L-selectin-negative CCR7- effector and memory CD8+ T cells enter reactive lymph nodes and kill dendritic cells. *Nat. Immunol.* 8: 743-752.

98. Middleton, J., S. Neil, J. Wintle, I. Clark-Lewis, H. Moore, C. Lam, M. Auer, E. Hub, and A. Rot. 1997. Transcytosis and surface presentation of IL-8 ky venular endothelial cells. *Cell* 91: 385-395.

99. Stein, J. V., A. Rot, Y. Luo, M. Narasimhaswamy, H. Nakano, M. D. Gunn, A. Matsuzawa, E. J. Quackenbush, M. E. Dorf, and U. H. Von Andrian. 2000. The CC chemokine thymus-derived chemotactic agent 4 (TCA-4, secondary lymphoid tissue chemokine, 6Ckine, exodus-2) triggers lymphocyte function-associated antigen 1-mediated arrest of rolling T lymphocytes in peripheral lymph node high endothelial venules. *J. Exp. Med.* 191: 61-75.

100. Baekkevold, E. S., T. Yamanaka, R. T. Palframan, H. S. Carlsen, F. P. Reinholt, U. H. Von Andrian, P. Brandtzaeg, and G. Haraldsen. 2001. The CCR7 ligand elc (CCL19) is transcytosed in high endothelial venules and mediates T cell recruitment. *J. Exp. Med.* 193: 1105-1112.

101. Palframan, R. T., S. Jung, G. Cheng, W. Weninger, Y. Luo, M. Dorf, D. R. Littman, B. J. Rollins, H. Zweerink, A. Rot, and U. H. Von Andrian. 2001. Inflammatory chemokine transport and presentation in HEV: a remote control mechanism for monocyte recruitment to lymph nodes in inflamed tissues. *J. Exp. Med.* 194: 1361-1373.

102. Kruizinga, R. C., J. Bestebroer, P. Berghuis, C. J. de Haas, T. P. Links, E. G. de Vries, and A. M. Walenkamp. 2009. Role of chemokines and their receptors in cancer. *Curr. Pharm. Des* 15: 3396-3416.

103. Muller, A., B. Homey, H. Soto, N. Ge, D. Catron, M. E. Buchanan, T. McClanahan, E. Murphy, W. Yuan, S. N. Wagner, J. L. Barrera, A. Mohar, E. Verastegui, and A. Zlotnik. 2001. Involvement of chemokine receptors in breast cancer metastasis. *Nature* 410: 50-56.

104. Buonamici, S., T. Trimarchi, M. G. Ruocco, L. Reavie, S. Cathelin, B. G. Mar, A. Klinakis, Y. Lukyanov, J. C. Tseng, F. Sen, E. Gehrie, M. Li, E. Newcomb, J. Zavadil, D. Meruelo, M. Lipp, S. Ibrahim, A. Efstratiadis, D. Zagzag, J. S. Bromberg, M. L. Dustin, and I. Aifantis. 2009. CCR7 signalling as an essential regulator of CNS infiltration in T-cell leukaemia. *Nature* 459: 1000-1004.

105. Arigami, T., S. Natsugoe, Y. Uenosono, S. Yanagita, H. Arima, M. Hirata, S. Ishigami, and T. Aikou. 2009. CCR7 and CXCR4 expression predicts lymph node status including micrometastasis in gastric cancer. *Int. J. Oncol.* 35: 19-24.

106. Xiang, Z. L., Z. C. Zeng, Z. Y. Tang, J. Fan, P. Y. Zhuang, Y. Liang, Y. S. Tan, and J. He. 2009. Chemokine receptor CXCR4 expression in hepatocellular carcinoma patients increases the risk of bone metastases and poor survival. *BMC. Cancer* 9: 176.

107. Trentin, L., C. Agostini, M. Facco, F. Piazza, A. Perin, M. Siviero, C. Gurrieri, S. Galvan, F. Adami, R. Zambello, and G. Semenzato. 1999. The chemokine receptor CXCR3 is expressed on malignant B cells and mediates chemotaxis. *J. Clin. Invest.* 104: 115-121.

108. Jahnke, K., S. E. Coupland, I. K. Na, C. Loddenkemper, U. Keilholz, A. Korfel, H. Stein, E. Thiel, and C. Scheibenbogen. 2005. Expression of the chemokine receptors CXCR4, CXCR5, and CCR7 in primary central nervous system lymphoma. *Blood* 106: 384-385.

109. Mori, M., C. Manuelli, N. Pimpinelli, B. Bianchi, C. Orlando, C. Mavilia, P. Cappugi, E. Maggi, B. Giannotti, and M. Santucci. 2003. BCA-1, A B-cell chemoattractant signal, is constantly expressed in cutaneous lymphoproliferative B-cell disorders. *Eur. J. Cancer* 39: 1625-1631.

110. Barone, F., M. Bombardieri, M. M. Rosado, P. R. Morgan, S. J. Challacombe, V. S. De, R. Carsetti, J. Spencer, G. Valesini, and C. Pitzalis. 2008. CXCL13, CCL21, and CXCL12 expression in salivary glands of patients with Sjogren's syndrome and MALT lymphoma: association with reactive and malignant areas of lymphoid organization. *J. Immunol.* 180: 5130-5140.

111. Vermi, W., S. Lonardi, D. Bosisio, M. Uguccioni, G. Danelon, S. Pileri, C. Fletcher, S. Sozzani, F. Zorzi, G. Arrigoni, C. Doglioni, M. Ponzoni, and F. Facchetti. 2008. Identification of CXCL13 as a new marker for follicular dendritic cell sarcoma. *J. Pathol.* 216: 356-364.

112. de, L. L., D. S. Rickman, C. Thielen, A. Reynies, Y. L. Huang, G. Delsol, L. Lamant, K. Leroy, J. Briere, T. Molina, F. Berger, C. Gisselbrecht, L. Xerri, and P. Gaulard. 2007. The gene expression profile of nodal peripheral T-cell lymphoma demonstrates a molecular link between angioimmunoblastic T-cell lymphoma (AITL) and follicular helper T (TFH) cells. *Blood* 109: 4952-4963.

113. Flaishon, L., S. Becker-Herman, G. Hart, Y. Levo, W. A. Kuziel, and I. Shachar. 2004. Expression of the chemokine receptor CCR2 on immature B cells negatively regulates their cytoskeletal rearrangement and migration. *Blood* 104: 933-941.

114. Vanbervliet, B., N. Bendriss-Vermare, C. Massacrier, B. Homey, O. de Bouteiller, F. Briere, G. Trinchieri, and C. Caux. 2003. The inducible CXCR3 ligands control plasmacytoid dendritic cell responsiveness to the constitutive chemokine stromal cell-derived factor 1 (SDF-1)/CXCL12. *J. Exp. Med.* 198: 823-830.

115. Badr, G., G. Borhis, E. A. Lefevre, N. Chaoul, F. Deshayes, V. Dessirier, G. Lapree, A. Tsapis, and Y. Richard. 2008. BAFF enhances chemotaxis of primary human B cells: a particular synergy between BAFF and CXCL13 on memory B cells. *Blood* 111: 2744-2754.

116. Struyf, S., P. Proost, J. P. Lenaerts, G. Stoops, A. Wuyts, and J. Van Damme. 2001. Identification of a blood-derived chemoattractant for neutrophils and lymphocytes as a novel CC chemokine, Regakine-1. *Blood* 97: 2197-204.

117. Krug, A., R. Uppaluri, F. Facchetti, B. G. Dorner, K. C. Sheehan, R. D. Schreiber, M. Cella, and M. Colonna. 2002. IFN-producing cells respond to CXCR3 ligands in the presence of CXCL12 and secrete inflammatory chemokines upon activation. *J. Immunol.* 169: 6079-6083.

118. Gouwy, M., S. Struyf, J. Catusse, P. Proost, and D. J. Van. 2004. Synergy between proinflammatory ligands of G protein-coupled receptors in neutrophil activation and migration. *J. Leukoc. Biol.* 76: 185-194.

119. Struyf, S., M. Gouwy, C. Dillen, P. Proost, G. Opdenakker, and D. J. Van. 2005. Chemokines synergize in the recruitment of circulating neutrophils into inflamed tissue. *Eur. J. Immunol.* 35: 1583-1591.

120. von, H. P., R. R. Koenen, M. Sack, S. F. Mause, W. Adriaens, A. E. Proudfoot, T. M. Hackeng, and C. Weber. 2005. Heterophilic interactions of platelet factor 4 and RANTES promote monocyte arrest on endothelium. *Blood* 105: 924-930.

121. Paoletti, S., V. Petkovic, S. Sebastiani, M. G. Danelon, M. Uguccioni, and B. O. Gerber. 2005. A rich chemokine environment strongly enhances leukocyte migration and activities. *Blood* 105: 3405-3412.

122. Sebastiani, S., G. Danelon, B. Gerber, and M. Uguccioni. 2005. CCL22-induced responses are powerfully enhanced by synergy inducing chemokines via CCR4: evidence for the involvement of first beta-strand of chemokine. *Eur. J. Immunol.* 35: 746-756.

123. Lasagni, L., M. Francalanci, F. Annunziato, E. Lazzeri, S. Giannini, L. Cosmi, C. Sagrinati, B. Mazzinghi, C. Orlando, E. Maggi, F. Marra, S. Romagnani, M. Serio, and P. Romagnani. 2003. An alternatively spliced variant of CXCR3 mediates the inhibition of endothelial cell growth induced by IP-10, Mig, and I-TAC, and acts as functional receptor for platelet factor 4. *J. Exp. Med.* 197: 1537-1549.

124. Pervushina, O., B. Scheuerer, N. Reiling, L. Behnke, J. M. Schroder, B. Kasper, E. Brandt, S. Bulfone-Paus, and F. Petersen. 2004. Platelet factor 4/CXCL4 induces phagocytosis and the generation of reactive oxygen metabolites in mononuclear phagocytes independently of Gi protein activation or intracellular calcium transients. *J. Immunol.* 173: 2060-2067.

125. Koenen, R. R., H. P. von, I. V. Nesmelova, A. Zernecke, E. A. Liehn, A. Sarabi, B. K. Kramp, A. M. Piccinini, S. R. Paludan, M. A. Kowalska, A. J. Kungl, T. M. Hackeng, K. H. Mayo, and C. Weber. 2009. Disrupting functional interactions between platelet chemokines inhibits atherosclerosis in hyperlipidemic mice. *Nat. Med.* 15: 97-103.

126. Okada, T., V. N. Ngo, E. H. Ekland, R. Forster, M. Lipp, D. R. Littman, and J. G. Cyster. 2002. Chemokine requirements for B cell entry to lymph nodes and Peyer's patches., 196 ed.

127. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401: 708-712.

128. Warnock, R. A., J. J. Campbell, M. E. Dorf, A. Matsuzawa, L. M. McEvoy, and E. C. Butcher. 2000. The role of chemokines in the micro environmental control of T versus B cell arrest in Peyer's patch high endothelial venules. *J. Exp. Med.* 191: 77-88.

129. Reif, K., E. H. Ekland, L. Ohl, H. Nakano, M. Lipp, R. Forster, and J. G. Cyster. 2002. Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position. *Nature* 416: 94-99.

130. Lazarus, N. H., E. J. Kunkel, B. Johnston, E. Wilson, K. R. Youngman, and E. C. Butcher. 2003. A common mucosal chemokine (mucosae-associated epithelial chemokine/CCL28) selectively attracts IgA plasmablasts. *J. Immunol.* 170: 3799-3805.

131. Kunkel, E. J., C. H. Kim, N. H. Lazarus, M. A. Vierra, D. Soler, E. P. Bowman, and E. C. Butcher. 2003. CCR10 expression is a common feature of circulating and mucosal epithelial tissue IgA Ab-secreting cells. *J. Clin. Invest* 111: 1001-1010.

132. Haddad, W., C. J. Cooper, Z. Zhang, J. B. Brown, Y. Zhu, A. Issekutz, I. Fuss, H. O. Lee, G. S. Kansas, and T. A. Barrett. 2003. P-selectin and P-selectin glycoprotein ligand 1 are major determinants for Th1 cell recruitment to nonlymphoid effector sites in the intestinal lamina propria. *J. Exp. Med.* 198: 369-377.
133. MacLennan, I. C. 1994. Germinal centers. *Annu. Rev. Immunol.* 12: 117-139.
134. Kuppers, R., U. Klein, M. L. Hansmann, and K. Rajewsky. 1999. Cellular origin of human B-cell lymphomas. *N. Engl. J. Med.* 341: 1520-1529.
135. Stevenson, F., S. Sahota, D. Zhu, C. Ottensmeier, C. Chapman, D. Oscier, and T. Hamblin. 1998. Insight into the origin and clonal history of B-cell tumors as revealed by analysis of immunoglobulin variable region genes. *Immunol. Rev.* 162: 247-259.
136. Kuppers, R., and R. Dalla-Favera. 2001. Mechanisms of chromosomal translocations in B cell lymphomas. *Oncogene* 20: 5580-5594.
137. Toyama, H., S. Okada, M. Hatano, Y. Takahashi, N. Takeda, H. Ichii, T. Takemori, Y. Kuroda, and T. Tokuhisa. 2002. Memory B cells without somatic hypermutation are generated from Bcl6-deficient B cells. *Immunity.* 17: 329-339.
138. William, J., C. Euler, S. Christensen, and M. J. Shlomchik. 2002. Evolution of autoantibody responses via somatic hypermutation outside of germinal centers. *Science* 297: 2066-2070.
139. Weller, S., M. C. Braun, B. K. Tan, A. Rosenwald, C. Cordier, M. E. Conley, A. Plebani, D. S. Kumararatne, D. Bonnet, O. Tournilhac, G. Tchernia, B. Steiniger, L. M. Staudt, J. L. Casanova, C. A. Reynaud, and J. C. Weill. 2004. Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. *Blood* 104: 3647-3654.
140. Hauser, A. E., T. Junt, T. R. Mempel, M. W. Sneddon, S. H. Kleinstein, S. E. Henrickson, U. H. Von Andrian, M. J. Shlomchik, and A. M. Haberman. 2007. Definition of germinal-center B cell migration in vivo reveals predominant intrazonal circulation patterns. *Immunity.* 26: 655-667.
141. Jacob, J., G. Kelsoe, K. Rajewsky, and U. Weiss. 1991. Intraclonal generation of antibody mutants in germinal centres. *Nature* 354: 389-392.
142. Klein, U., Y. Tu, G. A. Stolovitzky, J. L. Keller, J. Haddad, Jr., V. Miljkovic, G. Cattoretti, A. Califano, and R. Dalla-Favera. 2003. Transcriptional analysis of the B cell germinal center reaction. *Proc. Natl. Acad. Sci. U. S. A* 100: 2639-2644.
143. Shaffer, A. L., A. Rosenwald, E. M. Hurt, J. M. Giltnane, L. T. Lam, O. K. Pickeral, and L. M. Staudt. 2001. Signatures of the immune response. *Immunity.* 15: 375-385.
144. Phan, R. T., and R. Dalla-Favera. 2004. The BCL6 proto-oncogene suppresses p53 expression in germinal-centre B cells. *Nature* 432: 635-639.
145. Hu, B. T., S. C. Lee, E. Marin, D. H. Ryan, and R. A. Insel. 1997. Telomerase is up-regulated in human germinal center B cells in vivo and can be re-expressed in memory B cells activated in vitro. *J. Immunol.* 159: 1068-1071.
146. Liu, Y. J., D. E. Joshua, G. T. Williams, C. A. Smith, J. Gordon, and I. C. MacLennan. 1989. Mechanism of antigen-driven selection in germinal centres. *Nature* 342: 929-931.
147. Liu, Y. J., D. Y. Mason, G. D. Johnson, S. Abbot, C. D. Gregory, D. L. Hardie, J. Gordon, and I. C. MacLennan. 1991. Germinal center cells express bcl-2 protein after activation by signals which prevent their entry into apoptosis. *Eur. J. Immunol.* 21: 1905-1910.
148. Martinez-Valdez, H., C. Guret, B. O. de, I. Fugier, J. Banchereau, and Y. J. Liu. 1996. Human germinal center B cells express the apoptosis-inducing genes Fas, c-myc, P53, and Bax but not the survival gene bcl-2. *J. Exp. Med.* 183: 971-977.
149. Shaffer, A. L., X. Yu, Y. He, J. Boldrick, E. P. Chan, and L. M. Staudt. 2000. BCL-6 represses genes that function in lymphocyte differentiation, inflammation, and cell cycle control. *Immunity.* 13: 199-212.
150. Niu, H., G. Cattoretti, and R. Dalla-Favera. 2003. BCL6 controls the expression of the B7-1/CD80 costimulatory receptor in germinal center B cells. *J. Exp. Med.* 198: 211-221.

151. Parekh, S., J. M. Polo, R. Shaknovich, P. Juszczynski, P. Lev, S. M. Ranuncolo, Y. Yin, U. Klein, G. Cattoretti, F. R. Dalla, M. A. Shipp, and A. Melnick. 2007. BCL6 programs lymphoma cells for survival and differentiation through distinct biochemical mechanisms. *Blood* 110: 2067-2074.

152. Shapiro-Shelef, M., K. I. Lin, L. J. McHeyzer-Williams, J. Liao, M. G. McHeyzer-Williams, and K. Calame. 2003. Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells. *Immunity.* 19: 607-620.

153. Allman, D., A. Jain, A. Dent, R. R. Maile, T. Selvaggi, M. R. Kehry, and L. M. Staudt. 1996. BCL-6 expression during B-cell activation. *Blood* 87: 5257-5268.

154. Saito, M., J. Gao, K. Basso, Y. Kitagawa, P. M. Smith, G. Bhagat, A. Pernis, L. Pasqualucci, and R. Dalla-Favera. 2007. A signaling pathway mediating downregulation of BCL6 in germinal center B cells is blocked by BCL6 gene alterations in B cell lymphoma. *Cancer Cell* 12: 280-292.

155. Phan, T. G., D. Paus, T. D. Chan, M. L. Turner, S. L. Nutt, A. Basten, and R. Brink. 2006. High affinity germinal center B cells are actively selected into the plasma cell compartment. *J. Exp. Med.* 203: 2419-2424.

156. Blink, E. J., A. Light, A. Kallies, S. L. Nutt, P. D. Hodgkin, and D. M. Tarlinton. 2005. Early appearance of germinal center-derived memory B cells and plasma cells in blood after primary immunization. *J. Exp. Med.* 201: 545-554.

157. Klein, U., S. Casola, G. Cattoretti, Q. Shen, M. Lia, T. Mo, T. Ludwig, K. Rajewsky, and R. Dalla-Favera. 2006. Transcription factor IRF4 controls plasma cell differentiation and class-switch recombination. *Nat. Immunol.* 7: 773-782.

158. Sciammas, R., A. L. Shaffer, J. H. Schatz, H. Zhao, L. M. Staudt, and H. Singh. 2006. Graded expression of interferon regulatory factor-4 coordinates isotype switching with plasma cell differentiation. *Immunity.* 25: 225-236.

159. Basso, K., U. Klein, H. Niu, G. A. Stolovitzky, Y. Tu, A. Califano, G. Cattoretti, and R. Dalla-Favera. 2004. Tracking CD40 signaling during germinal center development. *Blood* 104: 4088-4096.

160. Liu, Y. J., and J. Banchereau. 1997. Regulation of B-cell commitment to plasma cells or to memory B cells. *Semin. Immunol.* 9: 235-240.

161. Shan, H., M. Shlomchik, and M. Weigert. 1990. Heavy-chain class switch does not terminate somatic mutation. *J. Exp. Med.* 172: 531-536.

162. Cobaleda, C., A. Schebesta, A. Delogu, and M. Busslinger. 2007. Pax5: the guardian of B cell identity and function. *Nat. Immunol.* 8: 463-470.

163. Delogu, A., A. Schebesta, Q. Sun, K. Aschenbrenner, T. Perlot, and M. Busslinger. 2006. Gene repression by Pax5 in B cells is essential for blood cell homeostasis and is reversed in plasma cells. *Immunity.* 24: 269-281.

164. Nera, K. P., P. Kohonen, E. Narvi, A. Peippo, L. Mustonen, P. Terho, K. Koskela, J. M. Buerstedde, and O. Lassila. 2006. Loss of Pax5 promotes plasma cell differentiation. *Immunity.* 24: 283-293.

165. Kallies, A., J. Hasbold, K. Fairfax, C. Pridans, D. Emslie, B. S. McKenzie, A. M. Lew, L. M. Corcoran, P. D. Hodgkin, D. M. Tarlinton, and S. L. Nutt. 2007. Initiation of plasma-cell differentiation is independent of the transcription factor Blimp-1. *Immunity.* 26: 555-566.

166. Reimold, A. M., N. N. Iwakoshi, J. Manis, P. Vallabhajosyula, E. Szomolanyi-Tsuda, E. M. Gravallese, D. Friend, M. J. Grusby, F. Alt, and L. H. Glimcher. 2001. Plasma cell differentiation requires the transcription factor XBP-1. *Nature* 412: 300-307.

167. Mittrucker, H. W., T. Matsuyama, A. Grossman, T. M. Kundig, J. Potter, A. Shahinian, A. Wakeham, B. Patterson, P. S. Ohashi, and T. W. Mak. 1997. Requirement for the transcription factor LSIRF/IRF4 for mature B and T lymphocyte function. *Science* 275: 540-543.

168. Dogan, I., B. Bertocci, V. Vilmont, F. Delbos, J. Megret, S. Storck, C. A. Reynaud, and J. C. Weill. 2009. Multiple layers of B cell memory with different effector functions. *Nat. Immunol.* 10: 1292-1299.

169. Davis, R. E., K. D. Brown, U. Siebenlist, and L. M. Staudt. 2001. Constitutive nuclear factor kappaB activity is required for survival of activated B cell-like diffuse large B cell lymphoma cells. *J. Exp. Med.* 194: 1861-1874.

170. Alizadeh, A. A., M. B. Eisen, R. E. Davis, C. Ma, I. S. Lossos, A. Rosenwald, J. C. Boldrick, H. Sabet, T. Tran, X. Yu, J. I. Powell, L. Yang, G. E. Marti, T. Moore, J. Hudson, Jr., L. Lu, D. B. Lewis, R. Tibshirani, G. Sherlock, W. C. Chan, T. C. Greiner, D. D. Weisenburger, J. O. Armitage, R. Warnke, R. Levy, W. Wilson, M. R. Grever, J. C. Byrd, D. Botstein, P. O. Brown, and L. M. Staudt. 2000. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 403: 503-511.

171. Lenz, G., R. E. Davis, V. N. Ngo, L. Lam, T. C. George, G. W. Wright, S. S. Dave, H. Zhao, W. Xu, A. Rosenwald, G. Ott, H. K. Muller-Hermelink, R. D. Gascoyne, J. M. Connors, L. M. Rimsza, E. Campo, E. S. Jaffe, J. Delabie, E. B. Smeland, R. I. Fisher, W. C. Chan, and L. M. Staudt. 2008. Oncogenic CARD11 mutations in human diffuse large B cell lymphoma. *Science* 319: 1676-1679.

172. Compagno, M., W. K. Lim, A. Grunn, S. V. Nandula, M. Brahmachary, Q. Shen, F. Bertoni, M. Ponzoni, M. Scandurra, A. Califano, G. Bhagat, A. Chadburn, R. Dalla-Favera, and L. Pasqualucci. 2009. Mutations of multiple genes cause deregulation of NF-kappaB in diffuse large B-cell lymphoma. *Nature* 459: 717-721.

173. Staudt, L. M., and S. Dave. 2005. The biology of human lymphoid malignancies revealed by gene expression profiling. *Adv. Immunol.* 87: 163-208.

174. Lenz, G., G. W. Wright, N. C. Emre, H. Kohlhammer, S. S. Dave, R. E. Davis, S. Carty, L. T. Lam, A. L. Shaffer, W. Xiao, J. Powell, A. Rosenwald, G. Ott, H. K. Muller-Hermelink, R. D. Gascoyne, J. M. Connors, E. Campo, E. S. Jaffe, J. Delabie, E. B. Smeland, L. M. Rimsza, R. I. Fisher, D. D. Weisenburger, W. C. Chan, and L. M. Staudt. 2008. Molecular subtypes of diffuse large B-cell lymphoma arise by distinct genetic pathways. *Proc. Natl. Acad. Sci. U. S. A* 105: 13520-13525.

175. Mann, R. B. 1999. Are there site-specific differences among extranodal aggressive B-cell neoplasms? *Am. J. Clin. Pathol.* 111: S144-S150.

176. Krol, A. D., C. S. le, S. Snijder, J. C. Kluin-Nelemans, P. M. Kluin, and E. M. Noordijk. 2003. Primary extranodal non-Hodgkin's lymphoma (NHL): the impact of alternative definitions tested in the Comprehensive Cancer Centre West population-based NHL registry. *Ann. Oncol.* 14: 131-139.

177. Hussell, T., P. G. Isaacson, J. E. Crabtree, and J. Spencer. 1993. The response of cells from low-grade B-cell gastric lymphomas of mucosa-associated lymphoid tissue to Helicobacter pylori. *Lancet* 342: 571-574.

178. Bayerdorffer, E., A. Neubauer, B. Rudolph, C. Thiede, N. Lehn, S. Eidt, and M. Stolte. 1995. Regression of primary gastric lymphoma of mucosa-associated lymphoid tissue type after cure of Helicobacter pylori infection. MALT Lymphoma Study Group. *Lancet* 345: 1591-1594.

179. Lenze, D., E. Berg, R. Volkmer-Engert, A. A. Weiser, A. Greiner, C. Knorr-Wittmann, I. Anagnostopoulos, H. Stein, and M. Hummel. 2006. Influence of antigen on the development of MALT lymphoma. *Blood* 107: 1141-1148.

180. Ferreri, A. J., M. Ponzoni, M. Guidoboni, C. C. De, A. G. Resti, B. Mazzi, A. A. Lettini, J. Demeter, S. Dell'Oro, C. Doglioni, E. Villa, M. Boiocchi, and R. Dolcetti. 2005. Regression of ocular adnexal lymphoma after Chlamydia psittaci-eradicating antibiotic therapy. *J. Clin. Oncol.* 23: 5067-5073.

181. Rosado, M. F., G. E. Byrne, Jr., F. Ding, K. A. Fields, P. Ruiz, S. R. Dubovy, G. R. Walker, A. Markoe, and I. S. Lossos. 2006. Ocular adnexal lymphoma: a clinicopathologic study of a large cohort of patients with no evidence for an association with Chlamydia psittaci. *Blood* 107: 467-472.

182. Navarro, W. H., and L. D. Kaplan. 2006. AIDS-related lymphoproliferative disease. *Blood* 107: 13-20.

183. Gridley, G., J. K. McLaughlin, A. Ekbom, L. Klareskog, H. O. Adami, D. G. Hacker, R. Hoover, and J. F. Fraumeni, Jr. 1993. Incidence of cancer among patients with rheumatoid arthritis. *J. Natl. Cancer Inst.* 85: 307-311.

184. Knight, A., J. Askling, and A. Ekbom. 2002. Cancer incidence in a population-based cohort of patients with Wegener's granulomatosis. *Int. J. Cancer* 100: 82-85.

185. Pedersen, R. K., and N. T. Pedersen. 1996. Primary non-Hodgkin's lymphoma of the thyroid gland: a population based study. *Histopathology* 28: 25-32.

186. Batchelor, T., and J. S. Loeffler. 2006. Primary CNS lymphoma. *J. Clin. Oncol.* 24: 1281-1288.

187. Ferreri, A. J., L. E. Abrey, J. Y. Blay, B. Borisch, J. Hochman, E. A. Neuwelt, J. Yahalom, E. Zucca, F. Cavalli, J. Armitage, and T. Batchelor. 2003. Summary statement on primary central nervous system lymphomas from the Eighth International Conference on Malignant Lymphoma, Lugano, Switzerland, June 12 to 15, 2002. *J. Clin. Oncol.* 21: 2407-2414.

188. Eby, N. L., S. Grufferman, C. M. Flannelly, S. C. Schold, Jr., F. S. Vogel, and P. C. Burger. 1988. Increasing incidence of primary brain lymphoma in the US. *Cancer* 62: 2461-2465.

189. Kadan-Lottick, N. S., M. C. Skluzacek, and J. G. Gurney. 2002. Decreasing incidence rates of primary central nervous system lymphoma. *Cancer* 95: 193-202.

190. Cote, T. R., A. Manns, C. R. Hardy, F. J. Yellin, and P. Hartge. 1996. Epidemiology of brain lymphoma among people with or without acquired immunodeficiency syndrome. AIDS/Cancer Study Group. *J. Natl. Cancer Inst.* 88: 675-679.

191. Kasamon, Y. L., and R. F. Ambinder. 2005. AIDS-related primary central nervous system lymphoma. *Hematol. Oncol. Clin. North Am.* 19: 665-vii.

192. Miller, D. C., F. H. Hochberg, N. L. Harris, M. L. Gruber, D. N. Louis, and H. Cohen. 1994. Pathology with clinical correlations of primary central nervous system non-Hodgkin's lymphoma. The Massachusetts General Hospital experience 1958-1989. *Cancer* 74: 1383-1397.

193. Fine, H. A., and R. J. Mayer. 1993. Primary central nervous system lymphoma. *Ann. Intern. Med.* 119: 1093-1104.

194. Herrlinger, U., M. Schabet, M. Clemens, R. D. Kortmann, D. Petersen, B. E. Will, R. Meyermann, and J. Dichgans. 1998. Clinical presentation and therapeutic outcome in 26 patients with primary CNS lymphoma. *Acta Neurol. Scand.* 97: 257-264.

195. Braaten, K. M., R. A. Betensky, L. L. de, Y. Okada, F. H. Hochberg, D. N. Louis, N. L. Harris, and T. T. Batchelor. 2003. BCL-6 expression predicts improved survival in patients with primary central nervous system lymphoma. *Clin. Cancer Res.* 9: 1063-1069.

196. Abrey, L. E., T. T. Batchelor, A. J. Ferreri, M. Gospodarowicz, E. J. Pulczynski, E. Zucca, J. R. Smith, A. Korfel, C. Soussain, L. M. DeAngelis, E. A. Neuwelt, B. P. O'Neill, E. Thiel, T. Shenkier, F. Graus, M. van den Bent, J. F. Seymour, P. Poortmans, J. O. Armitage, and F. Cavalli. 2005. Report of an international workshop to standardize baseline evaluation and response criteria for primary CNS lymphoma. *J. Clin. Oncol.* 23: 5034-5043.

197. Camilleri-Broet, S., E. Criniere, P. Broet, V. Delwail, K. Mokhtari, A. Moreau, M. Kujas, M. Raphael, W. Iraqi, C. Sautes-Fridman, P. Colombat, K. Hoang-Xuan, and A. Martin. 2006. A uniform activated B-cell-like immunophenotype might explain the poor prognosis of primary central nervous system lymphomas: analysis of 83 cases. *Blood* 107: 190-196.

198. Ponzoni, M., F. Berger, C. Chassagne-Clement, M. Tinguely, A. Jouvet, A. J. Ferreri, S. Dell'Oro, M. R. Terreni, C. Doglioni, J. Weis, M. Cerati, M. Milani, P. Iuzzolino, T. Motta, A. Carbone, E. Pedrinis, J. Sanchez, J. Y. Blay, M. Reni, A. Conconi, F. Bertoni, E. Zucca, F. Cavalli, and B. Borisch. 2007. Reactive perivascular T-cell infiltrate predicts survival in primary central nervous system B-cell lymphomas. *Br. J. Haematol.* 138: 316-323.

199. Montesinos-Rongen, M., R. Siebert, and M. Deckert. 2009. Primary lymphoma of the central nervous system: just DLBCL or not? *Blood* 113: 7-10.

200. Alderson, L., M. R. Fetell, M. Sisti, F. Hochberg, M. Cohen, and D. N. Louis. 1996. Sentinel lesions of primary CNS lymphoma. *J. Neurol. Neurosurg. Psychiatry* 60: 102-105.

201. Drillenburg, P., and S. T. Pals. 2000. Cell adhesion receptors in lymphoma dissemination. *Blood* 95: 1900-1910.

202. Springer, T. A. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: The multistep paradigm. *Cell* 76: 301-314.

203. Smith, J. R., R. M. Braziel, S. Paoletti, M. Lipp, M. Uguccioni, and J. T. Rosenbaum. 2003. Expression of B-cell-attracting chemokine 1 (CXCL13) by malignant lymphocytes and vascular endothelium in primary central nervous system lymphoma. *Blood* 101: 815-21.

204. McCann, K. J., M. Ashton-Key, K. Smith, F. K. Stevenson, and C. H. Ottensmeier. 2009. Primary central nervous system lymphoma: tumor-related clones exist in the blood and bone marrow with evidence for separate development. *Blood* 113: 4677-4680.

205. Bashir, R., H. Coakham, and F. Hochberg. 1992. Expression of LFA-1/ICAM-1 in CNS lymphomas: possible mechanism for lymphoma homing into the brain. *J. Neurooncol.* 12: 103-110.

206. Aho, R., H. Kalimo, M. Salmi, D. Smith, and S. Jalkanen. 1997. Binding of malignant lymphoid cells to the white matter of the human central nervous system: role of different CD44 isoforms, beta 1, beta 2 and beta 7 integrins, and L-selectin. *J. Neuropathol. Exp. Neurol.* 56: 557-568.

207. Smith, J. R., K. M. Falkenhagen, S. E. Coupland, T. J. Chipps, J. T. Rosenbaum, and R. M. Braziel. 2007. Malignant B cells from patients with primary central nervous system lymphoma express stromal cell-derived factor-1. *Am. J. Clin. Pathol.* 127: 633-641.

208. Fischer, L., A. Korfel, S. Pfeiffer, P. Kiewe, H. D. Volk, H. Cakiroglu, T. Widmann, and E. Thiel. 2009. CXCL13 and CXCL12 in central nervous system lymphoma patients. *Clin. Cancer Res.* 15: 5968-5973.

209. Miller, C. G., D. N. Cook, and G. J. Kotwal. 1996. Two chemotactic factors, C5a and MIP-1α, dramatically alter the mortality from zymosan-induced multiple organ dysfunction syndrome (MODS): C5a contributes to MODS while MIP-1α has a protective role. *Mol. Immunol.* 33: 1135-1137.

210. Mittra, R. A., J. S. Pulido, G. A. Hanson, A. Kajdacsy-Balla, and C. F. Brummitt. 1999. Primary ocular Epstein-Barr virus-associated non-Hodgkin's lymphoma in a patient with AIDS: a clinicopathologic report. *Retina* 19: 45-50.

211. Montesinos-Rongen, M., A. Brunn, S. Bentink, K. Basso, W. K. Lim, W. Klapper, C. Schaller, G. Reifenberger, J. Rubenstein, O. D. Wiestler, R. Spang, R. la-Favera, R. Siebert, and M. Deckert. 2008. Gene expression profiling suggests primary central nervous system lymphomas to be derived from a late germinal center B cell. *Leukemia* 22: 400-405.

212. Larocca, L. M., D. Capello, A. Rinelli, S. Nori, A. Antinori, A. Gloghini, A. Cingolani, A. Migliazza, G. Saglio, S. Cammilleri-Broet, M. Raphael, A. Carbone, and G. Gaidano. 1998. The molecular and phenotypic profile of primary central nervous system lymphoma identifies distinct categories of the disease and is consistent with histogenetic derivation from germinal center-related B cells. *Blood* 92: 1011-1019.

213. Thompsett, A. R., D. W. Ellison, F. K. Stevenson, and D. Zhu. 1999. V(H) gene sequences from primary central nervous system lymphomas indicate derivation from highly mutated germinal center B cells with ongoing mutational activity. *Blood* 94: 1738-1746.

214. Montesinos-Rongen, M., R. Kuppers, D. Schluter, T. Spieker, R. D. Van, C. Schaller, G. Reifenberger, O. D. Wiestler, and M. Deckert-Schluter. 1999. Primary central nervous system lymphomas are derived from germinal-center B cells and show a preferential usage of the V4-34 gene segment. *Am. J. Pathol.* 155: 2077-2086.

215. Rosenwald, A., G. Wright, W. C. Chan, J. M. Connors, E. Campo, R. I. Fisher, R. D. Gascoyne, H. K. Muller-Hermelink, E. B. Smeland, J. M. Giltnane, E. M. Hurt, H. Zhao, L. Averett, L. Yang, W. H. Wilson, E. S. Jaffe, R. Simon, R. D. Klausner, J. Powell, P. L. Duffey, D. L. Longo, T. C. Greiner, D. D. Weisenburger, W. G. Sanger, B. J. Dave, J. C. Lynch, J. Vose, J. O. Armitage, E. Montserrat, A. Lopez-Guillermo, T. M. Grogan, T. P. Miller, M. LeBlanc, G. Ott, S. Kvaloy, J. Delabie, H. Holte, P. Krajci, T. Stokke, and L. M. Staudt. 2002. The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. *N. Engl. J. Med.* 346: 1937-1947.

216. Rubenstein, J. L., J. Fridlyand, A. Shen, K. Aldape, D. Ginzinger, T. Batchelor, P. Treseler, M. Berger, M. McDermott, M. Prados, J. Karch, C. Okada, W. Hyun, S. Parikh, C. Haqq, and M. Shuman. 2006. Gene expression and angiotropism in primary CNS lymphoma. *Blood* 107: 3716-3723.

217. Pasqualucci, L., P. Neumeister, T. Goossens, G. Nanjangud, R. S. Chaganti, R. Kuppers, and R. Dalla-Favera. 2001. Hypermutation of multiple proto-oncogenes in B-cell diffuse large-cell lymphomas. *Nature* 412: 341-346.

218. Montesinos-Rongen, M., R. D. Van, C. Schaller, O. D. Wiestler, and M. Deckert. 2004. Primary diffuse large B-cell lymphomas of the central nervous system are targeted by aberrant somatic hypermutation. *Blood* 103: 1869-1875.

219. Papavasiliou, F. N., and D. G. Schatz. 2002. Somatic hypermutation of immunoglobulin genes: merging mechanisms for genetic diversity. *Cell* 109 Suppl: S35-S44.

220. Hochberg, F. H., G. Miller, R. T. Schooley, M. S. Hirsch, P. Feorino, and W. Henle. 1983. Central-nervous-system lymphoma related to Epstein-Barr virus. *N. Engl. J. Med.* 309: 745-748.

221. Epeldegui, M., Y. P. Hung, A. McQuay, R. F. Ambinder, and O. Martinez-Maza. 2007. Infection of human B cells with Epstein-Barr virus results in the expression of somatic hypermutation-inducing molecules and in the accrual of oncogene mutations. *Mol. Immunol.* 44: 934-942.

222. Mead, G. M., N. M. Bleehen, A. Gregor, J. Bullimore, D. Shirley, R. P. Rampling, J. Trevor, M. G. Glaser, P. Lantos, J. W. Ironside, T. H. Moss, M. Brada, J. B. Whaley, and S. P. Stenning. 2000. A medical research council randomized trial in patients with primary cerebral non-Hodgkin lymphoma: cerebral radiotherapy with and without cyclophosphamide, doxorubicin, vincristine, and prednisone chemotherapy. *Cancer* 89: 1359-1370.

223. Brada, M., D. Hjiyiannakis, F. Hines, D. Traish, and S. Ashley. 1998. Short intensive primary chemotherapy and radiotherapy in sporadic primary CNS lymphoma (PCL). *Int. J. Radiat. Oncol. Biol. Phys.* 40: 1157-1162.

224. Batchelor, T., K. Carson, A. O'Neill, S. A. Grossman, J. Alavi, P. New, F. Hochberg, and R. Priet. 2003. Treatment of primary CNS lymphoma with methotrexate and deferred radiotherapy: a report of NABTT 96-07. *J. Clin. Oncol.* 21: 1044-1049.

225. Herrlinger, U., W. Kuker, M. Uhl, H. P. Blaicher, H. O. Karnath, L. Kanz, M. Bamberg, and M. Weller. 2005. NOA-03 trial of high-dose methotrexate in primary central nervous system lymphoma: final report. *Ann. Neurol.* 57: 843-847.

226. Pels, H., I. G. Schmidt-Wolf, A. Glasmacher, H. Schulz, A. Engert, V. Diehl, A. Zellner, G. Schackert, H. Reichmann, F. Kroschinsky, M. Vogt-Schaden, G. Egerer, U. Bode, C. Schaller, M. Deckert, R. Fimmers, C. Helmstaedter, A. Atasoy, T. Klockgether, and U. Schlegel. 2003. Primary central nervous system lymphoma: results of a pilot and phase II study of systemic and intraventricular chemotherapy with deferred radiotherapy. *J. Clin. Oncol.* 21: 4489-4495.

227. Abrey, L. E., J. Yahalom, and L. M. DeAngelis. 2000. Treatment for primary CNS lymphoma: the next step. *J. Clin. Oncol.* 18: 3144-3150.

228. Reni, M., F. Zaja, W. Mason, J. Perry, E. Mazza, M. Spina, R. Bordonaro, F. Ilariucci, M. Faedi, G. Corazzelli, P. Manno, E. Franceschi, A. Pace, M. Candela, A. Abbadessa, C. Stelitano, G. Latte, and A. J. Ferreri. 2007. Temozolomide as salvage treatment in primary brain lymphomas. *Br. J. Cancer* 96: 864-867.

229. Fischer, L., E. Thiel, H. A. Klasen, J. Birkmann, K. Jahnke, P. Martus, and A. Korfel. 2006. Prospective trial on topotecan salvage therapy in primary CNS lymphoma. *Ann. Oncol.* 17: 1141-1145.

230. Enting, R. H., A. Demopoulos, L. M. DeAngelis, and L. E. Abrey. 2004. Salvage therapy for primary CNS lymphoma with a combination of rituximab and temozolomide. *Neurology* 63: 901-903.

231. Herrlinger, U., W. Brugger, M. Bamberg, W. Kuker, J. Dichgans, and M. Weller. 2000. PCV salvage chemotherapy for recurrent primary CNS lymphoma. *Neurology* 54: 1707-1708.

232. Ferreri, A. J., M. Reni, F. Pasini, A. Calderoni, U. Tirelli, A. Pivnik, G. M. Aondio, F. Ferrarese, H. Gomez, M. Ponzoni, B. Borisch, F. Berger, C. Chassagne, P. Iuzzolino, A. Carbone, J. Weis, E. Pedrinis, T. Motta, A. Jouvet, T. Barbui, F. Cavalli, and J. Y. Blay. 2002. A multicenter study of treatment of primary CNS lymphoma. *Neurology* 58: 1513-1520.

233. Ferreri, A. J., S. Dell'Oro, D. Capello, M. Ponzoni, P. Iuzzolino, D. Rossi, F. Pasini, A. Ambrosetti, E. Orvieto, F. Ferrarese, G. Arrigoni, M. Foppoli, M. Reni, and G. Gaidano. 2004. Aberrant methylation in the promoter region of the reduced folate carrier gene is a potential mechanism of resistance to methotrexate in primary central nervous system lymphomas. *Br. J. Haematol.* 126: 657-664.

234. Illerhaus, G., R. Marks, G. Ihorst, R. Guttenberger, C. Ostertag, G. Derigs, N. Frickhofen, F. Feuerhake, B. Volk, and J. Finke. 2006. High-dose chemotherapy with autologous stem-cell transplantation and hyperfractionated radiotherapy as first-line treatment of primary CNS lymphoma. *J. Clin. Oncol.* 24: 3865-3870.

235. Hanahan, D., and J. Folkman. 1996. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 86: 353-364.

236. Bishop, J. M. 1997. Cancer: what should be done? *Science* 278: 995.

237. Vogelstein, B., and K. W. Kinzler. 1993. The multistep nature of cancer. *Trends Genet.* 9: 138-141.

238. Mantovani, A., P. Allavena, A. Sica, and F. Balkwill. 2008. Cancer-related inflammation. *Nature* 454: 436-444.

239. Balkwill, F., and A. Mantovani. 2001. Inflammation and cancer: back to Virchow? *Lancet* 357: 539-545.

240. Pardoll, D. 2003. Does the immune system see tumors as foreign or self? *Annu. Rev. Immunol.* 21: 807-839.

241. Balkwill, F., K. A. Charles, and A. Mantovani. 2005. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell* 7: 211-217.

242. Coussens, L. M., and Z. Werb. 2002. Inflammation and cancer. *Nature* 420: 860-867.

243. Paik, S., S. Shak, G. Tang, C. Kim, J. Baker, M. Cronin, F. L. Baehner, M. G. Walker, D. Watson, T. Park, W. Hiller, E. R. Fisher, D. L. Wickerham, J. Bryant, and N. Wolmark. 2004. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. *N. Engl. J. Med.* 351: 2817-2826.

244. Dave, S. S., G. Wright, B. Tan, A. Rosenwald, R. D. Gascoyne, W. C. Chan, R. I. Fisher, R. M. Braziel, L. M. Rimsza, T. M. Grogan, T. P. Miller, M. LeBlanc, T. C. Greiner, D. D. Weisenburger, J. C. Lynch, J. Vose, J. O. Armitage, E. B. Smeland, S. Kvaloy, H. Holte, J. Delabie, J. M. Connors, P. M. Lansdorp, Q. Ouyang, T. A. Lister, A. J. Davies, A. J. Norton, H. K. Muller-Hermelink, G. Ott, E. Campo, E. Montserrat, W. H. Wilson, E. S. Jaffe, R. Simon, L. Yang, J. Powell, H. Zhao, N. Goldschmidt, M. Chiorazzi, and L. M. Staudt. 2004. Prediction of survival in follicular lymphoma based on molecular features of tumor-infiltrating immune cells. *N. Engl. J. Med.* 351: 2159-2169.

245. Bunt, S. K., L. Yang, P. Sinha, V. K. Clements, J. Leips, and S. Ostrand-Rosenberg. 2007. Reduced inflammation in the tumor microenvironment delays the accumulation of myeloid-derived suppressor cells and limits tumor progression. *Cancer Res.* 67: 10019-10026.

246. Mantovani, A., S. Sozzani, M. Locati, P. Allavena, and A. Sica. 2002. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol.* 23: 549-555.

247. Gordon, S. 2003. Alternative activation of macrophages. *Nat. Rev. Immunol.* 3: 23-35.

248. Mantovani, A., A. Sica, S. Sozzani, P. Allavena, A. Vecchi, and M. Locati. 2004. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol.* 25: 677-686.

249. Wang, T., G. Niu, M. Kortylewski, L. Burdelya, K. Shain, S. Zhang, R. Bhattacharya, D. Gabrilovich, R. Heller, D. Coppola, W. Dalton, R. Jove, D. Pardoll, and H. Yu. 2004. Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells. *Nat. Med.* 10: 48-54.

250. Kortylewski, M., M. Kujawski, T. Wang, S. Wei, S. Zhang, S. Pilon-Thomas, G. Niu, H. Kay, J. Mule, W. G. Kerr, R. Jove, D. Pardoll, and H. Yu. 2005. Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. *Nat. Med.* 11: 1314-1321.

251. Yu, H., M. Kortylewski, and D. Pardoll. 2007. Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. *Nat. Rev. Immunol.* 7: 41-51.

252. Galon, J., A. Costes, F. Sanchez-Cabo, A. Kirilovsky, B. Mlecnik, C. Lagorce-Pages, M. Tosolini, M. Camus, A. Berger, P. Wind, F. Zinzindohoue, P. Bruneval, P. H. Cugnenc, Z. Trajanoski, W. H. Fridman, and F. Pages. 2006. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 313: 1960-1964.

253. Mihm, M. C., Jr., C. G. Clemente, and N. Cascinelli. 1996. Tumor infiltrating lymphocytes in lymph node melanoma metastases: a histopathologic prognostic indicator and an expression of local immune response. *Lab Invest* 74: 43-47.

254. Lipponen, P. K., M. J. Eskelinen, K. Jauhiainen, E. Harju, and R. Terho. 1992. Tumour infiltrating lymphocytes as an independent prognostic factor in transitional cell bladder cancer. *Eur. J. Cancer* 29A: 69-75.

255. Rilke, F., M. I. Colnaghi, N. Cascinelli, S. Andreola, M. T. Baldini, R. Bufalino, P. G. Della, S. Menard, M. A. Pierotti, and A. Testori. 1991. Prognostic significance of HER-2/neu expression in breast cancer and its relationship to other prognostic factors. *Int. J. Cancer* 49: 44-49.

256. Nacopoulou, L., P. Azaris, N. Papacharalampous, and P. Davaris. 1981. Prognostic significance of histologic host response in cancer of the large bowel. *Cancer* 47: 930-936.

257. Boon, T., P. G. Coulie, B. J. Van den Eynde, and P. van der Bruggen. 2006. Human T cell responses against melanoma. *Annu. Rev. Immunol.* 24: 175-208.

258. Rosenberg, S. A. 2001. Progress in human tumour immunology and immunotherapy. *Nature* 411: 380-384.

259. Pardoll, D. M., and S. L. Topalian. 1998. The role of CD4+ T cell responses in antitumor immunity. *Curr. Opin. Immunol.* 10: 588-594.

260. Wang, R. F., G. Peng, and H. Y. Wang. 2006. Regulatory T cells and Toll-like receptors in tumor immunity. *Semin. Immunol.* 18: 136-142.

261. Sakaguchi, S. 2005. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nat. Immunol.* 6: 345-352.

262. Viguier, M., F. Lemaitre, O. Verola, M. S. Cho, G. Gorochov, L. Dubertret, H. Bachelez, P. Kourilsky, and L. Ferradini. 2004. Foxp3 expressing CD4+CD25(high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. *J. Immunol.* 173: 1444-1453.

263. Curiel, T. J., G. Coukos, L. Zou, X. Alvarez, P. Cheng, P. Mottram, M. Evdemon-Hogan, J. R. Conejo-Garcia, L. Zhang, M. Burow, Y. Zhu, S. Wei, I. Kryczek, B. Daniel, A. Gordon, L. Myers, A. Lackner, M. L. Disis, K. L. Knutson, L. Chen, and W. Zou. 2004. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat. Med.* 10: 942-949.

264. Sanchez-Beato, M., A. Sanchez-Aguilera, and M. A. Piris. 2003. Cell cycle deregulation in B-cell lymphomas. *Blood* 101: 1220-1235.

265. Eray, M., V. Postila, J. Eeva, A. Ripatti, M. L. Karjalainen-Lindsberg, S. Knuutila, L. C. Andersson, and J. Pelkonen. 2003. Follicular lymphoma cell lines, an in vitro model for antigenic selection and cytokine-mediated growth regulation of germinal centre B cells. *Scand. J. Immunol.* 57: 545-555.

266. Schattner, E. J., J. Mascarenhas, I. Reyfman, M. Koshy, C. Woo, S. M. Friedman, and M. K. Crow. 1998. Chronic lymphocytic leukemia B cells can express CD40 ligand and demonstrate T-cell type costimulatory capacity. *Blood* 91: 2689-2697.

267. Haxhinasto, S. A., and G. A. Bishop. 2004. Synergistic B cell activation by CD40 and the B cell antigen receptor: role of B lymphocyte antigen receptor-mediated kinase activation and tumor necrosis factor receptor-associated factor regulation. *J. Biol. Chem.* 279: 2575-2582.

268. Kern, C., J. F. Cornuel, C. Billard, R. Tang, D. Rouillard, V. Stenou, T. Defrance, F. Ajchenbaum-Cymbalista, P. Y. Simonin, S. Feldblum, and J. P. Kolb. 2004. Involvement of BAFF and APRIL in the resistance to apoptosis of B-CLL through an autocrine pathway. *Blood* 103: 679-688.

269. Nishio, M., T. Endo, N. Tsukada, J. Ohata, S. Kitada, J. C. Reed, N. J. Zvaifler, and T. J. Kipps. 2005. Nurselike cells express BAFF and APRIL, which can promote survival of chronic lymphocytic leukemia cells via a paracrine pathway distinct from that of SDF-1alpha. *Blood* 106: 1012-1020.

270. Stevenson, F. K., and F. Caligaris-Cappio. 2004. Chronic lymphocytic leukemia: revelations from the B-cell receptor. *Blood* 103: 4389-4395.

271. Ghia, P., and F. Caligaris-Cappio. 2000. The indispensable role of microenvironment in the natural history of low-grade B-cell neoplasms. *Adv. Cancer Res.* 79: 157-173.

272. Alvaro, T., M. Lejeune, M. T. Salvado, R. Bosch, J. F. Garcia, J. Jaen, A. H. Banham, G. Roncador, C. Montalban, and M. A. Piris. 2005. Outcome in Hodgkin's lymphoma can be predicted from the presence of accompanying cytotoxic and regulatory T cells. *Clin. Cancer Res.* 11: 1467-1473.

273. Carreras, J., A. Lopez-Guillermo, B. C. Fox, L. Colomo, A. Martinez, G. Roncador, E. Montserrat, E. Campo, and A. H. Banham. 2006. High numbers of tumor-infiltrating FOXP3-positive regulatory T cells are associated with improved overall survival in follicular lymphoma. *Blood* 108: 2957-2964.

274. Lee, N. R., E. K. Song, K. Y. Jang, H. N. Choi, W. S. Moon, K. Kwon, J. H. Lee, C. Y. Yim, and J. Y. Kwak. 2008. Prognostic impact of tumor infiltrating FOXP3 positive regulatory T cells in diffuse large B-cell lymphoma at diagnosis. *Leuk. Lymphoma* 49: 247-256.

275. Tzankov, A., C. Meier, P. Hirschmann, P. Went, S. A. Pileri, and S. Dirnhofer. 2008. Correlation of high numbers of intratumoral FOXP3+ regulatory T cells with improved survival in germinal center-like diffuse large B-cell lymphoma, follicular lymphoma and classical Hodgkin's lymphoma. *Haematologica* 93: 193-200.

276. Marshall, N. A., L. E. Christie, L. R. Munro, D. J. Culligan, P. W. Johnston, R. N. Barker, and M. A. Vickers. 2004. Immunosuppressive regulatory T cells are abundant in the reactive lymphocytes of Hodgkin lymphoma. *Blood* 103: 1755-1762.

277. Sanchez-Aguilera, A., C. Montalban, P. de la Cueva, L. Sanchez-Verde, M. M. Morente, M. Garcia-Cosio, J. Garcia-Larana, C. Bellas, M. Provencio, V. Romagosa, A. F. de Sevilla, J. Menarguez, P. Sabin, M. J. Mestre, M. Mendez, M. F. Fresno, C. Nicolas, M. A. Piris, and J. F. Garcia. 2006. Tumor microenvironment and mitotic checkpoint are key factors in the outcome of classic Hodgkin lymphoma. *Blood* 108: 662-668.

278. Oudejans, J. J., N. M. Jiwa, J. A. Kummer, G. J. Ossenkoppele, H. P. van, J. W. Baars, P. M. Kluin, J. C. Kluin-Nelemans, P. J. van Diest, J. M. Middeldorp, and C. J. Meijer. 1997. Activated cytotoxic T cells as prognostic marker in Hodgkin's disease. *Blood* 89: 1376-1382.

279. Kusmartsev, S., and D. I. Gabrilovich. 2005. STAT1 signaling regulates tumor-associated macrophage-mediated T cell deletion. *J. Immunol.* 174: 4880-4891.

280. Alvaro, T., M. Lejeune, F. I. Camacho, M. T. Salvado, L. Sanchez, J. F. Garcia, C. Lopez, J. Jaen, R. Bosch, L. E. Pons, C. Bellas, and M. A. Piris. 2006. The presence of STAT1-positive tumor-associated macrophages and their relation to outcome in patients with follicular lymphoma. *Haematologica* 91: 1605-1612.

281. Parsonnet, J., S. Hansen, L. Rodriguez, A. B. Gelb, R. A. Warnke, E. Jellum, N. Orentreich, J. H. Vogelman, and G. D. Friedman. 1994. Helicobacter pylori infection and gastric lymphoma. *N. Engl. J. Med.* 330: 1267-1271.

282. Gunn, M. D., V. N. Ngo, K. M. Ansel, E. H. Ekland, J. G. Cyster, and L. T. Williams. 1998. A B-cell-homing chemokine made in lymphoid follicles activates Burkitt's lymphoma receptor-1. *Nature* 391: 799-803.

283. Mueller, S. N., K. A. Hosiawa-Meagher, B. T. Konieczny, B. M. Sullivan, M. F. Bachmann, R. M. Locksley, R. Ahmed, and M. Matloubian. 2007. Regulation of homeostatic chemokine expression and cell trafficking during immune responses. *Science* 317: 670-674.

284. St John, A. L., and S. N. Abraham. 2009. Salmonella disrupts lymph node architecture by TLR4-mediated suppression of homeostatic chemokines. *Nat. Med.* 15: 1259-1265.

285. Smith, J. P., G. F. Burton, J. G. Tew, and A. K. Szakal. 1998. Tingible body macrophages in regulation of germinal center reactions. *Dev. Immunol.* 6: 285-294.

286. Hanayama, R., M. Tanaka, K. Miyasaka, K. Aozasa, M. Koike, Y. Uchiyama, and S. Nagata. 2004. Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. *Science* 304: 1147-1150.

287. Tun, H. W., D. Personett, K. A. Baskerville, D. M. Menke, K. A. Jaeckle, P. Kreinest, B. Edenfield, A. C. Zubair, B. P. O'Neill, W. R. Lai, P. J. Park, and M. McKinney. 2008. Pathway analysis of primary central nervous system lymphoma. *Blood* 111: 3200-3210.

288. van Kempen, M. J., G. T. Rijkers, and P. B. Van Cauwenberge. 2000. The immune response in adenoids and tonsils. *Int. Arch. Allergy Immunol.* 122: 8-19.

289. Junt, T., E. A. Moseman, M. Iannacone, S. Massberg, P. A. Lang, M. Boes, K. Fink, S. E. Henrickson, D. M. Shayakhmetov, N. C. Di Paolo, R. N. van, T. R. Mempel, S. P. Whelan, and U. H. Von Andrian. 2007. Subcapsular sinus macrophages in lymph nodes clear lymph-borne viruses and present them to antiviral B cells. *Nature* 450: 110-114.

290. Phan, T. G., I. Grigorova, T. Okada, and J. G. Cyster. 2007. Subcapsular encounter and complement-dependent transport of immune complexes by lymph node B cells. *Nat. Immunol.* 8: 992-1000.

291. Graeber, M. B., W. J. Streit, and G. W. Kreutzberg. 1990. The third glial cell type, the microglia: cellular markers of activation in situ. *Acta Histochem. Suppl* 38: 157-160.

292. Benveniste, E. N., V. T. Nguyen, and G. M. O'Keefe. 2001. Immunological aspects of microglia: relevance to Alzheimer's disease. *Neurochem. Int.* 39: 381-391.

293. Koopman, G., R. M. Keehnen, E. Lindhout, W. Newman, Y. Shimizu, G. A. van Seventer, G. C. de, and S. T. Pals. 1994. Adhesion through the LFA-1 (CD11a/CD18)-ICAM-1 (CD54) and the VLA-4 (CD49d)-VCAM-1 (CD106) pathways prevents apoptosis of germinal center B cells. *J. Immunol.* 152: 3760-3767.

294. McCandless, E. E., L. Piccio, B. M. Woerner, R. E. Schmidt, J. B. Rubin, A. H. Cross, and R. S. Klein. 2008. Pathological expression of CXCL12 at the blood-brain barrier correlates with severity of multiple sclerosis. *Am. J. Pathol.* 172: 799-808.

295. Li, M., and R. M. Ransohoff. 2008. Multiple roles of chemokine CXCL12 in the central nervous system: a migration from immunology to neurobiology. *Prog. Neurobiol.* 84: 116-131.

296. Magliozzi, R., S. Columba-Cabezas, B. Serafini, and F. Aloisi. 2004. Intracerebral expression of CXCL13 and BAFF is accompanied by formation of lymphoid follicle-like structures in the

meninges of mice with relapsing experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* 148: 11-23.

297. Aloisi, F., S. Columba-Cabezas, D. Franciotta, B. Rosicarelli, R. Magliozzi, R. Reynolds, E. Ambrosini, E. Coccia, M. Salvetti, and B. Serafini. 2008. Lymphoid chemokines in chronic neuroinflammation. *J. Neuroimmunol.* 198: 106-112.
298. Brustle, A., S. Heink, M. Huber, C. Rosenplanter, C. Stadelmann, P. Yu, E. Arpaia, T. W. Mak, T. Kamradt, and M. Lohoff. 2007. The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. *Nat. Immunol.* 8: 958-966.
299. Huber, M., A. Brustle, K. Reinhard, A. Guralnik, G. Walter, A. Mahiny, L. E. von, and M. Lohoff. 2008. IRF4 is essential for IL-21-mediated induction, amplification, and stabilization of the Th17 phenotype. *Proc. Natl. Acad. Sci. U. S. A* 105: 20846-20851.
300. Zangani, M. M., M. Froyland, G. Y. Qiu, L. A. Meza-Zepeda, J. L. Kutok, K. M. Thompson, L. A. Munthe, and B. Bogen. 2007. Lymphomas can develop from B cells chronically helped by idiotype-specific T cells. *J. Exp. Med.* 204: 1181-1191.
301. Kim, C. H., H. W. Lim, J. R. Kim, L. Rott, P. Hillsamer, and E. C. Butcher. 2004. Unique gene expression program of human germinal center T helper cells. *Blood* 104: 1952-1960.

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Conferences/Posters:

2005

18th Annual Immunology Graduate Group Retreat Nov 18-20	Lancaster , USA

2006

Annual SGAI Meeting , March 30-31	Zürich, CH
Europ. School of Oncology: "Leukemia and Lymphoma", Apr 29-30	Ascona, CH
Gordon Research Conferenc: "Chemotactic Cytokines", Sep 17-22	Aussois, FRA
I INNOCHEM Meeting, Oct 02-04	Bellinzona, CH

2007

Keystone Symposia: "Linking Inflammation and Cancer", Feb 10-15	Santa Fe, USA
International workshop on "Inflammation and Cancer", Nov 06-10	Milan, ITA
Novartis Biotech Leadership Meeting, Aug 28-30	Basel, CH

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22nd EMDS/DC-THERA Meeting, Sep 18-19	Brescia, ITA
6th European Mucosal Immunology Group Meeting, Oct 08-10	Milan, ITA

2009

Keystone Symposia: "Immunity to Intestinal Microbiota", Jan 13-18	Taos, USA
Patenting in Life sciences, Jun 04-05	Les Diablerets, CH
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Publications:

Perivascular Expression of CXCL9 and CXCL12 in Primary Central Nervous System Lymphoma: T Cell Infiltration and Positioning of Malignant B Cells
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C-terminal processing of CXCL13 by cathepsin B results in increased chemoattractant potency
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(submitted for publication)

Languages:

German:	fluent (mother tongue)
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Interests:

Outdoor sports (hiking, mountain-biking snowboarding, skiing, windsurfing, kitesurfing)
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“Try not to become a man of success but rather to become a man of value” (*Albert Einstein*)

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