Doctoral Thesis

Role of chemokines in antiviral immunity and immunopathology

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Role of chemokines in antiviral immunity and immunopathology

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1. SUMMARY

Chemokines are small (8-14 kDa) proteins with high structural homology. The main task of chemokines is to help to establish local microenvironments of the immune system by the attraction of defined subsets of cells. Functionally, chemokines fall into two groups: a large group of inflammatory chemokines which help to build up the inflammatory environment after an insult in the periphery, and a smaller group of constitutive chemokines which are mainly produced within secondary lymphoid organs and help to form the specialized microenvironments within these organs. Chemokines thus play a central part in compartmentalizing and orchestrating the immune response.

The constitutive chemokines produced within secondary lymphoid organs are CCL19 (ELC; Epstein-Barr virus-induced molecule 1 Ligand Chemokine), CCL21 (SLC; Secondary Lymphoid organ Chemokine) and CXCL13 (BLC; B Lymphocyte Chemoattractant). CCL19 and CCL21 ligate the chemokine receptor CCR7 while CXCL13 ligates the chemokine receptor CXCR5.

These key players organize the lymphoid microstructure, and studies in mice with genetically engineered or natural mutations have evidenced their morphogenetic role. CCR7^{-/} mice and a natural mouse mutant strain lacking lymphoid CCL19 and CCL21 (plt/plt) show a similar phenotype in that splenic T cell zones are absent and the number of T cells within lymph nodes is severely diminished. CXCR5^{-/} mice show an aberrant organization of the splenic B cell zone with ectopic germinal centers forming around central arterioles of the spleen.

Although the morphogenetic role of CXCR5, CCR7 and their respective ligands was well characterized in vivo, their functional role in vivo, particularly during virus infections, had not been sufficiently investigated. Mice lacking lymph nodes (aly/aly) or certain compartments within lymphoid organs, e.g. the marginal zone, are highly susceptible to virus infections and fail to clear the virus. To learn more about the cellular interactions within secondary lymphoid organs during virus infections and to define the importance of lymphoid microstructures, we infected plt/plt, CCR7^{-/} and CXCR5^{-/} mice with the noncytopathogenic Lymphocytic Choriomeningitis Virus (LCMV) and the cytopathogenic Vesicular Stomatitis Virus (VSV).

Surprisingly, plt/plt mice did differ in their ability to protect against both viruses, indicating that B and T cell responses can be mounted normally in the absence of a
functional T cell zone. However, CCR7−/− mice, an apparently complementary mouse model, displayed slower kinetics in their antiviral immune response. We hypothesize that in the absence of CCR7 there is this a more pronounced defect in the migration of dendritic cells (DC) from the periphery to secondary lymphoid organs. In addition, CCR7−/− mice allowed us to study the induction and maintenance of antiviral memory. Memory CTL from all organs show the same efficacy of target lysis and interferon production, independently of their CCR7 expression. This challenges the concept of “central” and “peripheral” memory and defines CCR7 as a mere marker for CTL localization rather than a correlate of CTL function.

We found that the impaired immunoglobulin class switch in CXCR5−/− mice is especially pronounced when antigen is limiting, i.e. in the memory phase, after clearance of the virus or after immunization with protein. These findings provide evidence for the fact in cases of limited T and B cell expansion, the induction of functional immune responses relies especially on their correct positioning along chemokine gradients.

Taken together, we conclude that constitutive chemokines represent an innate immune mechanism increasing the probability of contacts between APC and lymphocytes in secondary lymphoid organs.
ZUSAMMENFASSUNG

Chemokine sind 8-14 kDa grosse Proteine von hoher struktureller Homologie. Ihre Hauptaufgabe besteht darin, Mikroumgebungen des Immunsystems durch Chemoattraktion bestimmter Zelltypen zu schaffen. Sie lassen sich in zwei funktionelle Gruppen unterteilen: zum einen in eine grosse Gruppe inflammatorischer Chemokine, die das entzündliche Milieu nach einer Verletzung in der Peripherie schaffen, und zum anderen in eine kleine Gruppe konstitutiver Chemokine, die vor allem von sekundären lymphatischen Organen gebildet werden und dort für die Organisation spezialisierter Mikroumgebungen mitverantwortlich sind. Chemokine spielen also eine zentrale Rolle bei der Orchestrierung und Kompartmentalisierung von Immunreaktionen.

Die konstitutiven Chemokine, die von sekundären lymphatischen Organen produziert werden heissen CCL19 (ELC; Epstein-Barr virus-induziertes Molekül 1 Ligand-Chemokin) und CCL21 (SLC; Sekundäres-Lymphoides Organ Chemokin), beides Liganden für den Chemokinrezeptor CCR7. Ein weiteres konstitutives Chemokin, CXCL13, (BLC; B Lymphozyten Chemoattraktant) bindet den Chemokinrezeptor CXCR5.

Diese Schlüsselmoleküle organisieren die lymphoiden Mikroumgebungen und Untersuchungen von genetisch veränderten Mäusen und natürlichen Mausmutanten haben ihre morphogenetische Rolle offengelegt. CCR7+/− Mäuse und eine natürliche Mausmutante, der CCL19 und CCL21 fehlen (plt/plt), zeigen einen ähnlichen Phänotyp: die T Zell-Zonen der Milz fehlen und die Anzahl der T Zellen in den Lymphknoten ist deutlich verringert. CXCR5−/− Mäuse hingegen haben eine aberrante Organisation der B Zell-Zone, denn die Keimzentren sind ektopisch um die Zentralarteriole angeordnet.

Obwohl die morphogenetische Funktion von CXCR5, CCR7 und den jeweiligen Liganden bereits gut charakterisiert war, war doch ihre funktionelle Rolle in vivo, besonders nach Virusinfektionen, schlecht definiert. Mäuse ohne Lymphknoten (aly/aly) oder Mäuse ohne gewisse Kompartimente innerhalb von lymphatischen Organen (z.B. der Marginalzone), sind sehr anfällig für Virusinfektionen und können das Virus nicht aus dem Körper eliminieren. Um mehr über die zellulären Interaktionen während einer Virusinfektion zu erfahren und um die zentrale Bedeutung von lymphoiden Substrukturen zu untersuchen, haben wir plt/plt Mäuse,
CCR7⁻ Maus und CXCR5⁻ Maus nach Infektion mit dem nicht cytopathogenen Lymphozytischen Choriomeningitis Virus (LCMV) und dem cytopathogenen Vesikulären Stomatitis Virus (VSV) untersucht.

Ueberraschenderweise zeigten plt/plt Mäuse keinen Unterschied in der antiviralen Protektion gegenüber beiden Viren, was belegt, dass B und T Zell-vermittelte Immunantworten auch ohne eine funktionelle T Zell-Zone normal induziert werden können. Ein auf den ersten Blick komplementäres Mausmodell, die CCR7⁻ Maus, zeigte jedoch eine verlangsamae antivirale Protektion. Wir nehmen an, dass dies durch einen ausgeprägteren Defekt in der Rekrutierung peripherer dendritischer Zellen in die sekundären lymphatischen Organe verursacht wird. Ausserdem konnten wir mit Hilfe von CCR7⁻ Mäusen die Induktion und die Aufrechterhaltung des antiviralen immunologischen Gedächtnisses untersuchen. Memory-Zellen von allen untersuchten Organen zeigten die gleiche Fähigkeit zur direkten Zellyse und zur Interferonproduktion, unabhängig von ihrer CCR7-Expression. Dieser Befund stellt das Konzept von "zentralem" und "peripherem Memory" in Frage und definiert CCR7 eher als einen Marker für die Lokalisation zytotoxischer T Zellen, der aber für ihre Funktion selbst bedeutungslos ist.

Im Falle der CXCR5⁻ Maus fanden wir, dass der Immunoglobulin-Klassenwechsel in CXCR5⁻ Mäusen vor allem dann beeinträchtigt ist, wenn Antigen limitierend ist, d.h. in der Memory-Phase oder nach Immunisierung mit Protein. Diese Daten belegen die Annahme, dass insbesondere in Fällen einer eingeschränkten T und B Zell-Expansion die produktive Induktion von Immunantworten von der korrekten Lage dieser Zellen zueinander abhängt.

Zusammenfassend sehen wir uns zur Annahme berechtigt, dass konstitutive Chemokine eine grundlegende Bedeutung in der angeborenen Immunantwort spielen, da sie die Wahrscheinlichkeit spezifischer Zell-Zell-Kontakte in den sekundären lymphatischen Organen entscheidend steigern.
3. INTRODUCTION

3.1 Secondary lymphoid organs

Specific cognate interactions between antigen presenting cells (APC) and lymphocytes are a prerequisite for the generation of efficient immune responses. As these interactions are based on cell-to-cell contact, specific microenvironments have evolved to foster these contacts. These sites are secondary lymphoid organs, which comprise the spleen, the lymph nodes, Peyer’s patches and the more disperse mucosa-associated lymphoid tissue (MALT). However, the evolution of specialized organs for the induction of immune responses creates another challenge for the organism in that an efficient connection of these organs to the periphery and efficient communication systems of the periphery with these organs have to exist. Pathogens, which invade the body from distal sites, have to be carried rapidly into secondary lymphoid organs, naïve lymphocytes of rare specificities have to recirculate rapidly to find their cognate antigen on APC, and primed specific lymphocytes need to find the peripheral site of pathogenic insult immediately in order to contain the infection.

It is therefore clear that elaborate mechanisms of cell migration must have evolved to guide the cells of the immune system throughout the body, thereby fostering the immune response. All the processes of lymphocyte migration and redistribution within and between secondary lymphoid organs are regulated by adhesive and chemotactic cues: the concert of membranous adhesion molecules and soluble chemoattractant cytokines, the chemokines.
3.2. Microstructure of secondary lymphoid organs

The basic unit of secondary lymphoid organs is the lymphoid follicle, which has a defined microstructure containing T cell, B cell and marginal zone (MZ) (Gretz et al., 1996). The distinction between T and B cell zones was discovered through the observation that some lymphoid compartments were depleted after neonatal thymectomy (Parrott et al., 1966), and that cells isolated from the thymus accumulate at lymphoid sites different than cells isolated from the bone marrow (Parrott and De Sousa, 1971). The marginal zone was first described as a zone of phagocytic activity, which later was attributed to macrophages (Humphrey and Sundaram, 1985).

T cell zones contain CD4+ and CD8+ T cells and interdigitating DC, and B cell zones contain CD4+ T cells, B cells, follicular dendritic cells (FDC) and tingible body macrophages (Cyster, 2000; Cyster et al., 2000). The marginal zone contains dendritic cells, macrophages and some marginal zone B cells (Fig. 1 and Martin and Kearney, 2002).

In the spleen, lymphoid follicles form around penicillary arterioles. Their entity is referred to as white pulp. All other secondary lymphoid organs are conglomerates of lymphoid follicles. They are dynamic structures, connected to each other by the bloodstream and the lymph.

3.2.1. The marginal zone

The marginal zone is rich in metallophilic macrophages (MM, detected by the antibody MOMA1) and marginal zone macrophages (MZM, detected by the antibody ERTR9) (Dijkstra et al., 1985; Kraal and Janse, 1986). Both, MM and MZM function during the immune response to capture blood-borne antigens (Martin and Kearney, 2000) via the complement receptors CR3 and CR4. In addition, the marginal zone contains long-lived IgMhighCD21highCD23lowIgDlow marginal zone B cells (Oliver et al., 1997). The MZ is not fully formed until 2-3 weeks after birth in mice (MacLennan et al., 1985), which most likely contributes to the immaturity of the immune system in newborn mice.
Figure 1: Fluorescent micrograph of lymphoid compartments of the spleen (A) and of mesenteric lymph nodes (B) in naïve mice.
Metallophilic macrophages are stained with MOMA-FITC (green), B cells are stained with B220-PE (red), CD4+ T cells are stained with CD4-biotin/streptavidin-Cy5 (purple) and nuclei are counterstained with DAPI (blue). Magnification 65x.

Most humoral and cellular immune responses can be induced in the marginal zone. This has been evidenced by reduced T and B cell-mediated immune responses when
the marginal zone was selectively damaged by depletion of macrophages or in mice lacking macrophages of the marginal zone (Oehen et al., 2002; Seiler et al., 1997). B cells of the marginal zone have been characterized as IgM producers in the T cell-independent (TI) early phase of the immune response, as they have a low activation threshold (Bachmann et al., 1997; Guinamard et al., 2000). This low threshold might be due to the trimerization of the B cell co-receptor (CD19, CD21, CD81) after binding of complement-coated antigen to CD21 (Fearon and Carter, 1995). An alternative way of pre-activating marginal zone B cells may be through induction of the B lymphocyte stimulator BLyS (BAFF) on interferon-activated macrophages, monocytes or dendritic cells (Mackay et al., 1999; Moore et al., 1999) which links its receptors BAFF-R, TACI and BCMA on B cells. This non-specific B cell activation might even be so strong that immunoglobulin class switch occurs without cognate T help (Maloy et al., 1998). It has also been observed that some activated APC of the marginal zone can migrate to B cell follicles (Yu et al., 2002), possibly enhancing (Berney et al., 1999) or even directly activating B cells (Batista et al., 2001).

Efficient IgM production in the marginal zone also critically depends on pre-existing natural antibodies, which aggregate antigen (Ochsenbein et al., 1999a) and target them to the MZM via complement receptors (Ochsenbein and Zinkernagel, 2000). The marginal zone is thus a site where the innate and adaptive immune systems are linked (Fagarasan and Honjo, 2000).

### 3.2.2. The B cell zone

The B cell zone (or the B cell follicle) is the site of B cell differentiation during the later phase of an immune response (Berek et al., 1991). In the course of an immune response, B cell follicles change their morphological appearance from resting, loosely populated primary follicles to active, densely populated secondary follicles. The latter consists of two distinct zones: an inner region (the germinal center, GC) and a sparsely populated rim (the mantle zone).

Some of the B cell blasts, which were activated in the marginal zone, do not differentiate to extrafollicular IgM producing plasma cells but instead receive signals for immunoglobulin class switch via CD40L (MacLennan, 1994) and cytokines (Kupfer et al., 1994), from CD4⁺ T cells. To this end, B cells must present antigenic
peptide-MHCII complexes to primed T helper cells (Lanzavecchia, 1990) in the presence of co-stimulation, such as CD80/86-CD28 interactions (Linsley and Ledbetter, 1993) or ICOS-B7h interactions (Hutloff et al., 1999; Yoshinaga et al., 1999).

T-B interaction is usually a prerequisite for the formation of GC (McHeyzer-Williams et al., 2001), although they can form without T helper cells if the frequency of specific B cell precursors is very high (de Vinuesa et al., 2000). GC are specialized environments for the genetic modification of immunoglobulins based on antigen affinity (affinity maturation). In the dark zone of the GC, the entering B cells (centrocytes) proliferate (clonal expansion) and give rise to centroblasts. During expansion, B cells randomly mutate their Ig receptor (somatic hypermutation) at defined hot spots of the Ig sequence (Papavasiliou and Schatz, 2002), by an activation-induced B cell-specific cytidine deaminase (AID) followed by error-prone DNA repair mechanisms (Di Noia and Neuberger, 2002; Muramatsu et al., 1999; Petersen-Mahrt et al., 2002). High-affinity B cells are then selected out of the pool of centroblasts by antigen bound on FDC. FDC can fix antigen in the form of FcγRIIB-bound immune complexes (Qin et al., 2000) or via the complement receptors CD21 and CD35 (Tew et al., 2001). Low-affinity centrocytes are pushed to the GC light zone where they die by neglect and are rapidly removed by tingible body macrophages. The potency of affinity maturation is underscored by the fact that HC1 mice, in which all complementary determining regions (CDR) of the antibody except CDR3 are genetically fixed, show a normal Ig repertoire (Xu and Davis, 2000).

Memory B cells also arise also from the germinal center reaction. They continue to recirculate between GC whereas antibody-forming cells eventually locate to the bone marrow (Bachmann et al., 1994).

3.2.3. The T cell zone

When antigen enters at a peripheral site of the body, it can reach secondary lymphoid organs in an isolated form or associated with DC. Resting DC are found in all peripheral organs and have the capacity to engulf and process antigens. Upon an inflammatory stimulus, such as IL-1 or TNFα, or by triggering of Toll-like receptors by pathogenic constituents, such as LPS, GpC or ssRNA, DC undergo maturation (Cella
et al., 1997). This includes upregulation of MHC I and II, upregulation adhesion (ICAM-1) and co-stimulatory molecules (CD80, CD86) (Banchereau and Steinman, 1998), and migration towards the T cell zone of secondary lymphoid organs via the afferent lymph (Butcher et al., 1999). In the T cell zone, DC form immunological synapses with naïve CD4 and CD8 positive T cells (Grakoui et al., 1999) and initiate T cell dependent immune responses. Upon priming, T cells proliferate and differentiate to various types of effector cells depending on the TCR affinity or the local milieu of the lymph node. High concentrations of IL-12 during priming can lead to a Th1- or Tc-1 like bias, whereas high concentrations of IL-4 induce a Th2- or Tc2 bias (Cerwenka et al., 1998; Mosmann and Sad, 1996; Sad et al., 1995). Th1 cells secrete IL-2 and IFN-γ while Th2 cells produce IL-4, IL-5, IL-6 and IL-13. The amount of antigen and the intensity of co-stimulation available during the priming event have also been described to further fine-tune the immune response, shifting the balance from T cell unresponsiveness to effector cell responses or even activation-induced cell death.

Newly primed T cells gain the effector mechanisms for target cell lysis or B cell help and change their array of surface molecules (adhesion molecules and chemokine receptors) allowing them to leave secondary lymphoid organs and relocate to peripheral tissues (Cerwenka et al., 1999a; Oehen and Brduscha-Riem, 1998). Upon encounter with their cognate antigen, T cells exert their effector function. Some T cells, however, differentiate to T memory cells. It is still a matter of debate whether memory T cells represent a separate lineage or derive directly from survivors of the primary response (Manjunath et al., 2001). Furthermore, the issue of antigen persistence as prerequisite for the persistence of memory cells has not yet been resolved (Zinkernagel, 2000) and is still a matter of debate. However, it has become clear that survival of memory T cell pools also depends on the availability of lymphoid niches, because activation and expansion of T cells leads to the “attrition” of “old” memory T cell populations (Selin et al., 1999).
3.3. Lymphocyte recirculation and homing

To efficiently survey the body for pathogens, the cells of the immune system need to recirculate through the body. Naïve cells home to secondary lymphoid organs using blood and lymph as conduits; they make one or two complete circuits per day (Smith and Ford, 1983). Effector and memory lymphocytes, on the other hand, efficiently home to peripheral sites of inflammation. During an inflammatory reaction, it has been observed that effector/memory cells extravasate antigen-independently (Kalish and Johnson, 1990). Indeed, the mediators of inflammatory responses and the adhesion molecules of inflamed endothelia act on lymphocytes of different specificities. After the acute phase of the immune reaction, effector cells maintain their preference to localize to the tissue type where the antigen has been encountered initially via the expression of specific adhesion molecules and chemokine receptors (Campbell and Butcher, 2002). This is thought to reflect a mechanism to save energy, as this is likely to be the site where the same antigen will enter at a later timepoint.

Lymphocyte homing always involves an extravasation event through the endothelium of postcapillary venules. This minimizes the effect of leukocyte traffic on metabolite exchange at capillaries and on tissue perfusion, which is regulated by the arteriolar diameter. Homing of naïve lymphocytes to lymph nodes occurs across specialized endothelial structures, termed high endothelial venules (HEV).

3.3.1. High Endothelial Venules

High endothelial venules were discovered in the first half of the 20th century (Dabelow, 1939), but only 60 years later it was appreciated that radiolabeled cells were able to leave the blood at HEV (Gowans, 1959).

HEV are found within the T cell zones of lymph nodes, Peyer's patches and all lymphoid foci of the MALT (Woodruff et al., 1987) but they are absent from B cell zones (Claesson et al., 1971). They are typically composed of three layers: a thick, cuboidal endothelial wall, a thick basement membrane, and a prominent perivascular sheath which anchors the vessel to the surrounding reticular connective tissue (Fig.2
and Kraal and Mebius, 1997). The narrow lumen of HEV leads to an increased turbulence within the bloodstream. This enhances the probability of leukocyte contacts with the endothelial wall, which are necessary for their transmigration.

Lymphocyte homing through HEV was first assessed by Stamper and Woodruff in the late 1970s (Stamper and Woodruff, 1977) by overlaying tissue sections of secondary lymphoid organs with lymphocyte suspensions to assess the adhesion potential of lymphocyte subsets to particular lymphoid structures. B cells preferentially adhered to HEV of Peyer’s patches and T cells to HEV of lymph nodes. Later, it became established that the particular pattern of cell adhesion in vitro correlated exactly to the pattern of cell migration in vivo after adoptive transfer of radiolabeled lymphocytes (Kraal et al., 1983; Stevens et al., 1982). This implicated the presence of specialized adhesion and/or chemoattraction processes taking place at these sites.

A multistep process via adhesion molecules and chemokines was found to be responsible for the specificity pattern of cell transmigration across endothelia (Springer, 1994). Multiple recognition events have several advantages as they finely tune homing specificity, overcoming possible mutations in single-step recognition pathways, and they serve to oppose the enormous shear stress of the blood (appr. 50 dyn/cm² Firrell and Lipowsky, 1989).

Figure 2: Microanatomical structure of High Endothelial Venules (HEV) showing the cuboidal endothelium and various stages of lymphocyte transmigration. The endothelium is lined by reticulocytes of the connective tissue which anchor the HEV to the lymph node stroma (Adapted from Kraal1997).
Extravasation or homing of cells across an endothelium involves four phases (Fig. 3):

1. Initially, cells *tether* loosely and reversibly to the endothelium by the ligation of selectins.

2. Recruitment of more adhesion molecules makes this interaction stronger and the lymphocytes start *rolling* along the vessel wall.

3. The specific action of chemokines from the endothelium then leads to intracellular *activation* of the rolling cell. This results in the expression of additional adhesion molecules, mainly integrins on the rolling cell itself. Integrin ligation leads to lymphocyte *arrest*.

4. The attached cell then *diapedese* across the endothelial wall which opens up around it. This action is probably mediated by CD31.

Every step of this process is a prerequisite for the subsequent step. Successful extravasation and homing of leukocytes thus requires several checkpoints, which ensures a gradually narrowed specificity ("area-code-principle"). By this combinatorial process, a limited number of adhesion molecules can account for a large array of homing specificities. Furthermore, novel homing specificities can simply arise during evolution by establishing new combinations (Butcher and Picker, 1996).

### 3.4. Adhesion Molecules

During the late 1980s many lymphocyte homing receptors and their endothelial counterparts, the vascular addressins, have been characterized (Tab.1).

#### 3.4.1. Selectins and mucins

With the help of a modified Stamper-Woodruff assay, the monoclonal antibody MEL-14 was generated which specifically interfered with the binding of the 38C-13 lymphoma to HEV (Gallatin et al., 1983). This finding was then generalized for other cell populations and binding of MEL-14 to the cell surface generally abolished HEV-
binding of T lymphocytes (Gallatin et al., 1986; Reichert et al., 1986). MEL-14 was subsequently shown to recognize CD62L (L-selectin), a member of the selectin family (Bevilacqua et al., 1991; Lasky, 1992). Selectins (Fig. 4) are composed of a distal carbohydrate binding site, an epidermal growth factor domain, and two (L-selectin), six (CD62E/E-selectin) or eight to nine (CD62P/P-selectin) complement regulatory domains (CRD). The selectins are genetically closely linked and the two inner domains display a 60-70% amino acid homology.

![Diagram of lymphocyte transmigration across HEV](image)

**Figure 3: Recognition events during lymphocyte transmigration across HEV.**

Constitutive chemokines are involved in lymphocyte activation leading to integrin-mediated arrest (see below).
All selectins are involved in the lymphocyte transmigration processes; CD62L is expressed on lymphocytes while CD62E and CD62P are expressed on activated endothelium. Therefore, only CD62L plays a role in the homeostatic recirculation of lymphocytes, although it may also support extravasation under inflammatory conditions (Lewinsohn et al., 1987).

Selectins recognize their ligands via hydrophobic residues in the CRD in a Ca\textsuperscript{2+}-dependent manner (McEver, 1994). Lymphocytes alter their recirculation behavior after activation and this is paralleled by downregulation of CD62L on the mRNA level (Picker et al., 1993) leading to exclusion of the lymphocyte from secondary lymphoid organs.

All selectins appear to recognize a sialylated carbohydrate determinant on their counterparts (Lasky, 1992; Rosen, 1993; Varki, 1994; Vestweber and Blanks, 1999). E-selectin and P-selectin recognize distinct, but closely related carbohydrate structures related to the tetrasaccharide sialyl Lewis\textsuperscript{x} (Fig. 4). The L-selectin ligand is related to sialyl-Lewis\textsuperscript{x}, but is additionally sulfated and glycosylated. In every instance these carbohydrate moieties are O-linked to threonine- and serine-rich mucin-like protein backbones.

As early as 1964, carbohydrates were implicated in efficient lymphocyte homing, as glucosidases (Gesner, 1964) inhibited binding of lymphocytes to HEV. Addition of competing monosaccharides (Stoolman and Rosen, 1983) established the identity of the interacting saccharide moieties. Mice deficient in fucosyltransferase VII who cannot synthesize the correct sialyl Lewis\textsuperscript{x}, suffer from a selective lymphocyte homing defect to lymph nodes but not to Peyer’s patches (PP, Maly et al., 1996).

Using a L-selectin-lg fusion protein in an immunoprecipitation approach (Watson et al., 1990), two mucin-like binding partners were identified, one of 50 kDa (glycosylation-dependent cell adhesion molecule-1/ GlyCAM-1 (Lasky, 1992) that could be secreted, and one of 90 kDa (CD34, Baumhueter et al., 1994). Both of these ligands could also be precipitated with MECA79, an antibody which blocks the \textit{in vivo} homing of lymphocytes to HEV of peripheral lymph nodes but not to PP (Berg et al., 1991; Imai et al., 1991; Streeter et al., 1988). These molecules were called peripheral node vascular addressins (PNAd). GlyCAM-1 is selectively expressed by HEV of peripheral lymph nodes, but CD34 is expressed in a more widespread manner. It was thus assumed that post-translational modification leads to the specific L-selectin-CD34-interaction on HEV (Baumhueter et al., 1994). Subsequently,
immunoprecipitation with an antibody blocking homing of lymphocytes to HEV of PP but not of peripheral lymph nodes led to identification of the dimeric PSGL-1 (P-selectin glycoprotein ligand-1, Moore et al., 1992) (Sako et al., 1993).

Selectins are involved in the first steps of lymphocyte transmigration: tethering and rolling. This correlates with their exposed site of expression on lymphocyte microvilli (von Andrian et al., 1995). The interaction of the selectins with their ligands are transient due to proteolytic shedding of these molecules (Miethke et al., 1993).

![Structure of adhesion molecules.](image)

**Figure 4:** Structure of adhesion molecules.

### 3.4.2. Integrins and integrin ligands (ICAM, VCAM, MadCAM)

LFA-1 (Lymphocyte Function-related Antigen 1) is the prototype of the integrins (Stewart et al., 1995). LFA-1 was initially discovered because it was the target of a monoclonal antibody which blocks the CTL-target cell interaction in a killing assay (Davignon et al., 1981). It is expressed, however, by all leukocytes (Kurzinger et al., 1981). LFA-1 is a typical integrin in that it is a noncovalently linked heterodimer of an \( \alpha \)- and a \( \beta \)-subunit of approximately 1,100 and 750 amino acids, respectively (Kurzinger et al., 1982). Eight different \( \beta \)-subunits and 16 different \( \alpha \)-subunits have been described. Further structural characteristics of the integrins revealed a
conserved RGD-motif (Arg-Gly-Asp) of the β-chain and it is at this precise site, where
the β-chain is thought to interact with the α-chain. Integrin α-chains have three or
four tandem repeats of a putative divalent EF-hand cation-binding motif and require
Ca\(^{2+}\) or Mg\(^{2+}\) for function. It has been proposed that the divalent cation binds to the
Asp residue in the RGD motif (Fig. 4 and Corbi et al., 1987).

For the interaction of leukocytes with the endothelium, three groups of integrins are
important: the β1, β2 and β7 integrin subfamilies. LFA-1 belongs to the β2 (CD18)
subfamily which is expressed on leukocytes, and is also called αLβ2 integrin
(CD11aCD18).

The receptor for LFA-1 on the target cell is ICAM-1 or ICAM-2 (intercellular cell
adhesion molecule-1/-2), both of which are five-domain members of the Ig
superfamily (Marlin and Springer, 1987). ICAM-1 was found by immunizing mice with
LFA-1\(^{+/+}\) cells and selecting monoclonal antibodies that would inhibit homotypic
adhesion of LFA-1\(^{+/+}\) cells to each other (Rothlein et al., 1986). ICAM-1 can be
expressed by a wide variety of cells and is upregulated during inflammatory
processes. ICAM-2 was identified due to the observation that anti-LFA-1 but not anti-
ICAM-1 could inhibit lymphocyte binding in some adhesion assays (Staunton et al.,
1989). ICAM-2 is expressed by endothelial cells and not regulated upon
inflammation.

Other members of the β2 (CD18) family include Mac-1 (CD11bCD18), which is
mainly expressed on macrophages and CD11c/CD18, which is mainly expressed by
dendritic cells.

In the β1 family of integrins, the α4β1 integrin (very late activation antigen, VLA-4,
CD49dCD29 heterodimer) is an important molecule for interaction of effector and
memory lymphocytes with inflamed endothelia by ligating its receptor VCAM-1
(vascular cell adhesion molecule, Elices et al., 1990) (Elices et al., 1990; Osborn et
al., 1989), which is then upregulated. Additionally, VCAM-1 is constitutively
expressed on HEV. Involvement of VLA-4 in T cell-mediated killing (Takada et al.,
1989) and homotypic adhesion (Bednarczyk and McIntyre, 1990) suggests some
functional redundancy with LFA-1.
## Table 1: Adhesion molecules involved in leukocyte traffic.

<table>
<thead>
<tr>
<th>ADHESION MOLECULE</th>
<th>EXPRESSION</th>
<th>REGULATION OF EXPRESSION</th>
<th>LIGANDS</th>
<th>FUNCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Selectins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Selectin (CD62L)</td>
<td>All leukocytes, except effector/memory stages</td>
<td>Shed on activation</td>
<td>PNAd, MAdCAM-1</td>
<td>Homing to LN and PP</td>
</tr>
<tr>
<td>E-Selectin (CD62E)</td>
<td>Endothelial cells</td>
<td>Induced by pro-inflammatory cytokines</td>
<td>CLA on skin-homing lymphocytes</td>
<td>Homing of effector/memory lymphocytes to sites of inflammation (mainly skin)</td>
</tr>
<tr>
<td>P-Selectin (CD62P)</td>
<td>Endothelial cells, platelets</td>
<td>Stored in granules, rapid activation by histamine/thrombin</td>
<td>PSGL-1</td>
<td>Homing of activated platelets to sites of inflammation</td>
</tr>
<tr>
<td><strong>Selectin ligands</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sialyl Lewis X (sCD15)</td>
<td>HEV, myeloid cells</td>
<td>Expression in leukocytes needs fucosyltransferase VII</td>
<td>All selectins</td>
<td>Function depends on presenting mucin stalk</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>All leukocytes</td>
<td></td>
<td>P- (L-, E-) selectin</td>
<td>Binding to activated platelets</td>
</tr>
<tr>
<td>Peripheral Node Adressin (PNAd)</td>
<td>HEV (LN, chronic inflammation)</td>
<td>Sialyl Lewis X-like sugar, presented by GlyCAM and CD34</td>
<td>L-selectin, (P-selectin on platelets)</td>
<td>Homing of naive lymphocytes to LN, induced in de novo formed lymphoid tissue.</td>
</tr>
<tr>
<td>CLA</td>
<td>Skin-homing lymphocytes</td>
<td>Induced by pro-inflammatory cytokines</td>
<td>E-selectin</td>
<td>Homing of effector/memory lymphocytes to sites of inflammation</td>
</tr>
<tr>
<td><strong>ß2 Integrins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>αLβ2 (LFA-1, CD11aCD18)</td>
<td>All leukocytes</td>
<td>Enhanced expression in effector/memory cells</td>
<td>ICAM</td>
<td>Homing of lymphocytes across HEV of LN and PP</td>
</tr>
<tr>
<td>αMβ2 (Mac-1, CD11bCD18)</td>
<td>Myeloid cells</td>
<td>Up-regulation upon cell activation</td>
<td>ICAM, C3b</td>
<td>?</td>
</tr>
<tr>
<td>αXβ2 (CD11cCD18)</td>
<td>DC</td>
<td>Constitutive expression</td>
<td>C3b</td>
<td>?</td>
</tr>
<tr>
<td><strong>α4-Integrins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α4β1 (VLA-4)</td>
<td>Leukocytes, except neutrophils</td>
<td>Enhanced expression in effector/memory cells</td>
<td>VCAM-1</td>
<td>Homing of effector/memory lymphocytes to sites of inflammation (mainly skin)</td>
</tr>
<tr>
<td>α4β7</td>
<td>Most leukocytes</td>
<td>Enhanced expression on gut-homing cells</td>
<td>MAdCAM-1</td>
<td>Homing to gut-associated lymphoid tissue</td>
</tr>
<tr>
<td><strong>Immunoglobulin superfamily</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICAM-1 (CD54)</td>
<td>ubiquitous</td>
<td>Up-regulation by pro-inflammatory cytokines</td>
<td>αLβ2-integrin</td>
<td>Critical ligand for [L2 integrins]</td>
</tr>
<tr>
<td>VCAM-1 (CD106)</td>
<td>Endothelium, BM stroma</td>
<td>Induced by pro-inflammatory cytokines</td>
<td>α4-Integrins</td>
<td>Homing of effector/memory lymphocytes to sites of inflammation (skin)</td>
</tr>
<tr>
<td>MAdCAM-1</td>
<td>HEV in gut-associated lymphoid tissue</td>
<td>Constitutive expression in HEV</td>
<td>α4β7 integrin, L-selectin</td>
<td>Homing to gut-associated lymphoid tissue</td>
</tr>
<tr>
<td>CD44</td>
<td>Activated leukocytes</td>
<td></td>
<td></td>
<td>Tethering of effector/memory lymphocytes to sites of inflammation</td>
</tr>
</tbody>
</table>

Table 1: Adhesion molecules involved in leukocyte traffic.
The β7 family of integrins contains one important member, α4β7, which confers specificity to the mucosal homing process (Bargatze et al., 1995; Hamann et al., 1994). It is expressed by lymphocytes homing to PP and appendix and ligates MAdCAM-1 on HEV of PP.

Immunoprecipitation with the anti-selectin MECA367 antibody, which blocks homing of lymphocytes to HEV of PP but not to HEV of peripheral lymph nodes (Streeter et al., 1988), yielded a fraction that bound to lymphocytes (Nakache et al., 1989). It was characterized as MAdCAM-1 (mucosal adressin cell adhesion molecule-1), a member of the immunoglobulin superfamily (Briskin et al., 1993). It also contains a mucin-like serine/threonine-rich region with many sites for the addition of O-linked sugars. MAdCAM-1 is thus a receptor for both L-selectin and integrins. Most integrins mediate firm lymphocyte arrest, although the ligation of L-selectin to MAdCAM-1 is involved in tethering and rolling of gut-homing lymphocytes.

### 3.4.3. CD44 and hyaluronate

It has been observed that antibodies against CD44 block binding of lymphocytes to HEV of PP but not to HEV of peripheral lymph nodes (Jalkanen et al., 1987). CD44 is expressed by most cells of hematopoietic origin and binds hyaluronate of endothelial cells (Aruffo et al., 1990). The phenotype of CD44 may be very heterogeneous due to mRNA splicing and post-translational modification. After activation of the cell, CD44 gets strongly upregulated (Rodrigues et al., 1992) and it was thus correlated with migration of activated lymphocytes to inflamed tissue (DeGrendele et al., 1997).

### 3.4.4. CD31

Homophilic ligation of CD31 (platelet-endothelial cell adhesion molecule-1, PECAM-1) on leukocytes and on endothelium has been implicated in lymphocyte diapedesis across the endothelial sheath and the basal membrane (Liao et al., 1999; Muller and Randolph, 1999). Ligation of CD31 leads to the activation of β1 and β2 integrins on the transmigrating cells (Tanaka et al., 1992), allowing for a more efficient interaction of the cells with extracellular matrix components such as laminin and fibronectin.
3.5. Chemokines

The activation of homing leukocytes is controlled by the action of chemokines (Thelen, 2001) (Mackay, 2001). Chemokines are a group consisting of approximately 50 small (8-14 kDa) structurally related proteins (Zlotnik and Yoshie, 2000) with 20-90 % sequence identity.

3.5.1. Primary Structure and Classification

Chemokines are defined by four invariant cysteine residues that form disulfide bonds. The first cysteine in the sequence forms a covalent bond with the third and the second forms a bond with the fourth. The chemokine family is subclassified on the basis of the local sequence at the N-terminal two cysteines. Chemokines, with one amino acid positioned between the N-terminal cysteines are called CXC or α-chemokines. Chemokines where these cysteines are immediately adjacent, are called CC or β-chemokines. The CX3C or γ-chemokine subfamily consists of only one unusual member, CX3CL1 (fractalkine). CX3CL1 forms the N-terminal domain of the receptor neurotactin and is followed by a mucin-like stalk, a transmembrane helix and a cytoplasmic domain (Bazan et al., 1997; Pan et al., 1997). The C or δ-chemokine lymphotactin is an exception to the four-cysteine rule.

The genes for chemokines are designated SCY (small secreted cytokine) with SCYA corresponding to the CC (β) subfamily, SCYb to the CXC (α) subfamily, SCYc to lymphotactin (C or δ-family) and SCYd to fractalkine (CX3C or γ-family). The nomenclature of chemokines has recently been revised. The systematic names refer again to the chemokine subfamilies (CC, CXC, CX3C or XC), followed by an “L” for “ligand” and the number of the respective SCY gene (Murphy et al., 2000).
3.5.2. Secondary and Tertiary Structure

From their N- to C-terminus, chemokines contain the following structural features (Fig.5 and Fernandez and Lolis, 2002):

1. An extended, floppy sequence N-terminal of the first cysteine.
2. A flexible loop of approximately ten amino acids (N-loop)
3. A single-turn $3_{10}$ helix.
4. Three antiparallel $\beta$-strands forming a $\beta$-pleated sheet
5. A C-terminal $\alpha$-helix oriented at 75° to the plane of the $\beta$-sheet

Each secondary structural unit is connected by loops of three to four amino acids (30s, 40s, 50s – reflecting the numbering of residues in the mature protein). The 30s and the 50s loops possess the latter of the four cysteines. The two disulfide bridges and hydrophobic interactions between the $\alpha$-helix and the $\beta$-sheet stabilize the chemokine tertiary structure and function.

![Figure 5: Chemokine structure.](image)

3.5.3. Quaternary structure

Most chemokines dimerize at high concentrations or in the presence of molecules of the extracellular matrix, such as heparin (Baldwin et al., 1991) (Clore et al., 1990). Interestingly, dimers of CXC chemokines show a different quaternary structure than
dimers of CC chemokines (Clore and Gronenborn, 1995; Covell et al., 1994). The
dissociation constant of chemokines is in the micromolar range, but their
physiological concentrations in serum are in the nanomolar range (Burrows et al.,
1994; Clark-Lewis et al., 1995). However, chemokines can associate with
glycosaminoglycans (GAG), such as heparin and proteoglycans (Hoogewerf et al.,
1997), which might retain them at high local concentrations allowing their
dimerization. The functional unit of a chemokine in vivo, however, remains to be
determined.

Importantly, chemokines act along a gradient. If this gradient is dissipated, such as
by a widespread transgenic over-expression (Nakamura et al., 1995), the effect on
cell migration cannot be seen.

### 3.5.4. Genetic clusters of chemokines

Most chemokines probably arose through gene duplication and diversification
(Baggiolini and Dahinden, 1994). The initial observation that many CC chemokines
act on monocytes and many CXC chemokines act on neutrophils was found to
correlate with a different clustering of the respective genes: The SCYa locus of CC
chemokines is on mouse chromosome 11 (human chromosome 17) and the SCYb
locus of CXC chemokines is on mouse chromosome 5 (human chromosome 4).
Within these clusters, functional redundancy of the respective chemokines can be
observed (Yoshie et al., 2001). Interestingly, the more recently discovered CC and
CXC chemokines acting on lymphocytes (e.g. CCL19, CCL21 and CXCL14) tend to
be encoded at different chromosomal sites and display less receptor promiscuity
(Yoshie et al., 1997). Their respective receptors (CCR7, CXCR4) are also encoded
outside the chemokine receptor clusters, pointing out to a specialized function.

A phylogenetic relationship between mammalian chemokines has been established
(http://cytokine.medic.kumamoto-u.ac.jp) by sequence homologies.

Functionally, chemokines fall into two groups:

- a) inducible or inflammatory chemokines which build up the inflammatory milieu
  at peripheral tissues.
b) constitutive or lymphoid chemokines which mediate the homeostatic recirculation of lymphocytes and DC through secondary lymphoid organs (Cyster, 2000). These cytokines also govern lymphoid organ development during ontogeny (Ansel and Cyster, 2001) (Tab.2)

3.6. Chemokine receptors

The current nomenclature for chemokine receptors is based on the chemokine subfamily designation (CC, CXC, CX3C or XC) followed by an “R” for “receptor” and a number. Chemokine receptors are all seven-transmembrane, G protein-coupled proteins of about 350 amino acids. They share some structural features, the most prominent of which is the DRYLAIV-motif at the second intracellular loop (Murphy, 1994). Chemokine receptors have four intracellular (N-terminal) domains (E1-E4), which collaborate in signal transduction, and four extracellular (C-terminal) domains (C1-C4), which act in concert with the N-terminal extension to bind the ligand.

The extended N-terminus of chemokines is believed to interact with the receptor as mutations in this region lead to receptor antagonism (Crump et al., 1997; Proudfoot et al., 1996; Struyf et al., 1998). In many CXC chemokines, which act on neutrophils, a three-residue motif Glu-Leu-Arg (the ELR motif) immediately prior to the first cysteine is indispensable for activity (Baggiolini et al., 1997). Chemically synthesized N-terminus-like peptides, however, have a 10 to 1000-fold reduced activity if compared with the complete chemokine, highlighting the importance of other regions of the chemokine (Elisseeva et al., 2000; Loetscher et al., 1998; Luo et al., 1999). Kationic residues of the second β-strand are related to GAG-binding (Koopmann et al., 1999; Koopmann and Krangel, 1997).

The intracellular signals of chemokine receptors have been elucidated mainly for CXCR4, CCR5 and CCR2 (Mellado et al., 2001) in cell lines, but the findings might be extended to other homologous chemokine receptors as well. The downstream effect of chemokine receptor signaling is cellular migration through cytoskeleton remodeling (Howard and Oresajo, 1985). These events take place at the focal adhesion areas of the cytoskeleton and lead to cell polarization with chemokine
receptors concentrating at the leading edge of the cell (Nieto et al., 1997; Vicente-Manzanares et al., 1998).

Most chemokine receptors dimerize upon chemokine binding (Rodriguez-Frade et al., 2001; Rodriguez-Frade et al., 1999). As a first downstream event, a Janus kinase (JAK) family member phosphorylates the tyrosine of the DRYLAIV-motif (Mellado et al., 1998). The JAK protein remains associated with the receptor and activates a STAT family member. As a parallel event, chemokine binding activates the heterotrimeric $G_\text{i}$ protein, which then dissociates into the $G_\beta_\gamma$ subunit complex and the GTP-bound $G_\alpha_1$-subunit. Treatment with the $G_\text{i}$-protein inhibitor pertussis toxin leads to impaired cell migration in vitro and in vivo (Cyster and Goodnow, 1995).

There is a certain redundancy of the chemokine-chemokine receptor system in that one chemokine receptor may have several ligands and one chemokine might act on several receptors.

### 3.7. Chemokines in T cell responses

The current paradigm states that naïve T cells and mature antigen-bearing DC meet in the T cell zone of secondary lymphoid organs for priming (Cyster, 2000). The chemokine receptor CCR7 and its chemokine ligands are fundamentally involved in co-localizing DC and naïve T cells in this particular compartment (Forster et al., 1999; Gunn et al., 1999a). CCR7 is expressed on naïve T and B cells (Tangemann et al., 1998; Yoshida et al., 1997) and binds the chemokines CCL19/ELC and CCL21/SLC. CCL19 and CCL21 activate lymphocytes to express $\alpha_L\beta_2$ and $\alpha_4\beta_7$ integrins, which, in turn, mediate their arrest on high endothelial venules (Gunn et al., 1998; Stein et al., 2000). Depending on the lymphoid organ and the lymphocyte subset, CCR7-mediated homing might be further modulated by CXCR4 or CXCR5 (Okada et al., 2002).

CCL19 and CCL21 form a distinct cluster on mouse chromosome 4 (human chromosome 9) and are structurally closely related (Yoshie et al., 1997). CCL21, however, contains a highly basic C-terminal extension of 30 amino acids that is absent in CCL19. This might reflect the differential capacity of these chemokines to be retained by acidic moieties of the extracellular matrix. Indeed, tissue levels of
CCL21 protein are higher than those of CCL19 even when the expression of both chemokines was comparable at the mRNA level. This might explain why CCL21 is more potent than CCL19 in inducing tertiary lymphoid tissue (Luther et al., 2002). In addition, CCL21 is expressed by T stromal cells and HEV (Gunn et al., 1998; Luther et al., 2000b; Willimann et al., 1998), whereas the expression of CCL19 is limited to the T cell zone (Kim et al., 1998; Ngo et al., 1998). CCL21 exists in two isoforms that differ at one amino acid in position 65 (Vassileva et al., 1999) with the serine-isoform (CCL21-Ser) being expressed on T cell stromal cells and the leucine-isoform (CCL21-Leu) being expressed in lymphatic vessels (Luther et al., 2000b).

Immature DC express high levels of CCR1 and intermediate levels of CCR2, CCR5 (Sallusto et al., 1998b) and CCR6 (Dieu et al., 1998), which might attract them to peripheral sites of inflammation. Indeed, the CCR6-ligand CCL21/LARC is strongly expressed by mucosal epithelium and keratinocytes (Charbonnier et al., 1999; Nakayama et al., 2001; Tanaka et al., 1999), and this correlates with the localization of immature DC to the body surfaces. In addition, ß-defensins, antimicrobial peptides of the innate immune response, can attract immature DCs directly by binding to CCR6 (Yang et al., 1999).

Early after maturation, DC downregulate CCR6 and induce CCR7 (Dieu et al., 1998; Sozzani et al., 1998; Yanagihara et al., 1998; Yang et al., 1999) which correlates to the localization of mature DC to T cell zones of secondary lymphoid organs. Late after maturation, DC upregulate CCL19 but do not downregulate CCR7 through desensitization (Sallusto et al., 1999c). This could be a mechanism by which the DC recruits more naïve T cells for efficient priming. Moreover, mature DCs express CCL17/TARC (thymus and activation-regulated chemokine) and CCL22/MDC (macrophage-derived chemokine), which might attract CCR4-positive memory cells (Schaniel et al., 1999; Tang and Cyster, 1999).

T cells, in turn, lose CCR7 upon priming and up-regulate inflammatory chemokine receptors allowing for their efficient emigration to the periphery (Potsch et al., 1999; Sallusto et al., 1999a). Depending on the polarization of T cells (Th1/ Tc1 versus Th2/ Tc2), a different set of chemokine receptors is expressed. Th1 cells express CCR2, CCR5 and CXCR3 whereas Th2 cells express CCR4, CCR8 and in some instances CCR3 (Bonecchi et al., 1998; D'Ambrosio et al., 1998; Sallusto et al., 1997; Siveke and Hamann, 1998; Annunziato et al., 1999; Yamamoto et al., 2000; Zingoni
et al., 1998). This was shown to correlate with differential localization in vivo after virus infection (Maloy et al., 2000).

Furthermore, Th1 and Th2 cells show differential expression of CCR7 (Randolph et al., 1999). Th1 cells express high levels of CCR7 and localize to the PALS whereas Th2 cells express this chemokine receptor at lower levels which juxtaposes them to B cell zones. This correlates to the more pronounced role of Th2 cells in providing B cell help.

Two subgroups of memory T cells have been described: CD44+CD62L+CCR7+ central memory cells and CD44+CD62L−CCR7− peripheral memory cells (Sallusto et al., 1999b). In this model, central memory cells are localized in secondary lymphoid organs where they may be restimulated by persistent antigen, whereas effector memory cells are localized in peripheral organs where they can exert rapid effector function. Effector memory cells are high expressors of inflammatory chemokine receptors whereas central memory cells express these receptors at low levels. Effector memory cells localizing in peripheral organs have been shown to mediate rapid cytolytic function (Masopust et al., 2001), even after infection with the non-persisting Vesicular Stomatitis virus (VSV).

As terminally differentiated effector T cells still can up-regulate CCR7 after reactivation (Sallusto et al., 1999a), it has been hypothesized that there is a constant back-flow of effector/memory T cells from the periphery to secondary lymphoid organs.

The paradigm regarding the action of CCR7 on T cells and DC received further support from in vivo models. Mice with the paucity of lymph node T cells mutation (Plt/plt) lack lymphoid CCL19 and CCL21 (Nakano et al., 1998). In these mice, Langerhans cells of the skin migrated with lower efficiency to secondary lymphoid organs. CCR7−/− mice revealed a strikingly similar morphological phenotype: DCs are recruited less efficiently to secondary lymphoid organs and defined lymphoid T cell zones are absent (Forster et al., 1999).
3.8. Chemokines in B cell responses

CXCR5 was first described as Burkitt’s Lymphoma Receptor 1 (Dobner et al., 1992) and later, its expression was found on B lymphocytes and a subset of memory T lymphocytes (Forster et al., 1994). The CXCR5 ligand CXCL13/BLC (B lymphocyte chemoattractant) is constitutively expressed by FDC in the B cell zones of secondary lymphoid organs (Gunn et al., 1998; Legier et al., 1998). CXCR5 critically impacts on B cell responses because it specifically regulates the homeostatic homing of activated T and B cells. B cells, for example, engage CXCR5 for homing across HEV of PP follicles (Okada et al., 2002). In addition, localization of B1 cells to the peritoneum is mediated via CXCR5 and CXCL13 with marginal zone macrophages and the omentum being the source of CXCL13 (Ansel et al., 2002).

Early after activation by protein antigens, B cells are released from the marginal zone through the loss of αLβ2 and α4β1 integrin-mediated adhesion (Lu and Cyster, 2002). They concomitantly upregulate CCR7 without modulation of CXCR5 (Reif et al., 2002). This localizes them to the border of T cell zones, where they can receive T help. After differentiation to plasma cells, B cells downregulate CXCR5 and CCR7, but not CXCR4 (Hargreaves et al., 2001; Wehrli et al., 2001). This correlates with the emigration of plasma cells from the B cell zone and their relocation to the bone marrow, where the CXCR4-ligand CXCL12/SDF-1α is expressed.

A subset of CD44hiCXCR5hi CD4+ cells arises during the course of a B cell dependent immune reaction. The localization of this subset near the B cell zone, their reduced responsiveness to CCL19 and CCL21 (Ansel et al., 1999), and the fact that these cells enhance the production of switched immunoglobulin isotypes in vitro (Breitfeld et al., 2000; Schaeferli et al., 2000) led to their classification as follicular helper cells. They are found in the mantle zone and at follicular HEV, which may point out to their direct recruitment into B cell follicles. In addition, in vitro stimulation of CD4+ T cells by anti-CD3 and anti-CD28 in the presence of OX40L on antigen-activated B cells, leads to the upregulation of CXCR5 and IL-4 (Flynn et al., 1998). CXCR5 expression on primed T helper cells precedes their effector function and after full differentiation CXCR5 expression is irreversibly lost (Schaeferli et al., 2001). T cell contact to B cells in follicles has therefore been postulated to be required for full helper differentiation (Homann et al., 1998; Macaulay et al., 1998; Moser et al., 2001).
This is further evidenced by the fact that the B cell helping capacity is focused only within a subset of CXCR5⁺CD4⁺ T cells in humans (Kim et al., 2001). CXCR5 is also involved in the trafficking of APC. Macrophages of the marginal zone can upregulate CXCR5 upon activation with LPS and then can localize to B cell follicles (Yu et al., 2002). Similarly, a subset of skin DC expresses CXCR5 and localizes directly to the B cell zones of draining lymph nodes (Saeki et al., 2000). These DC can most likely modulate B cell responses, as antigen-pulsed CXCR5-transfected skin DC augmented IgM and IgG levels when compared with antigen-pulsed nontransfected DC (Wu and Hwang, 2002). The chemokines CCL22/MDC and CCL17/TARC, which are expressed by activated DC (Tang and Cyster, 1999) and B cells (Schaniel et al., 1999) may also play a role in regulating B cell immune responses because T helper cells can be attracted by these chemokines via CCR4 (Melchers et al., 1999).

Mice deficient in CXCR5 (Forster et al., 1996) or CXCL13 (Ansel et al., 2000) have a similar phenotype in that they lack follicular networks and a defined B cell zone. Induction of ectopic germinal centers and affinity maturation, however, is fully functional in CXCR5⁻/⁻ mice (Voigt et al., 2000).

### 3.9. Chemokines in virus infections

Chemokines and chemokine receptors are involved in antiviral immune responses in three different ways:

a) **Inflammatory chemokines attract effector lymphocytes to the site of virus infection and thus support antiviral immune responses.**

b) **Therefore, some viruses have evolved which express proteins that can bind to chemokines and quench their effect.** This generates a selective advantage of the virus over the host.

c) **Furthermore, some viruses exploit chemokine receptors as co-receptors for entry into the cell.**
3.9.1. Inflammatory chemokines attract effector lymphocytes

In general, specific CD8+ and CD4+ lymphocytes are primed during the course of antiviral immune responses. After it was established that T cell activation leads to the upregulation of several inflammatory chemokine receptors (Sallusto and Lanzavecchia, 2000), their expression was extensively studied in vivo on virus-specific T lymphocytes. Following intracerebral infection with the lymphocytic choriomeningitis virus (LCMV), a marked up-regulation of CCR1, CCR2 and CCR5 in brain tissue was observed (Nansen et al., 2000). CCR1 was mainly expressed by macrophages, whereas CCR2 and CCR5 was detected on antiviral CTL. A similar pattern of chemokine expression could be observed in LCMV-infected footpads (Maloy et al., 2000).

This combination of chemokine receptors and their respective ligands is characteristic of a Th1/Tc1-dominated inflammation (Sallusto et al., 1998a) and it is typical for many antiviral immune responses. These chemokine receptors act in concert to lead antiviral effector cells to the infected periphery (Cerwenka et al., 1999b; Maloy et al., 2000), although their relative importance varies with the model infection.

CCR5−/− mice, for example, did not show any marked defect in immune responses against LCMV (Nansen et al., 2002), most probably because other chemokine receptors could compensate for the defect. CCR5, however, seemed essential for the immune response against coronavirus (Glass et al., 2001). A murine model of influenza A infection revealed the cross-talk between CCR5 and CCR2 (Dawson et al., 2000): CCR5−/− mice expressed increased levels of the CCR2-ligand CCL2/MCP-1 (monocyte chemoattractant protein-1) and showed increased mortality, possibly due to the effect of CCL2 on macrophages. CCL3/MIP-1α (macrophage inflammatory protein-1α), a shared ligand for CCR5 and CCR1 also has an impact on the outcome of some virus infections. Infection of MIP-1α−/− mice with murine cytomegalovirus led to decreased antiviral NK-cell responses (Salazar-Mather et al., 1998) and infection of these mice with coxsackie virus led to milder immunopathological myocarditis (Cook et al., 1995). In concordance with these data, infection of MIP-1α−/− mice led to
a higher viral load and accelerated mortality (Domachowske et al., 2000). When MIP-1α mice were infected with LCMV, however, no difference in antiviral CTL activity could be observed, most probably due to redundancy of inflammatory chemokines (T. Junt unpublished results).

IFN-γ is an important antiviral effector molecule (Huang et al., 1993; Leist et al., 1989). The three CXCR3-ligands CXCL9/Mig (Monokine induced by interferon gamma), CXCL10/IP-10 (interferon-inducible protein 10) and CXCL11/I-TAC (interferon-inducible T cell alpha chemoattractant), which are all inducible by IFN-γ, therefore are relevant to antiviral protection. After intracerebral infection with LCMV or a neurotropic strain of mouse hepatitis virus (MHV), CXCL10/IP-10 is strongly up-regulated (Asensio et al., 1999; Lane et al., 1998).

IP-10 mice infected with MHV showed a weaker recruitment of T lymphocytes to the brain and a milder immunopathology (Dufour et al., 2002) and pre-treatment of mice with CXCL10/IP-10-neutralizing antibodies led to a significant delay in MHV clearance. CXCL9/Mig and CXCL10/IP-10 are both induced after infection of mice with vaccinia virus (Amichay et al., 1996; Maloy et al., 2000), and athymic nude mice, which die after infection with vaccinia virus, survive an infection with CXCL9/Mig- or CXCL10/IP-10-recombinant vaccinia viruses (Mahalingam et al., 1999). Similarly, IP-10 and Mig are essential in host defense against a neurotropic coronaivrus (Liu et al., 2001).

Taken together, the ligands of CCR1, CCR2, CCR5 and CXCR3 are of major relevance for antiviral protection, although viruses can induce a wide range of chemokines.

3.9.2. Modulation of inflammatory chemokines by viruses

Especially large DNA viruses, such as poxviruses (e.g. vaccinia virus) and herpesviruses (e.g. CMV, HHV-8) have evolved strategies to subvert the host immune system by counteracting inflammatory chemokines (Lalani et al., 2000;
There are three modes of chemokine modulation through viral proteins:

a) virus-encoded secreted chemokine homologs (vCKs) which function as agonists or antagonists,

b) virus encoded cell-surface chemokine receptor homologs (vCKRs)

c) virus-encoded secreted chemokine-binding proteins (vCKBPs).

Chemokine and chemokine receptor homologues might have been acquired by the virus as has been described for viral oncogenes. The fact that viral chemokines and chemokine receptor homologues interfere with the chemoattraction of effector leukocytes is currently being evaluated for potential therapeutical application (Chen et al., 1998).

3.9.3. Chemokine receptors as virus co-receptors

HIV enters cells by binding to CD4 on the cell surface (Klatzmann et al., 1984). This induces conformational changes in the envelope glycoprotein gp120, allowing for a secondary interaction with chemokine receptors before membrane fusion. Macrophage (M)-tropic non-syncytium-inducing (NSI) strains, which prevail during the initial phase of infection, use CCR5 as a co-receptor and thus are termed R5 (Choe et al., 1996; Deng et al., 1996; Dragic et al., 1996), whereas T cell-tropic, syncytium-inducing (SI) strains, which arise at later stages, use CXCR4 as a co-receptor and thus are termed X4 (Feng et al., 1996; Simmons et al., 1996). CCR3 and CCR8 have also been described to function as co-receptors for HIV (Choe et al., 1996; Horuk et al., 1998), although their relevance for in vivo infection remains unclear.

The discovery of chemokine receptors as co-receptors for HIV boosted research in the chemokine field. The CCR5 ligands MIP-1α, MIP-1β and RANTES (regulated upon activation, normal T cell expressed and secreted) were shown to block HIV entry (Cocchi et al., 1995) and individuals bearing the loss-of-function mutation Δ32
in CCR5 (Huang et al., 1996; Liu et al., 1996) are resistant to infection with HIV-R5. Mutations in the CCR5 promoter, possibly leading to enhanced CCR5 expression levels (Martin et al., 1998), accelerated disease progression. A mutation in the CXCR4/ CXCL14 receptor-ligand pair, which leads to an altered disease pattern, has been identified. A common nucleotide exchange variant in the 3’ untranslated region (position 801) of a splicing variant transcript of CXCL14 was discovered (SDF1-3’A) which conferred slow disease progression in homozygous individuals (Winkler et al., 1998).

Interestingly, also myxoma virus can use various chemokine receptors as co-receptors (Lalani et al., 1999) for cell entry.
3.10. Chemokines in chronic inflammation and autoimmune disease

In most instances, chronic autoimmune diseases are initiated by an acute inflammatory reaction, which may be linked to an infection (Regner and Lambert, 2001; Zinkernagel, 2002). This acute process might be chronically maintained and shift to overt disease under the following circumstances (Ludewig et al., 2001):

a) if immunologically ignored self-antigens are presented in secondary lymphoid organs (Kurts, 2000; Ohashi et al., 1991),

b) if lymphocytes directed against an unrelated infectious agent cross-react with otherwise ignored self-antigens (Molecular mimicry, Oldstone, 1987),

c) if an acute immune reaction, e.g. a viral infection, leads to an extensive activation of autoreactive CD4+ T cells (Bystander activation, Horwitz et al., 1998).

Both the acute phase of autoimmune reactions and the chronic phase of autoimmune diseases correlate with the expression of inflammatory chemokines and chemokine receptors. Animal models provided further evidence for the involvement of chemokines in the disease process (Proudfoot, 2002) and a distinct pattern of chemokines is associated with autoimmune diseases in humans.

In multiple sclerosis (MS), a Th-1-associated disease, the chemokine receptors CCR1, CCR5 and CXCR3 (Balashov et al., 1999; Sorensen et al., 1999) were shown to be highly expressed in brain samples from MS patients after autopsy. Antagonizing (Liang et al., 2000) or genetically ablating (Rottman et al., 2000) CCR1 resulted in a significant decrease in the clinical score in a rat EAE model of MS (Liang et al., 2000).

Large numbers of monocytes accumulate in atherosclerotic lesions (Ross, 1999). Monocytes and macrophages express CCR2, and the CCR2-ligand CCL2/MCP-1 was found to be expressed in atherosclerotic lesions of patients (Yla-Herttuala et al., 1991). In addition, CCR2−/− mice show a reduced size of atherosclerotic plaques after crossing on the apoE-deficient background (Boring et al., 1998; Dawson et al., 1999).
In atopic dermatitis, enhanced expression of the CCR4 ligands CCL17/TARC and CCL22/MDC was observed (Vestergaard et al., 1999) and enhanced expression of CCL17 was demonstrated in bronchial epithelium of asthma patients (Sekiya et al., 2000). Blocking these chemokines by neutralizing antibodies effectively suppressed lung infiltration of T cells in murine asthma models (Gonzalo et al., 1999; Kawasaki et al., 2001).

The chemokines and chemokine receptors which were associated with a Th1- or Th2- bias in vitro (Sallusto et al., 1998a) were thus also found to be significant for the related diseases in vivo.

3.10.1. De novo formation of lymphoid tissue in chronic inflammations

The constitutive chemokines CCL19, CCL21, CXCL14 and CXCL13 help to maintain the microenvironment of secondary lymphoid organs. Constitutive chemokines are also induced on endothelial cells during the course of chronic autoimmune inflammations (Christopherson et al., 2002) or in the lesions themselves (Hjelmstrom et al., 2000). Expression of constitutive chemokines was found to be associated with artherosclerosis (Reape et al., 1999), ulcerative colitis (Mazzucchelli et al., 1999), Sjögren's syndrome (Amft et al., 2001), autoimmune thyroid disease (Armengol et al., 2001) and rheumatoid synovitis (Takemura et al., 2001).

Constitutive chemokines probably play a role in organizing the newly formed lymphoid tissue that develops during a sustained autoimmune reaction (Hjelmstrom, 2001). The role of this organized tertiary lymphoid tissue is still unclear, but it might support the exacerbation of autoimmune diseases as it may contain self-reactive lymphocytes (Armengol et al., 2001).

The induction of organized lymphoid tissue in RIP-CXCL13 mice (Luther et al., 2000a) as well as in RIP-CCL21 mice (Fan et al., 2000) provide additional evidence for the role of constitutive chemokines as key players in the formation of ectopic tertiary lymphoid tissue (see below).

The link between inflammation and the induction of secondary lymphoid tissue is made by cytokines of the tumor necrosis factor (TNF) superfamily (Chaplin and Fu, 1998). TNF family members have a dual function, they are both pro-inflammatory and morphogenetic. Both processes involve the migration of leukocytes (Sedgwick et al.,
and therefore the induction of adhesion molecules and of inflammatory or constitutive chemokines (Cuff et al., 1999; Cuff et al., 1998; Ngo et al., 1999).

Initially, two secreted, homotrimeric members of the tumor necrosis factors (TNF) superfamily, TNFα (now: TNF) and TNFβ (now: lymphotixin-α/LTα) were described, both produced by activated macrophages and T cells (Bazzoni and Beutler, 1996) (Vassalli, 1992). There is also a membrane-bound form of TNF from which the soluble form is generated through the metalloproteinase TNF converting enzyme (TACE) (Moss et al., 1997). The soluble form of TNF has a more pro-inflammatory and the membranous form a more morphogenetic role (Ruuls et al., 2001). Two TNF receptors exist, TNFR1/p55 and TNFR2/p75. Both TNF and LTα can bind to either receptor (Ruddle, 1992).

With the discovery of lymphotoxin-β (LTβ) (Browning et al., 1993), it became clear that LTα can also form LTα1β2 heterotrimers, which are anchored to the cell membrane. LTα1β2 exclusively binds to the lymphotoxin-β-receptor (LTβR) (Crowe et al., 1994). Unlike TNFR1 and TNFR2, which are expressed very broadly on lymphoid cells, the LTβR is expressed on lymphoid stromal cells.

TNF receptors signal via TNF receptor-associated factors (TRAF) and NFκB and recruitment of distinct NFκB pathways induces the pro-inflammatory versus the morphogenetic effect (Dejardin et al., 2002).

Therefore, the same positive feedback loop may come into play during chronic inflammation, which was described for the generation of secondary lymphatic tissue during ontogeny (Ansel and Cyster, 2001; Ansel et al., 2000): The induction of TNF and LT at the site of inflammation augments the local production of constitutive chemokines which attract T and B cells. B cells are a major source of LTα1β2, which, in turn further increases the local concentration of CCL19, CCL21 and CXCL13.

### 3.11. Vesicular Stomatitis Virus (VSV)

Vesicular Stomatitis Virus (VSV) is a bullet-shaped, enveloped (-)-RNA virus and is a member of the *Rhabdoviridae* family (Fig. 6). The name of this virus family is derived from their elongated structure (gr. *rhabdos* = rod). The rhabdoviridae can be further subdivided into the *Lyssavirus* genus, to which the rabies virus belongs, and the *Vesiculovirus* genus of which VSV is the prototype member (Fields and Knipe, 1985).
Most rhabdoviruses infect a wide range of mammals as well as insects, which are most probably their vector of transmission. VSV derives its name from the vesicular disease caused on tongues and feet of infected cattle, horse and swine. VSV is enzootic in subtropical areas of the Americas (Walton et al., 1987). Transmission to humans occurs occasionally but clinical manifestation of disease is usually mild. Two serotypes of VSV have been described due to the non-crossreactive characteristic of the respective neutralizing antibodies, Indiana (VSV-IND) and New Jersey (VSV-NJ) (Keil and Wagner, 1989).

Structurally, VSV is composed of the nucleocapsid core, the matrix protein (M), a membrane derived from host cell lipids, and regularly spaced monomeric glycoprotein (GP) spikes protruding outward. The nucleocapsid consists of a single strand of (-)RNA which is tightly associated with the nucleoprotein (NP) and the large (L) and nonstructural (NS) proteins. The NS and L proteins are associate with the viral RNA polymerase to transcribe the (-)RNA to a (+)RNA upon infection. The M protein connects the nucleocapsid to the membrane and the GP is involved in attachment to the host cell. It contains the single epitope for neutralizing antiviral antibodies (Kelley et al., 1972), whereas CTL can also recognize internal components such as the nucleoprotein (Puddington et al., 1986).
3.12. The Immune Response against VSV

3.12.1. Antibody responses to VSV

VSV is a cytopathic virus that does not persist in an infectious form after peripheral infection of adult mice (Hecht and Paul, 1981). It is highly neurotropic and may cause neurological disease and death, especially in mice devoid of an appropriate B cell response (Brundler et al., 1996; Thomsen et al., 1997) or lacking a functional IFN system (Muller et al., 1994).

In normal mice, VSV induces both a cytotoxic T cell (Rosenthal and Zinkernagel, 1980) and a neutralizing antibody response (Kelley et al., 1972). The neutralizing IgG
and not the CTL response seems to be crucial for recovery from primary infections (Gobet et al., 1988; Leist et al., 1987). After intravenous injection, VSV induces an early short-lived neutralizing IgM response peaking around day four, known to be largely T helper-cell independent (TI) (Bachmann and Zinkernagel, 1997; Charan and Zinkernagel, 1986): This is followed by a virtually lifelong, strictly T helper cell-dependent (TD) neutralizing IgG response (Charan and Zinkernagel, 1986; Leist et al., 1987) starting from day six to eight after infection and reaching plateau levels after three weeks (Fig. 6).

VSV-G is the only protein in the envelope and induces a strong neutralizing antibody response with one immunodominant neutralizing epitope (Wagner et al., 1970). Neutralization is most likely mediated by prevention of the virus docking to cellular receptors. VSV-G may be used experimentally in three different forms with varying degrees of viral envelope organization (Bachmann and Zinkernagel, 1996).

- VSV particles contain ca. 1200 identical glycoprotein molecules that form a regular and densely ordered (“quasicrystalline”) pattern of spike tips spacing 5-10 nm. This two-dimensional spacing has been shown to be optimal for TI induction of B cells (Feldmann and Easten, 1971). The induction of IgM-producing plasma cells has been shown to occur in the marginal zone of the spleen (Ochsenbein et al., 1999a): After infection, VSV attaches to pre-existing neutralizing serum antibodies or complement. These complexes are then directed to MZM. This fixation of the virus allows an efficient presentation of the virus to B cells and the induction of TI-1 IgM responses before a TD production of IgG.

UV-inactivated VSV maintains the same three-dimensional structure, the Ig response, is of the same type but weaker, as it is a non-replicating antigen.

- Vaccinia virus recombinant for VSV-Indiana-G (VV-IND-G) itself does not contain any VSV-G, but, after productive infection of host cells, VSV-G is expressed in the lipid bilayer in a randomly spaced fashion (Johnson et al., 1981). VV-IND-G replicates abortively, such that the administered amount of antigen increases moderately in the host. The IgM response against VV-IND-G is TI-2, followed by a TD production of IgG.
• VSV-G expressed in vitro by recombinant baculovirus-infected Spodoptera frugiperda (Sf9) cells forms micelles with a low degree of organization. In this form, Baculo-VSV-G basically behaves like any other protein preparation. Depending on the route of the administration, even the IgM production might be TD, whereas the IgG production is always TD (Ochsenbein et al., 2000a).

3.12.2. CTL responses to VSV

In the immune response against VSV, CTL are induced against glycoprotein (G)- and nucleoprotein (N)-derived epitopes (Yewdell et al., 1986) only after the virus has been neutralized by the antibody response. This might be due to the fact that antigen-activated B cells, but not resting B cells can prime this response (Ciavarra and Burgess, 1988) and that this response was strongly dependent on CD4+ T cell activation (Ciavarra and Tedeschi, 1994). In the acute phase of VSV infection, CTL thus do not play a protective role.

3.13. Lymphocytic choriomeningitis virus (LCMV)

Lymphocytic choriomeningitis virus (LCMV) is the prototype member of the Arenaviridae family, which is subdivided into the old world (LCMV, Lassa, Mopeia, Mobala, Ippy) and new world viruses (Hunin, Machupo, Guanarito, Tacaribe, Amapari, Flexal, Pichinde, Latino, Paraná Tamiami). The name of this virus family is derived from their granulated microscopical appearance (lat. arenosus = sandy). These granula were shown to be ribosomes of the host cell which were included into the virion during packaging (Farber and Rawls, 1975; Pedersen and Königshofer, 1976; Vezza et al., 1978). LCMV particles are polymorphic in shape and of variable size (50-300 nm) (Dalton et al., 1968) but infectivity is mostly associated with particles of intermediate size of appr. 100 nm (Blechschmidt and Thomssen, 1976) (Fig. 6).
The natural host of LCMV is the mouse. Infectious particle are transmitted from mother to offspring via the transplacental or congenital route or via the milk, although infection can also occur via other body secretions. Between three and 20 % of wild rodents display antibodies against LCMV (Ackermann et al., 1964), depending on the population. Zoonotic LCMV infections of humans have also been described. They range from mostly mild respiratory tract symptoms tract to lethal disease (Gregg, 1975; Hinman et al., 1975).

LCMV contains two single-stranded RNA species, one large (L, 8 kb) and one small (S, 3.5 kb). The LCMV genome is organized in an ambisense fashion (Auperin et al., 1984) such that it contains (+)-RNA sequences as well as (-)-RNA sequences. The (+)-sequences, however, do not directly participate in protein synthesis, but the genome serves as a template for an RNA antigenome. Genome and antigenome then serve as templates for mRNA synthesis.

The L-RNA encodes the 200 kDa L-polymerase and an 11-14 kDa zinc-binding protein (Harnish et al., 1983; Salvato and Shimomaye, 1989). The S-RNA encodes the 75 kDa GP-precursor (GP-C) and the 63 kDa NP.

Globular NP units associate with viral RNA and give the two circular nucleocapsids a beaded appearance (Fields and Knipe, 1985).

Posttranslational cleavage of GP-C into GP-1 (44 kDa) and GP-2 (35kDa) (Bruns et al., 1983; Buchmeier and Oldstone, 1979; Wright et al., 1990) involves two steps: First, the signal sequence which is responsible for insertion of the nascent protein into the endoplasmic reticulum (ER) is removed by the signal peptidase. Subsequently, the remainder of GP-C is further processed in the late ER or the early Golgi compartment by an endopeptidase from the subtilase family (SKI-1/S1P, (Lenz et al., 2001)). The cleavage site for the GP-C of LCMV (RRLS-GTFT) is located between Ser-265 and Gly-266, (W.Beyer, unpublished data and Buchmeier et al., 1987). GP-C contains nine potential N-linked glycosylation sites of which five are used in GP-1 and three in GP-2 (Wright et al., 1990).

GP-1 forms a globular homotetramer that interacts with the N-terminus of the GP-2 homotetrameric helix. The C-terminus of the GP-2 tetramer is anchored in the virion by interaction with the nucleocapsid (Bruns et al., 1986; Burns and Buchmeier, 1991).

GP homotetramers are present on the surface of the virions and form irregularly spaced club-shaped projections of 5-10 nm in length (Compans and Bishop, 1985;
LCMV-GP was shown to interact with α-Dystroglycan, the cellular receptor of LCMV and Lassa virus (Cao et al., 1998).

A comparison of the binding affinities of different strains of LCMV to α-Dystroglycan revealed that two groups can be distinguished: (1) high-affinity binders which possess a leucin at position GP260 and (2) low-affinity binders which have a phenylalanine at the same position. In addition, the mutation S153F within LCMV-GP abolished the binding to α-Dystroglycan completely (Smelt et al., 2001). These different binding affinities were correlated with different tropisms of the corresponding strains. The high-affinity binders Clone 13, WE54 and Traub are preferentially found in the white pulp of the spleen whereas the low-affinity binders ARM53b and E350 mainly infiltrate the splenic red pulp. Interestingly, the high-affinity binding strains were also found to be aggressive strains, leading to immunosuppression and viral persistence (Sevilla et al., 2000).

3.14. The immune response against LCMV

LCMV is a non-cytopathogenic virus. Clearance of primary infection is mostly dependent on perforin-mediated cytolysis by CTL (Kagi et al., 1994), protection from secondary infection mostly relies on the action of antiviral neutralizing antibodies (Zinkernagel et al., 2001). LCMV can establish a multitude of virus-host relationships in the mouse, depending on the dose of infection and the administration route. It is thus a useful tool to study the murine immune system and the kinetics of viral infections (Fig. 6 and Zinkernagel, 1996).

3.14.1. CTL responses to LCMV

Infection of LCMV is followed by immediate activation of MZM (Seiler et al., 1997) and strong induction of interferons (Huang et al., 1993). Primary infection is essentially controlled by CTL, which reach their maximal activity on day eight after infection and clear the virus fifteen to eighteen days after infection (Byrne and
Oldstone, 1984). Depending on the infectious dose and the strain of LCMV used, virus titers reach a maximum of $10^7$ to $10^8$ plaque forming units (pfu) per gram tissue on day 4-6 and drop below detectable levels by day ten (Lehmann-Grube et al., 1985).

In mice of the H-2$^b$ haplotype, the three immunodominant CTL epitopes are presented by the H-2D$^b$ molecule. The gp33-41 and gp276-286 epitopes are located in the GP leader sequence and in GP-2, respectively (Gairin et al., 1995; Oldstone et al., 1988). The third immunodominant epitope, np396-404, is located in the NP (Schulz et al., 1989). Studies employing mutant strains of LCMV devoid of these immunodominant epitopes led to the discovery of two additional, H-2D$^b$-restricted, subdominant epitopes, gp92-101 and gp117-125 (Gallimore et al., 1998; van der Most et al., 1998). In addition, a shorter version of gp33-41, gp34-41, can be presented by the H-2K$^b$ molecule (Hudrisier et al., 1997).

**a) CTL responses to intracerebral infection**

If immunocompetent mice are infected intracerebrally (i.c.) with a low dose of LCMV, symptoms of lymphocytic choriomeningitis develop within five and six days after infection. Symptoms include hunched back, ruffled fur, tremor and convulsions. Mice succumb to the infection one to three days later due to a destructive inflammatory immunopathology in the brain (Cole et al., 1972). The lethal outcome of meningitis can be attributed to the action of CTL (Buchmeier et al., 1980; Lehmann-Grube et al., 1988).

**b) CTL responses to peripheral infection**

The outcome of peripheral infection, subcutaneous (s.c.) or intravenous (i.v.), critically depends on the virus strain and dose used for infection. Infection with a low dose of LCMV (200 pfu) or with a slowly replicating strain such as LCMV-Armstrong (LCMV-ARM) leads to virus clearance and lifelong immunity. Virus clearance is mainly mediated by CTL (Cole et al., 1972; Kagi et al., 1994; Moskophidis et al., 1987). Virus clearance after low dose infection might, however, not lead to the complete removal of LCMV antigen from the host. LCMV can persist below the detection limit of the standard focus-forming assay (Battegay et al., 1991),
either as free viral RNA (Ciurea et al., 1999) or as cDNA integrated into the host genome (Kleinerman et al., 1997). Although the significance of these persistent forms remains elusive, a continuous low-level production of LCMV antigen within the host most likely has an impact on the maintenance of CTL memory.

Infection with intermediate doses of LCMV ($2 \times 10^4$ pfu) generally lead to a lymphoid immunopathology when infected APC get eliminated by specific CTL. This results in heavy damage of the lymphoid microarchitecture and temporary immunosuppression (Althage et al., 1992; Odermatt et al., 1991).

Infection with a high dose ($\geq 2 \times 10^6$ pfu) of rapidly replicating strains of LCMV such as LCMV-Docile (LCMV-DOC) leads to an overwhelming infection and results in lifelong persistence of the virus within the host. This so-called carrier state is virtually asymptomatic because specific T cells of the periphery have been deleted by exhaustion (Moskophidis et al., 1993a; Moskophidis et al., 1993b) and, once LCMV has reached the thymus, its antigens are perceived as neo-self antigens and all newly generated LCMV-specific T cells are deleted by negative selection.

c) CTL responses after neonatal or transplacental infection

A carrier state can equally be established if LCMV reaches the immature immune system of newborn mice.

3.14.2. Antibody responses to LCMV

a) Neutralizing antibodies

Neutralizing antibodies against LCMV develop late, between day 60 and 80 following infection (Bruns et al., 1983; Buchmeier et al., 1980). Most of these antibodies are directed against a single neutralizing epitope of GP-1, GP-1A (Parekh and Buchmeier, 1986). Studies of B cell-deficient or -depleted mice have revealed that neutralizing antibodies are dispensable for the clearance of a primary infection (Cerny et al., 1986; Cerny et al., 1988).

Neutralizing antibodies directed against LCMV develop faster in the absence of CTL (Battegay et al., 1993), suggesting that antibody-producing B cells may become a target for CTL-mediated lysis (Planz et al., 1996). Alternatively, the absence of CTL
might attenuate the LCMV-related immunosuppression and increase the viral load, which might enhance the humoral part of the immune response.

The role of neutralizing antibodies is fourfold. (1) Neutralizing antibodies serve to control virus over extended periods of time (Planz et al., 1997; Thomsen et al., 1996). If CD4 positive T cells or B cells are missing, virus reappears 50 to 200 days after an initial phase of virus control, eventually resulting in a carrier state. (2) Neutralizing antibodies decrease viral spread and are able to protect from immunopathology or exhaustion (Seiler et al., 1998; Wright and Buchmeier, 1991). This effect involves the Fc portion of the antibody and the complement system, suggesting virus fixation to MZM as for VSV. A further indication that neutralizing antibodies may help to concentrate virus in secondary lymphoid organs comes from B cell-deficient mice in which more virus could be recovered from blood and organs during the acute phase of infection. (3) Neutralizing antibodies protect suckling mice from the establishment of a persistent LCMV infection (MacPherson personal communication and Baldridge and Buchmeier, 1992). (4) Neutralizing antibodies protect adult mice against re-infection (Thomsen and Marker, 1988).

b) Non-neutralizing antibodies

Six to eight days post LCMV infection, antibodies against NP are formed (Kyburz et al., 1993). These antibodies participate in clearing viral components from the circulation, but do not play a role in antiviral protection.

Taken together, VSV and LCMV are well-studied model infections, which serve to explore functional deficiencies of the immune system in immunocompromised mice. The particular advantage of LCMV is that it can lead to a plethora of disease patterns and the advantage of VSV is that VSV-G is available in different preparations, which allows to fine tune the impact on the immune system after immunization.

Mice deficient in components of the innate immune system, such as interferons (Muller et al., 1994) or mice with an altered lymphoid architecture (Karrer et al., 1997) immediately establish a carrier state after low-dose infection with LCMV. Mice with more subtle defects of the adaptive immune system, such as co-stimulatory molecules (Andreasen et al., 2000) or single homing receptors (Bartholdy et al., 2000) however, can completely clear LCMV infections. LCMV thus can be used as a tool to establish an “order of importance” of the various components of an immune
reaction *in vivo*. Immunizations with VSV and related preparations of VSV-G can reveal the degree of impairment that a given defect confers to a mouse strain.
4. CENTRAL QUESTION

The main aim of this thesis was to investigate how relevant the lymphoid microarchitecture as given by the expression of constitutive chemokines was to the capacity of the body to cope with virus infections. I expected to observe a profound immune defect in mice with a defect in lymphoid morphology (plt/plt, CCR7⁻⁻⁻, CXCR5⁻⁻⁻, Fig. 7, Tab. 2). Interestingly, the lymphoid compartments and the co-ordinate movement of cells along gradients of constitutive chemokines appear to be relevant to the outcome of an immune response only in conditions of limiting antigen. Constitutive chemokines and the lymphoid compartments therefore may only serve to optimize the immune response. Viruses, which have the capacity to spread over the whole organism, will eventually infect a sufficient number of APC, which will be sufficiently close to a naïve lymphocyte within secondary lymphoid organs to induce a potent immune response.
Figure 7: Splenic architecture of the mouse strains used in this thesis. 
Immunohistochemical stain for B220 indicates the localization of the B cell zone, stains for CD4 and CD8, respectively, indicate the localization of the T cell zone in naive mice. The splenic architecture of naive C57BL/6 mice is given as a control.

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Table 2: Constitutive chemokines and their receptors
5. RESULTS

5.1. Part I: Antiviral immune responses in the absence of organized lymphoid T cell zones in plt/plt mice

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Running title: Antiviral immunity in the absence of organized T cell zones

Key words: CCR7, CCL21/SLC, CCL19/ELC, lymphocyte traffic, antiviral immunity

Abbreviations used in this paper: BSS, balanced salt solution, DC, dendritic cell, LCMV-GP, glycoprotein of the lymphocytic choriomeningitis virus, HBSS, Hanks balanced salt solution, LCMV-NP, nucleoprotein of the lymphocytic choriomeningitis virus; MACS, magnet assisted cell sorting; MOI, multiplicity of infection; plt, paucity of LN T cells; PP, Peyer’s patch; VSV, Vesicular Stomatitis Virus; VV, vaccinia virus

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5.1.1. Abstract

The paucity of lymph node T cells (plt) mutation in mice results in strongly reduced T cell numbers in lymph nodes and homing defects of both dendritic cells and naïve T cells. We investigated here the functional significance of the plt phenotype for the generation of antiviral immune responses against cytopathic and noncytopathic viruses. We found that dendritic cell-CD8+ T cell contacts and the initial priming of virus-specific T cells in plt/plt mice occurred mainly in the marginal zone of the spleen and in the superficial cortex of lymph nodes. The magnitude of the initial response and the maintenance of protective memory responses in plt/plt mice was only slightly reduced compared to plt/+ controls. Furthermore, plt/plt mice mounted rapid neutralizing antiviral B cell responses and displayed normal immunoglobulin class switch. Our data indicate that the defective homing of dendritic cells and naïve T cells resulting from the plt/plt mutation results in a small, but not significant effect on the induction of protective antiviral T and B cell immunity. Overall, we conclude that the spatial organization of secondary lymphoid T cell zones via the CCR7-CCL19/CCL21 pathway is not an absolute requirement for the initial priming and the maintenance of protective T and B cell responses in the course of viral infections.
5.1.2. Introduction

Secondary lymphoid organs, such as spleen, lymph nodes (LN), Peyer’s patches (PP) and the mucosa-associated lymphoid tissue (MALT), sample antigen through drainage of the afferent lymph and provide the microenvironment for optimal antigen presentation to naïve lymphocytes. The importance of lymph nodes for the generation of protective antiviral T and B cell responses and for the induction of antitumor immunity has been shown, for example, in alymphoblastic (aly/aly) mice which lack LN and PP (Karrer et al., 1997; Ochsenbein et al., 1999b). Absence of the spleen, which filters antigens from the blood, is less dramatic but may lead to deficient induction of immunoglobulins against non-replicating agents (Amlot and Hayes, 1985; Ochsenbein et al., 2000a). In humans, splenectomy can pose the risk of overwhelming bacterial infection (Diamond, 1969), although the induction of antiviral immune responses may be unimpaired (Chan et al., 1989) or even enhanced, if the spleen is a site of extensive viral replication (Katzenstein et al., 1983; Tsoukas et al., 1998).

Within lymphoid organs, induction and maintenance of antiviral immune responses depend on correctly formed lymphoid compartments. Early trapping of virus in the marginal zone leads to the initial extrafollicular induction of antiviral B cells against T cell independent antigens. Marginal zone macrophages have also been shown to be involved in induction of antiviral CTL responses as it has been shown that only weak, non-protective antiviral CTL responses could be initiated after depletion of marginal zone macrophages (Seiler et al., 1997). The environment of germinal centers (GC) is required for the maintenance of antiviral B cell memory in mice (Karrer et al., 2000) and patients with X-linked hyper-IgM-syndrome lacking germinal centers due to an impaired CD40-CD40L interaction show hypogammaglobulinemia and insufficient B cell and CTL activation leading to increased susceptibility to infections (Jain et al., 1999; Seyama et al., 1998).

The localization of lymphocytes within secondary lymphoid organs is controlled by constitutive chemokines differentially expressed in the B and T cell zones (Cyster, 2000). Primary B cell follicles, most probably follicular dendritic cells, produce CXCL13 (B lymphocyte chemoattractant, BLC, Gunn et al., 1998) which attracts mature resting B cells and a T cell subpopulation recently described as follicular helper T cells via CXCR5 (Ansel et al., 1999). T cell zone stromal cells express both
the serine isoform of CCL21-Ser/SLC-Ser and CCL19/ELC (Ngo et al., 1998; Willmann et al., 1998). These chemokines act via the CCR7 receptor and are capable of attracting naïve T cells and mature dendritic cells and thus co-ordinate their interaction within the T cell zone (Saeki et al., 1999; Sallusto et al., 1998b). T cells downregulate CCR7 after TCR triggering and concomitant to their evasion to the periphery (Sallusto et al., 1999a), suggesting that the CCR7-CCL19/CCL21 interaction may be involved in T cell priming (Forster et al., 1999; Potsch et al., 1999). However, only little is known about the role of constitutive chemokines in the modulation of antiviral immune responses.

The paucity of lymph node T cells (plt) mutation, which arose as a spontaneous recessive mutation in mice (Nakano et al., 1997), was recently mapped to the chemokine locus chromosome 4 (Cklc4) and results in loss of both the only functional CCL19/ELC and the CCL21-Ser/SLC-Ser genes and in an aberrantly formed lymphoid T cell zone (Luther et al., 2000b; Nakano and Gunn, 2001; Vassileva et al., 1999). These mice are therefore an excellent model to investigate the induction and maintenance of immune responses in a situation where the recruitment of naïve T cells and DC to the T cell zone is defective (Gunn et al., 1999b).

Infection of mice with the lymphocytic choriomeningitis virus (LCMV) is a well characterized model system for the investigation of antiviral T and B cell responses in vivo. LCMV can infect various cells of the immune system, such as B cells, macrophages and DC. In particular, the tropism of different LCMV strains to DC determines whether the virus infection results in induction of protective CTL responses and clearance, or leads to exhaustion of virus-specific CTL and establishment of persistent infection (Sevilla et al., 2000; Smelt et al., 2001). We used here the LCMV system and other well established viral infectious models, Vesicular Stomatitis virus (VSV) and vaccinia virus, to investigate the impact of defective DC and T cell homing on the induction and maintenance of antiviral T and B cell responses in plt/plt mice. We found that T and B cell priming in plt/plt mice occurred mainly in the marginal zone of the spleen and in superficial cortical areas of lymph nodes. Furthermore, both antiviral T and B cell responses were comparable in plt/plt and plt/+ control mice suggesting that the co-ordinate interaction of DC and T cells in the lymphoid T cell zone may be of minor importance in virus infections.
5.1.3. Materials and Methods

Mice

B6-plt/plt mice were bred at the Institut für Labortierkunde (University of Zürich, Switzerland). They were backcrossed to C57BL/6 5 to 7 times and used for experiments in sex-matched groups with heterozygous littermates at the age of 8-12 weeks. Plt/plt mice were typed by PCR using the D4Mit286 and D4Mit237 primer pairs as described (Nakano et al., 1998). B6PL-Thy1.1. mice were obtained from the Jackson Laboratories (Bar Harbor, Maine) and bred to the LCMV-gp33-specific TCR transgenic line 318 (Pircher et al., 1989). Heterozygous F1 animals (318 × Thy1.1.) served as donors of CD8+ T cells in adoptive transfer experiments.

Viruses and peptides

Lymphocytic choriomeningitis virus (LCMV), WE strain, originally obtained from Dr. F. Lehmann-Grube (Hamburg, Germany), was propagated on L929 cells at a low multiplicity of infection (MOI) and was plaqued as previously described (Battegay et al., 1991). Vesicular Stomatitis virus, Indiana strain (VSV-IND, Mudd-Summers isolate), was originally obtained from Prof. D. Kolakofsky (University of Geneva, Switzerland). VSV-IND was propagated on baby hamster kidney 21 (BHK-21) cells and plaqued on Vero cells. For some experiments, UV-inactivated VSV-IND was obtained using UV irradiation (7UV 15W, Philips) for 5 min in a thin layer of liquid in a 60 mm petri dish.

The vaccinia virus recombinant for the LCMV-GP (VV-G2) was originally obtained from Dr. D. Bishop (Institute of Virology, Oxford, UK) and was propagated on BSC40 cells (Hany et al., 1989).

The LCMV-GP peptide KAVYNFATM (gp33) and the LCMV-NP peptide FQPGNGQFI (np396) were purchased from Neosystem (Strasbourg, France).

Cytotoxic T cell response

Specific cytotoxicity was determined ex vivo in a standard \(^{51}\)Cr release assay as described (Hany et al., 1989). Briefly, cell suspensions were prepared from spleens or lymph nodes of immunized mice at the indicated time point after priming. EL4 cells were labeled with gp33 (10\(^{-6}\) M) and 250 mCi \(^{51}\)Cr for 1.5 h at 37°C. 10\(^4\) target cells
per well were incubated for 4 h in 96-well round-bottom plates with 3-fold serial dilutions of spleen effector cells, starting at an E:T ratio of 90:1. EL-4 cells without peptide served as controls. The supernatants of the cytotoxicity assay cultures were counted in a Cobra II Gamma Counter (Canberra Packard). Percentage of specific lysis was calculated as \((\text{experimental release} - \text{spontaneous release}) / (\text{total release} - \text{spontaneous release}) \times 100\). Spontaneous release was always below 20%.

CTL were restimulated on peptide-labeled, irradiated (25 Gy) spleen cells as previously described (Ludewig et al., 1998) and tested in a conventional \(^{51}\text{Cr}\) release-assay using gp33-labeled EL-4 target cells.

**LCMV-NP specific ELISA**

The LCMV nucleoprotein-specific enzyme-linked immunosorbent assay (ELISA) has been described previously (Battegay et al., 1993) using LCMV-NP expressed by Spodoptera frugiperda 9 (Sf9) cells after infection with a recombinant baculovirus.

**VSV-specific serum neutralization test**

Neutralizing antibody titers of sera were determined as described (Roost et al., 1990). Sera were prediluted 40-fold in supplemented MEM and heat-inactivated for 30 min at 56°C. Serial twofold dilutions were mixed with equal volumes of virus diluted to contain 500 plaque forming units (pfu)/ml. The mixture was incubated for 90 min at 37°C in an atmosphere containing 5% CO\(_2\). One hundred ml of the serum-virus mixture were transferred onto Vero cell monolayers in 96-well plates and incubated for 1 h at 37°C. The monolayers were then overlaid with 100 ml DMEM containing 1% methyl cellulose. After incubation for 24 h at 37°C the overlay was removed and the monolayer was fixed and stained with 0.5% crystal violet. The highest dilution of the serum that reduced the number of plaques by 50% was taken as the neutralizing titer. To determine IgG titers, undiluted serum was first pre-treated with an equal volume of 0.1 M 2-ME in saline.

**Footpad swelling reaction**

The indicated amounts of LCMV-WE were injected in a volume of 50 ml in balanced salt solution (BSS) into both hind footpads in experimental groups of 3 mice. The footpad thickness was measured at the indicated time points with a spring-loaded caliper (Moskophidis and Lehmann-Grube, 1989).
Construction of tetrameric class I-peptide complexes and flow cytometry

MHC class I (H-2D\textsuperscript{b}) tetramers complexed with gp33 were produced as previously described (Altman et al., 1996). Briefly, H2-D\textsuperscript{b} and human b2-microglobulin molecules were recombinantly expressed in E. coli (the plasmids were kindly provided by John Altman, Emory University, Atlanta). Biotinylated H2-D\textsuperscript{b} peptide complexes were purified using and Äkta Explorer 10 chromatography system (Pharmacia, Sweden) and tetramerized by addition of streptavidin-PE (Molecular Probes, Eugene, OR). At the indicated time points after immunization, animals were bled and single cell suspensions were prepared of spleen and lymph nodes. Aliquots of 5\times10\textsuperscript{5} cells or 3 drops of blood were stained using 50 ml of a solution containing tetrameric class I-peptide complexes at 37°C for 10 min followed by staining with anti-CD8-FITC (Pharmingen) at 4°C for 20 min. Erythrocytes in blood samples were lysed with FACS lysis solution (Becton Dickinson) and the cells were analyzed on a FACScan flow cytometer (Becton Dickinson) after gating on viable leukocytes. For the determination of absolute cell counts, the number of total viable leukocytes was assessed in an improved Neubauer chamber. For blood, the number of total viable leukocytes was automatically determined in an Advia counter (Bayer, Germany) in the Central Hematology Laboratory of the University Hospital Zürich.

Intracellular cytokine staining

Spleens were removed at the indicated time points after infection with LCMV. Single cell preparations of 1 \times 10^{6} splenocytes were incubated for 5 h at 37°C in 96-well round-bottom plates in 200 ml culture medium containing 25 U/ml IL-2 and 5 mg/ml Brefeldin A (Sigma) Splenocytes were stimulated with phorbolmyristateacetate (PMA, 50 ng/ml) and ionomycin (500 ng/ml) as a positive control or left untreated as a negative control. For analysis of peptide-specific responses, 1\times10^{6} splenocytes were stimulated by adding 10^{-6} M gp33. After stimulation, splenocytes were surface stained with anti-CD8b-PE (53-5.8, Pharmingen) in FACS-buffer (PBS+ 2% FCS + 20 mM EDTA + 0.03% NaN\textsubscript{3}) overnight at 4°C. Splenocytes were washed once with FACS-buffer, fixed with 100 ml 4% paraformaldehyde in PBS for 5 min at 4°C, and permeabilized with 2 ml of permeabilization buffer (FACS-buffer + 0.1% saponin) for 5 min at 4°C. Cells were then stained intracellularly with anti-IFN-\gamma-FITC (AN18, Prat et al., 1984) in permeabilization buffer for 30 min at 4°C. Cells were washed twice
with permeabilization buffer, and the percentage of IFN-γ-producing cells was
determined after gating on CD8+ cells using a FACScan flow cytometer.

*Fluorescence microscopy and immunohistochemistry*

Spleens and lymph nodes of infected or non-infected animals were removed at the
indicated time points, immersed in Hanks Balanced Salt Solution (HBSS) and snap-
frozen in liquid nitrogen. 6 mm cryostat sections were fixed in acetone for 10 min and
dried for 30 min. After blocking of Fc-receptors with 10 mg/ml 2.4G2 (Pharmingen)
antibody in PBS 1% PBS for 30 min, LCMV-NP was detected using FITC- or TRITC-
labeled VL-4 antibody (Battegay et al., 1991). Adoptively transferred Thy1.1.+CD8+ T
cells from 318 x Thy1.1. mice cells were detected using an anti-CD90.1-FITC
antibody (Pharmingen). CD11c-positive cells were detected using N418 hybridoma
supernatant (Metlay et al., 1990) and rat-anti-hamster-TRITC second stage antibody
(Jackson). VL4-TRITC, VL4-FITC, anti-CD90.1-FITC and rat-anti-hamster-TRITC
were used at 10 mg/ml in PBS 1% FCS, the N418-supernatant was used at a 1:5
dilution in PBS 1% FCS. The slides were treated for 1 h with each antibody and
washed extensively with PBS between incubations. The fluorescence was monitored
on a Zeiss Axiophot microscope with a JVC KYF70 camera, using the Analysis
software (Soft Imaging System, Münster, Germany).

For immunohistochemistry, cryostat sections were fixed in acetone for 10 min. Then,
they were incubated with anti-mouse B220 (RA3-3A1/6.1, American Type Culture
Collection, Rockville, MD) and VL-4. A goat-anti-rat antibody (Caltag, Burlingame,
CA) was used as a secondary reagent. It was subsequently detected with a donkey-
anti goat antibody (Jackson) and the substrate for the red color reaction was AS-BI
phosphate/New Fuchsin. Sections were counterstained with hemalum.

5.1.4. Results

*Clonal expansion of LCMV-GP specific CD8+ T cells in plt/plt mice*

After immunization with LCMV-WE, CD8+ T cells directed against the
immunodominant epitope gp33 undergo a vigorous systemic clonal expansion. To
test the effect of defective DC and T cell homing on the initiation of LCMV-specific T
cell responses, we used MHC class I tetramers complexed with the immunodominant
Results

CTL epitope gp33 derived from the LCMV-GP (tet-gp33, Altman et al., 1996) (Gallimore et al., 1998) At different time points after i.v. infection with 200 pfu LCMV-WE, the proportion of tet-gp33-positive cells in the CD8+ T cell pool (Fig. 8, first row), total cell numbers of CD8+ T cells (Fig. 8, second row) and total cell numbers of gp33-specific CTL (CD8⁺tet-gp33⁺, Fig. 8 third row) were assessed from blood, spleen, and inguinal lymph nodes. Naive plt/plt mice display a significant paucity of total CD8+ T cell numbers in lymph nodes (Fig. 8F). Following LCMV infection, the expansion of CD8+ T cells and the gp33-specific CTL response in both plt/plt and plt/+ mice peaked on day 8. The kinetics of the relative numbers of tet-gp33⁺ CTL revealed marked differences in the anti-LCMV response between plt/plt and control mice, particularly in peripheral blood (Fig. 8 A-C). However, the total numbers of tet-gp33+ CD8+ CTL were comparable in plt/plt and plt/+ mice (Fig. 8 G-l). This discrepancy may be explained by the fact that the expansion of CD8+ T cells in the respective compartments of plt/plt mice was slightly faster than in plt/+ mice (Fig. 8, D-F) thus counterbalancing the differences in relative numbers.

Different LCMV strains induce CTL responses of different magnitude. The slowly replicating strain Armstrong (ARM) elicits weaker CTL responses and is rapidly cleared whereas fast replicating strains such as Docile (DOC) induce exhaustive immune responses and tend to persist in the host. It has been suggested that the α-dystroglycan-dependent DC-tropism of LCMV is critical for the strength of the CTL response and therefore determines whether the virus is controlled by the immune system or can persist (Sevilla et al., 2000; Smelt et al., 2001). To assess whether the distinct DC-tropism of different LCMV strains affects the induction of CTL responses when DC homing to secondary lymphoid organs is impaired, we compared gp33-specific CTL responses in spleens of plt/plt and plt/+ control mice after infection with ARM, WE, and DOC (Fig. 9). The peak response on day 8 after infection with 200 pfu of ARM or DOC was comparable in plt/plt and plt/+ mice both in percentages of tet-gp33⁺ CTL within the CD8+ pool (Fig. 9A) and in total numbers of CD8⁺tet-gp33⁺ CTL (Fig. 9B).
Figure 8. Expansion of LCMV-gp33 specific CD8+ T cells in plt/plt and plt/+ mice.

Plt/plt (filled triangles) and plt +/− mice (open boxes) were infected i.v. with 200 pfu LCMV-WE. The proportion of tet-gp33 positive cells in the CD8+ T cell pool (A-C), total cell numbers of CD8+ T cells (D-F) and total cell numbers of CD8+tet-gp33+ CTL were assessed for blood, spleen and inguinal lymph nodes (ILN) at the indicated time points by FACS analysis. Given values represent mean ± SD (n=6-8 per data point).

The proportion of tet-gp33+ CTL in the CD8+ T cell pool of plt/+ mice after LCMV-WE infection was slightly higher than in plt/plt mice (Fig. 9A, middle row); however, like in the previous experiments, the total numbers of tet-gp33+ CTL were comparable in plt/plt and plt/+ mice (Fig. 9B).

Taken together, these data show that the clonal expansion of gp33-specific CD8+ T cells in plt/plt mice after infection with LCMV mice is not impaired.
**Results**

![Graph](image)

**Figure 9.** Expansion of gp33-specific CD8+ T cells in plt/plt and plt/+ mice after infection with different LCMV strains. plt/plt mice and plt/+ mice were infected i.v. with 200 pfu of LCMV-ARM, WE or DOC. Eight days later, gp33-specific CD8+ T cells were visualized in spleens using MHC class I tetramers (A) Representative FACS-stainings from plt/plt mice and plt/+ mice with mean percentage of CD8+ T cells specific for gp33 (+SD) indicated in the corresponding upper right quadrant. (B) Total numbers of CD8+ tet-gp33+ T cells (±SD) on day 8 post infection in spleens from plt/plt and plt/+ mice. Pooled data with values from 6 mice from two independent experiments.

**LCMV-specific CTL activity and antiviral protection against systemic and peripheral challenge infection**

To examine the functional status of LCMV-specific CTL in plt/plt mice in more detail, the cytolytic activity of CTL was assessed in a 51Cr release assay using cells from spleens and mesenteric lymph nodes on day 8 after LCMV infection. CTL responses against the LCMV-NP-derived peptide np396 and the LCMV-GP-derived peptide gp33 were comparable in both compartments (Fig. 10A). Virus dissemination and clearance in different organs were also determined on days 4 and 8 after infection.
As shown in Fig. 10B, virus titers in various organs of plt/plt and plt/+ mice showed no significant differences at either time point. Similar results were obtained for LCMV-ARM and LCMV-DOC (data not shown).

An important function of antiviral CTL is their potential to migrate through peripheral tissue. To further characterize the functional activity of antiviral CTL in plt/plt mice, we used the intra footpad route of infection and monitored the CTL-mediated footpad swelling reaction. The kinetics and degree of the swelling reaction directly correlate with the vigour and the kinetics of the antiviral CTL response (Hany1989). Interestingly, plt/plt mice responded with a slightly accelerated, but overall weaker footpad swelling reaction (Fig. 11A), also when LCMV-ARM or LCMV-DOC were used (data not shown).

To distinguish between a peripheral immune reaction initiated and maintained in draining lymph nodes and a generalized immune response with contribution of the spleen, plt/plt and plt/+ mice were splenectomized and infected with 50 pfu LCMV-
Results

WE into both hind footpads. The overall footpad swelling after splenectomy was reduced in both plt/plt and control mice (Fig. 11B); however, the overall kinetics were similar to non-splenectomized mice suggesting that the aberrantly structured draining lymph nodes of plt/plt mice alone were capable to initiate and maintain an efficient antiviral immune response. Although the T cell numbers of the LNs of plt/plt mice are dramatically reduced (Nakano et al., 1998), splenectomized plt/plt mice were also capable of mounting a good systemic LCMV-specific CTL response after peripheral infection.

Figure 11 Footpad swelling reaction and expansion of LCMV-GP specific CD8+ T cells. (A) plt/plt (filled triangles) and plt/+ mice (open squares) were infected i.f.p. with 50 pfu or 10⁵ pfu LCMV-WE into both hind footpads. (B) Footpad infection of splenectomized plt/plt (filled triangles) and plt/+ mice (open squares) with 50 pfu LCMV-WE. The footpad swelling reaction was measured with a spring-loaded caliper at the indicated time points. (C) Proportion of tet-gp33+ cells in the CD8+ T cell pool, total cell numbers of CD8+ T cells and gp33-specific CTL (CD8+tet-gp33+) were assessed for blood and inguinal lymph nodes from LCMV-WE footpad infected splenectomized mice at the indicated time points. Values represent mean ± SD from 4 mice per data point.
This is shown by the fact that the expansion kinetics of gp33-specific CTL in blood and inguinal lymph nodes were equivalent in splenectomized plt/plt and plt/+ mice (Fig. 11C). Overall, these results indicate that the functional activity of antiviral CTL in plt/plt mice is normal and that CTL responses mediating rapid protection against systemic and peripheral LCMV infection can be generated in plt/plt mice, even in the absence of the splenic compartment.

**Antiviral CTL responses against cytopathic Vaccinia virus**

To evaluate whether plt/plt mice are able to mount protective antiviral CTL responses against acytopathic virus, we infected plt/plt and plt/+ mice with $2 \times 10^6$ pfu of LCMV-GP recombinant Vaccinia virus (Vacc-G2). Seven days later, spleens were harvested and cytotoxicity was determined after restimulation for 5 days with irradiated, gp33-pulsed splenocytes. As shown in Fig. 12, plt/plt mice mounted a normal CTL response against the cytopathic Vacc-G2. Furthermore, Vacc-G2 was cleared from ovaries in plt/plt and plt/+ mice with similar kinetics (Fig. 12B), indicating that the plt-defect has no major impact on the CTL-mediated control of a cytopathic virus.

![Figure 12](image-url)

**Figure 12** CTL induction and viral clearance in plt/plt mice after infection with the LCMV-GP recombinant Vaccinia virus Vacc-G2.

Plt/plt (filled triangles) and plt/+ mice (open squares) were infected i.v. with $2 \times 10^6$ pfu Vacc-G2. (A) On day 5 after immunization, splenocytes were restimulated on irradiated gp33-loaded spleen cells and gp33-specific CTL activity was measured on $^{51}$Cr-labeled EL4 cells pulsed with gp33. (B) Vacc-G2 titers were measured in ovaries at the indicated time points. Mice were used in groups of 3. One representative of two independent experiments is shown.
Antiviral antibody responses in plt/plt mice

The rapid induction of neutralizing antiviral antibodies is crucial for protection against infection with cytopathic viruses. It appears that viral pathogens have the common characteristic to be T cell-independent for IgM production, whereas isotype switching to IgG is mainly dependent on cognate help delivered by the CD4+ T helper cell subset (Bachmann and Zinkernagel, 1997). In the following set of experiments, the induction of neutralizing IgM and IgG antibodies was assessed in plt/plt mice and plt/+ controls after i.v. infection with either $2 \times 10^6$ pfu of live Vesicular Stomatitis virus of the Indiana strain (VSV-IND; Fig. 13A) or with $1 \times 10^8$ pfu UV-inactivated VSV-IND (Fig. 13B). Both the replication competent (Fig. 13A) and the inactivated VSV-IND (Fig. 13B) elicited comparable neutralizing antibody responses in both groups of mice. Similarly, after i.v. infection with 200 pfu LCMV-WE, antinucleoprotein (NP) antibodies of the IgM (Fig. 13C) and IgG class (Fig. 13D) were induced in plt/plt mice and plt/+ controls to a similar extent.

![Graphs showing antibody responses](image)

Figure 13 Induction of neutralizing anti-VSV and binding anti-LCMV-NP antibodies in plt/plt mice.
(A) plt/plt (filled triangles) and plt/+ mice (open squares) were infected i.v. with $2 \times 10^6$ pfu of VSV-IND or (B) $5 \times 10^8$ pfu UV-inactivated VSV-IND. Neutralizing antibody titers were determined from serum at the indicated time points. (day 4: IgM; after day 4: IgG).

Similar to LCMV, the peripheral route of infection for VSV ($2 \times 10^6$ pfu VSV-IND into both hind footpads) did not reveal any significant differences between plt/plt and plt/+ mice in the kinetics of antibody formation or class switch (data not shown). Induction
of anti-LCMV-NP responses was also not dependent on the route of infection; also i.p. and intra footpad infection of plt/plt mice with LCMV-WE elicited anti-NP IgG titers comparable to those found in plt/+ controls (data not shown).

Thus, the generation of T cell-dependent and -independent antiviral B cell responses in plt/plt appears not to be affected by the homing defect of DC and naïve T helper cells.
Maintenance of functional CTL memory responses in plt/plt mice

The notion has been put forward that memory T cell populations cells can be distinguished by their level of CCR7 expression (Sallusto et al., 1999b). We tested here whether a disturbance of the CCR7/CCL19/CCL21 system by the absence of the CCR7 ligands CCL19 and CCL21-Ser has an influence on the recirculation of memory CTL through secondary lymphoid organs and their activity. Fig. 14A shows that LCMV-specific CTL memory in plt/plt mice was not impaired even more than 300 days after infection with 200 pfu LCMV-WE. The total numbers of CD8+tet-gp33+ CTL (Fig. 14A, upper panel) and gp33-specific IFN-γ producing CTL (Fig. 14A, lower panel) in different lymphoid compartments were comparable in plt/plt and plt/+ controls. These results indicate that the numbers of virus-specific memory CTL, their distribution in lymphoid compartments and peripheral blood and their ability to differentiate rapidly into IFN-γ producing effector cells is not affected by the plt mutation.

Furthermore, we tested whether the kinetics of a recall memory response would be affected in plt/plt mice. To this end, groups of plt/plt and plt/+ mice were i.v. infected with 2 × 10^6 pfu Vacc-G2 to generate gp-33 specific memory CTL. The acute activity of these gp33-specific cells was comparable in plt/plt mice and plt/+ mice (see above). Fourteen, 40 or 140 days after the initial immunization with Vacc-G2, mice were challenged i.v. with 200 pfu LCMV-WE and 4 days after the challenge infection, virus titers were measured in spleen, liver, kidney, inguinal lymph nodes and thymus. LCMV could not be detected in plt/plt and plt/+ mice in any of the organs in these experiments (data not shown), indicating that the Vacc-G2-induced GP-specific CTL memory population was capable of protecting the mice against a challenge infection. In addition, total counts of CD8+tet-gp33+ CTL were determined at day 4 after LCMV-WE challenge in spleen and mesenteric lymph nodes.
Figure 14 LCMV-specific memory responses in plt/plt mice.

(A) The number of gp33-tet specific CD8+ T cells was assessed for blood, spleen and mesenteric lymph nodes on day 240 after i.v. infection with 200 pfu LCMV-WE in plt/plt (black bars) and plt/+ mice (white bars) by tetramer analysis (upper panel) and by intracellular IFNγ-staining (lower panel). (B) Anti-LCMV recall responses were determined in plt/plt (black bars) and plt/+ mice (white bars) after i.v. infection with 2 × 10^6 pfu Vacc-G2. At the indicated time points, mice were i.v. challenged with 200 pfu LCMV-WE and the total numbers of CD8+tet-gp33+ T cells in spleens and mesenteric lymph nodes were determined on day 4 post LCMV-WE infection by FACS analysis. Pooled data from two experiments with 3 mice per group are shown.

As shown in Fig. 14B, the clonal expansion of gp33-specific CTL was comparable in plt/plt and plt/+ mice in both compartments. The weaker clonal expansion observed in MLN shortly after Vacc-G2 infection may be explained by a higher residual activity of the recently primed memory population facilitating the elimination of LCMV, and therefore eliciting only a minor expansion of gp33-specific CTL.

Localization of initial CTL activation in plt/plt mice

The above experiments established that the chemokine-driven interaction of DC and naïve T cells in lymphoid T cell zones in plt/plt mice is not an exclusive prerequisite for the induction of rapid protective antiviral immune responses. In order to determine the site of primary activation of antiviral T cell responses in plt/plt mice, we visualized the co-localization of LCMV-specific CTL and virus-infected DC in lymphoid organs. To this end, 1.5 × 10^7 MACS-sorted Thy1.1-positive gp33-specific TCR transgenic
CD8+ T cells were adoptively transferred into plt/plt mice. Twenty-four hours later, the mice were intravenously infected with $2 \times 10^4$ pfu LCMV-WE, and spleens were removed and processed for immunohistochemistry 3 days post infection. In the spleens of both plt/plt and control mice, LCMV antigen was largely confined to the marginal zone (Fig. 15A, 8B). Double-staining for virus antigen and the DC marker CD11c revealed that a significant proportion of the marginal zone DC in plt/plt mice were infected with LCMV (Fig. 15A insert, arrowhead), whereas in plt/+ mice marginal zone DC (Fig. 15B, right insert, arrowhead) as well as T cell zone DC (Fig. 15B, left insert, arrow) harbored LCMV antigen. LCMV-specific 318 x Thy1.1. CD8+ T cells were mainly localized in the red pulp and in the marginal zone of plt/plt mice (Fig. 15C). These virus-specific CTL formed foci in close contact with infected cells exclusively at the marginal zone of plt/plt mice (Fig. 15C insert, arrowhead). In plt/+ mice, however, virus-specific cells homed preferentially to the T cell zone and had contact with virus-infected cells both from the side of the T cell zone (Fig. 15D, insert, arrow) and from the side of the marginal zone (Fig. 15D, insert arrowhead). The precise localization of LCMV antigen was also demonstrated by immunohistochemistry. Sequential spleen sections of plt/plt and plt/+ mice were stained for LCMV-NP (Fig. 15E, F) and B220 (Fig. 15G, H) and antigen was found to be confined mainly to the marginal zone (Fig. 15E, F, arrows). Only in plt/+ mice, a fraction of LCMV antigen could be detected in the T cell zone (asterisk).

Similarly, an immunohistological analysis of mesenteric lymph nodes of LCMV-infected plt/plt mice and plt/+ mice revealed that, in both groups of mice, LCMV-antigen positive DC and Thy1.1. positive gp33-specific antiviral CTL were in close contact in superficial cortical areas (data not shown). Taken together, these data suggest that the contact between virus-infected DC and naïve virus-specific T cells in the splenic marginal zone and in superficial cortical areas of lymph nodes of plt/plt mice was sufficient to generate potent antiviral immune responses.
Figure 15 Histological analysis of LCMV-specific CTL responses.  
(A, B) Double stain for LCMV antigen (green) and the DC marker CD11c (red). LCMV-infected DC appear yellow (arrowhead) in plt/plt (A) and plt/+ mice (B). The inserts in (A) and (B) show the magnification of LCMV-infected DC in the boxed areas. (C, D) Co-localization of LCMV-specific TCR-transgenic CTL (green) and virus infected cells (red) was assessed in plt/plt (C) and plt/+ mice (D) on day 3 after infection with 2 x 10^4 pfu LCMV-WE. The close contact of LCMV-specific CTL and infected cells (arrowheads, arrow) is shown in inserts in (C) and (D). (E-H) Localization of LCMV-NP and B220 in consecutive spleen sections of plt/plt (E, G) and plt/+ mice (F, H) shows that infectivity is confined to the marginal zone (arrows) and, to some extent, to the T cell zone of plt/+ mice (asterisk). RP: Red pulp; B: B cell zone; T: T cell zone; MZ: Marginal zone; CA: Central arteriole. Magnification A-H: 65-fold, inserts: 110-fold.
5.1.5. Discussion

Chemokines and lymphokines coordinate the development of secondary lymphoid organs and the cellular interactions that generate their distinct functional compartmentalization. In the present study, we have analyzed the role of the coordinate chemokine-driven interaction of DC and naïve T cells in the T cell zone of secondary lymphoid organs for the induction of protective antiviral immune responses. Our experiments with plt/plt mice indicate that normal antiviral T and B cell responses can be generated even in the absence of the T cell zone organized by the constitutive chemokines CCL21-Ser/SLC-Ser and CCL19/ELC. As the genomic locus affected by the plt mutation is rather large (Nakano et al., 1997), it cannot be ruled out, however, that other genes than CCL21a and CCL19 lead to the phenotype observed in plt/plt mice. The morphological similarity of this mouse to the CCR7 knockout mouse (Potsch et al., 1999) strongly suggests, however, that the absence of these chemokines has a major impact on the phenotype of plt/plt mice.

Functional consequences of altered CCR7-SLC/ELC interaction for the induction of antiviral immune responses.

It has been speculated (Cyster, 1999; Sallusto and Lanzavecchia, 2000) that CCR7 and its ligand chemokines CCL19/ELC and CCL21/SLC not only influence T cell and DC homeostasis but also have a fundamental impact on priming and maintenance of immune reactions. This hypothesis is supported by a number of studies describing the function of the CCR7-ELC/SLC system either in vitro (Dieu et al., 1998; Sallusto et al., 1999a) or based on correlations between the in vivo and in vitro migration pattern of T cells (Potsch et al., 1999). In vivo, the ablation of CCR7 expression results not only in severely impaired migration of naïve T cells and activated DC, but also in a reduced immune responsiveness such as decreased contact hypersensitivity (Forster et al., 1999). Furthermore, reduced responsiveness to hapten sensitization after in vivo blockade of CCL21/SLC suggested that selective interference with DC and naïve T cell homing may down-modulate adaptive immune responses (Engeman et al., 2000).

An earlier report on the in vivo significance of the CCL19/CCL21-driven DC and naïve T cell migration for the induction of antiviral immunity showed an enhanced susceptibility of BALB/c-plt/plt mice to mouse hepatitis virus (Gunn et al., 1999b).
BALB/c mice express the MHVR1 gene which renders them susceptible to MHV infection (Ohtsuka and Taguchi, 1997). The divergence in response to MHV among susceptible strains, however, depends mainly on the susceptibility of macrophages (Taguchi et al., 1976; Wijburg et al., 1997) which constitute the primary target of the virus. Since the F4/80+ macrophage compartment appears to be less prominent in spleens and lymph nodes of plt/plt mice (T. Junt, unpublished data), the increased susceptibility of plt/plt mice to MHV may be at least partly due to differences in target cell availability. However, the altered lymphoid microenvironment of plt/plt mice did not influence the replication kinetics of LCMV, which is also a virus targeting macrophages, amongst other APCs.

Protection against LCMV (Kagi and Hengartner, 1996), and Vaccinia virus (Hany et al., 1989) is mediated primarily by CTL. In view of the fact that CTL also control MHV infection (Heemskerk et al., 1995; Lavi and Wang, 1995) and that plt/plt mice showed increased susceptibility to MHV infection, we expected a significant impairment of the protective immunity in plt/plt mice after infection with the non-cytopathic LCMV or the cytopathic Vaccinia Virus. However, both the kinetics of virus-specific CTL, i.e. acute and memory responses, and their function were not significantly altered in plt/plt mice. The major difference between plt/plt and plt/+ control mice was found in the relative proportion of virus-specific CTL in the CD8 compartment (see Fig. 8 and 9). Nevertheless, these differences were leveled out by a slight overshoot in the CTL expansion rate and differences in the cellular distribution between lymphoid compartments. The reduction in T cell numbers is much more severe in lymph nodes than in the spleen of plt/plt mice (Gunn et al., 1999b; Nakano et al., 1997). It is therefore interesting to note that even after splenectomy, plt/plt mice generated equivalent CTL responses compared to plt/+ controls (see Fig. 10). Furthermore, our study describes the quality of humoral responses in plt/plt showing that beside the largely intact T cell responses, plt/plt mice also generated normal antiviral B cell responses.

A recently published study by Mori et al. (Mori et al., 2001) investigated immune responsiveness in plt/plt mice in a model of contact hypersensitivity and described that the plt mutation results in fully functional T cell responses which only differ in their kinetics. Our data corroborate and significantly extend this previous study because we establish that the altered spatial organization of secondary lymphoid T
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Cell zones in plt/plt mice is probably not crucial for initial priming and maintenance of protective T and B cell responses in the course of viral infections.

Where are antiviral immune responses induced when DC and naïve T cell can not meet in the T cell zone?

Complexes of virus-infected cells and specific T cells were found in plt/plt mice only in the marginal zone, whereas these clusters were distributed throughout the white pulp and in the splenic marginal zone of control mice. This suggests that priming of antiviral T cells in plt/plt mice occurred in the marginal zone. Mori et al. (Mori et al., 2001) have shown that immunization of plt/plt mice with ovalbumin was followed by a remodeling of lymphoid organs indicating that T cell responses against non-replicating proteinaceous antigens may also be elicited efficiently in the splenic marginal zone. Our data are also in line with a recent study by Ciavarra et al. (Ciavarra et al., 2000) showing that after selective depletion of phagocytic marginal zone dendritic cells, the remaining interdigitating dendritic cells were able to trap antigen but failed to prime T cell responses against VSV.

Stein et al. have demonstrated (Stein et al., 2000) that peripherally injected CCL21 may accumulate on high endothelial venules of plt/plt mice and thus lead to an enhanced activation of lymphocyte transmigration. As plt/plt mice do express CCL21b outside lymphoid organs, it may be that this peripherally expressed chemokine has some effect on T cell and DC migration into lymph nodes by this mechanism. This may account for the small differences observed between plt/plt and plt/+ mice. However, CCL21 could not detected on the high endothelial venules in this study, but this may have been because this chemokine accumulated at biologically significant levels below the detection level of the anti CCL21-antibody. CCL21b, however, which is still expressed by plt/plt mice is found to a very low level in lymph nodes and spleen (Chen et al., 2002) and may contribute to the functional antiviral immune response in these mice. Although the mechanism of the mildly impaired antiviral immune reactions remains elusive, it is surprising that the morphological absence of a defined T cell zone leads to a rather mild effect on the function of the immune system.

It remains to be resolved whether splenic marginal zone dendritic cells and their lymph node equivalents alone contribute to the priming of antiviral immune responses in plt/plt mice. We found that LCMV antigen in the marginal zone of the
spleen and in the superficial cortex of the lymph nodes was also associated with CD11c-negative cells. It may well be that marginal zone macrophages not only function as “antigen trapping structures” and transmit virus to adjacent dendritic cells, but they may also contribute directly to the priming of antiviral T cells. This notion is supported by previous findings showing that functional marginal zone macrophages are crucial for the induction of anti-LCMV T cell responses (Seiler et al., 1997). Overall, our study supports the concept that the splenic marginal zone and its lymph node equivalent are crucial structures for the rapid generation of antiviral immune responses.

5.1.6. Acknowledgements

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5.2. Part II: Impact of CCR7 on function and distribution of antiviral effector and memory cytotoxic T lymphocytes

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Abbreviations: CCL, CC chemokine ligand; CCR, CC chemokine receptor; DC, dendritic cell; DTH, delayed-type hypersensitivity; GP, glycoprotein; LCMV, lymphocytic choriomeningitis virus; LT, lymphotoxin; PMA, phorbol myristate acetate.
5.2.1. Abstract

The chemokine receptor CCR7 is a key factor in the coordinate migration of T cells and dendritic cells (DC) to and their localization within secondary lymphoid organs. In addition, the expression of CCR7 has been used to define functionally distinct types of memory T cells. Here we show that acutely lymphocytic choriomeningitis virus (LCMV) infected CCR7-deficient mice (CCR7−/−) generated functionally normal and protective responses of cytotoxic T lymphocytes (CTL). However, the overall number of virus-specific CTL was reduced in all lymphoid and non-lymphoid organs tested, suggesting that the absence of CCR7 impacts on the magnitude of the acute virus-specific CTL response. CCR7−/− mice maintained a stable antiviral memory CTL population and rapidly mounted protective CTL responses against a challenge infection with LCMV-recombinant Vaccinia virus. CCR7-deficient and -competent antiviral memory CTL derived from lymphoid or peripheral non-lymphoid organs showed comparable cytolytic activity, but antiviral memory CTL in CCR7−/− mice predominantly accumulated in non-lymphoid organs. These data indicate a role for CCR7 in the efficient expansion of antiviral CTL and the homeostatic recirculation of memory T cells. However, CCR7 appears to be of minor importance for the development of functional effector T cells and the maintenance of central or peripheral memory CTL function.
5.2.2. Introduction

Naïve T cells proliferate and differentiate into effector cells following antigen encounter on professional antigen presenting cells (APC) within secondary lymphoid organs. Acquisition of effector cell function is accompanied by distinct changes in the expression of surface molecules which facilitates T cell emigration from secondary lymphoid organs and their relocation to peripheral tissues (Sprent, 1997). Changes of surface markers normally comprise modulation of adhesion molecules (Oehen and Brduscha-Riem, 1998), as well as inflammatory and constitutive chemokine receptors (Sallusto et al., 1999a).

Following antigen clearance, the effector T cell population rapidly declines leaving a stable memory T cell pool. Hyper-responsiveness of such memory T cells following re-encounter with antigen (Rogers et al., 2000; Veiga-Fernandes et al., 2000) facilitates rapid generation of an effector T cell population. However, it is still a matter of debate as to whether memory T cells are derived only from fully differentiated effector T cells (Jacob and Baltimore, 1999), or whether they represent a mixture of distinct subtypes derived from differentially stimulated precursors (Manjunath et al., 2001).

The chemokine receptor 7 (CCR7) and its ligands CCL19 and CCL21 are crucial for the traffic of T cells and dendritic cells (DC) to and their positioning within T cell zones of secondary lymphoid organs (Forster et al., 1999; Gunn et al., 1999b; Luther et al., 2000b). This is evidenced by the fact that in the absence of CCR7 (Forster et al., 1999) or its ligands (Gunn et al., 1999b) lymphoid T cell zones do not form correctly. Naïve T cells express high levels of CCR7 and its downregulation is closely associated with T cell activation (3, 9). Furthermore, it has been suggested, that memory T cell populations can be functionally distinguished by the presence or absence of CCR7. CCR7\text{high}CD62L\text{high} “central memory” cells that are retained within secondary lymphoid organs are thought to be antigen-experienced nonpolarized cells lacking immediate effector function, whereas CCR7\text{low}CD62L\text{low} “effector memory” cells preferentially migrate to peripheral non-lymphoid organs (Lanzavecchia and Sallusto, 2000; Sallusto et al., 1999b) and express immediate effector function.

Numerous studies support the notion that CCR7 and its chemokine ligands CCL19 and CCL21 also have a fundamental impact on priming and maintenance of immune reactions by influencing T cell and DC migration (Forster et al., 1999; Gunn et al.,...
1999; Engeman et al., 2000). We have recently shown that mice lacking the CCR7 ligands CCL19 and CCL21a mount rapid antiviral T and B cell responses and exhibit normal formation of memory CTL (Junt et al., 2002). In the present study, we used CCR7-deficient mice to further dissect the role of this receptor on the migration and function of antiviral effector and memory cytotoxic T cells in lymphoid (central) and nonlymphoid (peripheral) organs.

Protective immune responses against the non-cytopathogenic lymphocytic choriomeningitis virus (LCMV) depend largely on the induction of antiviral CTL which destroy infected cells in a contact-dependent and perforin-mediated manner (Kagi and Hengartner, 1996). We examined the role of CCR7 during the effector and memory phase of antiviral immune responses using the LCMV model system. Virus-induced LCMV-specific CTL could be detected and quantified in both lymphoid and non-lymphoid organs of CCR7/− mice using tetrameric complexes of MHC class I molecules plus specific peptide ligand (Altman et al., 1996) (Gallimore et al., 1998). Our results suggest that the structural defects of secondary lymphoid organs in the absence of CCR7 impair the maximal expansion of antiviral CTL but indicate that CCR7 is not directly involved in the differentiation process of effector CTL within these organs. Furthermore, the absence of CCR7 does not influence the function of antiviral memory CTL, but crucially affects memory T cell distribution between lymphoid and non-lymphoid organs.

### 5.2.3. Materials and Methods

**Mice**

CCR7/− mice were bred at the Institut für Labortierkunde (University of Zürich, Switzerland) and typed by PCR as described (Forster et al., 1999). All mice were backcrossed onto the 129/Ola background for at least five generation. Experiments consisted of sex-matched groups of CCR7+/− mice and control heterozygous littermates at the age of 8-12 weeks.
Antibodies
Anti-CD8-FITC, anti-CD8-PE, anti-IFN-γ-FITC, anti-CD62L-FITC, anti-CD8-PerCP, anti-CCR5-PE, anti-CD43-PE and Streptavidin-APC were obtained from BD PharMingen (Basel, Switzerland). To assess CCR7 expression, lymphocytes were incubated for 1 h at 4°C with 80 ml COS cell supernatant containing 1 mg/ml CCL19-Ig which was generated as described (Hargreaves et al., 2001) with minor modifications (S. Krautwald, manuscript in preparation). Cells were washed and incubated for 30 min at 4°C with Bio-SP-conjugated goat-anti-human IgG (Fcγ-specific, Jackson Labs, West Grove, PA), followed by Streptavidin-APC and other directly labeled antibodies. If a gp33-tetramer stain (see below) was included, it was performed before addition of the CCL19-Fc supernatant. For PBL samples, erythrocytes were lysed with FACS® Lysing Solution (BD PharMingen). Cells were analyzed with a FACScalibur® flow cytometer using the CellQuest software (BD Biosciences).

Viruses and peptides
Lymphocytic choriomeningitis virus (LCMV), WE strain, originally obtained from Dr. F. Lehmann-Grube (Hamburg, Germany), was propagated on L929 cells at a low multiplicity of infection (MOI) and titrated as previously described (Battegay et al., 1991). Recombinant Vaccinia virus expressing gp33 as a minigene (VV-gp33C) was kindly provided by Dr. Maries van den Broek (University of Zurich, Switzerland) and titrated as described (Hany et al., 1989). The LCMV-GP peptides KAVYNFATM (gp33) and FQPGNGQFI (np396) were purchased from Neosystem (Strasbourg, France).

Cytotoxic T cell response
Specific ex vivo cytotoxicity was determined in a standard ⁵¹Cr release assay as described (Hany et al., 1989). The supernatants of the cytotoxicity assay cultures were counted in a Cobra II Gamma Counter (Canberra Packard, Mississauga, Ontario). Percentage of specific lysis was calculated as (experimental release – spontaneous release)/(total release – spontaneous release) x 100. Spontaneous release was always below 20%.
Isolation of liver, lung and splenic white pulp lymphocytes

Perfused livers were smashed through a metal grid. Lymphocytes were purified by Ficoll (Biochrom, Berlin, Germany) gradient centrifugation (600 x g, 15 min). Lungs were minced with razor blades and incubated in balanced salt solution (BSS) containing 1 mg/ml DNase (Fluka) and 2 mg/ml collagenase I (Sigma) at 37°C for 30 min. Cell aggregates were dispersed by passing the digest through a 18G syringe and lymphocytes were isolated by Ficoll gradient centrifugation. The white pulp compartment of the spleen was isolated by digestion with collagenase V and III as described (Nolte et al., 2000).

Construction of tetrameric class I-peptide complexes and flow cytometry

MHC class I (H-2Db) monomers complexed with gp33 were produced as described (Altman et al., 1996) and tetramerized by addition of streptavidin-PE (Molecular Probes, Eugene, OR). At the indicated time points following immunization, animals were bled and single cell suspensions were prepared from spleen and lymph nodes. Aliquots of 5 x 10^6 cells or 3 drops of blood were stained using 50 ml of a solution containing tetrameric class I-peptide complexes at 37°C for 10 min followed by staining with anti-CD8-FITC (BD Pharmingen) at 4°C for 20 min. The cells were analyzed by flow cytometry gating on viable leukocytes. Absolute cell counts were determined by counting leukocytes in an improved Neubauer chamber and, for blood samples, by using an automated Advia® counter (Bayer, Germany) in the Central Hematology Laboratory of the University Hospital Zürich.

Intracellular cytokine staining

Spleens were removed at the indicated time points following infection with LCMV. Single cell suspensions of 1 x 10^6 splenocytes, lymph node cells, liver or lung lymphocytes were incubated for 5 h at 37°C in 96-well round-bottom plates in 200 ml culture medium containing 25 U/ml IL-2 and 5 mg/ml Brefeldin A (Sigma). Cells were stimulated with phorbolmyristateacetate (PMA, 50 ng/ml) and ionomycin (500 ng/ml) as positive control or left untreated as a negative control. For analysis of peptide-specific responses, 10^6 cells were stimulated with 10^{-6} M gp33 peptide and then surface stained as described elsewhere (Junt et al., 2002). The percentage of CD8^+ T cells producing IFN-γ was determined using a FACScalibur flow cytometer.
5.2.4. Results

**CCR7 on CD8^+ T cells gets modulated during the immune response against LCMV**

Differential expression of CCR7 and CD62L has been reported to characterize effector and memory T cells in both humans (Sallusto et al., 1999b) and mice (Manjunath et al., 2001). We thus assessed the surface phenotype of virus-specific CTL in lymphoid and non-lymphoid compartments following infection of heterozygous CCR7^{+/−} mice with LCMV (Fig. 16). Due to the very low numbers of LCMV-specific CTL in naïve animals, CCR7 and CD62L expression of total CD8^+ T cells was assessed (Fig. 16, upper row). As expected, CD8^+ T cells were uniformly found to be CCR7^{high}, even in peripheral organs such as lung and liver. Furthermore, CD62L was highly expressed on CD8^+ T cells in all organs tested, except the lung where 67% of the cells had down-regulated CD62L.

To characterize the phenotype of LCMV-specific effector CTL, we used MHC class I tetramers complexed with the immunodominant CTL epitope gp33 derived from the LCMV glycoprotein (tet-gp33) (Altman et al., 1996; Gallimore et al., 1998). During the peak CTL response against LCMV (day 8 post infection), CD8^+ tet-gp33^+ effector CTL from peripheral organs, such as liver and lung, were largely found to be CD62L^{low}CCR7^{low} (Fig. 16, middle row). Surprisingly, an additional CD62L^{high}CCR7^{high} population of gp33-specific CTL could be observed in spleens and lymph nodes, possibly representing CD8^+ T cells that had not yet undergone complete differentiation to the CTL effector state. In memory mice (day 80 post infection), two populations, “central memory”-like CD62L^{high}CCR7^{high} and “effector memory”-like CD62L^{low}CCR7^{low}, CD8^+ tet-gp33^+ CTL were found in spleen, blood, liver and lung (Fig. 16, lower row). Cells of the CD62L^{low}CCR7^{low} phenotype were largely excluded from lymph nodes and in the splenic white pulp suggesting that the entry of memory CTL into lymph nodes and the splenic white pulp relies on the expression of CD62L and/or CCR7.
Figure 16. Phenotypic characterization of naïve, effector and memory CTL in spleen, mesenteric lymph node, liver, lung, and blood during the course of LCMV infection.

Expression of CD62L and CCR7 was examined on CD8-positive lymphocytes from naïve heterozygous CCR7+/+ mice (upper row), and from gp33-specific CD8+ T cells on days 8 (effector, middle row) and 80 (memory, bottom row) after i.v. infection with LCMV. Representative plots are shown with mean percentages derived from at least 3 mice.

**CCR7−/− mice display a reduced clonal burst of effector CTL**

CCR7−/− mice show aberrantly formed lymphoid T cell zones, strongly reduced T cell numbers in lymph nodes and homing defects of DC and naïve T cells (Forster et al., 1999). To assess the functional significance of this phenotype for the generation of acute LCMV-specific CTL responses, CCR7−/− and CCR7+/+ mice were infected with 200 pfu LCMV-WE and the number and surface phenotype of LCMV-specific, tet-gp33-positive cells in the CD8 T cell pool was determined in the indicated organs by flow cytometry (Fig. 17). In all organs tested, CCR7−/− mice displayed a clear reduction in relative (Fig. 17A) and absolute numbers of CD8+tet-gp33+ CTL (Fig. 17B). However, the distribution of effector CTL in CCR7−/− mice resembled that observed in CCR7+/+ mice, indicating that the absence of CCR7 did not lead to an aberrant localization of effector CTL during the acute phase of infection.
Figure 17. Effect of CCR7-deficiency on CTL clonal burst following LCMV infection. 

(A and B). Eight days after LCMV infection, lymphocytes were isolated from spleen, mesenteric lymph node (MLN), liver, lung, and blood and analyzed for expression of CD8 and for reactivity with gp33-tetramer. Values in (A) represent the percent of CD8+ T cells staining positive for the gp33 tetramer and (B) represent absolute numbers of tet-gp33+CD8+ cells ±SD in the indicated organ (n=6). Pooled data from two separate experiments are shown. (C) Expression of different activation markers on tet-gp33+CD8+ cells from acutely LCMV infected CCR7−/− (thick line) and CCR7+/+ (thin line) mice are shown.

Furthermore, the surface phenotype (CD62LlowCD44highCCR5lowCD43high) of gp33-specific effector CTL from CCR7−/− and CCR7+/+ mice was identical (Fig. 17C). These data suggest that the lack of CCR7 plays a role in determining the overall magnitude of antiviral effector CTL responses, but does not affect effector CTL localization or differentiation.
**Results**

**Figure 18. Induction of functional LCMV-specific effector CTL in the absence of CCR7.**

(A) Lymphocytes were isolated from the indicated organs and analyzed for cytolytic activity in a 5 h $^{51}$Cr release assay at day 8 post infection. *Ex vivo* CTL activity of lymphocytes from spleen and mesenteric lymph node from CCR7$^{-/-}$ (open symbols) and CCR7$^{+/+}$ (closed symbols) mice was tested on $^{51}$Cr-labeled EL4 cells pulsed with gp33. E:T values were corrected for the number of tet-gp33$^{+}$CD8$^{+}$ cells (see Fig 17A) and indicate mean ± SD of 3 to 4 mice per group. (B) CD8$^{+}$ T cells from the indicated organs of CCR7$^{-/-}$ (black line) and CCR7$^{+/+}$ mice (grey area) were gated by flow cytometry and analyzed for LCMV-gp33 specific IFN-γ-production at day 14 post infection. Values indicate means ±SD of 3 mice. Data shown in A and B is from one experiment and is representative of two separate experiments. (C) LCMV-titers were determined in different organs on days 4, 8, and 13 following infection with 200 pfu LCMV-WE. Values represent organ titers of individual mice. Pooled data from three separate experiments are shown.

**CTL induced in the absence of CCR7 exert normal effector function**

To further substantiate the finding that the differentiation of effector CTL is independent of CCR7, the cytolytic activity of LCMV-specific CTL on the single cell level was assessed using a standard chromium release assay. Direct cytolytic activity of CCR7$^{-/-}$ was comparable to CCR7$^{+/+}$ mice following normalization of the values for the numbers of tetramer-positive cells present in spleen and lymph node cell preparations (Fig. 18A). Moreover, IFN-γ production by individual gp33-specific CD8$^{+}$ T cells in response to cognate peptide was not impaired by the lack of CCR7 when assessed on day 14 post infection (Fig. 18B).
Unlike in CCR7\textsuperscript{+/-} mice, LCMV was not eliminated from lymphoid and non-lymphoid organs of CCR7\textsuperscript{-/-} mice on day 8 post infection (Fig. 18C), most probably due to the reduced numbers of effector CTL in these mice. However, virus was eventually cleared from these mice, supporting the notion that CCR7-deficient CTL can display full effector function and are able to mediate antiviral protection.

\textit{Memory T cells in the absence of CCR7 are aberrantly distributed but protective}

We next set out to examine the role of CCR7 in the distribution of memory CTL following viral infection. For this purpose we determined the absolute numbers of gp33-specific CTL in different lymphoid and non-lymphoid organs during the memory phase of the LCMV infection. Absolute numbers of gp33-specific memory CTL at day 80 post infection were lower in all organs of CCR7\textsuperscript{-/-} mice compared to CCR7\textsuperscript{+/-} mice (Fig. 19A-E, first data point). Comparison of these values with the expansion of CTL during the acute phase of LCMV infection, (Fig. 17B) and on day 40 post infection (data not shown), indicated that CTL memory responses in CCR7\textsuperscript{-/-} mice did not decline more rapidly than in CCR7\textsuperscript{+/-} mice. Indeed, plotting the memory (day 80) values as percent of the initial clonal burst (day 8) of gp33-specific CTL revealed that numbers of memory CTL in CCR7\textsuperscript{-/-} mice were decreased in lymph nodes (Fig. 19F, upper panel), but increased in spleen, blood and non-lymphoid organs. These data indicate that the absence of CCR7 results in an altered memory T cell distribution.
Figure 19. Distribution of LCMV-specific memory CTL in CCR7-deficient mice.

The first data point in A – E indicates the number gp33-specific CTL ± SD for (A) spleen, (B) mesenteric lymph nodes, (C) liver, (D) lung, and (E) blood on day 80 following infection of CCR7-/- (filled triangles) and CCR7+/+ mice (open squares) with 200 pfu LCMV-WE. The second data point in A – E (day 85 after primary LCMV infection) indicates the expansion of tet-gp33+CD8+ memory cells ± SD on day 5 following i.v. challenge infection with 2x10⁵ pfu VV-gp33C. Data shown are from one experiment and are representative of two separate experiments using 3 mice per group. (F) Relative changes in the distribution of tet-gp33+CD8+ memory T cells in CCR7-/- (black bars) and CCR7+/+ (open bars) mice on day 80 following primary LCMV infection (upper panel) and on day 5 post challenge infection with VV-gp33C (lower panel). The absolute number of gp33-specific CTL on day 8 post LCMV infection was set as 100%.

We next tested whether CCR7-/- mice were still able to mount a protective response against secondary infection with virus. For this purpose, LCMV-immune CCR7-/- and CCR7+/+ mice were intravenously challenged with 2 x 10⁶ pfu VV-gp33C on day 80 following primary infection. The second data point in Fig. 19A-E indicates the expansion of CCR7-/- and CCR7+/+ memory CTL following challenge infection. In all organs except lymph nodes, gp33-specific CTL lacking CCR7 expanded to almost identical or even increased numbers if compared with CCR7-competent gp33-specific CTL (Fig. 19A-E, second data point) and both groups of mice had completely eliminated the Vaccinia virus already by day 3 post challenge (data not shown). Thus, memory CTL from CCR7-/- mice demonstrated a high protective antiviral
capacity. Interestingly, CCR7-deficient memory CTL remained mostly excluded from lymph nodes (Fig. 19B and 4F, lower panel) even following secondary infection with VV-gp33C.

**CCR7 expression does not correlate with the activity of antiviral memory CTL from lymphoid versus non-lymphoid organs**

Memory CTL from peripheral non-lymphoid organs exert rapid effector function (Masopust et al., 2001) and are thought to provide a mechanism for rapid pathogen containment. It has been suggested that the presence of such "effector memory" T cells in peripheral organs is associated with loss of CCR7 expression and gain of effector function (Sallusto et al., 1999b). We therefore assessed the immediate IFN-γ production of CCR7-deficient or –competent memory CTL from different lymphoid and nonlymphoid organs on day 80 following infection with LCMV-WE after a short-term in vitro stimulation with gp33. Both CCR7−/− and CCR7+/− gp33-specific memory CTL from all organs tested readily produced IFN-γ (Fig. 20A). This data indicate that memory CTL from both lymphoid and non-lymphoid organs can mount immediate effector function, and that this function is not dependent on CCR7 expression. Although the absolute numbers of gp33-specific memory CD8+ T cells producing IFN-γ after short-term peptide stimulation was decreased in CCR7−/− mice if compared to CCR7+/− mice (Fig. 20B), these numbers did not significantly differ from the values obtained for the respective groups by tetramer analysis (compare Fig. 19A-E). When memory CTL from lymphoid versus peripheral organs were tested in a 5 h 51Cr-release assay, both memory CTL from spleens and lungs of CCR7−/− mice and CCR7+/− controls showed efficient target lysis (Fig. 20C). Furthermore the expression of the cell surface markers CD44, CD62L, CCR5 and the activation-associated isoform of CD43 (1B11) was similar on gp33-specific memory CTL from lymphoid and non-lymphoid organs of CCR7−/− mice and CCR7+/− (Fig. 20D). Taken together, these data further support the notion that CCR7 is important for the distribution of memory CTL in lymphoid and non-lymphoid organs, but that CCR7 expression it does not correlate to their functional differentiation.
Figure 20. Analysis of LCMV-specific memory responses in CCR7$^{-/-}$ mice.

(A, B) Relative and absolute numbers of CD8$^+$ T cells producing IFN-$\gamma$ after short-term in vitro restimulation with gp33 were assessed for spleen, mesenteric lymph node, liver, and lung on day 80 following i.v. infection of CCR7$^{-/-}$ (black bars) and CCR7$^{+/+}$ mice (white bars) with 200 pfu LCMV-WE. Numbers in (A) represent percent of IFN-$\gamma$ positive cells of CD8$^+$ T cells ± SD. (C) Ex vivo CTL activity of lymphocytes isolated from spleen and lung of CCR7$^{-/-}$ (squares) and CCR7$^{+/+}$ (triangles) mice, as assessed by lysis of $^{51}$Cr-labeled EL4 cells pulsed with gp33 (closed symbols) or unpulsed control cells (open symbols). Data indicate values derived from pooled samples of 3-6 mice. E:T ratios were adjusted for percentage of CD8$^+$/tet-gp33$^+$ cells to compensate for the reduced numbers of gp33-specific CTL in CCR7$^{-/-}$ mice. Data from one experiment are shown and are representative of two similar experiments. (D) T cell activation markers on tet-gp33$^+$CD8$^+$ cells from LCMV memory mice were assessed by flow cytometry as described (CCR7$^{-/-}$, thick line, CCR7$^{+/+}$, thin line).

5.2.5. Discussion

The role of CCR7 in the induction and maintenance of antiviral effector and memory CTL responses was examined in CCR7$^{-/-}$ mice using LCMV-specific tetramers and
analysis of effector function on a single cell level. These experiments revealed that CCR7-deficiency mainly impacts on the magnitude of virus-specific effector and memory CTL responses, and the distribution of these cells between lymphoid and non-lymphoid compartments. In contrast, the function of effector and memory CTL from CCR7-deficient mice was functionally normal on the single cell level. Together, these data indicate that CCR7 is crucial for the coordinate migration and expansion of antiviral effector and memory CTL, but not for their differentiation or function.

In vitro studies using polarized T cells, or T cell lines, have shown a correlation between effector function and CCR7 expression (Sallusto et al., 1999a) (Roman et al., 2002). In accordance with these findings we found that CCR7 is highly expressed on naïve CTL in lymphoid and non-lymphoid organs, and that virus-specific CTL in blood and peripheral organs of mice had down-regulated CCR7 during acute virus infection. However, it is interesting to note that a significant proportion of LCMV-specific CTL in spleen and lymph nodes had not lost CCR7 and CD62L expression during the effector phase yet retained normal effector function. These data corroborate a recent study by Unsoeld et al. (Unsoeld et al., 2002) suggesting that during the acute phase of an antiviral immune response both CCR7-positive and -negative CTL from secondary lymphoid organs can exert full effector function. Thus it appears that the CCR7\textsuperscript{low} phenotype corresponds to acute effector function in peripheral organs but not in secondary lymphoid organs. During the memory phase of an anti-LCMV immune response, a significant proportion of specific CTL retained in peripheral organs had down-regulated both CCR7 and CD62L, whilst those found in lymph nodes and splenic white pulp exhibited mainly the "central memory"-like CD62L\textsuperscript{high}CCR7\textsuperscript{high} phenotype. Our in vivo data are therefore compatible with the concept that CD62L\textsuperscript{low}CCR7\textsuperscript{low} "effector memory" CTL reside primarily in the periphery and that CD62L\textsuperscript{high}CCR7\textsuperscript{high} "central memory" CTL localize mainly to secondary lymphoid organs. However, the observed functional capacity of those cell subsets differ from that proposed in the original model of central versus effector memory (Sallusto et al., 1999b). Antiviral CTL from both peripheral non-lymphoid and secondary lymphoid organs rapidly produced IFN-γ following specific restimulation, and displayed significant cytolytic activity. This confirms previous studies showing that immediate effector function of memory T cells is not strictly associated with down-regulation of CCR7 (Kim et al., 2001; Roman et al., 2002; Unsoeld et al., 2002).
We have shown here that CCR7 plays a key role in homing of antiviral memory CTL. It does, however, not seem that the strict differentiation between resting central vs. active effector memory T cells based on their CCR7 expression indeed reflects function in a complex \textit{in vivo} situation as for example during a viral infection.

CCR7-deficiency has been shown to result in impaired T cell responses, such as the complete abolishment of delayed type hypersensitivity (DTH) reactions against FITC or KLH after a short (4 day) priming period (Forster et al., 1999). DTH reactions depend strongly on DC-mediated antigen transport from the site of inoculation to secondary lymphoid organs. Thus we consider it likely that severely impaired DTH reactions in CCR7$^{-/-}$ mice result from a nearly complete blockade of DC migration from skin to local lymph nodes. In contrast, in our studies at least part of the viral inoculum may reach secondary lymphoid organs independently from DC trafficking (e.g. via the bloodstream or the lymphatic route).

CCR7-deficient mice and plt/plt mice which lack the CCR7-ligand chemokines CCL21-Ser and CCL19 within secondary lymphoid organs (Gunn et al., 1999b) show a similar morphological phenotype of lymph nodes and spleen. However, it is interesting to note that plt/plt mice are able to mount nearly unimpaired DTH reactions (Mori et al., 2001) and antiviral CTL responses (Junt et al., 2002). Plt/plt mice express the leucine-isoform of CCL21 outside lymphoid tissues in lymphatic vessels (Luther et al., 2000; Chen et al., 2002) and the gradient of this chemokine may mediate some limited CCR7-dependent migration of mature DC and transport of antigen towards secondary lymphoid organs, whilst DC of CCR7$^{-/-}$ mice remain sequestered in peripheral tissues. The drastically reduced availability of mature, antigen-presenting DC and the reduced number of CTL precursors in secondary lymphoid organs of LCMV-infected CCR7$^{-/-}$ mice thus could be the critical factors resulting in decreased recruitment of LCMV-specific CTL to the immune response. Nevertheless, it is noteworthy that fully functional antiviral effector CTL can be primed in the complete absence of organized T cell zones in CCR7$^{-/-}$ mice as well as in plt/plt mice (this study and Junt et al., 2002). As in plt/plt mice, an alternative priming site for antiviral CTL in CCR7$^{-/-}$ mice (i.e. the compartment where infected DC and virus-specific CTL interact) is most likely the marginal zone of the spleen or the marginal sinus of lymph nodes (Junt et al., 2002).

CCR7-mediated lymphocyte and DC migration is closely linked to lymphoid organogenesis. For example, ectopic expression of CCL21 (Fan et al., 2000) in
pancreatic islet cells results in formation of organized secondary lymphoid structures, a process which is dependent on signals transmitted via the lymphotoxin (LT) beta receptor (Luther et al., 2002). It is interesting to note that LTα-deficient and LTβ-deficient mice, which have a severely impaired lymphoid microstructure, generate diminished T cell responses following LCMV infection and show delayed viral clearance (Berger et al., 1999; Suresh et al., 2002), whilst the function of antiviral effector T cells in LTα and LTβ-deficient mice is not affected at the single-cell level. There is a close correlation between the structural defect of secondary lymphoid organs and clonal expansion of CTL following virus infection, although a non-morphological factor of LT might also contribute to this effect. In mice with less profound structural alterations such as CCR7+/− or plt/plt mice, the same effect is observed but appears to be milder.

In summary, our data underscores the importance of organized lymphoid structures for the generation of primary immune responses, and further delineates the role of CCR7-mediated lymphocyte and DC migration in the induction and maintenance of antiviral immunity.

5.2.6. Acknowledgements

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5.3. Part III: Impaired immunoglobulin class switch in CXCR5−/− mice
– evidence for a defect in T helper cells

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5.3.1. Abstract

The chemokine receptor CXCR5 and its ligand CXCL13 (BLC) define the follicular structure within secondary lymphoid organs. CXCR5 is expressed by naïve B cells and by activated follicular T helper cells. We here show that CXCR5 deficient mice (CXCR5<sup>−/−</sup>) have normal lymphoid B cell compartments but are functionally impaired in the T help-dependent phase of the B cell response. Following immunization of CXCR5<sup>−/−</sup> mice with the glycoprotein of Vesicular stomatitis virus (VSV), no immunoglobulin class switch is observed. Similarly, immunization of CXCR5<sup>−/−</sup> mice with UV-inactivated VSV or infection with lymphocytic choriomeningitis virus (LCMV) led to lower IgG levels. Infection with live VSV, however, resulted in a complete class switch and the formation of morphologically normal but aberrantly localized germinal centers, and an impaired maintenance of long-term IgG titers. The help-dependent phase of the immune response against LCMV or VSV correlated to a strong induction of CXCR5<sup>+</sup>CD4<sup>+</sup> follicular helper T cells. In line with these data, CXCR5<sup>−/−</sup> mice had a limited capacity to expand and retain specific T helper cells within their spleens limiting the availability of T help for B cell responses. We thus provide evidence that the CXCR5 is critically involved in providing sufficient numbers of T helper cells within the white pulp areas, which is particularly important in augmenting immunoglobulin levels when antigen becomes limiting.
5.3.2. Introduction

It is one of the paradigms in B cell immunology that those antigens induce good immunoglobulin responses, which spatially link antigens for T helper cells and B cells. This implicates a close collaboration of T and B cells for immunoglobulin production. After the discovery of receptor pairs such as OX40-OX40L (Stuber and Strober, 1996) and CD40-CD40L (Oxenius et al., 1996) which enhance T-B collaboration, more recent studies focused on the anatomical site of T-B interaction, mostly by adoptive transfer studies (Garside et al., 1998; Pape et al., 1997) and in vivo microscopy (Miller et al., 2002). T cells and B cells relocate after their activation by modulation of chemokine receptor expression levels (Hargreaves et al., 2001; Schaeerli et al., 2000). One chemokine receptor of particular interest in this context is CXCR5, which ligates CXCL13/BLC (B Lymphocyte Chemoattractant, Moser et al., 2002). CXCL13 is expressed by B cell follicles (Gunn et al., 1998), and CXCR5 is expressed by naïve B cells and a subset of CD4+ helper T cells (Forster et al., 1996). Therefore, CXCR5 was suggested to be involved in T-B collaboration during immunoglobulin class switch and affinity maturation. It came as a surprise, however, that affinity maturation is normal in the absence of CXCR5 (Voigt et al., 2000). The functional relevance of CXCR5 during T-dependent antibody responses in vivo, is not yet consistently clear. After initial activation of CD4+ T cells, they start to express CXCR5 and move towards B cell follicles (Ansel et al., 1999). CD4+CXCR5+ T helper cells were characterized in vitro to enhance the production of IgG and IgA but not of IgM (Breitfeld et al., 2000; Schaeerli et al., 2000). Due to their potential to localize in B cell follicles, they were termed “follicular helper T cells”. The apparent involvement of CXCR5+ T helper cells in the early phase of an immune response, however, was in sharp contrast to the findings in CXCR5-/- mice which produce normal levels of switched Ig isotypes in vivo following immunization with DNP-KLH in adjuvant (Forster et al., 1996).

To better understand the role of CXCR5 in T-dependent B cell responses in vivo, we immunized CXCR5-/- mice with the WE strain of LCMV and with different preparations of VSV (Bachmann and Zinkernagel, 1996). VSV is a cytopathogenic rhabdovirus, which elicits a potent B cell response directed against a single neutralizing glycoprotein (G) epitope. The quasicrystalline structure of VSV accounts for an
immediate, largely, T-independent induction of neutralizing IgM after trapping of the
virus in the marginal zone (Ochsenbein et al., 1999a) followed by a later phase of T-
dependent IgG production. An attenuated preparation of live VSV is obtained by UV-
irradiation. It maintains its highly ordered structure, but is no longer replicative.
Recombinant VSV-G protein is an even weaker antigen, being neither replicative nor
ordered.

In this study we show that the capacity of CXCR5− mice to mount T-dependent
antibody responses is strongly impeded if the antigenic impact is low, i.e. following
immunization with non-replicating and/or weakly organized antigen. This could be
attributed to the limited capacity of CXCR5− to retain high numbers of specific CD4+
T cells within their spleens.
5.3.3. Materials and Methods

Mice
CXCR5−/− mice were bred at the Institut für Labortierkunde (University of Zürich, Switzerland). They were backcrossed to 129Sv 9 times and used for experiments in sex-matched groups with 129Sv mice at the age of 8-12 weeks. CXCR5−/− mice were typed by PCR as described (Forster et al., 1996).

Viruses, peptides and immunizations
Lymphocytic choriomeningitis virus (LCMV), WE strain, originally obtained from Dr. F. Lehmann-Grube (Hamburg, Germany), was propagated on L929 cells at a low multiplicity of infection (MOI) and was plaqued as described (Battegay et al., 1991). Vesicular Stomatitis virus, Indiana strain (VSV-IND, Mudd-Summers isolate) and New Jersey strain (VSV-NJ, Pringle isolate), were originally obtained from Prof. D. Kolakofsky (University of Geneva, Switzerland). VSV strains were propagated on baby hamster kidney 21 (BHK-21) cells and plaqued on Vero cells. For some experiments, UV-inactivated VSV-IND was obtained using UV irradiation (7UV 15W, Philips) for 5 min in a thin layer of liquid in a 60 mm petri dish. Recombinant vaccinia virus expressing the glycoprotein of VSV IND (VV-IND-G) was a generous gift of Dr. B. Moss, Laboratory of Viral Diseases, National Institutes of Health (Bethesda, MD, Mackett et al., 1985). Vaccinia virus was grown at a low multiplicity of infection on BSC 40 cells and plaqued on BSC 40 cells. Recombinant VSV-glycoprotein (VSV-G) was obtained from a culture of Spodoptera frugiperda 9 (Sf9) cells after infection with a recombinant baculovirus (Bailey et al., 1989). VSV-p8 (SSKAQVFEPHEPHIQAASQL) and LCMV-np309 peptide (SGEGWPIACRTSVEVGRAWE) were obtained from Neosystems (Strasbourg, France). For some s.c. immunizations, 20 μg VSV-G was injected together with 50 μg cholera toxin (CT). For p.o. immunizations, 1 mg ovalbumin (OVA) was gavaged in 15 μg CT three times at 10 day-intervals. Four days after the last gavage, serum and gut lavages were taken and tested by ELISA for specific and total IgA levels. For CD8-depletion, mice were injected with 1 mg of the anti-CD8 antibody YTS169.4 on day 3 and 1 prior to infection.
Antibodies and flow cytometry
B220-PerCP, anti-CD44-FITC, anti-CXCR5-PE, anti-CD4-PE, anti-CD4-APC, anti-B220-FITC and rat IgG2a-PE were purchased from BD Pharmingen. Aliquots of 5 x 10^5 cells or 3 drops of blood were stained at 4°C for 20 min and the cells were analyzed by flow cytometry gating on viable leukocytes. Before analysis of PBL, erythrocytes were lysed with FACS Lysing Solution (BD PharMingen). Cells were analyzed with a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA) using the CellQuest software (BD Biosciences).

ELISA for detection of IgA and serum antibodies against LCMV-NP
The LCMV nucleoprotein-specific enzyme-linked immunosorbent assay (ELISA) using LMCV-NP expressed by Sf9 cells after infection with a recombinant baculovirus was performed as described previously (Junt et al., 2002). The ELISA for CT-, OVA- and total IgA was performed as described (Macpherson et al., 2001), using a coating concentration of 1 mg/ml anti-mouse-IgA (Sigma), ovalbumin or cholera toxin in coating buffer.

VSV-specific serum neutralization test
Neutralizing antibody titers of sera were determined as described (Roost et al., 1990). Sera were prediluted 40-fold in supplemented MEM and heat-inactivated for 30 min at 56°C. Serial twofold dilutions were mixed with equal volumes of virus diluted to contain 500 plaque forming units (pfu)/ml. The mixture was incubated for 90 min at 37°C in an atmosphere containing 5% CO_2. One hundred μl of the serum-virus mixture were transferred onto Vero cell monolayers in 96-well plates and incubated for 1 h at 37°C. The monolayers were then overlaid with 100 μl DMEM containing 1% methyl cellulose. After incubation for 24 h at 37°C the overlay was removed and the monolayer was fixed and stained with 0.5% crystal violet. The highest dilution of the serum that reduced the number of plaques by 50% was taken as the neutralizing titer. To determine IgG titers, undiluted serum was first pre-treated with an equal volume of 0.1 M 2-ME in saline.
**Immunohistochemistry**

Freshly removed organs were immersed in HBSS and snap frozen in liquid nitrogen. Tissue sections of 5 µm thickness were cut in a cryostat, placed on siliconized glass slides, air dried, fixed with acetone for 10 min, and stored at -70°C. Secondary affinity-purified polyclonal anti-lg antisera were diluted in TBS (pH 7.4) containing 5% normal mouse serum. All other dilutions were made in TBS alone. Incubations were done at room temperature for 30 min; TBS was used for all washing steps. Alkaline phosphatase was visualized using naphthol AS-BI phosphate and New Fuchsin as substrate, which yields a red precipitate. Only for the detection of VSV-Ag-coupled alkaline phosphatase the more sensitive NBT/BCIP reaction was used. Endogenous alkaline phosphatase was blocked by levamisole. All color reactions were performed at room temperature for 15 min with reagents from Sigma (St. Louis, MO). Sections were counterstained with hemalum. Coverslips were mounted with glycerol and gelatin.

**LCMV-specific serum neutralization assay**

LCMV-specific neutralizing activity was measured from serum of infected mice using a focus reduction assay (Battegay et al., 1991). The neutralizing titer was defined as the dilution causing half-maximal reduction of plaques of LCMV when compared with the same amount of virus incubated with control sera from uninfected mice. Neutralizing IgG titers were determined after reduction of the serum with 0.1M β-mercaptoethanol.

**Intracellular cytokine stain**

Spleens were removed at the indicated time points following infection with LCMV. Single cell suspensions of 1 × 10⁶ splenocytes were incubated for 6 h at 37°C in 96-well round-bottom plates in 200 µl culture medium containing 1 µg/ml Brefeldin A (Sigma). Cells were stimulated with phorbolmyristateacetate (PMA, 50 ng/ml) and ionomycin (500 ng/ml) as positive control or left untreated as a negative control. For analysis of peptide-specific responses, 10⁶ cells were stimulated with 10⁻⁶ M p8 or np309 peptide and then surface stained with anti-CD4-PE and anti-B220-FITC (Pharmingen) in FACS-buffer (PBS+ 2% FCS + 20 mM EDTA + 0.03% NaN₃) for 20
min at 4°C. Splenocytes were washed twice with FACS-buffer, fixed with 100 μl 4% paraformaldehyde in PBS for 10 min at 4°C and washed twice with 2 ml of permeabilization buffer (FACS-buffer + 0.1% saponin). Cells were then stained intracellularly with anti-IFNγ-FITC (AN18, Metlay et al., 1990) in permeabilization buffer for 30 min at 4°C. Cells were washed twice with permeabilization buffer, and the percentage of IFNγ-producing cells was determined after gating on FITC-negative cells using a FACScan flow cytometer.

5.3.4. Results

**CXCR5**−/− mice have normal lymphoid B cell compartments and can trap VSV in the splenic marginal zone.

CXCR5 is expressed by naïve B cells (Forster et al., 1996) and ligation of CXCR5 can lead to integrin activation during lymphoid organ ontogeny (Finke et al., 2002). It was therefore necessary to examine the various lymphoid B cell compartments in naïve CXCR5−/− mice before infecting them with virus. Absolute numbers of B220+ B cells were comparable in CXCR5−/− mice and 129Sv controls in all lymphoid organs checked (Fig. 21A). As naïve CXCR5−/− mice have an altered morphology of the spleen, but not of lymph nodes, we next analyzed the composition of splenic B cell pools. Frequencies of marginal zone B cells (MZB) and follicular B cells (FB) were comparable in spleens of CXCR5−/− mice and 129Sv controls (Fig. 21B,C). Next, we wanted to assess the surface expression of IgM on MZB cells, because it was initially reported that it was higher in CXCR5−/− mice than in wild type controls (Forster et al., 1996). We found, however, comparable levels of IgM not only on the surface of MZB cells, but also in serum of CXCR5−/− and 129Sv mice (Fig. 21D and data not shown). We then assessed the expression levels of various integrins, CD18, CD29, LFA-1 (Fig. 21E-G) and α4β7 integrin (data not shown) on FB cells and MZB cells of CXCR5−/− and 129Sv mice and did not find a significant difference. From these data we conclude that, although CXCR5 was described to have a profound impact on B cell recirculation, all lymphoid B cell compartments of CXCR5−/− mice are normal.
Following infection with VSV, the virus gets trapped at the marginal zone of the spleen mediated by natural antibodies and complement (Ochsenbein et al., 2000b). Natural antibody titers were normal in CXCR5−/− mice (data not shown) and VSV gets conveyed to the marginal zone of the spleen 24 hours after infection as in 129Sv controls (Fig. 21H, I). This shows that the initial phase of the VSV infection occurs normally in CXCR5−/− mice.
CXCR5<sup>-/-</sup> mice form morphologically normal germinal centers at ectopic sites

The late phase of VSV infection is characterized by formation of VSV-specific germinal centers (Bachmann et al., 1996).

Figure 22: Induction of germinal centers in CXCR5<sup>-/-</sup> mice

Spleen sections of 129Sv and CXCR5<sup>-/-</sup> mice were analysed for the presence of (A,B) VSV-specific B cells, (C,D) for PNA<sup>+</sup> regions, (E,F) for CD4<sup>+</sup> T cells and (G,H) for 4C11<sup>+</sup> FDC 10 days following infection with 2x10<sup>6</sup> pfu VSV-IND i.v.. Note that CD4<sup>+</sup> T cells enter the germinal center areas in both mouse strains (Fig. 1E,F, arrowheads). Magnification 65x.

On day 10 following infection with 2x10<sup>6</sup> pfu VSV-IND, CXCR5<sup>-/-</sup> mice formed VSV-specific, PNA-positive germinal centers ectopically around the central arteriole (Fig. 22). CXCR5<sup>-/-</sup> mice contained CD4<sup>+</sup> T helper cells in their germinal centers (Fig. 1E,F, see arrowheads), which argues for the fact that T helper cells do not need to express CXCR5 to enter germinal centers. In addition, these data confirm that CXCR5 is not necessary for the induction of morphologically normal germinal centers.
Normal TI and TD B cell responses in CXCR5\textsuperscript{−} mice following infection with VSV-IND

To evaluate the consequences of impaired T and B cell homing via CXCR5 for TI and TD B cell responses, CXCR5\textsuperscript{−} mice and 129Sv mice were infected with 2x10\textsuperscript{6} pfu VSV-IND i.v. and neutralizing antibody titers were assessed from serum at various timepoints (Fig. 23A). Interestingly, the TI and the TD response were comparable in both mouse strains, indicating that the heavily damaged follicular architecture in CXCR5\textsuperscript{−} mice did not have a functional consequence for T-B collaboration after this virus infection. The VSV-specific neutralizing IgG titers, however, declined more rapidly in CXCR5\textsuperscript{−} mice during day 50 and day 220 following infection, whilst 129Sv controls maintain about 16-fold higher stable IgG levels over time. The weaker decline of IgG levels in 129Sv mice could not be caused by a prolonged persistence of the virus in these mice because even 5x10\textsuperscript{8} pfu VSV-IND i.v. became cleared already by day 4 post infection from CXCR5\textsuperscript{−} mice and 129Sv controls (data not shown). It could well be, however, that the aberrant follicular structure of CXCR5\textsuperscript{−} mice was responsible for this decline in the late phase of the response. After a secondary immunization with 2x10\textsuperscript{6} VSV-IND i.v., the recall IgG titers reached comparable levels in CXCR5\textsuperscript{−} mice and controls.

Non-replicating and structurally irregular antigens lead to an impaired class switch in CXCR5\textsuperscript{−} mice

UV-inactivated VSV or recombinant VSV-G are non-replicating VSV antigens with UV-VSV still maintaining a high level of structural organization. Both antigens, if administered i.v., induce a strong TI-neutralizing IgM response (Fig. 23B,C), reflecting no functional defect in direct B cell activation in CXCR5\textsuperscript{−} mice if compared to Sv129 controls. In contrast, CXCR5\textsuperscript{−} mice were not able to efficiently switch their immunoglobulin isotype to IgG following these immunizations. A similar impairment in immunoglobulin class switch was observed for CXCR5\textsuperscript{−} mice following infection with a vaccinia virus recombinant for VSV-G of the Indiana strain (VV-IND-G, data not shown). We thus concluded that CXCR5\textsuperscript{−} mice have a defect in the induction of TD immune responses.
Results

Figure 23: Tl and TD B cell responses in CXCR5⁻/⁻ mice
Neutralizing antibody titers against VSV-IND (A) following i.v. infection with 2x10⁶ pfu VSV-IND. On day 260 after primary infection, mice were re-infected with 2x10⁶ pfu VSV-IND, (B) following i.v. immunization with 10⁸ pfu UV-VSV, (C) following i.v. immunization with 20 μg VSV-G, (D) following s.c. immunization with 20 μg VSV-G. Neutralizing titers against LCMV-WE (E) following depletion of CD8⁺ T cells and i.v. infection with 200 pfu LCMV-WE. Data points are representative three animals per group.

It has been shown that IgM responses to VSV-G become T-dependent following s.c. immunization (Ochsenbein et al., 2000a). When CXCR5⁻/⁻ mice and 129Sv controls were immunized s.c. with 20 μg VSV-G, CXCR5⁻/⁻ mice even failed to induce IgM, proving that the T helper cell defect in CXCR5⁻/⁻ mice does not merely relate to the induction of IgG (Fig. 23D). LCMV-WE is another viral antigen that induces TD neutralizing antibodies (Planz et al., 1997). To test CXCR5⁻/⁻ mice for their ability to produce IgG in this system, we depleted them of CD8⁺ T cells and infected them with 200 pfu LCMV-WE i.v.. As expected, CXCR5⁻/⁻ mice produced lower levels of neutralizing IgG than 129Sv mice, indicating the impaired capacity of these mice to mount TD immune responses (Fig. 23E). CTL responses against LCMV and virus
clearance were functionally normal in spleens and lymph nodes in CXCR5−/− and 129Sv controls (data not shown).

We thus conclude that the deficiency of CXCR5−/− mice to induce TD immune responses is a general phenomenon. Only repetitively organized and replicating antigens, such as VSV, could overcome this defect.

![Graphs showing induction of T helper cells in CXCR5−/− mice](image)

**Figure 24: Induction of T helper cells in CXCR5−/− mice**

Frequency of CXCR5+ amongst CD4+CD44hi splenocytes of 129Sv mice was measured (A) following infection with 2x10⁶ pfu VSV-IND i.v. and (B) following infection with 200 pfu LCMV-WE i.v. Data points indicate means of 2 mice. (C) CD4+ T cells from spleens of 129Sv (left panels) and CXCR5−/− (right panels) mice were analysed for VSV-p8 specific IFN-γ-production at day 8 post infection (upper panels) or LCMV-np309 specific IFN-γ-production at day 12 post infection (lower panels). Values indicate means ±SD of 2 mice. (D) VSV-IND-specific neutralizing antibody titers were measured following i.v. infection of CXCR5−/− and 129Sv mice with 2x10⁶ pfu VSV-NJ and subsequent immunization with 20 μg recombinant VSV-IND-G i.v. seven days post infection. Values indicate means of three mice. For explanations: see text.
Induction of CXCR5 on primed T helper cells after virus infection

To further assess the relevance of CXCR5 for the localization of T helper cells during priming, we next followed the expression of CXCR5 on T helper cells after infection with 2x10^6 pfu VSV-IND i.v. or 200 pfu LCMV i.v., respectively. Activated (CD44^high) CD4^+ T cells induced optimal levels of CXCR5 at the timepoint of maximal CD4^+ T cell expansion, i.e., on day 8 following infection with 2x10^6 pfu VSV-IND i.v. (Fig. 24A) and on day 12 following infection with 200 pfu LCMV-WE i.v. (Fig. 24B). The induction of CXCR5 on CD4^+ T cells concomitant to Ig class switch led us to speculate that the induction of CXCR5 on activated CD4^+ T cells had a functional relevance for the induction of TD immunoglobulins in vivo.

Expansion of specific T helper cells is impaired in CXCR5^{-/-} mice

We next wanted to quantify the expansion of specific T helper cells in secondary lymphoid organs after immunization with 2x10^6 pfu VSV-IND i.v. or 200 pfu LCMV-WE i.v., respectively. To this end, we isolated splenocytes on day 8 following infection with VSV-IND and on day 12 following infection with LCMV-WE, restimulated them in vitro on the VSV-derived T helper peptide p8 and on the LCMV-derived T helper peptide np309 and assessed the peptide-specific IFN-γ production. Interestingly, CXCR5^{-/-} spleens contained significantly lower numbers of np309-specific CD4^+ T cells than 129Sv controls (Fig. 24C). This correlated with the failure of these mice to induce efficient TD responses against LCMV. On the other hand, the frequencies of p8-specific CD4^+ T cells of CXCR5^{-/-} mice and 129Sv controls were comparable (Fig. 24D), indicating that the capacity of CXCR5^{-/-} mice to perform immunoglobulin class switch directly depended on the frequency of specific T helper cells, which could be retained in their spleens.

Rescue of the T helper defect of CXCR5^{-/-} mice by maximal expansion of T helper cells before immunization

We next wanted to increase the frequencies of specific T helper cells in secondary lymphoid organs of CXCR5^{-/-} mice before assessing the capacity of these mice to
switch their Ig isotype in order to test whether Ig class switch could be restored in these mice.

The two serotypes of VSV, VSV-IND and VSV-NJ, do not induce cross-reactive antibodies but cross-reactive T helper cells (Roost et al., 1990). We made use of this fact to optimally expand the T helper population in CXCR5^−/− mice with 2x10^6 VSV-NJ i.v., seven days prior to immunization with 20 μg of recombinant VSV-IND-G i.v.. This dose would normally not induce class switch in CXCR5^−/− mice (see Fig. 23E). As in unprimed mice, CXCR5^−/− mice mounted normal IgM titers (Fig. 24D). The class switch to IgG, however, which was totally absent in unprimed CXCR5^−/− mice, was almost completely restored to wild-type levels in VSV-NJ primed CXCR5^−/− mice. A similar result was obtained if priming with VSV-NJ was done 4 weeks before immunization with VSV-G (data not shown).

We therefore conclude that an optimal expansion of the T helper compartment can overcome the defective T cell help in CXCR5^−/− mice.

Adiuvant can substitute for impaired T help in CXCR5^−/− mice

The data presented here are in apparent contrast to the findings by Förster et al (Forster et al., 1996) who described normal IgG1 production following immunization with DNP-KLH in CFA. It might have been that the non-specific immunostimulatory capacity of the adiuvant might have substituted for an impaired T helper cell expansion. Experiments to assess the capacity of CXCR5^−/− mice to induce IgG against VSV-G in the presence of adiuvant are underway.

In a similar approach, we used cholera toxin as an adiuvant for the induction of serum and intestinal IgA. After repetitive gavage of 1 mg ovalbumin in 15 μg CT, ovalbumin- and CT-specific IgA levels were determined from serum and gut lavages and standardized on total IgA levels. There was no difference in IgA levels between CXCR5^+/+ mice and 129 Sv controls in none of the compartments (Fig. 25A), indicating that cholera toxin as an adiuvant could substitute for cognate T helper activation for immunoglobulin class switch in gut-associated lymphoid tissue and lymph nodes, respectively.
5.3.5. Discussion

In this study, we have demonstrated a selective deficiency of CXCR5−/− mice in mounting TD immune responses, particularly in cases where the antigen was weak, i.e. non-replicating, structurally irregular, or both. This deficiency could be related to the reduced capacity of CXCR5−/− mice to expand or retain specific CD4+ T cells, and maximal expansion of these cells could restore the capacity of these mice to switch their Ig isotypes. Cognate T help could be replaced, at least for IgA induction in the gut, by administering the antigen in adjuvant maximizing non-cognate T help. This non-specific effect could have been the reason that initially CXCR5−/− mice were found to produce normal levels of IgG1 following immunization with protein in CFA, although this will need to be directly shown in our immunization model.

In summary, we have collected some evidence for the fact that CXCR5−/− mice may have a defect on the T helper cell level.
It has been proposed for the human system, that a special subset of CXCR5+ T helper cells, termed follicular helper cells, can sustain or induce IgG or IgA, but not IgM levels in tonsillar cultures (Breitfeld et al., 2000; Schaerli et al., 2000). We extend these in vitro findings by showing that IgM production may also be impaired in CXCR5− mice as soon as IgM production gets T help dependent. It was shown that, after immunization with ovalbumin, activated CD4+ T cells acquire CXCR5 and get relocalized close to B cell follicles (Ansel et al., 1999). We have demonstrated that also following virus infection CXCR5 gets induced on activated CD4+ cells and that there is a time correlation between this up-regulation and immunoglobulin class switch. Although the hypothesis needs further support, we would like to argue that the CXCR5+ population may be involved in immunoglobulin class switch after virus infections in vivo. The induction of CXCR5 on CD4+ T cells, however, does not seem to be an absolute requirement for the immunoglobulin class switch, as we observed normal IgG levels in the acute phase of VSV-IND infection and following secondary infection.

B cells express CXCR5 and the overall splenic morphology of CXCR5− mice is greatly altered. Therefore, it was important to rule out a possible effect of B cells in the observed functional phenotype. As all lymphoid B cell compartments and the TI IgM production seemed to be undisturbed in CXCR5− mice, these mice do not appear to have an intrinsic B cell defect, so that the availability of B cells did not seem to account for the observed difference.

Intriguingly, our functional data closely resemble the data obtained with TNFRI− mice (Karrer et al., 2000), where normal TI responses but impaired TD responses were observed in cases of non-replicating antigen. The splenic morphology of TNFRI− mice and CXCR5− mice is strikingly similar, with ectopic germinal centers forming around the central arteriole, and concentric circles of T cells and B cells forming around them. Like TNFRI− mice, CXCR5− mice could induce functional germinal centers (this study and Voigt et al., 2000) and showed a pronounced decline in memory IgG titers. This similarity could have several explanations: It has been shown that the ligands for TNFRI, TNF and LTα3, are potent inducers of CXCL13, which, in turn, is the only known ligand for CXCR5 (Ngo et al., 1999). These defects on the same axis of lymphoid morphogenesis may lead to the very similar structural defects
in splenic microarchitecture. TNFRI−/− mice, however, also display a partial lack of CCL19 and CCL21, so that the defect of TNFRI−/− mice might be more profound. An alternative explanation for the similar results in both mouse strains is that similar splenic morphology leads to similar outcomes of infection. If APCs and effector lymphocytes are separated by the same distances, the probability of their encounter will be similar, irrespective of the molecular mechanism that causes the alterations in splenic morphology. This explanation receives further support from data of LTα−/− mice and aly/aly mice which both lack all lymph nodes and retain a morphologically altered spleen. Although the molecular defect is different in both mouse strains, the lymph system of the organism, and, thus, their susceptibility to virus infection is comparable (Karrer et al., 1997; Suresh et al., 2002).

If antigen is replicating and highly organized, such as VSV, a slightly altered splenic morphology does not have a strong impact on the outcome of the immune response, because the virus spreads all over the organism infects a sufficient number of APCs for priming. In cases of non-replicating antigen, such as UV-VSV and VSV-G, the outcome of the immune response depends on the splenic architecture and the coordinated movement of APC and lymphocytes, as governed by chemokine receptors. This finding is of general importance as it correlates to what was described for the T cell zone in plt/plt mice (Junt et al., 2002; Mori et al., 2001). Immune responses against protein antigens in plt/plt mice were dependent on a correctly formed T cell zone whilst immune responses against viruses were not.

CXCR5 in particular regulates the encounter of T cells and B cells, and, more recently, a population of DC was described which, by means of CXCR5 could localize to B cell zones of secondary lymphoid organs (Saeki et al., 2000). A lack of CXCR5 thus could potentially disrupt a multitude of interactions during immune responses. We found a particular impact on the magnitude of specific T helper responses in CXCR5−/− mice. This could be caused by an impaired contact of CD4+ T cells with CXCR5+ DC, or an impaired retention of CXCR5+CD4+ T cells which arise concomitant to immunoglobulin class switch. Alternatively, the expansion of CD4+ T cells might have been hampered by the lack of T-B contacts, as it was shown that B cells could further augment T cell responses (Homann et al., 1998). These functional aspects are currently under investigation.
5.3.6. Acknowledgements

We want to thank Anja Nowotny and Silvia Sahner for immunohistochemistry and Kathy McCoy for critical reading of the manuscript.
5.4. Part IV: Chemokines in disease

5.4.1. CD4(+) T cell subsets during virus infection. Protective capacity depends on effector cytokine secretion and on migratory capability.


To analyze the antiviral protective capacities of CD4(+) T helper (Th) cell subsets, we used transgenic T cells expressing an I-A(b)-restricted T cell receptor specific for an epitope of vesicular stomatitis virus glycoprotein (VSV-G). After polarization into Th1 or Th2 effectors and adoptive transfer into T cell-deficient recipients, protective capacities were assessed after infection with different types of viruses expressing the VSV-G. Both Th1 and Th2 CD4(+) T cells could transfer protection against systemic VSV infection, by stimulating the production of neutralizing immunoglobulin G antibodies. However, only Th1 CD4(+) T cells were able to mediate protection against infection with recombinant vaccinia virus expressing the VSV-G (Vacc-IND-G). Similarly, only Th1 CD4(+) T cells were able to rapidly eradicate Vacc-IND-G from peripheral organs, to mediate delayed-type hypersensitivity responses against VSV-G and to protect against lethal intranasal infection with VSV. Protective capacity correlated with the ability of Th1 CD4(+) T cells to rapidly migrate to peripheral inflammatory sites in vivo and to respond to inflammatory chemokines that were induced after virus infection of peripheral tissues. Therefore, the antiviral protective capacity of a given CD4(+) T cell is governed by the effector cytokines it produces and by its migratory capability.
5.4.2. CC chemokine receptor 7-dependent and -independent pathways for lymphocyte homing: modulation by FTY720.


*(J Exp Med 2001 Dec 17;194(12):1875-81)*

Cognate interaction of chemokine receptor CCR7 on lymphocytes with its ligands CCL19 and CCL21 expressed on high endothelial venules (HEVs) is essential for effective migration of T and B cells across HEVs into secondary lymphoid organs. Plt mice, which lack expression of CCL19 and CCL21-ser, both ligands for CCR7 on HEVs, as well as CCR7-deficient mice, have a defective cell migration and reduced homing of lymphocytes. FTY720, a novel immunosuppressant, causes a reduction of lymphocytes in peripheral blood and tissues and their sequestration into lymphoid tissues. In this study we demonstrate that FTY720 rescues the homing defect in both CCR7(−/−) mice and plt mice. After FTY720 treatment, the number of CD4(+) and CD8(+) T cells as well as B cells in peripheral blood is reduced while pertussis toxin-sensitive homing into peripheral lymph nodes, mesenteric lymph node, and Peyer's patches is increased. Immunohistology demonstrates that FTY720 enables these cells to enter lymphoid tissue through HEVs. Thus, our data suggest an alternative G-alpha(i)-dependent, CCR7-CCL19/CCL21-independent mechanism for lymphocyte homing through HEVs which is strongly augmented in the presence of FTY720.

5.4.3. Beta cells are responsible for CXCR3-mediated T-cell infiltration in insulitis.


*(Nat Med. 2002 Dec;8(12):1414-20)*

T cell-mediated loss of insulin-secreting beta cells in the islets of Langerhans is the hallmark of type 1 diabetes. The molecular basis for the directed migration of autoreactive T cells leading to insulitis is presently unknown. Here we demonstrate that in response to inflammation, beta cells secrete the chemokines CXC ligand 10 and CXC ligand 9, which specifically attract T-effector cells via the CXC chemokine receptor 3. In mice deficient for this receptor, the onset of type 1 diabetes is
substantially delayed. Thus, in the absence of known etiological agents, CXC receptor 3 represents a novel target for therapeutic interference early in type 1 diabetes.

5.4.4. Speed of prion neuroinvasion is controlled by lymphoreticular microenvironment

Marco Prinz, Mathias Heikenwalder, Tobias Junt, Petra Schwarz, Markus Glatzel, Frank L. Heppner, Yang-Xin Fu, Martin Lipp, Adriano Aguzzi
(Nature, submitted)

After peripheral infection, rapid prion expansion in lymphoid organs precedes progressive neurological disease. But how do prions accomplish transfer from lymphoreticular stroma to the nervous system? Here we show that ablation of the chemokine receptor CXCR5 greatly accelerates the entrance into spinal cord of intraperitoneally administered prions, by shifting the locale of follicular dendritic cells (FDCs) towards the neighborhood of nerve terminals. Neuroinvasion velocity correlated exclusively with FDC location: transfer of CXCR5−/− bone marrow to wild-type mice induced perineural FDCs and enhanced neuroinvasion, whereas reciprocal transfer to CXCR5+/− mice abolished them and restored normal efficiency of neuroinvasion. Suppression of lymphotoxin signalling depleted FDCs and abolished splenic infectivity in CXCR5−/− mice. Therefore, prion neuroimmune transition occurs between FDCs and sympathetic nerves, and positioning of FDCs relative to splenic nerve endings controls the efficiency of peripheral prion infection. This suggests that manipulation of FDC locales may be exploited against prion propagation.
6. DISCUSSION

6.1. Staggered recruitment of secondary lymphoid organs to the immune response

If an organism gets infected, the body aims to contain the pathogen locally in order to avoid its uncontrolled dissemination via the bloodstream. To this end, the body uses three interconnected control circuits (Fig. 26):

a) Lymph nodes for the surveillance of external body surfaces,

b) The mucosa-associated lymphoid tissue (MALT) for the surveillance of internal body surfaces,

c) The spleen to monitor the blood for disseminating pathogens, once the two other more distal circuits have failed.

With these three systems, the adaptive immune system is organized for an efficient task sharing. Lymphoid organs thus get recruited to the immune reaction in a staggered fashion.

6.1.1. Lymph nodes and the surveillance of the periphery

Lymph nodes are connected to each other by the lymph system. On the way from the periphery to the bloodstream, the lymph passes a series of lymph nodes, where it is monitored for antigen, and, where, if necessary, a localized immune reaction is initiated. If a given lymph node is overwhelmed with the amount of entering pathogens, the next, more centrally situated, lymph node gets recruited to the immune reaction.
6.1.2. The MALT and the surveillance of internal surfaces

All surfaces that serve as entry sites of metabolites are critical sites for immune surveillance as the majority of non-self substances would pose a severe risk of disease. The MALT prevents entry of potentially hazardous material at these sites. It thereby complements the system of lymph nodes at a more distal site. The importance of the MALT is underscored by the fact that the majority of the body’s lymphocytes are located at mucosal surfaces. A continuous immune reaction is maintained at dispersed sites all along the gut, at Peyer’s patches and at isolated lymphoid follicles lining the mucosa. Once the MALT is overwhelmed by a pathogen, the system of lymph nodes is called into action before the pathogen reaches the bloodstream and the spleen.
6.1.3. The spleen and the surveillance of the blood

Once a pathogen has disseminated over the peripheral lymph nodes and has reached the blood, it has unlimited access to all tissues. Therefore, pathogens have to get cleared from the circulation as quickly as possible. The white pulp of the spleen filters pathogens from blood and initiates immune responses. If the capacity of all secondary lymphoid organs of the body, including the spleen, is exhausted, antigen reaches the thymus. If this occurs, all the antigen-specific effector cells get deleted due to negative selection, the pathogen is perceived as "self" and a permanent carrier state is established.

6.2. Lymphoid structures foster responses of the adaptive immune system

At the beginning of each primary immune response, APC have to establish cognate contacts to naive lymphocytes in secondary lymphoid organs. Therefore, efficient priming of an immune response depends on a high probability of encounter between APC and naïve lymphocytes. Genetically engineered mouse strains and natural mutations have revealed physiological alterations decrease the probability of APC-lymphocyte contacts:

a) APC and lymphocytes cannot meet efficiently within secondary lymphoid organs if lymphoid microenvironments are not formed correctly (e.g. plt/plt, BLTβ-ko). This defect plays a minor role for the efficient induction of antiviral immune responses, as secondary lymphoid organs can foster the encounter of APC and lymphocytes at ectopic sites, especially if the antigen replicates.

b) APC and lymphocytes cannot meet efficiently within secondary lymphoid organs if the cell migration to these organs is impaired (e.g. CCR7−/−, CXCR5−/−, L-selectin−/−). In this situation, a slightly slower kinetics of antiviral immune responses is observed, because the body needs more time to recruit sufficient
numbers of APC and naive lymphocytes for priming. Eventually, however, the antigen gets eliminated.

c) APC and lymphocytes cannot meet efficiently within secondary lymphoid organs if overall leukocyte numbers within the organism are reduced (e.g. RAG\(^{-}\), \(\mu\)MT). This situation leads to both malformed lymphoid architecture and decreased lymphocyte recruitment.

d) APC and lymphocytes cannot meet efficiently if some secondary lymphoid organs do not develop during ontogeny (e.g. aly/aly, LT\(\alpha^{+}\), LT\(\beta^{+}\)). In these cases, immune responses are heavily weakened because the body cannot provide APC and lymphocytes with a sufficient number niches for interaction.

Not only the probability of encounter between APC and lymphocytes, but also the capacity of antigen absorption of a given lymphoid organ has an impact on the outcome of an immune response. As antigen absorption depends on DC-mediated transport (i.e. a migration phenomenon) or on absorption via APC within secondary lymphoid organs (i.e. the APC number), the only four parameters defining the outcome of the adaptive immune response are:

- The number of APC in an organism,
- The number of potential effectors (i.e. the size of the T or B cell repertoire),
- The number of secondary lymphoid organs,
- The probability of leukocyte entry into secondary lymphoid organs, defined as migratory capacity of APC and lymphocytes. The results of this thesis show, however, that this parameter is only of minor importance, as sufficient numbers of lymphoid sites and leukocytes compensate for an effect of impaired leukocyte migration.
6.3. Lymphoid structures foster responses of the innate immune system

In the above quantitative considerations, components of the innate immune system were not taken into account. They modify the quality of the immune response, e.g. by maturing APC (IL-6, IL-1) or by augmenting the clonal burst of lymphocytes (IL-2). Their action relies on cell-cell contact or on diffusion over short distances, i.e. again on the probability of encounter between secreting/ signalling and receiving cell. Therefore, the efficacy of innate immune mechanisms also depends on an intact of lymphoid microenvironment. I thus would like to argue that the microarchitecture of secondary lymphoid organs is the most basic principle on which the efficiency of innate and adaptive immune systems rely.
7. APPENDIX

7.1. References


Appendix


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Appendix


Appendix


Appendix 133


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Appendix


Appendix


7.2. Abbreviations

\begin{itemize}
  \item \textit{aly} \quad \text{allymphoblastic}
  \item APC \quad \text{Antigen-presenting cell}
  \item BLC \quad \text{B lymphocyte chemoattractant}
  \item BLR \quad \text{Burkitt's Lymphoma Receptor}
  \item BSS \quad \text{Balanced Salt Solution}
  \item CCL \quad \text{CC chemokine ligand}
  \item CCR \quad \text{C chemokine receptor}
  \item CFA \quad \text{Complete Freund's adjuvant}
  \item CRD \quad \text{Complement regulatory domain}
  \item CT \quad \text{Cholera toxin}
  \item CTL \quad \text{Cytotoxic T lymphocyte}
  \item CXCL \quad \text{CXC chemokine ligand}
  \item CXCR \quad \text{CXC chemokine receptor}
  \item DC \quad \text{Dendritic cell}
  \item DTH \quad \text{Delayed-type hypersensitivity}
  \item EAE \quad \text{Experimental allergic encephalomyelitis}
  \item ELC \quad \text{Epstein-Barr virus-induced molecule I ligand chemokine}
  \item FDC \quad \text{Follicular dendritic cell}
  \item GAG \quad \text{Glycosaminoglycan}
  \item GC \quad \text{Germinal centers}
  \item GlyCAM \quad \text{Glycosylation-dependent cell adhesion molecule}
  \item GP \quad \text{Glycoprotein}
  \item HBSS \quad \text{Hanks Balanced Salt solution}
  \item HEV \quad \text{High endothelial venules}
  \item HIV \quad \text{Human immunodeficiency virus}
  \item ICAM \quad \text{Intercellular cell adhesion molecule}
  \item IP-10 \quad \text{Interferon-inducible protein 10}
  \item I-TAC \quad \text{Interferon-inducible T cell alpha chemoattractant}
  \item JAK \quad \text{Janus kinase}
  \item LCMV \quad \text{Lymphocytic choriomeningitis virus}
  \item LFA \quad \text{Lymphocyte function-related antigen}
  \item LPS \quad \text{Lipopolysaccharide}
  \item LT \quad \text{Lymphotoxin}
\end{itemize}
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<tr>
<th>Abbreviation</th>
<th>Explanation</th>
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<tr>
<td>MACS</td>
<td>Magnetic assisted cell sorting</td>
</tr>
<tr>
<td>MAAdCAM</td>
<td>Mucosal adressin cell adhesion molecule</td>
</tr>
<tr>
<td>MALT</td>
<td>Mucosa-associated lymphoid tissue</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein 1</td>
</tr>
<tr>
<td>MDC</td>
<td>Macrophage-derived chemokine</td>
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<tr>
<td>MHV</td>
<td>Mouse hepatitis virus</td>
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<tr>
<td>Mig</td>
<td>Monokine induced by interferon gamma</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>Macrophage inflammatory protein 1α</td>
</tr>
<tr>
<td>MM</td>
<td>Metallophilic macrophages</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
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<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
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<tr>
<td>MZM</td>
<td>Marginal zone macrophages</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>NP</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>NSI</td>
<td>Non-syncytium inducing</td>
</tr>
<tr>
<td>pLIt</td>
<td>Paucity of lymph node T cells</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>PNAd</td>
<td>Peripheral node vascular adressin</td>
</tr>
<tr>
<td>PP</td>
<td>Peyer's patch</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon activation normal T cell expressed and secreted</td>
</tr>
<tr>
<td>RIP</td>
<td>Rat insulin promoter</td>
</tr>
<tr>
<td>SCY</td>
<td>Small secreted cytokine</td>
</tr>
<tr>
<td>SDF-1</td>
<td>Stem cell derived factor 1</td>
</tr>
<tr>
<td>SI</td>
<td>Syncytium-Inducing</td>
</tr>
<tr>
<td>SLC</td>
<td>Secondary lymphoid organ chemokine</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TACE</td>
<td>TNF converting enzyme</td>
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<td>TARC</td>
<td>Thymus and activation regulated chemokine</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>TRAF</td>
<td>TNF receptor associated factor</td>
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<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
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<td>Vesicular stomatitis virus</td>
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<td>VV</td>
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7.5. Curriculum vitae

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<th>Description</th>
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| 1996 | Research project in immunology at the Weizmann Institute of Science, Rehovot, Israel (Prof. Gideon Berke)  
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