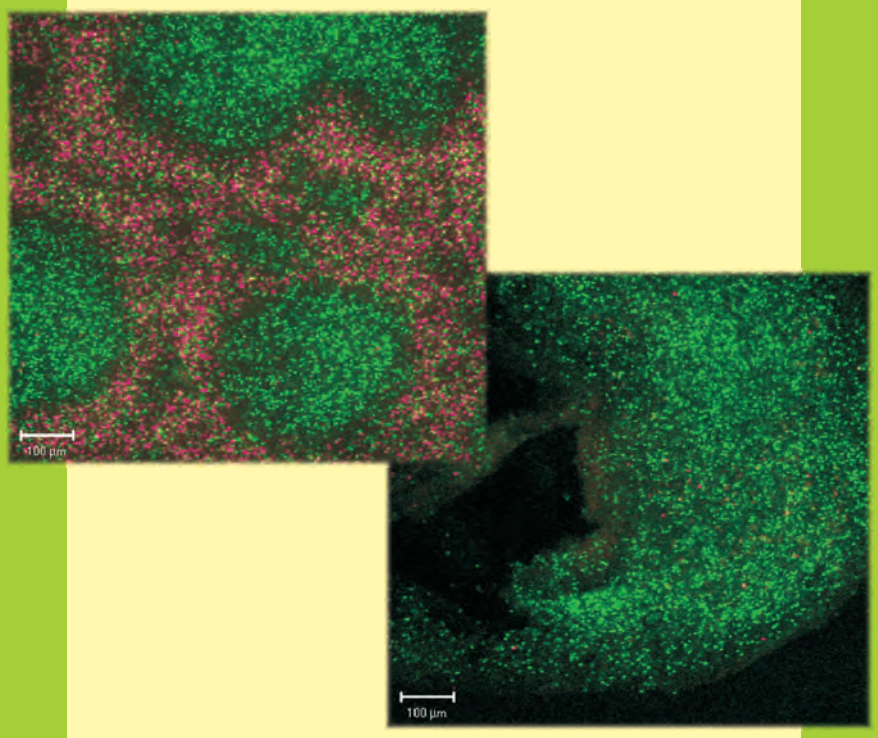
Type I interferon stimulation of lymphocytes



Elisabeth Kamphuis

INAUGURAL-DISSERTATION
zur Erlangung des Grades eines
Dr. med. vet.
beim Fachbereich Veterinärmedizin
der Justus-Liebig-Universität Gießen

édition scientifique
VVB LAUFERSWEILER VERLAG

1. Auflage 2007

1st Edition 2007

VVB LAUFERSWEILER VERLAG
édition scientifique

STAUFENBERGRING 15, D-35396 GIESSEN
Tel: 0641-5599888 Fax: 0641-5599890
email: redaktion@doktorverlag.de

www.doktorverlag.de

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eingereicht von

Elisabeth Kamphuis
Tierärztin aus Darmstadt

Gießen 2006

Mit Genehmigung des Fachbereichs Veterinärmedizin
der Justus-Liebig-Universität Gießen

Dekan: Prof. Dr. M. Reinacher

Gutachter: Prof. Dr. H.-J. Thiel
Prof. Dr. J. Löwer

Tag der Disputation: 13.12.2006

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Cover illustration:

Laser scan micrographs of spleen (upper left) and lymph node (lower right) tissue containing fluorescently labelled lymphocytes. Recipient mice were intravenously transferred with green-labelled control splenocytes and with purple-labelled splenocytes that had been treated with pertussis toxin to block G-protein-coupled receptors. Lymphoid organs were isolated twenty hours after adoptive transfer, and manual sections were analysed by laser scan microscopy. The micrographs represent optical sections of approximately 100 µm. In spleen, the typical meshwork of red and white pulp can be recognised. Of note, pertussis toxin-treated lymphocytes were unable to enter splenic white pulp and lymph node tissue. Since entry into secondary lymphoid organs is dependent on G-protein-coupled receptors, the data confirm the effective blockade of the respective receptors. (See also section 3.5.1.)

"No el mucho saber harta y satisface al ánima,
mas el sentir y gustar de las cosas internamente."

Siglo XVI
Ignacio de Loyola,
Ejercicios Espirituales, n^{o} 2

Meiner Mutter

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Abbreviations

α-	Anti-
Ab	Antibody
ABTS	2, 2´-azino-bis-(3-ethylbenziazoline-6-sulfonic acid)
AC	*Arteria centralis*
aq.dest.	*Aqua destillata*
APC	Antigen-presenting cell
APRIL	A proliferation-inducing ligand
BCR	B cell receptor
BL/6	C57/BL/6 mice, immunocompetent black wild-type mice
BLyS	B lymphocyte stimulator protein
BM	Bone marrow
bp	Base pair
BZ	B cell zone
CCR	Chemokine receptor of “CC” type
CD	Cluster of differentiation
CDR	Complementarity-determining region
CFSE	Carboxyfluorescein succinimidyl ester
CGG	Chicken gamma globulin
CLP	Common lymphoid precursor
CMP	Common myeloid precursor
CpG	Unmethylated structural element in DNA: cytidine - phosphate -guanosine
CTL	Cytotoxic T lymphocyte
CXCR	Chemokine receptor of “CXC” type
DC	Dendritic cell
DMEM	Dulbecco´s modified Eagle´s medium
DNA	Desoxribonucleic acid
dsRNA	Double-stranded ribonucleic acid

ELISA	Enzyme-linked immunosorbent assay
ES cells	Embryonal stem cells
env	Envelope proteins
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FDC	Follicular dendritic cell
FOB	Follicular B cell
FSC	Forward scatter in FACS, indicates particle size
FTY720	Given name of 2-amino-2-(2-[4-octylphenyl]ethyl)-1,3- propanediol
gag	Group-specific antigens
GC	Germinal centre
GPCR	G protein-coupled receptor
HEV	High endothelial venule
HRP	Horse radish peroxidase
Jak	Janus kinase
ICAM	Intracellular adhesion molecule
IFN	Interferon
IFNAR	Interferon-α/ß receptor
Ig	Immunoglobulin
IL	Interleukin
IRF	Interferon-regulatory factor
i.n.	Intranasal
i.p.	Intraperitoneal
i.v.	Intravenous
IVC	Individually ventilated cage
LFA-1	Lymphocyte function-associated antigen-1
LN	Lymph node
LPA	Lysophosphatidic acid

LPC	Lysophosphatidyl choline
M	Matrix protein of VSV
mAb	Monoclonal antibody
mDC	Myeloid dendritic cell
MACS	Magnetic adsorption cell sorting
MALT	Mucosa-associated lymphoid tissue
MHC	Major histocompatibility complex
MLV	Murine leukaemia virus
MS	Marginal sinus
MZ	Marginal zone
MZB	Marginal zone B cell
neo	Neomycin resistance
NK	Natural killer cell
OVA	Ovalbumin
PALS	Periarterial lymphatic sheath
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PFU	Plaque-forming unit
p.i.	Post infection
PNAd	Peripheral (lymph) node addressin
poly(I:C)	Poly inosine: cytidine
PP	Peyer´s patches
PRR	Pattern recognition receptor
PTX	Pertussis toxin
S1P	Sphingosine-1-phosphate
$S1P_1$	Sphingosine-1-phosphate receptor 1
s.c.	Subcutaneous

SD	Standard deviation
SDF-1	Stroma-derived factor-1
SLO	Secondary lymphoid organ
SPF	Specific pathogen-free
SSC	Side scatter in FACS, indicates particle granularity
ssDNA	Single-stranded deoxynucleotide
STAT	Signal transducer and activator of transcription
TAMRA	Carboxytetramethyl-rhodamine succinimidyl ester
TCR	T cell receptor
TD	T cell-dependent
Th	T helper cell
TI	T cell-independent
TK	Thymidine kinase
TLR	Toll-like receptor
TZ	T cell zone
VCAM	Vascular adhesion molecule
VI24	Given name of monoclonal antibody to VSV-G protein, IgG_{2a} subtype
VLP	Virus-like particle
VSV	Vesicular stomatitis virus
VSV-G	Glycoprotein of vesicular stomatitis virus

Publications

This dissertation has led to the following publications so far:

Kamphuis, E., Junt, T., Waibler, Z., Forster, R., and Kalinke, U. Type I interferons directly regulate lymphocyte recirculation and cause transient blood lymphopenia. *Blood*. 2006;108:3253-3261.

Bach, P., **Kamphuis, E.**, Odermatt, B., Sutter, G., Buchholz, C.J., and Kalinke, U. Vesicular Stomatitis Virus Glycoprotein Displaying Retrovirus-Like Particles Induce a Type I IFN Receptor-Dependent Switch to Neutralizing IgG Antibodies. *J.Immunol*. 2007;178:5839-5847.

Le Bon, A., Thompson, C., **Kamphuis, E.**, Durand, V., Rossmann, C., Kalinke, U., and Tough, D.F. 2006. Cutting edge: enhancement of antibody responses through direct stimulation of B and T cells by type I IFN. *J.Immunol.* 2006;176:2074-2078.

Le Bon, A., Durand, V. **Kamphuis, E.**, Thompson, C., Bulfone-Paus, S., Rossmann, C., Kalinke, U., and Tough, D.F. 2006. Direct stimulation of T cells by type I interferon enhances the CD8+ T cell response during cross-priming. *J.Immunol.* 2006;176:4682-4689.

1 Introduction

1.1 Immune system

During evolution, the immune system developed in order to defend complex organisms against invading pathogens. Recognition of the foreign, distinction from self and perception of danger are common features shared even by plants and animals. Generic receptors for pathogen-associated molecular patterns (PAMPs) and unspecific defence mechanisms comprise the so-called innate immune system, which protects the organism throughout life. However, innate immunity cannot distinguish between species of microorganisms and therefore does not have the potential to optimise the antigen-specific defence or to mount memory responses. For this, vertebrates developed a specific immune system capable of recognising even subtle differences between pathogens and adapting its defence in a flexible way to individual challenges. Consequently, the specific or "adaptive" immunity continuously evolves along with pathogen contacts throughout life, reflecting the personal infection history.

1.1.1 Innate and adaptive immunity

The innate immune system deploys pattern recognition receptors (PRRs)[1] such as Toll-like receptors (TLRs) to identify PAMPs including microbial nucleic acids such as single-stranded (ss) and double-stranded (ds) RNA and DNA as well as hypomethylated CpG motifs of bacterial DNA.[2;3] Furthermore, various TLRs recognise components of bacterial cell walls, such as lipopolysaccharide (LPS), peptidoglycan, lipoteichoic acid and flagellin (Fig. 1-1). A variety of serum proteins and their respective cleavage products, which constitute the so-called complement system, provide an effective innate tool to osmotically lyse intruders and infected cells. The complement system furthermore opsonises to-be-phagocytosed particles, i.e. marks deteriorated cells for degradation by phagocytes. Among leucocytes, the cells of the immune system, natural killer (NK) cells are specialised for the destruction of virally infected or neoplastic cells, whereas macrophages and different types of granulocytes clean up infectious detritus and microbes by ingestion.

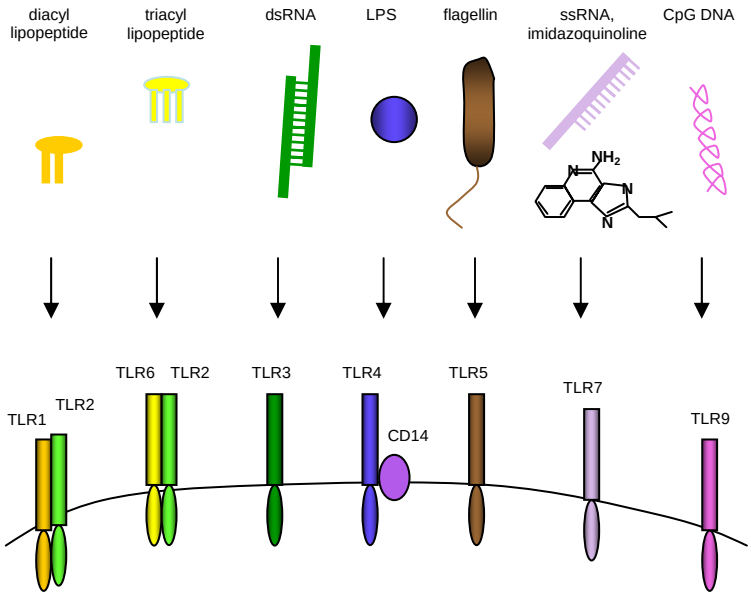


Fig. 1-1: Toll-like receptors and their ligands

Adapted from: Takeda K, Akira S. Toll receptors and pathogen resistance. Cell. Microbiol. 2003;5:143-153.

The adaptive immunity, however, is carried out by lymphocytes, which all differ from each other with respect to their individual lymphocyte receptors. B lymphocytes, also called B cells, can differentiate upon activation into plasma cells that are able to shed their specific B cell receptor (BCR) as antibody against invading pathogens. Among B cells, common B-2 cells undergo complex maturation steps upon specific antigen encounter to generate highly affine antibodies to virtually all foreign proteins, whereas $CD5^+$ B-1 cells show a restricted BCR repertoire focused on carbohydrate antigens. Specific antibodies, often referred to as immunoglobulins (Ig), can neutralise microorganisms or toxins to prevent infection or harmful effects on cells. Similar to complement components, antibodies can opsonise pathogens or infected cells to increment elimination by Ig receptor-carrying phagocytes or to enable antibody-dependent cellular cytotoxicity of NK cells. Antibodies consist of two heavy and two light chains that show variable and constant regions. According to their constant regions, they are classified in isotypes (classes IgM, IgD, IgG, IgA, IgE [and IgY in birds and reptiles]) and several IgG subtypes.

With the help of their individual T cell receptor (TCR), T cells react specifically to a fitting antigen presented on self molecules, so-called major histocompatibility complex (MHC) molecules. T cells divide into two major groups, the $CD4^+$ T helper (Th) cells and the $CD8^+$ cytotoxic T lymphocytes (CTL). Th cells coordinate immune responses by skewing them either

towards type 1, a cellular defence carried out by CTLs, or to type 2, a humoral response in form of specific antibodies. According to the former or the latter type of immunity induced, Th cells subdivide into Th1 and Th2 cells. Besides immune-stimulatory capacity, some $CD4^+$ T cells adopt regulatory functions to mediate tolerance, or suppressive activities to specifically inhibit immunity. $CD8^+$ T cells are responsible for the antigen-specific destruction of infected cells.

The presentation of foreign and degenerated self-derived materials is accomplished by antigen-presenting cells (APCs), amongst which dendritic cells (DCs) are the most efficient presenters while macrophages and especially B cells play minor roles only under certain circumstances. Depending on the target cell, subtype and the maturation state, DCs can induce tolerance or immunity and thus account for a critical control point in keeping the balance between host defence and autoaggression.[4;5] DCs digest foreign endocytosed antigens to present oligopeptides by MHC molecules of class II. In mice, constitutive expression of MHC class II is restricted to APCs, whereas several domestic animals also show spontaneous expression on T cells or various endothelia.[6] In contrast, virtually all nucleated cells are positive for MHC class I to present endogenously degraded cellular peptides. Thus, the peptide spectrum displayed by MHC class I molecules continuously mirrors the cellular metabolism and enables the detection of any abnormalities due to microbial infection or neoplastic transformation. According to their crucial role in foreign and cellular antigen presentation, MHC molecules control elimination of degenerated cells and rejection of transplanted tissues, which led to their given name. Importantly, adaptive immune responses induce abundant proliferation of antigen-specific lymphocytes to generate high amounts of short-lived effector cells, but simultaneously reserve a small fraction of differentiating B and T cells to become potentially long-lived memory cells. Thus, the adaptive immunity prepares for specific and fast defence upon re-encounter of pathogen.

1.1.2 Immune cells communicate in a language of cytokines

The communication between cells is achieved by secretion of cytokines, small soluble mediators that act in paracrine or sometimes autocrine fashion on their target cells. Cytokines are classified into major groups according to their cells of origin, targets and effects. Typically, the cytokine groups of interferons (IFNs), haematopoetins (including many interleukins (ILs)), the tumor necrosis factor family and chemokines are distinguished.[7] Production and release of cytokines as well as expression of their corresponding receptors are tightly regulated and characterise individual stages of development or activation. Many cytokine receptors share subunits, which can signal diverging functions according to their combination.[8] Both cytokines and their

receptors show pleitropism, i.e. exert different effects (on distinct cell types), and redundancy, i.e. their functions can overlap. Whereas most cytokines carry out functions on a rather limited target cell spectrum, the antiviral interferons can be sensed by virtually all non-immune tissues, too.

Cytokines can mediate stimulation of lymphocytes. To guarantee the very close contact of about 15 nm required for specific stimulation of target cells during immune responses, the reacting cells stick together tightly with the help of adhesion molecules such as lymphocyte function-associated antigen (LFA) and so-called costimulatory molecules and form a communicating interface, the immunological synapse.[9] Communicating cells then recruit cytokine receptors to the immunological synapse at the leading edge of the cell to efficiently take up secreted factors.

1.1.3 Links between the innate and adaptive immune system

Traditionally, innate and adaptive immune systems were considered independent branches of host immunity. This view has to be revised considering growing evidence that shows multiple links between innate and adaptive immunity.[10] Macrophages, for example, play an important role in the unspecific elimination of degraded cells and can also present foreign antigens to lymphocytes in order to elicit specific immunity. DCs are professional APCs that recognise PAMPs and induce the vast majority of adaptive immune responses, and at the same time secrete a panel of cytokines and chemokines, which stimulate B and T cells as well as NK cells and neutrophils. Due to generic induction upon virus infection, upregulation of MHC class I expression on all cells and stimulation of NK cell activity, type I IFN was regarded part of the innate immune system.[11] However, type I IFN is nowadays considered a crucial link to adaptive immunity[10] since it also regulates multiple functions of lymphocytes, as will be discussed in this work.

Taken together, the general and specific defence cooperate tightly during immune responses, with an emphasis on innate resistance during the first days before adaptive mechanisms set on. Upon recall infections, the kinetics are accelerated and specific memory takes over the main responsibilities already after few days.

1.1.4 Chemokines and chemoattractants guide cell movements

In contrast to conventional cytokines, chemokines determine cellular migration and localisation. Chemokines usually act in gradients set up by secreting cells to control physiological processes or inflammation.

Therefore, homeostatic and inflammatory chemokines can be distinguished,[12] albeit their functions do partially overlap. Chemokines can be produced constitutively in lymphoid organs and specialised high endothelial venules (HEV) to provide a selective milieu, which initiates the formation and controls the organisation of lymphoid tissues by attracting naïve circulating lymphocytes.[13] Following infection, it is the expression of inflammatory chemokines by endothelia and by migrating immune cells that actually recruits activated lymphocytes and other immune cells to infiltrate infected tissues and to cause inflammation.[12]

Chemokines (chemokine receptor ligands) comprise a growing family of more than 50 small proteins, classified into four subfamilies according to the position and distance (X) of the first two cysteine (C) residues (XCL, CCL, CXCL or CX_3CL). At least 17 chemokine receptors have been identified that are characterised by seven transmembrane domains and intracellular signalling coupled to G proteins. Most receptors bind to more than one ligand and some chemokine ligands show receptor redundancy. Several chemokines are important for guiding mature lymphocytes: CXCL12 binds to CXCR4 and plays essential homeostatic and inflammatory roles in B cell development,[14;15] generation of secondary lymphoid organs (SLOs)[13;16] and plasma cell localisation.[13;17;18] CXCL13, also called B zone chemokine due to its expression in B cell zones of SLOs, binds to CXCR5 to organise SLOs and control encounter of antigen-specific B cells with attracted T cells.[16;19;20] Finally, CCL19 and CCL21, which are also termed T zone chemokines, bind to CCR7. They are constitutively expressed in T cell zones and HEV to allow for immigration of DCs and lymphocytes into their corresponding sites within SLOs.[13;16;19-22]

Apart from classical chemokines, in the last years small phospholipids were recognised to participate in cell guidance. An emerging role concerning lymphocytes was identified for sphingosine-1 phosphate (S1P) that is produced during physiological cell metabolism and acts as an intracellular messenger.[23] Apparently, in mammals, some cells secrete large amounts of S1P so that a steep concentration gradient from micromolar levels in blood to nanomolar levels in lymphatic organs and lymph is established.[21;24;25] This abrupt S1P gradient controls T cell egress from thymus and lymph nodes (LN) and also licences circulation of B cells.[26-29] S1P binds to five receptors ($S1P_1$ to $S1P_5$) that were originally discovered for their roles in endothelial differentiation.[27] Indeed, $S1P_3$ has been identified to regulate cardiovascular development and chronotropic functions.[30] $S1P_1$ is expressed on lymphocytes and crucially controls B and T cell circulation.[27-29] In contrast, $S1P_4$, which was also found to be expressed on leucocytes, regulates T cell proliferation and cytokine secretion, but has not been implicated in mediating cell movements, so far.[31] Of note, $S1P_1$ is rapidly downregulated in presence of high ligand concentrations, suggesting that S1P does not act via progressive diffusion gradients such as

conventional chemokines.[32] In line with this, upon *in vitro* studies, S1P was hardly found to induce any B lymphocyte migration, which possibly reflects complete desensitisation of S1P-exposed cells. Instead, S1P-induced signalling might rather enable further steps including diapedesis through vessels and thus might licence egress from lymphoid organs.

Another lipid metabolite with a potential role in lymphocyte migration is lysophosphatidyl choline (LPC). Some reports demonstrated that LPC can induce *in vitro*-chemotaxis of activated T cells,[33] suggesting to contribute to T cell motility during immune responses. LPC binds to its specific receptor G2A expressed on activated T cells and macrophages.

Furthermore, lymphocytes were found to carry LPA_2, one of three receptors for lysophosphatidic acid (LPA).[34] However, it remains to be determined whether LPA can exert effects on lymphocyte migration.

1.1.5 CD molecules and immune nomenclature

Surface proteins of leucocytes determine the cellular reactivity. Various different receptors are used as so-called cluster differentiation (CD) molecules for cell and subtype classification. Due to historic reasons of identification, most CD molecules have several alternate denominations according to their functions. Generic CD molecules used for classification show identical or similar distribution among mammal species, whereas the expression of activation markers can differ to large extents between mice and humans.

Frequently, there are several alleles for one protein gene. The encoded allotypic proteins (allotypes) can be distinguished by the use of allotype-specific antibodies. According to current nomenclature in murine immunology, the allotype 2 is expressed by normal immunocompetent C57/BL6 (BL/6 or wild-type) mice. Several mouse lines are available, which carry allotype 1 either on BL/6 or on other backgrounds. Similar to genetically identical “syngeneic” animals, those mouse lines, which differ in only one allotype marker, do not reject transplanted grafts of the other allotype. They are therefore called “congenic” mice. For BM reconstitution of BL/6 mice, the pan-leucocyte marker CD45.2 can be deployed to distinguish recipient-derived white blood cells from $CD45.1^+$ donor leucocytes.

CD45R, usually called B220, is only expressed by murine B cells and is therefore used as a B cell-specific marker in mice. In contrast, in the human system, it does not exhibit B lineage specificity. In the last years, however, a novel population of plasmacytoid DCs (pDCs) was discovered in murine blood and lymphoid tissues that also expresses B220.[35-39] Due to the extremely rare frequency of these pDCs in blood (less than 0.5% in BL/6 mice)[39] and the very

high B220 expression on murine B cells, B220 is still widely used for the detection of murine B cells. Alternatively, CD19 is a specific marker for B cells that is expressed since very early developmental stages. However, CD19 shows slightly reduced expression as compared to B220. Both proteins are possibly involved in B cell receptor (BCR)-signalling.

A common marker for T cells is CD3, a hetero-tetrameric adaptor protein composed of single γ and δ chains and two ε subunits that participate in T cell receptor (TCR)-signalling.

In contrast to CD3, the T lymphocyte subset markers CD4 and CD8 show high expression on the respective cells. CD4 essentially stabilises the engagement of peptide-bearing MHC class II molecules with a suitable TCR and therefore restricts T helper cells to recognise MHC class II-presented antigens. Similarly, CD8 allows the firm engagement of the corresponding TCR with antigen-presenting MHC molecules of class I.[40] Since MHC class I show broad expression throughout the organism, cytotoxic T cells can be generated to specifically eliminate nearly all kinds of cells, whereas T helper cells can communicate only with a limited repertoire of immune cells.

1.1.6 Development of lymphocytes

The majority of leucocytes develop in fetal liver and in bone marrow (BM) of platitudinous bones, although maturation steps can follow in the peripheral tissues of their final destination. For example, circulating monocytes differentiate into tissue macrophages once having left the bloodstream. Lymphocytes, however, show developmental specialties and species differences.

The origin of B cells was first discovered in birds in the unique avian organ *bursa Fabricii*, for which they were denominated “B” cells. In mammals, however, B cells develop entirely in BM. On the contrary, T cells spend only very early stages in BM to continue their subsequent maturation in thymus. Notwithstanding, in ruminants, some Peyer´s patches were also shown to host developing lymphocytes.[6] All sites, where lymphocytes develop, are termed primary lymphoid organs.

In BM, leucocytes as well as erythrocytes originate from $CD34^+$ haematopoietic stem cells, which give rise to common lymphoid and myeloid precursor cells (CLP and CMP). They localise within specialised niches of the *spongiosa* meshwork, lined by stromal cells to nourish and control developing cells. Stroma-derived flt3 ligand and IL-7 induce c-kit^+ CLPs to proliferate and develop into B cells. Intermediate B cell developmental stages are classified by expression of surface proteins and lineage markers such as CD19 and surface IgM (sIgM) [41] into pre-progenitor (pre- pro-) B cells, pro-B cells, pre- B cells, immature and mature $sIgM^+$ B cells.

CXCL12, which was formerly termed stroma-derived factor-1 (SDF-1), retains developing B lymphocytes (and granulocytes) first at the *endosteum* and later in sinusoids along stromal processes of BM.[42] To finally get access to circulation,[43] mature B cells partially downmodulate CXCR4, the receptor for CXCL12. Besides, they upregulate CCR7 and regain the transiently lost expression of CXCR5[14] to prepare for subsequent circulation. Homeostatic expression of type I interferons in BM regulates B cell development,[44;45] possibly via controlling the expression of CXCL12.[46]

According to the crucial role of CXCL12 for BM retention, the development of CLPs into T cells is determined by lack of CXCR4 expression on a part of CLPs.[47] Ectopic expression of CXCR4 even abolishes normal T cell development and leads to hypoproliferative T cell lymphopenia.[48] T cell precursors are released into blood to continue maturation in thymus. Once having reached tymic cortex, $CD4^-$ $CD8^-$ double-negative T cells transiently express CXCR4[47;49] and differentiate into $CD4^+$ $CD8^+$ double-positive thymocytes. Hereby, they undergo a stringent positive selection process to guarantee self-tolerance of the arising TCR repertoire. After having developed into $CD4^+$ or $CD8^+$ single positive T cells in thymic cortex,[50;51] T cells pass a negative selection programme and downregulate CXCR4.[47] Guided by other chemokine receptors such as CCR4,[52] surviving, i.e. non-autoaggressive T cells cross the cortico-medullary junction[53;54] to complete maturation for about two weeks in thymic medulla. Therein, $CD4^+$ and $CD8^+$ T cells begin to acquire high levels of L-selectin (CD62L) and increase massively the expression CD69, which probably mediates T cell retention in thymus.[55] Consequently, to allow for release into blood, fully matured thymocytes downregulate CD69[29;55] and finally pass the medullary endothelium via CCR7[56] and $S1P_1$.[28;29]

1.2 Lymphocyte circulation

Lymphocytes dispose of numerous receptors, whose expression is well-controlled and strongly correlated to their maturation and activation stage.[14;17;18;28;47;57;58] Mature lymphocytes enter bloodstream and start their life-long recirculation through vessels and lymphoid tissues. B-1 cells home directly to pleural and peritoneal cavities, where they set up a first-line defence against invading pathogens.

To enable the encounter of the immunologically naïve B and T cells with their individually fitting antigen, lymphocytes have to routinely screen those sites, where antigens are presented. Only in secondary lymphoid organs (SLOs), i.e. spleen, lymph nodes (LNs) and various mucosa-associated lymphoid tissues (MALT) such as Peyer´s patches (PPs), a sufficiently high throughput of B and T cells is organised. This facilitates the rare encounter of the less than a

dozen antigen-specific lymphocytes with their antigen. Furthermore, SLOs provide a sophisticated unique compartmentalisation, which guarantees coordinated B and T cell responses.[8]

1.2.1 Structural differences between lymph nodes and spleen

Lymph collected from tributary tissues in several afferent lymphatic vessels flows into the LN via the subcapsular sinus and diffuses into the LN cortex (Fig. 1-2). Therein, B cell zones (BZ) and T cell zones (TZ, also called paracortex) can be distinguished. The BZ is represented by primary follicles, where naïve B cells pass through to screen for antigen, and by secondary follicles with an ongoing B cell response in so-called germinal centres (GCs). Follicles furthermore contain follicular DCs (FDCs), tingible-body macrophages and very few $CD4^+$ Th cells (see also Fig. 1-5). Follicles are surrounded by the paracortical TZ, in which many $CD4^+$ Th cells, some less $CD8^+$ Tc cells and interdigitating DCs are present. Circulating lymphocytes enter via specialised high endothelial venules (HEV) present in the paracortical region.

LN medulla is composed of medullary cords, which can host plasma cells, and medullary sinuses that coalesce to form the efferent lymphatic vessel, through which activated T lymphocytes and plasma cells finally return to circulation via the *ductus thoracicus*.[8] Porcine LNs, however show an inverse organisation, in which cortical and paracortical regions concentrate around afferent vessels, surrounded by the medulla areas.[6] The organisation of MALT resembles LNs and shows only slight variations according to the hosting organ.[8]

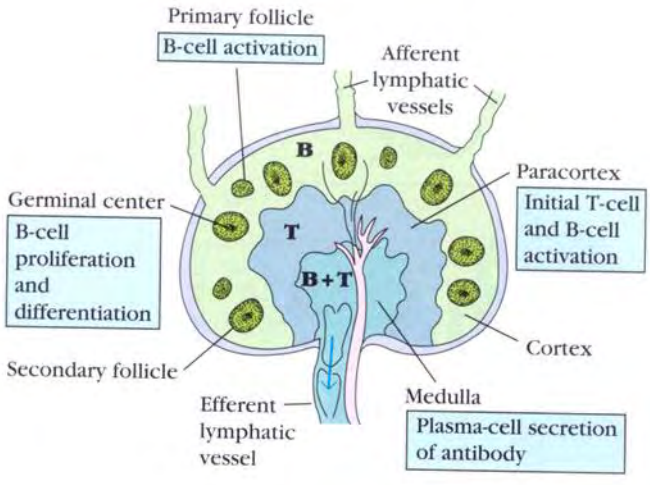


Fig. 1-2: Organisation and function of peripheral lymph nodes

Source: Goldsby RA, Kindt TJ, Osborne BA, Kuby J. Immunology. Fifth Edition. New York: W.H. Freeman and Company; 2003.

The spleen shows an independent and complex organisation that varies to some extent between species.[59] Splenic red pulp represents a unique compartment of partially open blood circulation through blind ending sinusoids. It belongs to the vascular system, albeit it regularly hosts some plasma cells.[60]

Splenic white pulp is organised along *arteriolae centrales* (AC) surrounded by periarterial lymphatic sheaths (PALS) that constitute the splenic TZ (Fig. 1-3). Usually associated with ramifications of arterioles, the splenic BZ appears as follicles with germinal centres (GC) within or growing out of the TZ. A so-called marginal zone (MZ) surrounds the BZ and the PALS and marks the border to the red pulp. Additionally, so-called marginal sinuses (MS) can be interspersed between MZ and BZ or TZ. In splenic sections, murine white pulp appears as round or longitudinal elements that can be distinguished macroscopically by its pale colour and microscopically by absence of erythrocytes and high cellular density.[8]

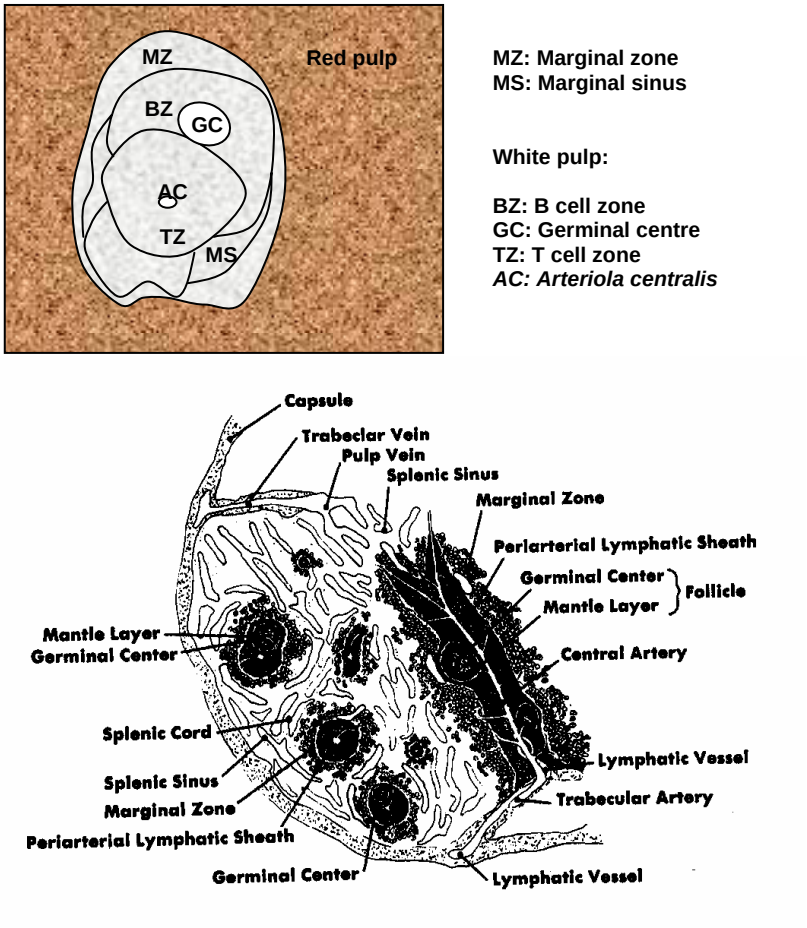


Fig. 1-3: Structural organisation of the spleen

Source: Paul, W.E. Fundamental Immunology. Fourth Edition. Philadelphia: Lipincott-Raven; 1999.

1.2.2 Entry into SLOs follows the multi-step model of leucodiapedesis

Except for red pulp, the vascular component of spleen, SLOs tightly regulate their access to white blood cell areas. Due to the anatomical differences of spleen, which lacks HEV, we will introduce an immigration concept focussing on mechanisms deployed by LNs and PPs. Lymphocyte entry into SLOs is termed “homing” and represents a multistep process of extravasation, so-called leucodiapedesis, which involves molecules on lymphocytes as well as on HEV (Fig. 1-4).[61-63]

L-selectin mediates the first loose contact of lymphocytes with endothelium in form of cellular protrusions, so-called tethers. Tethering and rolling on HEV gradually slow down lymphocytes against strong shear forces in blood.[64;65] L-selectin is recognised by a heterogeneous set of proteoglycans termed “peripheral node addressin” (PNAd)[66] in LNs or binds to MAdCAM-1 in PPs.[67] Secondly, the lymphocyte integrins $\alpha_L\beta_2$ (LFA-1 or CD11a/CD18), $\alpha_4\beta_1$ (VLA-4 or CD49d/CD29) or $\alpha_4\beta_7$ (in PPs) start binding to vascular adhesion molecules, including ICAM-1, ICAM-2 and ICAM-3, VCAM-1, MAdCAM-1 and fibronectin,[68] and initiate adhesion to endothelia. However, integrins of naïve lymphocytes show only low affinity and require triggering of G protein-coupled receptors (GPCRs)[63;69] to change into the activated high-affinity conformation. Hence, in a next step, cell-membrane linked chemokines CCL19, CCL21, CXCL12 and CXCL13 (in PPs), which are either directly produced in HEV[70-72] or transcytosed from nearby stroma,[73;74] bind to their corresponding receptors CCR7, CXCR4 and CXCR5 on naïve lymphocytes.[14;16] Chemokine receptor signalling then induces integrin-mediated firm arrest between lymphocytes and HEV.[75-77] $S1P_1$ might further contribute to amplify integrin activation.[26] Finally, lymphocytes cross HEV and start migrating along chemokine gradients within SLOs stroma.

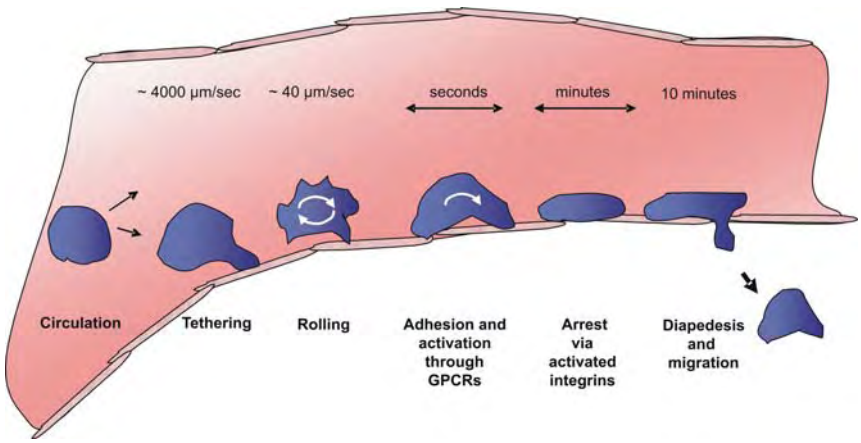


Fig. 1-4: The multi-step model of leucodiapedesis

Adapted from: Campbell JJ, Butcher EC. Chemokines in tissue-specific and microenvironment-specific lymphocyte homing. Curr Opin Immunol. 2000;12:336-341.

In spleen, additional migration and adhesion features exist according to the complex structure and abundant species differences. Some anatomically-focussed studies have even hypothesised the existence of open access without sinus- or MZ-endothelium.[78] Importantly, however, homing into splenic white pulp can be inhibited by treatment with pertussis toxin (PTX) that abolishes GPCR-signalling, and therefore follows the general multi-step model of leucodiapedesis.[79]

In certain cell subsets and under inflammatory conditions, several other molecules can mediate adhesion, such as the heterogeneous group of L-selectin ligands,[80;81] CD44,[82] CD43[83] and vascular adhesion protein-1.[84] Furthermore, receptor-ligand combinations can be interchangeable, so that contributions vary with the cell subset, activation stage and target organ.[85]

1.2.3 Microhoming within SLOs generates specialised compartments to mount immune responses

Despite structural differences, all SLOs are composed of B cell follicles and T cell zones. These highly-organised compartments are generated and maintained by local expression and balanced responsiveness to chemokines CXCL13, CCL19 and CCL21[13;20;21;86] and cytokines TNF-α and LT-$\alpha_1\beta_2$[87;88] that either nourish FDCs or attract the different immune cells. Thus, the coordinated contact between APCs and lymphocytes, which is required to initiate adaptive immune responses, is achieved. Once being primed efficiently within SLOs, activated B and T cells change their receptor equipment.[17;57] This enables them to immigrate into non-lymphoid organs or home to poorly-organised tertiary lymphoid tissues to carry out effector functions. In contrast to memory cells, which reside in splenic red pulp and LN medullary cords, the majority of plasma cells localises in BM and at mucosal surfaces.[17;60] Naïve lymphocytes, which have not encountered their specific antigen, recirculate and home to the next LN.

In spleen, a unique subset of $CD21^{high}CD23^{low}$ B cells (MZB) localises in MZ and does not participate frequently in recirculation. Long-term retention of these MZB cells is mediated by integrins $\alpha_L\beta_2$ (LFA-1 or CD11a/CD18) and $\alpha_4\beta_1$ (VLA-4 or CD49d/CD29),[89] which bind to stromally expressed ICAM-1 and VCAM-1. Due to the efficient perfusion of spleen and the immediate contact between MZ and red pulp, MZB cells and the also resident metallophilic macrophages easily scavenge circulating antigens and hence constitute an important first-line defence to viraemic and bacteriaemic infections.[90] IgM^+ B memory cells[91] and bacterial or autoantigen-specific B cells furthermore preferentially localise in the MZ.

1.2.4 Induction of humoral immunity

Pathogens encountered in the periphery are taken up by DCs and brought to LNs or MALT. During their migration, DCs undergo a maturation characterised by upregulation of MHC class II and costimulatory molecules CD80 and CD86 to efficiently process and present antigen. Expression of CCR7 directs DCs to TZs where they screen for T cells and provide a supportive cytokine milieu. Antigen-specific $CD4^+$ T cells form an immunological synapse[9] with APCs via LFA-1–ICAM-1, CD2–CD58 or DCSIGN–ICAM-3 and are then activated by the concomitant engagement of the TCR to antigen-presenting MHC class II molecules and by binding of CD80 and CD86 to CD28 on T cells. Directed by the cytokine pattern secreted by APCs, activated T cells differentiate into Th cells, which in turn start to screen for antigen-specific B cells.

Three signals, namely i) cytokine stimulation, ii) BCR engagement with APCs within the immunological synapse[9] and iii) costimulation via CD40 and B7-H2 on B cells with CD40 ligand (CD40L) and ICOS (inducible costimulator protein), respectively on T cells, activate B cells to form proliferating foci of plasmablasts at the TZ-BZ boundary. These blasts either differentiate into short-lived plasma cells or enter follicles via CXCR5 to initiate the germinal centre (GC) reaction. Being still in extrafollicular locations or already within the GC, IgM^+ B cells switch their isotype to IgG, IgA or IgE.[92] This switch continues for weeks p.i. and is controlled by Th cells or T cell-independently by the DC-derived cytokines “B lymphocyte stimulator protein” (BlyS, also called BAFF) and APRIL,[93] “a proliferation-inducing ligand”.[94]

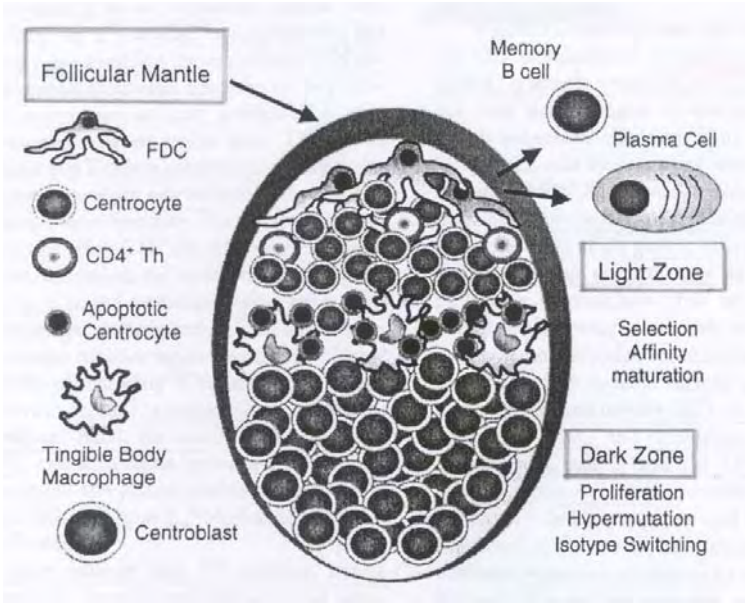


Fig. 1-5: Cells and compartments forming the germinal centre

Source: Wolniak KL, Shinall SM, Waldschmidt TJ. The germinal center response. Crit Rev Immunol. 2004;24:39-65.

The few GC founder B cells start multiplying massively and displace resident follicular B (FOB) cells so that a “mantle” is formed (Fig. 1-5). Follicular Th cells drive B cell proliferation to achieve B cell doubling times of 6-10 h. FDCs continuously present antigen to allow for Ig affinity maturation by a process called “somatic hypermutation”, which is largely restricted to the unique environment of the GC. Random point mutations are introduced into the variable parts and especially into the complementarity-determining regions (CDR) of the *bcr* genes. Subsequent RNA-editing can further contribute to somatic hypermutation.[95] Thus, the antigen-binding pockets of the antibody are progressively optimised. Those B cells, whose BCR looses affinity during somatic hypermutation or evolves autoaggressive, cease proliferation and undergo apoptosis. Therefore, the murine GC exhibits a dark zone of cycling centroblasts and a light zone of arrested and dying centrocytes, which are eliminated by so-called “tingible body macrophages”. Possibly controlled by OX40-OX40 ligand, highly affine B cells differentiate into long-lived plasma cells, which localise in BM, or into memory B cells that remain in SLOs. There, they await for repeated antigen encounter and to launch recall responses in the GC. In conclusion, the GC fashions the B cell repertoire.[92]

Those microbes, which have gained access to circulation, are filtered in splenic MZ and are subsequently presented to MZB cells by resident DCs and macrophages. MZB cells are specialised to quickly carry out T cell-independent IgM and IgG responses[96;97] and to initiate the early plasmablast wave during the first three days.[90] Due to their limited size, viruses can even reach the follicles and trigger FOB cells.[97] In general, GCs can be founded by both FOB and MZB blasts; however, the majority of MZB blasts remains in the MZ as plasma cells to facilitate IgM and IgG-mediated opsonisation of circulating pathogen.[97]

1.3 Peripheral blood lymphocyte numbers and lymphopenia

1.3.1 Clinical aspects affecting physiological lymphocyte numbers

The vast majority of peripheral blood lymphocytes are naïve B and T cells, whereas activated effector cells and immature lymphocytes account for only very minor percentages. In clinical laboratory diagnostics, lymphocytes are indicated in the differential blood count in percentage of total leucocytes. Physiological blood leucocytes levels vary among most domestic animal species between 6 to 12 x 10^6 cells/ml (Tab.1-1). Usually, cattle have a lower, small carnivores an intermediate blood count and pigs can show up to 20 x 10^6 leucocytes/ml. With respect to the percentage of lymphocytes of all peripheral blood leucocytes, domestic animals are classified in

two groups of an either lymphocyte or neutrophil–dominated differential blood count. In mice, we find absolute leucocyte numbers of 4-10 x 10^6 leucocytes/ml of which 60-85% account for lymphocytes, depending on the strain.[98] Absolute numbers of 6-8 weeks old BL/6 mice in this study ranged between 1600-4000 B cells and 400-1200 T cells per µl (personal observations).

Tab. 1-1: Leucocytes and lymphocytes in the differential blood count of domestic animals

Species[98]	Dog	Cat	Horse	Cattle	Swine	Mouse	Human (adult)[99]
Leucocytes [10^6/ml]	6-12	6-11	6-10	4-10	10-20	4-10	4-9
Lymphocytes [%]	13-30	15-30	13-30	45-60	50-85	approx. 60-85	25-40

Variations of blood lymphocyte counts within one species can be due to physiologic and pathologic conditions. Physiologic factors comprise age, gender, breed, excitement or stress and the endogenous cortisol levels, whose secretion underlies the circadian biorhythm set up by the hypothalamic-pituitary-adrenal axis.[100] Due to circadian secretion of cortisol, the levels of circulating lymphocytes peak at day and reach a minimum in the night. The stress-related endogenous messengers epinephrine and cortisol exert short and long-term effects on leucocyte numbers. Stress immediately increases numbers of lymphocytes and neutrophils, but causes lymphopenia one hour later or upon prolonged exposure.[100-103] In experimental immunology, BM reconstitution following lethal irradiation leads to anaemia-induced proliferation of both red and white blood cells, yielding more than five times higher B and T cell counts (personal observations).

Variations in lymphocyte numbers of up to factor 2 lie within the physiologic range and can occur easily between outbred individuals, i.e. the majority of domestic animals. However, alterations of about factor 10 indicate severe pathological conditions. An abnormal increase of lymphocyte numbers is called lymphocytosis that can occur upon chronic infections or in leukaemic leucosis, among many others. Massive reduction of blood lymphocyte numbers, termed lymphopenia, often goes along with further symptoms of disease and can be the consequence of hypoproliferation, for example following toxic or neoplastic suppression of BM, or of increased cell degradation due to lymphocyte infections, such as theileriasis. In absence of specific symptoms, however, lymphopenia is classically considered in Laboratory Medicine as an early marker for recent viral infection and serves to rule out differential diagnoses.[98]

1.3.2 Lymphopenia during early viral infection

Early viral infection of animals and humans is often associated with a profound redistribution of lymphocytes within the organism long before onset of clinical symptoms.[98;104]A well-known feature of the incubation time is a prominent, but transient lymphopenia in blood. Besides blood, the decrease in number of lymphocytes also affects lymph and cerebrospinal fluid [105;106], and was suspected to be consequence of lymph node "shut down" of efferent lymph. In some cases, "BM depression" of lympho- and myelopoiesis is observed as early inflammatory reaction of the haematopoetic system[107] and can go along with enhanced output of immature lymphocyte precursors, leading to a left shift of the lymphocyte lineage in blood.[46]

Previous experimental studies in mice infected with virus or treated with pathogen-mimicking TLR ligands attributed lymphopenia in blood and lymph to effects of cytokines, especially type I IFN,[105;108] rather than the administered agents themselves.[108] In line with this, lymphopenia is reported as a common side effect of human IFN-α/β therapy of multiple sclerosis, chronic hepatitis B and C and various neoplasias including hairy cell leukaemia, multiple myeloma and malign melanoma.[109-111] Furthermore, lymphopenia is also observed in the rather novel IFN-ω treatment of FIV and/or FeLV infection and of canine parvovirosis (Virbagen IFN-ω package insert).[112] Similarly, treatment with IFN-α, IL-2, TNF-α[109] IL-12[113] and various chemical compounds, including the clinically used immunosuppressant FTY720,[27] were also reported to cause a reduction of lymphocytes and other leucocyte subsets in patients´ blood.

In face of the largely unknown lymphocyte homing during lymphopenia,[114] several reports found a redistribution of leucocytes within SLOs following viral infection or challenge with the TLR ligand poly(I:C).[108;115] In essence, however, the mechanism of lymphopenia, lymphopenic homing and the cellular targets of cytokines and other factors remain elusive.

1.4 Interferons

1.4.1 Classification of interferons

Interferons (IFNs) were discovered on the basis of their antiviral activity[116] and are classified in two distinct families, IFN-α/β (or type I IFN) and IFN-γ (or type II IFN) by primary sequence homology, use of distinct cell surface receptors and direct induction upon virus infection or during immune responses. [117;118]

Type I IFN includes small peptides derived from up to 13 genes for IFN-α and one for IFN-β, depending on the species. Type I IFN further comprises IFN-δ in swine, IFN-κ, IFN-τ, IFN-ω and a factor known as limitin or IFN-ζ,[45] which are induced locally and play specialised roles such as IFN-κ in keratinocytes[119] and the trophoblast-derived IFN-τ and IFN-ω[6] that exert anti-luteolytic activity.[45;120] For therapeutic applications, natural interferons were modified chemically to prevent the quick renal excretion of small peptides. This led to the generation of so-called pegylated type I IFN that shows long-lasting effects in clinical use.
Type I IFN binds to the heterodimeric IFNAR composed of an α-chain, which is essential for signalling, and a β-chain. Expression of IFNAR was described to be moderately subjected to circadian biorhythm.[121]

1.4.2 Induction of IFNs

Microbial infections are sensed in form of "danger signals" of viral and bacterial PAMPs, which are expressed or even released by invading pathogens. Typical viral PAMPs are single-stranded and double-stranded RNA or DNA, which are massively produced upon viral replication and induce a set of cytokines in order to defend the host.[122] Whereas virtually all tissues respond to type I IFN with the expression of a wide array of IFN-inducible genes,[123] only few cells are specialised to produce large amounts of type I IFN *in vivo*.[36]

Upon infection with vesicular stomatitis virus, a specialised type of DCs of plasma cell-like phenotype, so-called plasmacytoid DCs (pDCs), represents the major source of type I IFN.[36] pDCs[36;38;39;124] reside in splenic MZ, where they can sense viral PAMPs by several pathways[125] including TLR7 and cytoplasmic protein kinases like PKR, RIG-I[126;127] and mda5.[128] Apart from pDCs, myeloid DCs (mDCs) can also produce type I IFN, probably deploying other pathways.[129] Apparently, the viral species determines the cell type to launch massive cytokine responses.[130]

Recognition of viral infection leads to phosphorylation of IFN regulatory factor-3 (IRF-3), which translocates into the nucleus to initiate transcription of IFN-β and IFN-α4 (Fig. 1-6). In a next step, these so-called early IFNs are secreted and bind to the IFNAR in an autocrine fashion. IFNAR-signalling via the Jak/STAT-pathway activates Janus kinases (Jak) and phosphorylates the adapter proteins "signal transducer and activator of transcription" (STAT) that join IRF-7 to start massive transcription of all other members of type I IFN. *In vivo*, pDCs can also induce high amounts of type I IFN in absence of IFNAR feedback-signalling.[36]

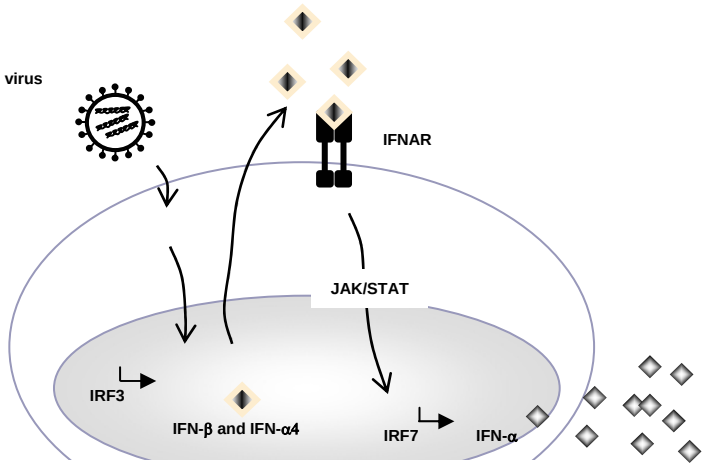


Fig. 1-6: Induction of type I IFN and IFNAR feedback-signalling

1.4.3 Control and inhibition of type I IFN induction

Potent induction of type I IFN is critical for resistance to viral infection; however, a prolonged stimulation with the pleitropic type I IFN can also result detrimental to the host. Therefore, a number of cellular inhibitory mechanisms control the extent and duration of type I IFN production in pDCs and mDCs.[131;132] On the other hand, viruses try to evade antiviral cytokines responses and have developed various strategies to interfere with the induction of type I IFN.[133] Numerous different type I IFN antagonists have been described[134-137] that either shut down or limit type I IFN responses. For example, influenza virus largely downregulates cytokine induction and even achieved to abuse the remnant NFkB activation for its own propagation.[138]

1.4.4 Effects of type I IFN on lymphocytes

In addition to anti-viral activity,[123;139] type I IFN exhibits pleiotropic effects that can have an impact on proliferation,[140] apoptosis[141] and expression of cytokines and cytokine receptors,[142-145] which link innate and adaptive immunity and can cause immune activation or modulation *in vivo*.[11;146;147]

Type I IFN exerts direct and indirect effects on lymphocytes. Many indirect effects of type I IFN are presumably mediated by the activation of APCs,[148-151] which in turn secrete various cytokines, such as IL-15 that can act on lymphocytes[145] and enhance upregulation of CD69 on T cells.[152]

In vivo, a critical role of type I IFN in $CD8^+$ T cell cross-priming was shown[153] which could recently be identified as a direct effect of type I IFN on $CD8^+$ T cells to promote clonal expansion and formation of memory T cells by enhancing cell survival.[154] Certainly, type I IFN does directly stimulate naïve T cells *in vitro* and may influence T cell development. Upon type I IFN administration, immune responses may be skewed towards the Th1 phenotype.[155;156]

On B cells, type I IFN seems to exert many direct effects. During B cell development, type I IFN inhibits IL-7-induced growth and survival of early B-lineage cells[44] and appears to set the stringency of B cell-repertoire selection.[157] Type I IFN-stimulated peripheral B cells upregulate CD69,[152] show an increased sensitivity to IgM receptor ligation[158;159] and are protected from apoptosis *in vitro*.[160] Furthermore, type I IFN acts on plasmablasts and thus promotes final plasma cell differentiation.[161] B cell responses *in vitro* may be both enhanced or inhibited,[158;161-164] whereas immunoglobulin production and isotype switch *in vivo* may be critically affected by type I IFN.[149;156;165] However, *in vivo,* the significance of direct type I IFN stimulation of B cells remains unclear.

Apart from activating B and T cells, type I IFN can influence lymphocyte homeostasis by suppressing haematopoiesis[107] or by enhancing output of lymphocyte precursors.[46] Moreover, it is conceivable that type I IFN affects circulation and homing of lymphocytes because it can induce chemokines and modulate adhesion molecules in human T cell lines[166] and endothelial cells.[167]

1.5 Vesicular stomatitis virus (VSV)

Vesicular stomatitis virus is a member of the genus *Vesiculovirus* and belongs to the family *Rhabdoviridae*. Several serotypes of VSV exists, including the experimentally used Indiana (VSV-IND) that shows a very broad host spectrum ranging from mammals and birds to insects. In horses, cattle and swine, VSV causes a disease characterised by vesicular lesions in the mouth that led to the name of “vesicular stomatitis”. Besides, VSV can cause erosions in the interdigital fissure of artiodactyls, especially swine, or at the coronary band of horses.[168] Since these vesicular lesions are indistinguishable from those caused by the highly contagious foot-and-mouth-disease virus, vesicular stomatitis is a notifiable disease listed in group A of infectious epizootics.

In humans, accidental inoculation of VSV can cause an influenza-like disease. In mice, intravenous (i.v.) infection is well-tolerated up to very high doses of 10^8 plaque-forming units (PFU). Due to the conserved neurotropism of the *Rhabdoviridae*, an intranasal (i.n.) challenge with more than 10^4 PFU VSV frequently results in lethal encephalitis.
VSV shows bullet-shaped morphology, contains negative ssRNA and measures about 170 nm in length and 70 nm in width (Fig. 1-7).[6]

Fig. 1-7: Electron micrograph of vesicular stomatitis virus
Micrograph from Frederick A. Murphy, School of Veterinary Medicine, University of California Davis, USA

The envelope of VSV is based on the matrix (M) protein inserted in a host cell-derived lipid bi-layer. Therein inserts the externally oriented glycoprotein (VSV-G). It forms the paracrystalline virion surface that is characterised by the tips of VSV-G which are arranged at a distance of 5-10 nm each.[169] This highly-repetitive pattern is able to cross-link IgM and thus provides a strong signal to induce the early IgM response without further need of T cell help.[169] The surface protein on VSV virions is therefore classified as a T cell-independent (TI) antigen. Since only the very tips of VSV-G are accessible by antibodies, the anti-VSV humoral response is directed nearly exclusively to one immuno-determinant on VSV-G.[170;171]

Besides potent humoral immunity with long-lasting IgG titres, VSV induces an effective CTL response. However, VSV infection is controlled by the concerted action of type I IFN and neutralising antibodies as indicated by the strongly increased susceptibility to lethal VSV infection of type I IFN receptor-deficient ($IFNAR^{-/-}$) mice[139] or B cell-deficient mice.[172] Passive immunisation alone suffices to protect $IFNAR^{-/-}$ mice against lethal VSV infection. However, metaphylactic transfer of neutralising antibodies is only effective if applied within 3 h following viral challenge. Adoptive transfer of VSV-G-specific memory B and T cells does not generate neutralising antibodies quickly enough to mediate protection in $IFNAR^{-/-}$ mice.[173]

1.6 Aim of the thesis

Lymphocytes are key players in the adaptive immunity to viral infections for generating antibodies and eliminating infected cells. Production of antiviral interferons (type I IFN) is a generic feature of viral infections, and there are multiple hints that type I IFN influences the adaptive immune system. For more than 100 years, Veterinary Sciences and Medicine reported massive lymphopenia during the incubation period of viral infections. Previous experimental studies in mice related this dramatic lymphopenia to type I IFN activity.

We hypothesised that type I IFN exerted direct effects on lymphocytes *in vivo*. The first part of the study focussed on the phenomenon of lymphopenia. The aim of the thesis was to identify the direct type I IFN stimulation of lymphocytes during lymphopenia; firstly by deploying a model of adoptive transfer of fluorescently labelled cells and secondly, with the use of novel conditionally-targeted mice with a B or T cell-specific deletion of the type I IFN receptor (IFNAR). For this, the IFNAR deletion of the conditionally-targeted mice was to be assessed on genetic and functional level. To count absolute numbers of lymphocytes in murine blood, a FACS-based technique was developed. Type I IFN production was either elicited by infection with vesicular stomatitis virus (VSV) or by treatment with the pathogen-related compounds poly(I:C) and R-848. Studies were designed to analyse whether lymphopenia was reversible or involved apoptosis and neo-formation of lymphocytes.

An important objective was to elucidate a possible role of type I IFN stimulation of lymphoid tissue stroma and endothelia, for which bone marrow-chimeric mice were generated. FACS analyses and confocal microscopy were pursued to study the lymphocyte homing targets during lymphopenia. Furthermore, the dissertation aimed at investigating the molecular mechanism of lymphopenia by using chemotaxis assays and FACS analyses to study receptors and ligands that control leucodiapedesis.

The second part of the work concentrated on how type I IFN modulates the humoral immunity. For this, we evaluated IgM and IgG responses towards the glycoprotein of VSV (VSV-G) in two antigen models of different immunogenicity. Firstly, systemic or peripheral infection with the highly immunogenic live VSV was studied. Secondly, recombinant virus-like particles (VLPs) displaying VSV-G were produced and characterised by electron microscopy. These non-replicative VLPs were then used for immunisation in presence or absence of IFN-α as an adjuvant. Antibody titres were analysed by enzyme-linked immunosorbent assays (ELISAs) and serum neutralisation tests (SNTs).

2 Materials and methods

2.1 Mice

C57BL/6 and C57BL/6 CD45.1 mice were purchased from Charles River. IFNAR$^{flox/flox}$ (see "Results"), IFNAR$^{\Delta ex10/\Delta ex10}$ mice (see "Results"), CD19-Cre,[174] CD4-Cre mice,[175] CD19Cre$^{+/-}$ IFNAR$^{flox/flox}$ and CD4Cre$^{+/-}$ IFNAR$^{flox/flox}$ (see "Results") were bred under specific pathogen-free (SPF)-conditions in individually ventilated cages (IVCs) at the mouse facility of the Paul-Ehrlich-Institut. All genetically modified mice used were 10-fold backcrossed to the C57BL/6 background (Tab.2-1). Experimental mouse work was performed under SPF conditions and mice were kept in IVCs or filter-isolated cages. Experimental animal work was conducted in compliance with the German federal and state legislation on animal experiments.

Tab. 2.1: Mouse strains used in this study

Mouse strain	**Provider**	**Strategy of genetic modification**	**Recombination of desired construct in genome**
C57BL/6	Charles River, PEI	-	
C57BL/6 CD45.1	Charles River	-	
IFNAR$^{-/-}$	PEI	Knock out via gene-targeting	Homologous
CD19Cre$^{+/-}$	PEI	Knock in via gene-targeting	Homologous
CD4Cre$^{+/-}$	PEI	Knock in of a transgene	Random
IFNAR$^{flox/flox}$	PEI	Conditional gene-targeting	Homologous
CD19Cre$^{+/-}$ IFNAR$^{flox/flox}$	PEI	Conditional IFNAR deletion in B cells	
CD4Cre$^{+/-}$ IFNAR$^{flox/flox}$	PEI	Conditional IFNAR deletion in T cells	

2.1.1 Mouse anaesthesia

Additional materials	Source
Isofluran	Curamed
Glass with a top	Schott

The glass was prepared with paper and 1 ml isofluran was added. The glass was covered and after 1 min, isofluran was evaporated. A mouse was set into the glass and observed for progressive stages of induction of anaesthesia. The mice first started to scratch their eyes and then lost conscience and fell to the side. After a few rapid breathings, the animals lost tension of the tail and did not react with defence movements when moving the glass. The operation stage of anaesthesia was achieved after usually 10 sec, when the mice started to breath profoundly and slowlier. The mice were then immediately taken out and manipulations could be performed for approximately 20 – 30 sec.

2.1.2 Mouse infections and injection procedures

Additional materials	Source
Omnican F 1 ml/0,01 ml (High precision dosing syringe, 0.3 mm diameter canula integrated into syringe)	Braun, “Ref: 09 16 15 03, PZN: 31 15 46 5”
Mouse restrainer (3 cm tube diameter)	PEI workshop
Pipette 10 µl	Eppendorf
Pipette tips (crystal)	Eppendorf

Mice were warmed up in their cages for 10 min by an infra-red lamp, which was set in 20 – 30 cm distance to the cage. Then, the mice were introduced into restrainers and injected with 2×10^6 or 2×10^7 PFU VSV into the lateral tail vein (i.v.) using a 0.3 mm syringe.

For i.n. infections, mice were anaesthesised, turned on the back and injected during inspiration with 5 µl of 10^4 PFU virus-containing PBS. After breathing few times, the mice were turned to the side and started to wake up.

2.1.3 Mouse treatments to induce lymphopenia

Additional materials	Source
Poly(I:C)	Sigma, # P 0913
R-848	3M, kindly provided by Heinfried Radeke, Uni Frankfurt
IFN-α	Kindly provided by D. Tough, Edward Jenner Institute, Compton, UK
IFN-ß	R&D, # 12 400-1

Mice were anaesthesised, turned on the back and injected i.p. with 200 µg poly(I:C) or 25 µg R-848 in 200 µl PBS. Alternatively, mice were treated s.c. in the right flank with 2 x 10^5 IU IFN-α or IFN-ß. Blood lymphocyte counts were assessed 16 to 20 h later.

2.1.4 Blood sampling for cell counting or serum analysis

Additional materials	Source
Glass capillaries	VWR, # 612-1701
Vacutainer-Microtainer, Plasma Li-heparin	BD via Döll Medizintechnik, Hofheim, # 36 59 66
Vacutainer-Microtainer, Serum	BD via Döll Medizintechnik, Hofheim, # 36 59 51

Mice were anaesthesised and laid on the left side. By gently grasping the skin of the neck close to the head, the blood flow in the jugular veins was blocked while the trachea was not suppressed to a major extent. The grasp further pushed forward the eyes, so that the capillary could be introduced behind the eye (retrobulbary bleeding). Under gentle turning, the ophthalmic venous plexus was opened and blood was collected in the capillary either directly behind the eye bulb or externally in from of drops. Few drops were collected for blood cell counting in heparinised micotainer tubes and approx. 150 µl were taken for serum sampling in serum vacutainers.

2.1.5 Isolation of splenocytes

Additional materials	Source
Set of surgical instruments	Hauptner/Herberholz
70% ethanol	PEI
PBS	PEI, Gibco
70 µm plastic cell strainer	BD Falcon, # 352350
2 ml syringe (or Norm-Ject 2 ml)	B. Braun Melsungen AG, # 460 60 27V or Henke Sass Wolf
Pipetboy	Pipetboy acu, Integra Bioscience
One way plastic pipettes	Greiner
Centrifuge	Heraeus Sepatech, Kendro

Mice were anaesthesised and sacrificed by atlanto-occipital dislocation. The bodies were bathed in 70% ethanol for disinfection when sterility was required for further procedure. Laying on the right side, the skin was cut behind the ribs and pulled away with coarse forceps. The abdominal wall was opened with a new pair of scissors and forceps to take out the spleen. The connecting ligaments were cut and the organ was put onto a 70 µm cell strainer that had been set onto a 50 ml Falcon tube. Some millilitres PBS were added and 2 spleens per strainer were squeezed with the plunger of a 2 ml syringe to open the splenic capsule. Crops of splenic tissue were flushed and carefully squeezed by moving the plunger up and down. Spleens were considered fully harvested when no red colour was visible any more. If staining samples containing about 2×10^5 cells had to be taken, 60 µl of the usually 15 ml splenic suspension were reserved.

The tubes were left for few minutes to sediment detritus. After decanting the suspensions into new 50 ml Falcon tubes, the cells were spun down at 800 rpm for 6 min.

2.1.6 Isolation of LN cells

Additional materials	Source
Block of polystyrene	(Package material)
Canulas	Henke Sass Wolf
PBS	PEI, Gibco PBS, # 100 10-015
70 µm plastic cell strainer	BD Falcon, # 352350
2 ml syringe (or Norm-Ject 2 ml)	B. Braun Melsungen AG, # 460 60 27V or

After sacrifice, the mice were turned on the back to extend and fix the limbs with needles on a polystyrene block. Skin was opened and pulled back to visualise the limbs. The *nodus lymphaticus axillaris* (laying close to *arteria* and *nervus axillaris*), the *nodus lymphaticus cervicalis superficialis* (close to the *angulus dorsalis scapulae*) and the *nodus lymphaticus inguinalis superficialis* (with a prominent vessel helping the identification) were removed. As for spleen, the LNs were squeezed and single cell suspensions prepared in PBS. Due to adherent fat tissue, the LN samples were spun down for 5 min at 1200 rpm and the pellets were resuspended in 1 ml.

For homing studies with CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ and CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice, spleens and two LN from the left side were prepared. In adoptive transfer experiments, at least three LNs were isolated from both sides. Single cell suspensions were prepared and stained for B and T cells and analysed by FACS.

2.1.7 Isolation of thymic cells

Mice were sacrificed and fixed after turning on the back. The thymus was visualised after opening the neck and thorax. The organ was grasped with forceps without touching neighbouring vessels to prevent contamination with blood. The thymi were squeezed on cell strainers to obtain single cell suspensions.

2.1.8 Isolation of BM cells

Additional materials	Source
Block of polystyrene	(Package material)
Canulas 26 G	Henke Sass Wolf
Petri dishes	Greiner
Syringes 10ml	Braun

After sacrifice of the mice, the hind limbs were taken off the corpse and femurs were purged from adherent muscles. Bones were washed in PBS in several Petri dishes. *Caput* and *condylus femoris* were clipped off and the BM was flushed out in PBS with a canula.

2.1.9 Generation of bone marrow-chimeric mice

Additional materials	Source
^{137}Cs-radiation machine	STS Steuerungstechnik und Strahlenschutz GmbH
Ventilated metal cage	STS Steuerungstechnik und Strahlenschutz GmbH

To study the impact of type I IFN stimulation of endothelium and lymphoid stroma, BM-chimeric mice with IFNAR-competent or IFNAR-deficient endothelia and immune cells, respectively, were generated. For this, lethal irradiation and BM reconstitution were performed.

Lethal irradiation of mice leads to abundant and irreversible damages in DNA, which especially affect proliferating cells such as BM stem cells, gastrointestinal epithelia and germ cells. Thus, a complete myelosuppression and severe alterations of inner organs and blood are observed in the irradiated organism, which cause a dose-dependent radiation syndrome. Myelosuppression first affects erythrocytes since they have the most rapid physiologic turnover among blood cells. Thus, the animals develop a severe hypoplastic anaemia from day 4 onwards, to which they finally would succumb without any treatment. However, all residing cells like stroma and endothelium are largely resistant to radiation.

To generate BM chimeras, 8-10 weeks old mice were lethally irradiated for 247 sec with γ-rays of a ^{137}Cs radiation source, a dose equivalent to 11 Gy. During irradiation, the mice were kept in a ventilated metal cage turning at the lowest frequency (<< 30rpm) within the radiation device. The following day, the mice were reconstituted with 5 x 10^6 BM cells, which were injected i.v. in 200 µl PBS.

To distinguish the lymphocytes of donors and recipients, we used congenic mice, which are genetically identical except for the leucocyte allotype marker CD45 (Ly5), which exists in two isoforms (see also 1.1.5). According to current nomenclature, wild-type BL/6 mice and all strains crossed to BL/6 background carry the isoform 2 (allotype CD45.2), whereas congenic BL/6 Ly5.1 mice express CD45.1. After BM reconstitution, mice were left to recover. After for approximately 6 weeks, the reconstitution efficiency was assessed by FACS analysis of CD45.1 and CD45.2 expression on peripheral blood lymphocytes (see Fig. 3-7A). $IFNAR^{-/-}$>$IFNAR^{-/-}$ chimeras could not be analysed since $IFNAR^{-/-}$ Ly5.1 mice were not available. The chimerism usually exceeded 95%, whereas typically 5% recipient-derived long-lived T cells were found in blood of the reconstituted chimeras.

2.2 Cells

2.2.1 Lysis of red blood cells

Additional materials	Source
Red blood cell lysing buffer	Sigma, # R7757
Alarm clock	neoLab 2-2002

After centrifuging splenic single cell suspensions, supernatant was taken off and the pellets were resuspended in 4 ml Sigma red blood cell lysing buffer. After incubation for 60 – 90 sec, the hypotonic solution was diluted with 15 ml PBS. If several mouse strains were used, suspensions of identical cell type were pooled and spun down at 1200 rpm for 6 min. Next, the cells were resuspended in the appropriate buffer or medium, according to the further procedure.

2.2.2 Manual counting of cells

Additional materials	Source
96 *well* round bottom plate	Nunc
Pipette (10 - 100 µl or 50 – 200 µl)	Eppendorf
Pipette tips yellow	Eppendorf
Trypan blue (0,4 % Trypan blue in PBS)	Sigma
Neubauer cell counting chamber	Labor Optik
Cover slip	Menzel-Gläser
Manual counter	Rettberg Laborgeräte
Microscope	Axiovert25, Axiolab, Zeiss

After shaking the cell suspension, 50 µl were taken and mixed 1:1 in a 96-well microtitre plate with trypan blue solution. Dilutions of 1:8 and 1:16 were prepared for analysis in a Neubauer counting chamber and covered with a slip. Vital lymphocytes - as characterised by pale grey colour - were counted manually per microscope in the four large squares at the corners of the chamber grid. Cell numbers were calculated according to the following:

Cells/ml = $n/4$ x dilution x 10^4 x volume [ml] of total suspension

n = counted cells in four squares

2.2.3 Purification of cells by Magnetic adsorption cell sorting (MACS)

2.2.3.1 Isolation of untouched B cells from murine spleens

Additional materials	Source
B cell isolation kit	Miltenyi, # 130-090-862
MACS separation columns (LS type, Midi)	Miltenyi, # 130- 042- 401
MACS – Multi Stand	Milteny Biotech
MACS magnet for LS columns	Milteny Biotech
5 ml tube with cap	Greiner
Steritop filters GP 0,22 µm	Fischer Scientific
Vacuum pump	Vacusafe, IBS Integra Biosciences
MACS buffer for murine B cell isolation kit	PBS
	0.5% BSA
	2 mM EDTA
Balance	Satorius LP 820
Refrigerator	Liebherr

Splenic single cell suspensions were counted and aliquotted at 10^8 per tube. During the MACS procedures, the cells were kept on ice, except for the incubation steps.

The suspensions were spun down for 10 min at 1800 rpm. MACS buffer was prepared and sterile-filtered. Supernatant was taken off completely and the cells were resuspended in 40 µl MACS buffer/10^7 cells. 10 µl of Biotin Cell Antibody/10^7 cells were added, mixed and incubated for 10 min in the fridge. Next, 30 µl of buffer/10^7 cells and 20 µl of Anti-Biotin-MicroBeads/10^7 cells were added. After 15 min incubation in the fridge, the cells were washed with 10 x labelling volume and spun down for 10 min at 1800 rpm.

Midi columns were placed into the magnets and a tube for the wash fluid was colocated beneath. The columns were prepared with 3 ml MACS buffer. Supernatant of centrifuged cells was taken off and the suspensions were resuspended in 0.5 ml MACS buffer. New tubes for the negative

fraction to be isolated, i.e. the untouched B cells, were placed and the cell suspension was applied onto the column.

After 3 times washing with 4 ml buffer, the suspension was spun down for 10 min at 1800 rpm. Supernatant was discarded, the cells were resuspended in the appropriate buffer for the next treatment and a dilution of 1: 16 was counted.

2.2.3.2 Positive selection of B cells with CD19 MicroBeads

Additional materials	Source
CD19 MicroBeads	Miltenyi, # 130-052-201
MACS separation columns (LS type, Midi) including plunger	Miltenyi, # 130- 042- 401
MACS buffer for CD19 MACS	PBS
	0.5% BSA

10^8 splenocytes per tube were spun down for 10 min at 1300 rpm. MACS buffer was prepared and sterile-filtered. Supernatant was taken off completely and the cells were resuspended in 90 µl MACS buffer/10^7 cells. 10 µl CD19 MicroBeads/10^7 cells were added and mixed thoroughly. After 15 min incubation in the fridge, the cells were washed with 10 x labelling volume and spun down for 10 min at 1300 rpm. Midi columns were placed and prepared with 3 ml MACS buffer. Supernatant of the centrifuged cells was taken off and the suspensions were resuspended in 0.5 ml MACS buffer for being applied onto the column.

After 3 times washing with 3 ml buffer, the column was taken off the magnet, placed onto a 15 ml Falcon tube and rinsed with 3 ml MACS buffer. The suspension containing the positive fraction, i.e. the $CD19^+$ B cells, was pressed out with the plunger and spun down for 10 min at 1300 rpm. Supernatant was discarded and the cells were resuspended in the appropriate buffer for the next treatment and a dilution of 1: 16 was counted.

2.2.4 Cell culture

2.2.4.1 Culture of primary B cells and *in vitro* stimulation with IFN-ß

Additional materials	Source
50 ml tissue culture flasks	Greiner
Incubators with supply of CO_2	Cytoperm, Heraeus
Sterile work bench	Steril Gard II Advance, The Baker Company
β-mercapto ethanol	Sigma
RPMI 1640	PEI, Gibco
Fetal calf serum (FCS)	Biochrom KG, Gibco
Glutamine	Gibco-BRL
ß-mercapto ethanol	Sigma, # M 7522
Murine B cell culture medium	RPMI 1640
	10% FCS
	1% Glutamine
	0.015 µM β-mercapto ethanol
IFN-ß	R&D, # 12 400-1

All cell culture work was performed under sterile conditions below laminar flow working benches of biosafety level II. The cell culture media for B cells were free of antibiotics. The MACS-purified murine B cells were cultured in 50 ml tissue culture flasks in murine B cell culture medium at 2 x 10^6 cells/ml. To stimulate the B cells, 10^3 IU IFN-ß were added. The cells were kept over night in an incubator in vapour-saturated atmosphere at 37°C and 5% CO_2.

Next day (after approx. 16 h), the cells were harvested. Due to strong non-specific adhesion of naïve B cells to uncoated plastic (personal and colleagues´ observations), the B cells were resuspended thoroughly by pipetting up and down and the cell culture flasks were washed three times with cold PBS.

2.2.4.2 Short-term culture of splenocytes to inhibit G protein-coupled receptors (GPCRs) with pertussis toxin (PTX)

Additional materials	Source
250 ml tissue culture flasks with filter top	Greiner
Pertussis toxin (50 µgin dilution)	Sigma, # P2980
37°C room	PEI
Shaker	Infors AG, Switzerland

Splenocytes were incubated for 3 h in 250 ml tissue culture flasks in murine B cell culture medium ± 20 ng/ml PTX at 2 x 10^6 cells/ml.

To prevent adhesion, the culture flasks were kept on a shaker at 37°C. After 3 h, the cells were harvested by resuspending thoroughly and washing the cell culture flasks with cold PBS.

2.3 Viruses

2.3.1 Vesicular stomatitis virus (VSV)

Additional materials	Source
Ultra low freezer (-80°C)	New Brunswick Scientific
Cryo conservation tubes (1.2 ml, 2.5 ml)	Nunc
VSV-Indiana (Mudd-Summers isolate), Wild-type virus	Originally obtained from D. Kolakofsky, University of Geneva, Switzerland
VSV-M2 (natural variant of HR strain)	Kindly provided by J. Bell, Canada

Stocks of VSV-Indiana, containing 10^{12} or 10^{9} PFU/ml in purified cell culture supernatants, were stored at -80° C. 50 µl aliquots were thawn on ice and discarded after usage. VSV-M2 was kept in stocks of 10^{9} PFU/ml.

2.3.2 Generation of virus-like particles (VLPs) expressing the VSV-G protein

Additional materials	Source
Lipofectamine 2000	Invitrogen, # 11 66 8-019
T175 cell culture flasks	Greiner
293T cells	Originally obtained from American Type Culture Collection (ACCT)
Dulbecco´s modified Eagle´s medium (DMEM)	Gibco
Penicillin/Streptomycin	Gibco-BRL
VSV-G displaying vector	Kindly provided by Christian Buchholz, PEI
Murine leukaemia virus (MLV)-based gag/pol expression plasmid pHit60	Plasmid factory
0.22 µm sterile filters	Qualilab
Cooling centrifuge	Heraeus Sepatech, Kendro

3×10^6 293T cells were seeded in T175 cell culture flasks in DMEM + 10% FCS containing antibiotics and glutamine.

After incubation over night, the cells were transfected with 5 µg of the VSV-G displaying vector and with 12.5 µg of the MLV-derived gag/pol expression plasmid pHit60 per cell culture flask. For this, the plasmids were mixed with 2 ml DMEM devoid of FCS, antibiotics or glutamine. In another reaction tube, 90 µl Lipofectamine 2000 were mixed with 2 ml pure DMEM. After incubation for 5 min at room temperature, the two mixtures were pooled and incubated for 20 min at room temperature. Subsequently, 6 ml DMEM with FCS and glutamine, but without antibiotics, were added to 4 ml transfection mix. Finally, the media were removed from the cell culture flasks and 10 ml transfection mix/flask were applied onto to the cells without washing. After 4 h incubation at 37°C, the medium was replaced with 20 ml DMEM + 10 % FCS containing antibiotics and glutamine.

The VLP-containing cell culture supernatants were harvested twice after 48 h and 72 h incubation and filtered through 0.2 µm sterile filters to remove clumps. The filtrate was spun down at 3600 rpm over night at 4°C. After discarding supernatants, the tubes were dried by standing upside down on absorptive paper for few minutes and subsequent manual removal of remnant liquids. By pipetting or gentle vortexing, the pellets were resuspended in 100 µl/flask of PBS containing 1% FCS. Finally, the VLPs preparations were shock-frozen at -80°C in 50 µl aliquots.

2.3.3 Labelling of VLPs for immune electron microscopy (EM) analysis

Additional materials	Source
Parafilm	American National Can
Carbon-vaporised and flamed 400-mesh Cu/Rh grids	Plano, adapted in PEI microscopy facility
Filter paper	Schleicher & Schuell
Polyclonal rabbit anti-VSV-G	Kindly provided by Bernhard Odermatt, CH
Goat anti-rabbit IgG labelled with 10 nm gold	BioZell
Uranyl acetate	Merck, prepared in PEI microscopy facility
Methylamine tungestate (Wolframat)	Agar Scientific
Transmission electron microscope EM 902	Carl Zeiss Jena

Immuno gold-labelling of the virus-like particles was performed with shock-frozen purified VLPs. Aliquots were thawn on ice and duplicates of 10 µl droplets of concentrated VLPs were set onto parafilm. Carbon-vaporised and flamed 400-mesh Cu/Rh grids were put onto the droplets to adsorp the particles.

All incubation steps were performed under humid chamber conditions by covering the grids with large Petri dishes. Following adsorption for 2 min, the grids were washed briefly. For this, a droplet of PBS was added and mixed by blowing carefully onto the suspension. Remnant liquids were removed by touching the border of the droplets with filter paper until the visible volume was just absorbed. The VLPs were stained with 1:1000-diluted polyclonal rabbit anti-VSV-G primary antibody and incubated for 15 min. Then, the grids were washed twice and remnant liquids were removed. After this, 1:50-diluted polyclonal goat anti-rabbit IgG antibody labelled with 10 nm gold particles, was added. Following 15 min incubation, the grids were washed once in PBS and once in aq. dest. for 1 min. For negative-contrasting, remnant liquids were removed and the grids were contrasted for 10 sec in uranyl acetate and methylamine wolframate.

The samples were analysed by electron microscope at magnifications of 20000.

2.4 Assays and techniques

2.4.1 Fluorescent labelling of cells for adoptive transfer studies

2.4.1.1 Labelling with TAMRA

Additional materials	Source
5-(6-) carboxytetramethyl-rhodamine succinimidyl ester, mixed isomers [5(6) TAMRA, SE]	Molecular Probes, # C 1171
DMF	Sigma
Freezer	Liebherr
1 M HEPES	PEI
Water bath	GFL

References:

Cytometry. 1997 Feb 1;27(2):145-52[175;176] and laboratory of Reinhold Förster, Hannover

A 5 mM stock solution of TAMRA (2,64 mg/ml) was prepared in DMF and stored at -20°C. (Later on, 50 mM stocks were used yielding a higher cell recovery.) Single cell suspensions were resuspended in a 50 ml Falcon tube at 1 x 10^7 cells in 5 ml pre-warmed RPMI supplemented with 125 µl 1 M Hepes solution (= 25 mM). (HEPES is critical to provide the correct pH for the labelling reaction.)

The cell suspensions and the TAMRA aliquot were warmed for 30 min at 37°C (critical to prevent formation of cell/dye flakes that would precipitate nearly all cells.). 10 µl TAMRA stock solution were added per 10^7 cells. The suspensions were incubated in the water bath at 37° for 10 min. Then, the cells were washed twice with 25 ml warm PBS, spun down at 1300 rpm for 6 min and resuspend properly. Finally, the cells were counted or immediately pooled with the CFSE-labelled fraction.

2.4.1.2 Labelling with CFSE

Additional materials	Source
Carboxyfluorescein succinimidyl ester (CSFE)	Molecular Probes, # C-1157
BSA (biotechn. Grade)	Serva, # 47 321
CFSE labelling buffer	PBS
	0.1% BSA
DMSO	Sigma

References:

Protocol from Charles Surh for thymocytes and LN cells[177;178]

A 5 mM stock solution of CFSE (2,78 mg/ml) was prepared in DMSO and stored in 40 µl aliquots at -20°C. Single cell suspensions were resuspended at $2x10^7$ cells/ ml pre-warmed CFSE labelling buffer.

Cells were labelled:

- for *in vivo* long term tracing by FACS analysis by adding **0.5 µl/ml cells 5 mM stock solution** (for few days tracing even only **0.25 µl/ml**).
- for *in vivo* tracing with Laser Scan Microscopy by adding **1.2 µl/ml cells 5 mM stock solution** (Viability of cells decreases due to DMSO, but less dye renders cells undetectable by LSM.)

The suspensions were incubated in the water bath at 37° for 10 min. Then, the cells were washed twice with plenty of cold labelling buffer and spun down at 1300 rpm for 6 min. Finally, the cells were counted or resuspended and immediately pooled with TAMRA-labelled cells in an injection volume of 200 µl PBS per recipient mouse. Few µl were sampled to assess the proportion of CFSE: TAMRA cells by FACS. 1 - 2 x 10^7 cells per labelling type were adoptively transferred i.v. into each recipient.

2.4.2 Cell stainings

2.4.2.1 Immuno-fluorescence staining of splenocytes or lymph node cells

Additional materials	Source
EDTA	PEI
Sodium azide (NaN_3)	Serva
FACS buffer (blood buffer), pH 8.0	PBS
	2 % BSA
	0,03 % NaN_3
	20 mM EDTA
Paraformaldehyde (PFA)	Fluka
Cell fixation solution FACS	PBS
	1% PFA
Vortexer	VF2, Janke & Kunkel IKA Labortechnik
FACS tubes large	BD
7-AAD	BD
Antibodies	
Anti-murine CD3e-FITC	Caltag Laboratories, # HM3401-3
Anti-murine CD18-FITC	Caltag Laboratories, # RM4004
Anti-murine CD69-FITC	BD, # 553236
Anti-murine Ly6C-FITC	Southern Biotech, # 1760-02
Anti-murine CD3-PE	BD, # 55 30 63 or
	Caltag Laboratories, # RM 3404-3
Anti-murine CD11a-PE	Caltag Laboratories, # RM3904
Anti-murine CD44-PE	BD, # 553134
Anti-murine CD49d-PE	BD, # 557420
Anti-murine CD62L-PE	Caltag, # RM 4304

Anti-murine CXCR4-PE	BD, # 551966
Anti-murine CXCR4-PE	BD, # 551966
Anti-murine CD45R/B220-PE Cy5	BD, # 553091
Anti-murine IgG_1k-FITC	BD, # 553953
(isotype of CD69-FITC)	
Anti-murine $IgG_{2a,}k$-FITC	Biozol, # 0117-02
(isotype of other FITC-labelled antibodies)	
Anti-murine $IgG_{2a,}k$-PE	Biozol, # 0117-09
(isotype of PE-labelled antibodies)	

Single cell suspensions were prepared and staining samples of 2 x 10^5 cells were aliquotted in large FACS tubes. The cells were kept on ice during the whole staining procedure. To achieve a constant staining volume throughout different experiments, the samples were filled up to a total volume of 50 – 100 µl with FACS buffer. The cells were stained with the following amounts of antibodies:

FITC-labelled antibodies: from BD and Caltag 1 µl/sample
PE-labelled antibodies: from BD and Caltag 0.5 µl/ sample, exception: CD3 from Caltag is less concentrated, thus 1 µl was added.
PE Cy5-labelled anti-murine CD45R/B220: A premix of 300 µl FACS buffer with 1.5 µl Ab was prepared. 10 µl of the mix were used for staining.

To stain for receptors involved in leucodiapedesis, α-CD3 and α-B220 were added to distinguish B and T cells, and a third antibody with yet a different dye was used for the detection of the receptor. To discriminate unspecific staining of antibodies, additional samples were stained with equal amounts of isotypes. (Since B or T lineage-specific markers were not expressed on other cells and did not underlie regulation, no isotypes were used for α-CD3 and α-B220.) The samples were vortexed and incubated for 20 min in the fridge. After washing with 1 ml FACS buffer, centrifugation at 1300 rpm for 6 min and discarding supernatant, the cells were resuspended in the last drops.

If the cells were measured within the following hour, the samples remained in the fridge without further treatment. To exclude dead cells (routinely when cultured B cells were analysed, optatively for fresh cells), those samples that had not been stained with anti-CD45R/B220-PE Cy5, were incubated for 10 min in the fridge with 1 µl 7-AAD and were then immediately submitted to FACS analysis.

If the cells were measured later, they were fixed by adding 100 µl PBS 2% PFA and the samples were kept in the fridge over night. Next day, they were washed with 1 ml FACS buffer.

2.4.2.2 Immuno-fluorescence staining of CCR7

Additional materials	Source
CCL19-Fc fusion protein	Kindly provided by Sanjiv Luther, Epalinges, Switzerland, formerly Cyster laboratory, UCSF, California
Anti-murine CD16/CD32 purified	Caltag Laboratories, # MM7400
Goat anti-human Fc $F(ab)_2$ – PE	Jackson Immunoresearch, # 109-116-098
Normal mouse serum	Sigma Aldrich, # S 3509
Normal rat serum	Sigma Aldrich, # S 7648
Anti-murine CD45R/B220-PE Cy5	BD, # 553091

Single cell suspensions were prepared on ice and staining samples of 4 x 10^5 cells were aliquotted in large FACS tubes. 25 µl of 1:100-diluted anti-CD16/CD32 Fc-blocking antibody were added. After incubation for 15 min on ice, the cells were washed twice for 6 min at 1400 rpm. 25 µl of 1:3-diluted CCL19-Fc was added and samples incubated for 30 min on ice or 20 min in the fridge.

In the meanwhile, the second antibody was pre-adsorbed with:

1 µl Fc $F(ab)_2$ – PE
1 µl mouse serum
1 µl rat serum
47 µl FACS buffer

and incubated 30 min on ice or in the fridge. The cell samples were washed and after discarding supernatant, 50 µl preadsorbed dilution of the secondary antibody (goat anti-human Fc $F(ab)_2$ – PE) was added together with 20 µl diluted α-B220-PE Cy5 (see above) and 2 µl α-CD69-FITC. The samples incubated for 30 min on ice or 20 min in the fridge. After two washing steps, the cells were measured directly with or without 7-AAD.

2.4.2.3 Immuno-fluorescence staining of CD69 on peripheral blood B and T cells

Additional materials	Source
Anti-murine CD3-PE	BD, # 55 30 63 or Caltag Laboratories, # RM 3404-3
Anti-murine CD45R/B220-PE Cy5	BD, # 553091
Anti-murine CD69-FITC	BD, # 553236
BD FACS Lysing solution	BD, # 34 92 02
Aqua destillata (aq.dest.)	PEI

15 µl heparinised blood were pipetted into large FACS tubes. The samples were stained with 1 µl α-CD3-PE for T cells and with 10 µl of α-B220-PECy5 dilution (see 2.4.2.1) for B cells. To assess the stimulation state of lymphocytes, 3 µl of 1:10-diluted α-CD69-FITC were used. The samples were vortexed gently and incubated for 15 min in the fridge (the time is very critical since CD69 stains unspecifically if incubated longer). To lyse erythrocytes, 1 ml of 1:10 aq. dest.-diluted BD FACS Lysing solution was added and samples were incubated for few minutes at room temperature. Consequently, the samples were washed with 3 ml FACS buffer and spun down gently at 600 rpm for 5 min. Supernatants were discarded and the samples were resuspended in the last droplet and measured by FACS.

2.4.3 FACS-based analysis of cells

2.4.3.1 Flow cytometric analysis (FACS)

Additional materials	Source
FACScan	BD
FACS Clean, Rinse, Flow	BD
CellQuestPro software	BD
WinList 5.0 software	Verity

The FACS machine was adapted by warming up the laser for 15 min while rinsing FACS flow. Samples were acquired when the machine display showed passing events in the detection chamber. The next sample was not put until a droplet of FACS flow had flushed the needle. Detritus and dead cells were excluded by setting a FSC threshold according to morphology or 7-AAD$^+$ events. The obtained data were analysed with CellQuestPro and WinList 5.0 software.

2.4.3.2 Counting absolute numbers of peripheral blood lymphocytes by FACS

Additional materials	Source
Anti-murine CD3-PE	BD, # 55 30 63 or Caltag Laboratories, # RM 3404-3
Anti-murine CD4-PE	BD, # 55 36 52
Anti-murine CD8-FITC	Biozol, # 1550-02 S
Anti-murine CD45.2-FITC	BD, # 553772
Anti-murine CD45.1-PE	BD, # 553776
Anti-murine CD45R/B220-PE Cy5	BD, # 553091
Caltag counting beads	Caltag, # PCB-100

In Veterinary Medicine, differential blood counts are usually performed on a Coulter Counter and measured in samples of at least 200 µl blood. In mice, however, the blood volume is too

small for reiterated sampling of such quantities. Furthermore, the Coulter Counter determines numbers of lymphocytes indirectly by calculating the percentage of lymphocytes based on the total blood cell count. For very small numbers of cells - as is the case in adoptive transfer and induction of lymphopenia - indirect calculations can inherit an error. Thus, to directly count absolute numbers of lymphocytes, a quantitative method on the base of reference counting beads was established.

15 µl of counting beads, which contained about 1000 beads/µl, were pipetted into large FACS tubes, and 15 µl of heparinised blood was added. The samples were stained with 1 µl α-CD3-PE for T cells and with 10 µl α-B220-PECy5 dilution (see 2.4.2.1) for B cells. To determine the reconstitution efficiency of BM-chimeric mice, 1 µl of each α-CD45.2-FITC and α-CD45.1-PE was added and the cells were further stained for B and T cells. All samples were vortexed gently and incubated for 20 min in the fridge. Erythrocytes were lysed in 1 ml 1:10 aq. dest.-diluted BD FACS Lysing solution and the samples were incubated for few min at room temperature. After washing with 3 ml FACS buffer, the samples were spun down gently at 600 rpm for 5 min. Supernatants were discarded and the samples were resuspended in the last droplet and measured by FACS.

To count absolute numbers of peripheral blood lymphocytes by FACS, the counting beads (consisting of two populations) were gated simultaneously in the forward scatter (FCS)/side scatter (SCC) (upper gate in the first plot, Fig. 2-1) as well as in the fluorescence 2/SSC (gate in the second plot), i.e. a linked gate with “and” function was created. Since erythrocyte detritus shows similar FSC/SSC properties as counting beads, a threshold was set in a density plot (vertical bar in the third plot, also applied in the first plot). The density plot further served to set a gate on the lymphocyte population (lower gate in the third plot, also applied in the first plot) and to adjust the beads gate. Depending on the intensity of lymphopenia in every sample, either dot or density plots were more appropriate to monitor data acquisition. The samples were measured for 5000 beads. Thus, all data files are equivalent to approximately 5 µl blood. The absolute numbers of B and T cells were derived from plots gated on lymphocytes (fourth plot).

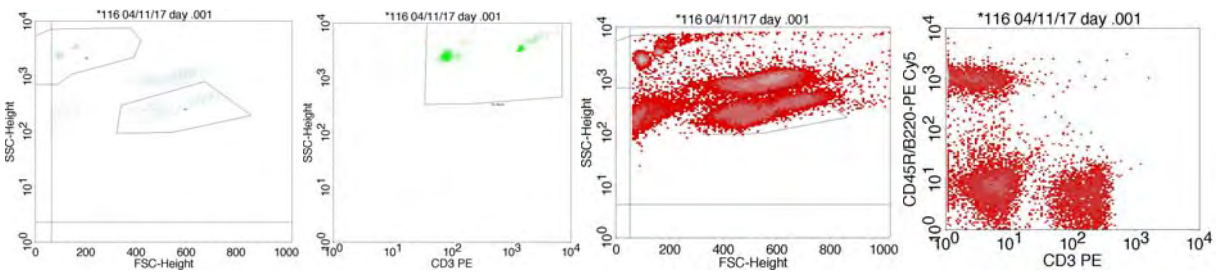


Fig. 2-1: Blood lymphocyte count by FACS (for description see page 58)

Fig. 2-1: Blood lymphocyte count by FACS (displayed on page 57)
Typical measurement: first plot: FSC/SSC dot plot with lymphocyte gate (lower gate) and FSC/SSC/fluorescence 2-linked beads gate for counting of pure beads (upper gate), second plot: fluorescence 2/SSC dot plot with FSC/SSC/fluorescence 2-linked beads gate; third plot: FSC/SSC density plot for adjustment of gates and threshold; fourth plot: fluorescence 2/ fluorescence 3 plot showing counted cells out of the lymphocyte gate

2.4.4 Confocal microscopy (Laser Scan Microscopy)

Additional materials	Source
Scalpel	Amefa
Laser Scan Microscope (LSM 510 Meta)	Zeiss
Axiovert 200 M connected to LSM	Zeiss
LSM 5 image browser software	Zeiss
Moviol	Calbiochem, Fluka

Preparation of Moviol:

20 g Moviol 4.88 was stirred in 80 ml PBS over night. Then, 40 ml glycerine were added and mixed. After centrifugation, the supernatant was aliquotted and stored at -20°C.

After adoptive transfer of CFSE and TAMRA-labelled splenocytes, mice were sacrificed for microscopic analysis and to confirm effective GPCR blockade. Lymphoid organs were isolated and cut manually with a scalpel blade. The sections were set onto microscope slides, embedded in droplets of moviol and covered for the analysis.

After adapting the Laser Scan Microscope for 15 min to warm up the lasers, the instrument was set up. Due to the excitation maxima of CFSE and TAMRA at 496 and 540-555 nm, respectively, the argon laser wavelength 488 nm and the He/Ne laser with 543 nm wavelength at 3% and 90% laser power, respectively, were chosen for excitation. A first beam splitter of 488/543 nm and a second splitter of 545 nm were selected. To detect the emitted light, a band pass filter of 505-530 nm in channel 2 and a long pass filter of 560 nm in channel 3 were used. The samples were screened thoroughly and micrographs were taken from representative areas and processed with LSM 510 image browser software.

2.4.5 Molecular biology techniques

2.4.5.1 Polymerase chain reaction (PCR) to detect *ifnar1* exon 10 deletion

Additional materials	Source
DNeasy Tissue Kit	Qiagen
Table centrifuge	Zentrifuge 5415C, Eppendorf
Cooling block	Eppendorf
Qiagen Taq PCR core kit (250 units)	Qiagen
10 mM deoxynucleotides (dNTP: dATP, dCTP, dGTP, dTTP)	New England Biolabs
Thermo-Tubes 0.2 ml	ABgene
PCR Cycler	Peltier Thermal Cycler 200, MJ Research
10 x TBE	PEI
10 x TBE	890 mM Tris Base
	890 mM Boric acid
	25 mM EDTA, pH 8,0
Microwave	Privileg 9025E
6 x DNA sampling buffer	0,25% bromphenol blue
	30% glycerine
	Aq. dest.
Ethidiumbromid (1% Aqua dest.)	Merck
DNA marker	Gibco, Invitrogen
Voltmeter	Power Pac 300, Bio-Rad

All materials for DNA techniques as well as the primers were kept on -20°C and were thawn and pipetted on a cooling block.

DNA was prepared from tail, spleen, thymus and MACS-purified splenic B cells of $IFNAR^{flox/flox}$, $IFNAR^{\Delta ex10/\Delta ex10}$ and $CD19Cre^{+/-}$ $IFNAR^{flox/flox}$ mice by Qiagen DNeasy Tissue Kit

according to the manufacturer´s instructions. The organs were incubated in lysing buffer supplemented with proteinase K. The cells and tissues were digested over night at 55°C. Ethanol was added, the samples were vortexed and passed over DNeasy Mini spin columns placed in a collection tube. After centrifugation for 1 min at 10000 rpm, the column was placed into a new collection tube and spun down again. Then, the column was placed onto an Eppendorf cup and eluted twice by centrifugation with the provided buffer.

The DNA samples were air-dried and dissolved in Qiagen Taq PCR core kit buffer. A master mix of the PCR reagents was pipetted under a sterile work bench:

Component	**Volume [µl]**
10 x buffer	5
Sense primer	5
Antisense primer	5
dNTPs (10 mM)	2
Q-solution	13
$MgCl_2$ (25 mM)	3
Aqua dest	11.8
Taq-polymerase (5 U/µl)	0.2

Primers were selected that bind outside the floxed region to distinguish the *floxed* and the *exon10-deleted* alleles.
Sense primer : GGT TAA GCT CCT TGC TGC TAT CTG G
Antisense primer : TTG GAG ATG CAA TCT GCT ACT CAG C

45 µl master mix were pipetted in Thermo-Tubes and 5 µl DNA per sample were added. One sample was prepared with a Taq-polymerase-free master mix. To run the PCR in a Peltier Thermal cycler, the tubes were adapted at 94°C for 4 min and then incubated for 35 cycles (at 94°C for 30 sec denaturation; at 58°C for 30 sec annealing; at 72°C for 120 sec elongation). After 35 cycles, the tubes were kept at 72°C for 10 min and were finally cooled down to 4 °C.

To analyse the amplification products, a 1% agarose gel was prepared by heating agarose in 70 ml 1 x TBE buffer supplemented with 2.22 µl ethidium bromide. After having cooled, the gel was kept in 1 x TBE buffer. 2 µl loading buffer were mixed with 10 µl PCR product solution to load the lanes of the gel. 10 µl DNA marker were added in an extra lane. The PCR products were separated for approximately 1 h at 110 V. Analysis was performed at UV light (245 nm) and gel photos were taken.

2.4.5.2 PCR-screening of CD19-Cre$^{+/-}$ IFNAR$^{flox/flox}$ and CD4-Cre$^{+/-}$ IFNAR$^{flox/flox}$ mice

CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice were bred among each other, giving rise to 25% CD19-Cre$^{+/+}$ mice (that could not be used for experiments due to a functional defect in B cells) and to 25% CD19-Cre$^{-/-}$ (wild-type) mice (that did not show IFNAR recombination). Therefore, CD19-Cre$^{+/-}$ IFNAR$^{flox/flox}$ mice were screened for the 50% heterozygous CD19-Cre$^{+/-}$ offspring with the primers:

#42 (5'-CCCAGAAATGCCAGATTACG-3'),
#46 (5'-AACCAGTCAACACCCTTCC-3') and
#47 (5'-CCAGACTAGATACAGACCAG-3'),

giving rise to a 452 base pair (bp) PCR product in the presence of a CD19 wt allele and a 525 bp PCR product for a CD19-Cre allele. Alternatively, the few CD19-Cre$^{+/+}$ IFNAR$^{flox/flox}$ mice available (due to reduced viability) were bred with IFNAR$^{flox/flox}$ mice to avoid screening, since all their progeny was CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$.

CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice were bred to IFNAR$^{flox/flox}$ mice, giving rise to 50% CD4-Cre$^{+/-}$ IFNAR$^{flox/flox}$ mice and to 50% CD4-Cre$^{-/-}$ IFNAR$^{flox/flox}$ mice. The CD4-Cre transgene was screened for with a Cre-specific PCR using the primers

#70 (5' GCCTGCATTACCGGTCGATGCAACGA 3') and
#71 (5' GTGGCAGATGGCGCGGCAACACCATT 3').

2.4.6 B cell chemotaxis assay

Additional materials	Source
Transwell plates [polycarbonate membranes]	Corning Costar, # 3421
BSA [low endotoxin, IgG free]	Sigma, # A-2058
Migration Medium	RPMI 1640
	0,5 % BSA
	10 mM HEPES
CCL19	R&D, # 440-M3-025
CCL21	R&D, # 457-6C-025
CXCL12	R&D, # 460-SD-050
CXCL13	R&D, # 470-BC-025

The chemotaxis assay is based on a diffusion gradient formed between the upper and the lower chamber of every single well.

Migrated cells are counted by FACS by an indirect method: The numbers of migrated cells that are measured from chemotaxis samples, are calculated by the rule of the three with those numbers measured from additional reference counting samples that contain known cell numbers. Thus, the total number of migrated cells and the percentage of chemotaxis are obtained.

Usefully, the reference counting wells are set up with a number corresponding to the estimated amount of migrating cells. In case of naïve lymphocytes, a mean of 20% migration of the control population can be expected. Hence, the chemotaxis wells are set up with a total of 10^6 cells, and the reference counting wells are filled directly with 2 x 10^5 cells. To assess the spontaneous migration, negative control wells are set up without adding any chemokines.

Purified B cell suspensions were prepared in B cell culture medium and counted. During the preparation of the migration plate, the B cell suspensions were kept in the incubator. The lower chambers of the reference count wells were filled with 500 µl pre-warmed B cell migration medium and the negative control wells with 600 µl. All chemotaxis dilution wells were filled with 600 µl pure migration medium and then the chemotaxis wells of the highest concentration were filled with 900 µl of the undiluted chemokine solution (see table 2.2). Serial 1:3 chemokine dilutions were prepared in triplicates or single wells according to the number of cells available.

Next, the B cell suspensions were spun down at 1200 rpm for 10 min, supernatants were taken off entirely and the cells were resuspended in migration medium at 10^7 cells/ml. 120 µl were taken out as reference count cells to be diluted with 480 µl migration medium. Of this, 100 µl containing 2 x 10^5 cells were added to the lower chamber of the reference count wells. Then, the transwell inserts were placed onto the chemotaxis and negative control wells. Finally, 100 µl of the concentrated cell suspension containing 10^6 B cells were carefully added into the upper chamber formed by the transwell inlay. The covered plates were kept in the incubator to let the cells migrate for 3 h.

Then, the transwell inlays were removed and the lower chambers were resuspended thoroughly. Samples of 550 µl were transferred into large FACS tubes and counted by FACS. For this, the FACS machine was prepared by rinsing with FACS clean and with aq. dest. for 5 min each. The samples were acquired for 60 sec without gating cells, but using a FCS/SCC detection threshold to exclude detritus. Between individual samples, the needle was rinsed with FACS flow.

Table 2.2: Chemokine concentrations for the preparation of chemotaxis assay plates

		For single wells		**For triplicates**	
Chemokine	**Conc. [µg/ml]**	**CXCL stock [µl]**	**Medium**	**CXCL stock [µl]**	**Medium**
CXCL12	0.3	2.7	897.3	8.1	2691.9
CXCL13	2.3	21	879	63	2637
CCL19	0.6	5.4	894.6	16.2	2683.8
CCL21	0.3	3	897	9	2673

	Single wells	**Triplicates**
Reference count wells	500	(Individually prepared)
Negative control wells	600	1800
Remnant dilution wells	600	1800

2.4.7 VSV Serum Neutralisation Assay

Additional materials	Source
Vero cells	Originally obtained from ACCT
96-well flat bottom cell culture plates	Nunc
96-well flat bottom plates	Greiner
10 x MEM	Gibco, #21435
280 mM ß-mercapto-ethanol	100 µl 14 M ß-mercapto-ethanol (concentrated)
	5 ml 0.9% Na Cl
Multichannel pipettes	Socorex
Magnetic stirrer	Combimag Ret, Janke& Kunkel
Methocel MC	Fluka #64620
Double-distilled H_2O	PEI
7.5% sodium bicarbonate	Gibco, # 25080-060
1% methylcellulose in 1 x MEM	(Preparation see below)
Crystal violet	0.5% Crystal violet
	5% formaldehyde
	0.8% NaCl
	Aq. dest.

Preparation of 1% methylcellulose in 1 x MEM:

15 g methocel were dissolved in 750 ml double-distilled H_2O (2%) over night under stirring in the cold room. After autoclavation, a methylcellulose block formed that dissolved at room temperature within 24 h. 250 ml of 2% methocel were mixed with 50 ml 10 x MEM. 30 ml 7.5% sodium bicarbonate was added. A final 1 x concentration of 0.44% was reached by a filling up to 500 ml with 170 ml double-distilled H_2O.

Sera of VSV-infected mice were analysed for their VSV-neutralisation capacity. For this, Vero cells were seeded at a density of 2 x 10^5 cells/ml in 100 µl/well MEM 5% FCS, using 96-well flat bottom cell culture plates. The plates were kept in the incubator to grow to confluency.

On day 2, sera were reduced for the determination of IgG by adding 10 µl 280 mM ß-mercapto-ethanol to 10 µl of the serum samples. The reduced sera were incubated for 1 h at room temperature. Untreated sera were used for determination of total Ig. Next, the sera were prediluted 1:40 by adding 380 µl MEM 5% FCS to the reduced sera and 390 µl MEM 5% FCS to the untreated sera. In order to destroy the complement system, the prediluted sera were heat-inactivated for 30 min at 56 ˚C.

For serial dilutions of the sera, lines 2 – 12 of new 96-well plates were filled with 100 µl MEM 5% FCS. Subsequently, 200 µl heat-inactivated serum dilutions were added to the first line of the wells and 1:2 titration steps were made. Next, 100 µl VSV were added at a concentration of 10^3 PFU/ml to all wells already containing 100 µl antibody dilution. These plates were left to incubate for 90 min at 37 ˚C without stapling.

Then, the medium of the confluently grown Vero cell culture plates was flicked off and 80 µl of the serum-VSV mixture was transferred onto the Vero cell layers by pipetting from front to back. The plates were incubated for 1 h at 37 ˚C and methylcellulose 1 x MEM was adapted to 37°C. For the next pipetting step, the tips were cut few millimetres. The plates were overlayed with 100 µl 1% methylcellulose 1x MEM and incubated over night at 37 ˚C.

On day 3, the medium was flicked off and the cultures were overlayed with crystal violet and incubated for 1 h at room temperature. Finally, the dye was carefully removed and the plates were washed extensively. The plates were air dried and the plaques were counted.

2.4.8 Enzyme-Linked Immuno-Sorbent Assay (ELISA) to detect VSV-specific serum antibodies

Additional materials	Source
Nunc Maxisorb plates	Nunc
Tween 20	Fluka
ELISA coating buffer	3.18 g/l Na_2CO_3
	5.88 g/l $NaHCO_3$
	Aqua dest.
	Adjust to pH 9.6 with 0.1 M $NaHCO_3$
ELISA blocking buffer	5% BSA
(Prepare immediately before use!)	0.1 % Tween 20
	PBS
ELISA washing buffer	0.1 % Tween 20
	PBS
ELISA serum and antibody dilution buffer	1 % BSA
	0.1 % Tween 20
	PBS
Goat anti-mouse IgG_1–horse radish peroxidase (HRP)	Southern Biotech, # 1070-05
Rabbit anti-mouse IgG_{2a}–HRP	Zymed, # 61-0220
Rabbit anti-mouse IgG_{2b}–HRP	Zymed, # 61-0320
Rat anti-mouse IgG_3 –HRP	Southern Biotech, # 1190-05
Rabbit anti-mouse IgM–HRP	Zymed, # 61-6820
Rabbit anti-mouse IgM,A,G–HRP	Zymed, # 61-6420
VI24	Prepared by U. Kalinke

ABTS (2, 2´-azino-bis-(3-ethylbenziazoline-6-sulfonic acid))	Roche
H_2O_2	Merck
ELISA reader	Tecan, Sunrise

Positive control for IgG_{2a}:
VI24 (mAb to VSV-G protein from Ulrich Kalinke, IgG_{2a}, 1 mg/ml in stocks; 1:2000 as initial dilution; the signal disappears at 1:30000)

Purified VSV was diluted 1:10000 in coating buffer (0.1 M $NaHCO_3$, pH 9.6) and 96-well Nunc Maxisorb plates were coated with 100 µl/well. The plates were incubated over night at 4˚C. The virus dilution was carefully flicked off and the plates were washed 2 x with 150 µl/well of PBS, 0.1 % Tween 20. To saturate unspecific protein binding, 100 µl/well blocking buffer were added. Then, the plates were incubated for 2 h at room temperature or alternatively over night at 4˚C. Subsequently, the VSV-coated plates were washed 2 x with 150 µl/well of washing buffer. Next, 150 µl of the usually 1:40-diluted serum samples were added to the first rows and 8 serial dilutions steps in 1:3 titration were performed in dilution buffer in the VSV-coated plates. After 2 h incubation at room temperature, the VSV coated plates were washed 3 x with 150 µl/well of washing buffer. To detect VSV-specific antibodies, 80 µl/well HRP-coupled detection antibodies (anti-IgG_{2a} and anti-IgG_{2b} 1:1000, anti-IgG_1 and anti-IgG_3 1:500-diluted in 1% BSA, PBS, 0.1 % Tween 20) were added and the plates were incubated for 1 h at room temperature.

In the meantime, aliquots of ABTS, the substrate of HRP, were thawn and activated by supplementing 20 µl 30 % H_2O_2 to 11 ml ABTS aliquots. Then, the incubated plates were washed 3 x, and 100 µl/ well of the activated substrate were added. After 1h of incubation at room temperature, the plates were read at 405 nm by an ELISA reader.

Serum IFN-α was detected by an IFN-α ELISA from R&D according to the manufacturer´s instructions (performed by Zoe Waibler, PEI).

2.4.9 Statistical analyses

Unless otherwise indicated, data are depicted as the mean ± SD. B: T cell ratios in SLOs were analysed with a Wilcoxon rank-sum test and differences were considered statistically significant when *P* values were less than 0.05.

3 Results

3.1 Virus-induced type I IFN alters lymphocyte recirculation

3.1.1 Following VSV infection, B and T cell counts are massively decreased in peripheral blood

In order to study viral infection-related lymphopenia, we injected C57BL/6 (BL/6) mice i.v. with 2 x 10^6 PFU VSV. Blood samples were collected at the indicated time points, and absolute numbers of peripheral blood lymphocytes were determined by a newly developed FACS method (Fig. 3-1, upper row). For this, 15 µl heparinised blood were mixed with the same volume of a counting beads suspension containing a defined number of spherical fluorescent beads (approximately 1000/µl). Following a standard staining procedure, FACS data equivalent to 5000 reference counting beads were acquired. Thus, all FACS plots shown in this study are representative for a defined volume of blood, i.e. approximately 5 µl (for further details see 2.4.3.2).

Already 18 h after VSV injection, blood cell numbers were massively decreased and reached minimum values around 36 h post infection (p.i.). At later time points, the cell counts returned to pre-infection levels. During lymphopenia, the few lymphocytes still found in blood showed an stimulated phenotype as characterised by high expression of CD69 (Fig. 3-1, lower left). Analysis of sera (in collaboration with Zoe Waibler, PEI) revealed an early and significant IFN-α production between 12 and 18 h post infection, whereas at later time points IFN-α levels declined and returned to background values within 3 days (Fig. 3-1, lower right). Thus, during systemic infection, a strong IFN-α response and concomitant upregulation of CD69 was observed prior to the appearance of lymphopenia, suggesting a connexion between the host cytokine response and the onset of lymphopenia.

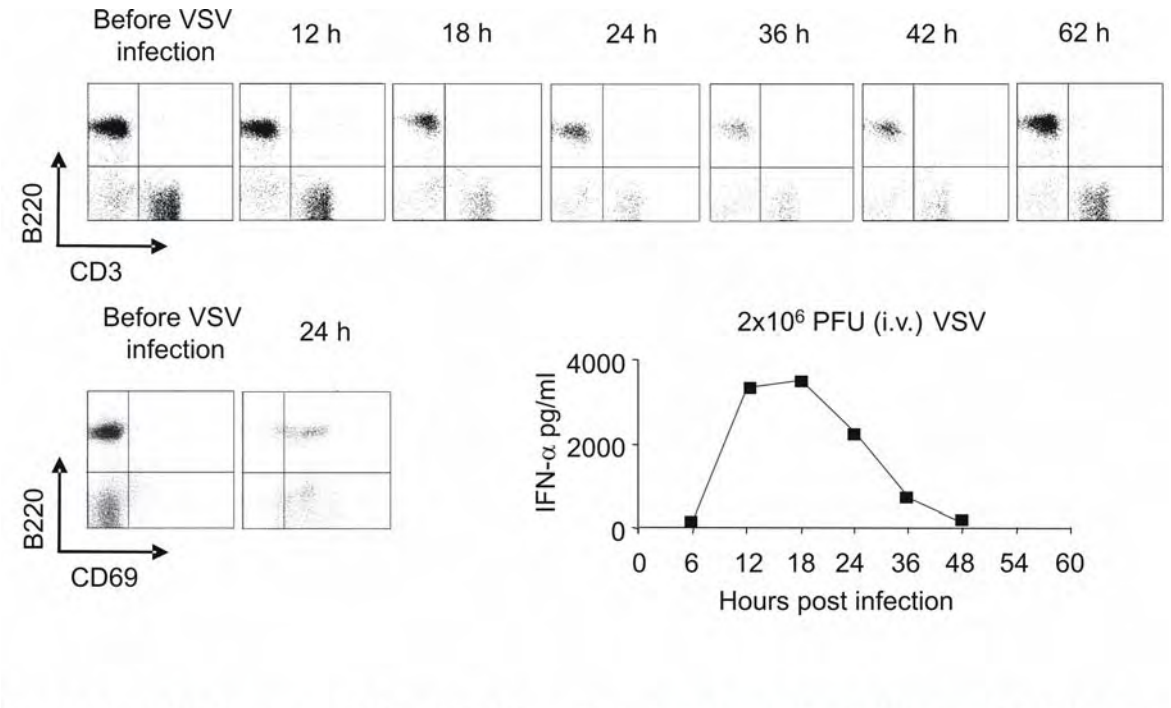


Fig. 3-1: Drastic decrease of B and T cell counts in peripheral blood following VSV infection. Mice were infected i.v. with $2x10^6$ PFU VSV and blood samples were taken at the indicated time points and stained for T cells (CD3-positive) and B cells (B220-positive). For FACS analysis, data equivalent to approximately 5 µl blood were acquired. Representative results of one out of two similar experiments are shown (upper panels).
24 h after VSV inoculation blood samples were stained for CD69 and B220 and FACS analysed. Representative data of one animal out of four are depicted (lower panel). Serum samples of mice infected with $2x10^6$ PFU VSV were taken at the indicated time points and analysed for IFN-α by ELISA. Three animals per time point were studied.

3.1.2 The infectious dose, administration route and the amount of induced host cytokines influence the onset of virus-induced lymphopenia

Since many natural infections are initiated locally, we next investigated the kinetics of lymphopenia in a model of intranasal (i.n.) inoculation. When BL/6 mice were infected i.n. with a sublethal dose of 10^4 PFU VSV, we observed pronounced lymphopenia only on day 2 (Fig. 3-2). Thus, the kinetics of lymphopenia were influenced by the infectious dose and the application route. To investigate whether the late onset of lymphopenia correlated with a delayed systemic cytokine production, we inoculated mice i.n. with 10^4 PFU VSV-M2 (also called VSV-AV1).[179;180] This virus variant expresses a mutant M protein with an M51R (methionine 51 to arginine 51) exchange that results in a less pronounced inhibition of cellular protein expression as compared to wild-type VSV because of a reduced sequestration of mRNA export. [181;182]

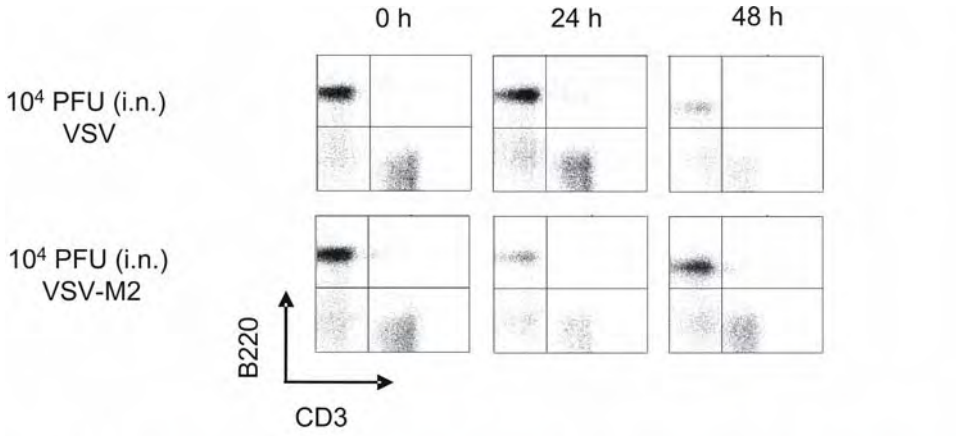


Fig. 3-2: Onset of lymphopenia is influenced by infection route and type of virus.
Mice were infected i.n. with 10^4 PFU VSV or VSV-M2, respectively, and blood samples were taken at the indicated time points p.i. to count B and T cells by FACS analysis. Data show representative animals out of five per group.

In vivo, VSV-M2 was shown to induce approximately 10-fold higher type I IFN responses than the wild-type virus (Zoe Waibler, data not shown[180]). In VSV-M2-treated mice, we found massive lymphopenia already on day 1 post infection (Fig. 3-2), and lymphocyte counts started to recover already by day 2 post infection (p.i.). Thus, VSV-M2 triggered the induction of lymphopenia earlier than VSV, suggesting that the onset of lymphopenia is determined by the viral capacity to trigger the host cytokine responses.

3.1.3 Similar to VSV infection, treatment with TLR3 or TLR7 agonists induces lymphopenia

To address whether IFN-α responses had an impact on lymphopenia, IFNAR-deficient mice ($IFNAR^{-/-}$),[139] which had been 10-fold backcrossed to the BL/6 background, and BL/6 controls were treated i.p. with the TLR3 ligand synthetic dsRNA (poly(I:C)), which is a strong type I IFN inducer. Reminiscent of VSV-infected mice, 20 h following poly(I:C) treatment, BL/6 mice showed massively reduced peripheral blood lymphocyte counts, whereas $IFNAR^{-/-}$ mice did not (Fig. 3-3). Since TLR7 might be involved in VSV-mediated type I IFN induction,[125] mice were also treated with the synthetic TLR-7 agonist R-848 which induced an IFNAR-dependent lymphopenia, too (Fig. 3-3).

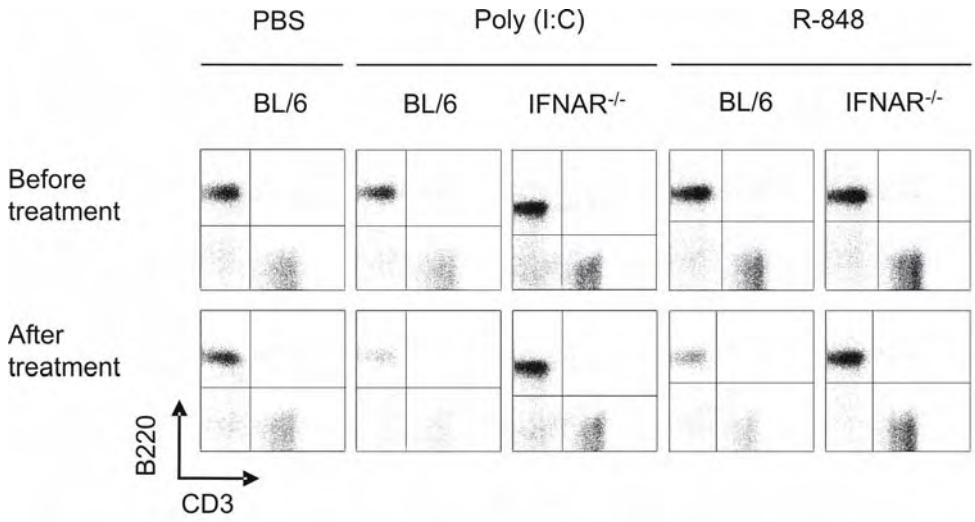


Fig. 3-3: Poly(I:C) and R-848-induced lymphopenia is dependent on IFNAR-signalling.
BL/6 and IFNAR$^{-/-}$ mice were treated i.p. with PBS, poly(I:C) or R-848, and lymphocytes equivalent to approximately 5 µl blood were analysed 20 h after treatment. Representative data of one out of two similar experiments are shown.

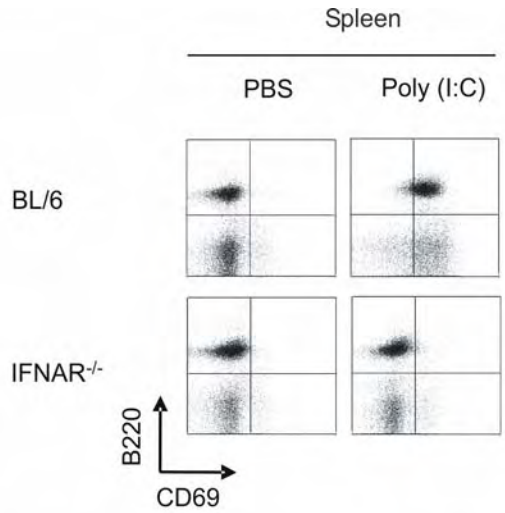


Fig. 3-4: Poly(I:C) induces IFNAR-dependent upregulation of CD69 in spleen.
Splenocytes of PBS or poly(I:C)-treated mice were stained for CD69 and B220 and FACS-analysed. Representative data out of three similar experiments are depicted.

Lymphocytes of poly(I:C) (Fig. 3-4) or R-848-treated (data not shown) BL/6 mice showed CD69 upregulation, which was absent in IFNAR$^{-/-}$ mice. In conclusion, IFNAR-signalling plays a critical role in the induction of lymphopenia and the upregulation of CD69 on lymphocytes.

3.1.4 Injection of type I IFN induces lymphopenia and stimulates lymphocytes

As poly(I:C) and R-848 induce many cytokines in addition to type I IFN, we checked whether injection of type I IFN alone was able to elicit lymphopenia. To this end, 2 x 10^5 IU IFN-α were administered subcutaneously (s.c.) to BL/6 mice (Fig. 3-5A). Unlike PBS-treated controls, one day after injection with IFN-α, the mice showed significantly reduced B and T cell numbers. Similar results were obtained upon injection of IFN-β and both $CD4^+$ and $CD8^+$ T cells underwent massive lymphopenia with similar efficiency (Fig. 3-5B). Thus, type I IFN stimulation is sufficient to induce lymphopenia.

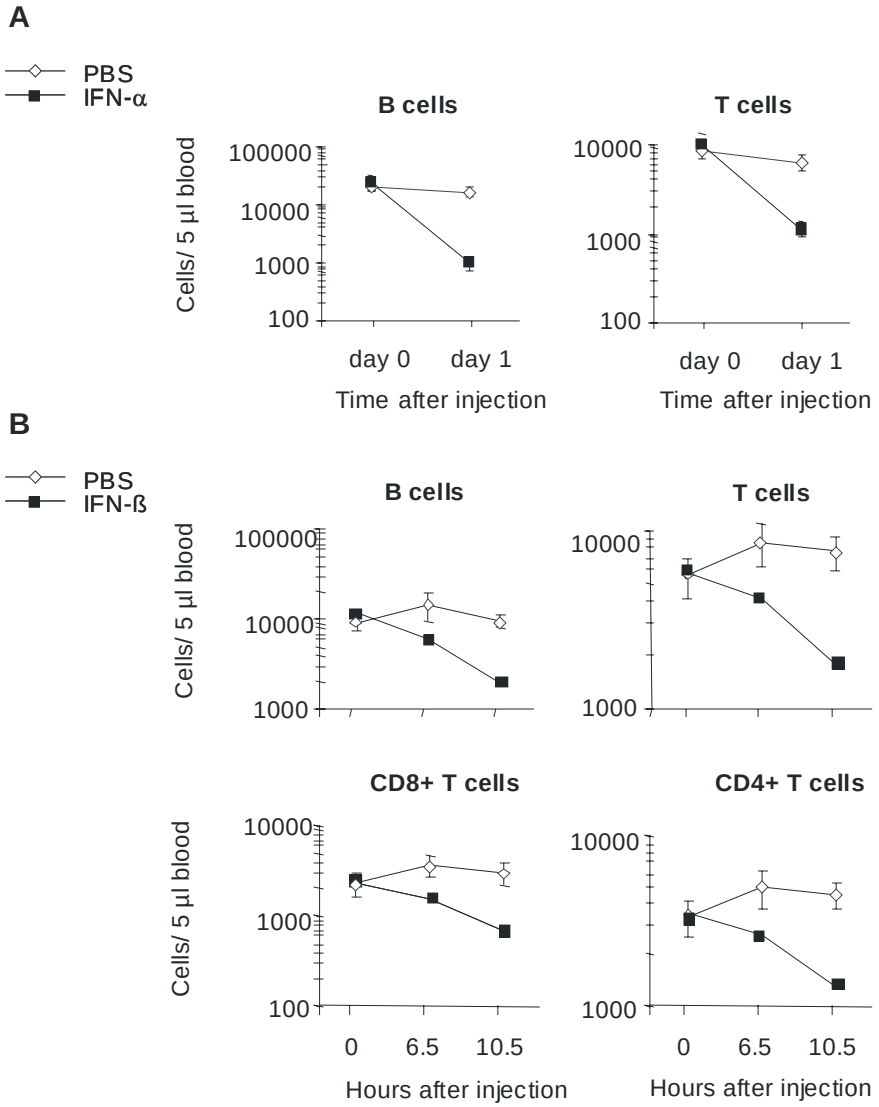


Fig. 3-5: Treatment of mice with type I IFN induces lymphopenia. BL/6 mice were treated s.c. with PBS, 2x10^5 IU IFN-α (A) or IFN-β (B). Blood lymphocyte counts were determined at the indicated time points. Results are expressed as mean ± SD for four mice per group (A). Treatment with IFN-β was performed in one mouse (B).

3.1.5 Type I IFN-induced lymphopenia is fully reversible

To analyse whether the reappearance of B and T cells in blood was related to redistribution of existing lymphocytes, to augmented BM output[46] or to increased *de novo* formation of lymphocytes, we adoptively transferred CFSE-labelled gender-matched splenocytes in BL/6 recipients and monitored the absolute numbers of labelled cells after treatment with poly(I:C) (Fig. 3-6). Throughout the experiment, the percentages of the adoptively transferred lymphocytes remained constant, demonstrating that CFSE-labelled cells were not rejected.

One day after the induction of lymphopenia, transferred and endogenous cells reappeared in blood and eventually reached similar numbers as in PBS-treated controls. Thus, lymphocytes were sequestered from blood and at later time points reappeared again, demonstrating that poly(I:C)-induced lymphopenia is completely reversible. As CFSE fluorescence intensity of labelled lymphocytes was not reduced after poly(I:C) treatment, cell division does not play a role in the reappearance of lymphocytes (data not shown).

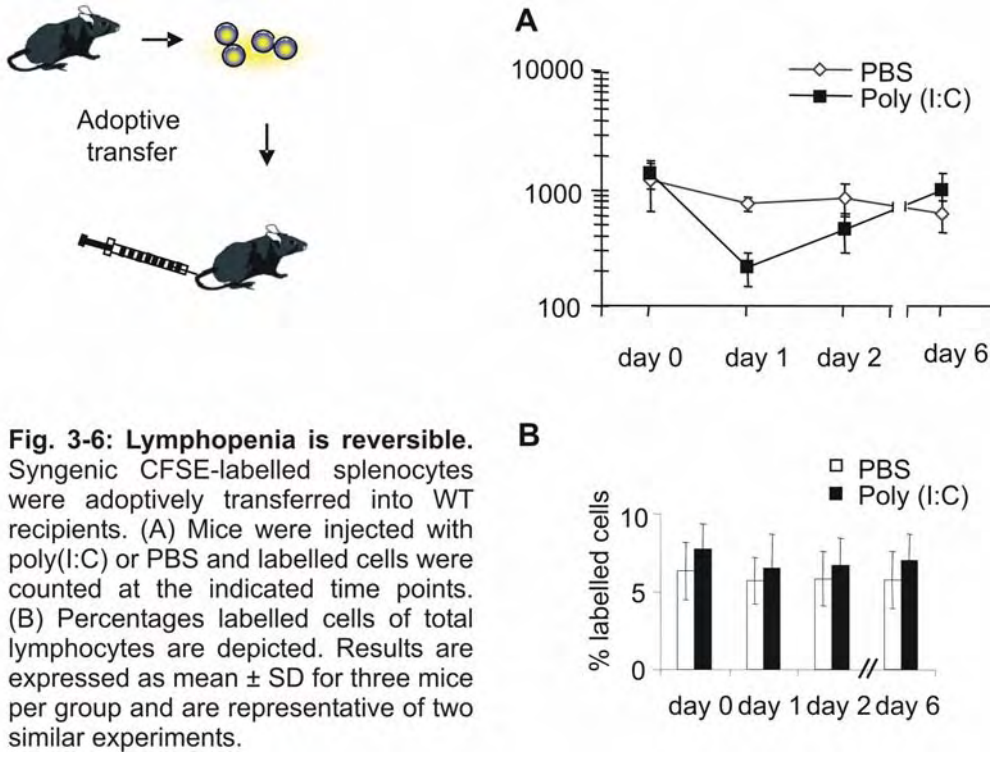


Fig. 3-6: Lymphopenia is reversible. Syngenic CFSE-labelled splenocytes were adoptively transferred into WT recipients. (A) Mice were injected with poly(I:C) or PBS and labelled cells were counted at the indicated time points. (B) Percentages labelled cells of total lymphocytes are depicted. Results are expressed as mean ± SD for three mice per group and are representative of two similar experiments.

3.2 Cellular targets for type I IFN

3.2.1 Type I IFN induces lymphopenia via stimulation of immune cells, but not through effects on endothelium or stroma

Lymphocytes circulate within the organism and transmigrate through vessels and within secondary lymphoid organs (SLOs). Since the endothelium was recently suggested to play a role in lymphopenia,[183] we examined whether type I IFN induces lymphopenia by acting on endothelium and stroma or on immune cells.

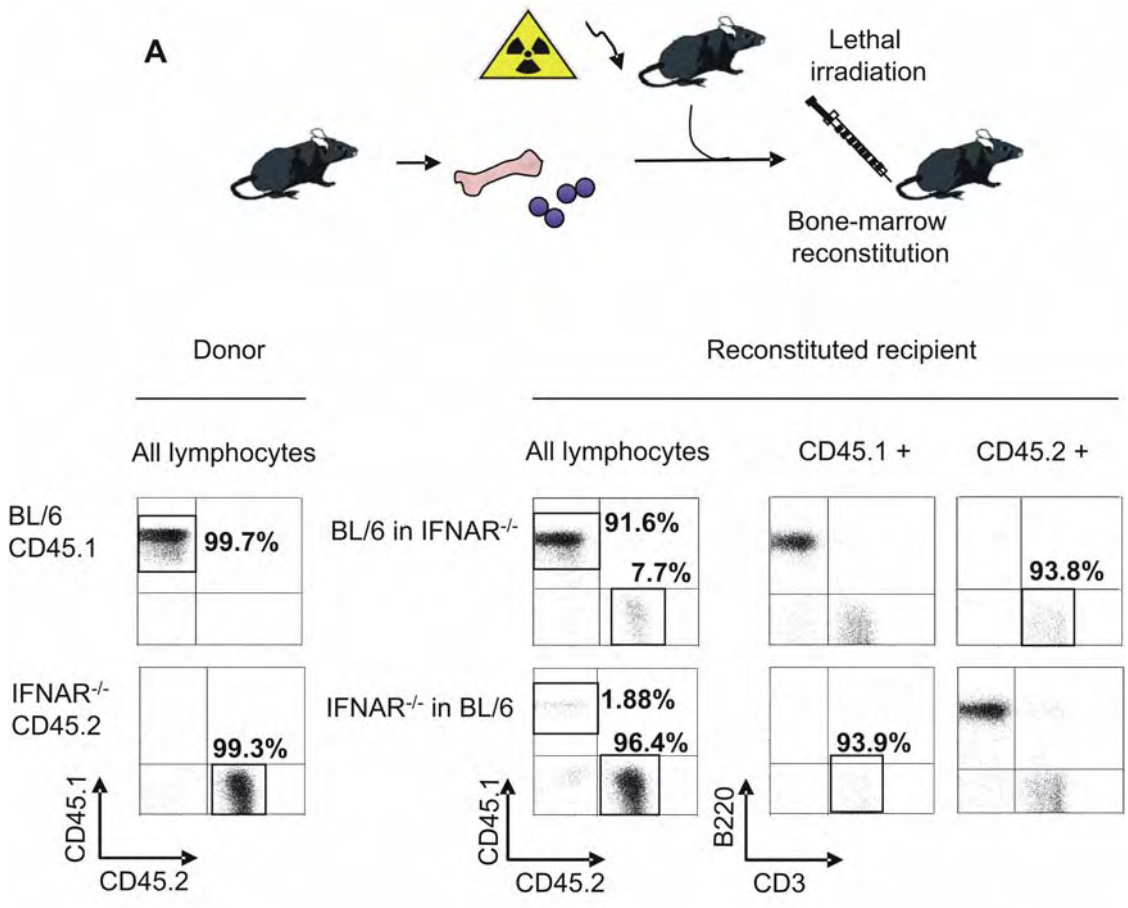


Fig. 3-7: Lymphopenia is induced by direct type I IFN stimulation of immune cells, but not of stroma or endothelium.
(A) Lethally irradiated recipient mice were reconstituted with congenic CD45.2$^+$ IFNAR$^{-/-}$ or CD45.1$^+$ BL/6 BM cells (IFNAR$^{-/-}$>WT and WT>IFNAR$^{-/-}$). As controls, IFNAR$^{-/-}$>IFNAR$^{-/-}$ and WT>WT mice were generated. Six weeks after grafting, reconstitution efficiency was assessed by FACS analysis of CD45.1 and CD45.2 expression on peripheral blood B and T cells.

For this, we lethally irradiated CD45.2^{+}IFNAR$^{-/-}$ mice and reconstituted them one day later with congenic CD45.1^{+}BL/6 wild-type (WT) bone marrow (BM) to obtain WT>IFNAR$^{-/-}$ BM chimeric mice. Similarly, CD45.1^{+} congenic WT mice were reconstituted with CD45.2^{+}IFNAR$^{-/-}$ BM (IFNAR$^{-/-}$>WT). Staining for the allotype markers CD45.1 and CD45.2 as well as for B and T cells revealed a reconstitution efficiency of about 95% and demonstrated that T cells accounted for more than 90% of these remnant recipient-derived lymphocytes (Fig. 3-7A).

Upon poly(I:C) treatment, only WT>WT positive controls and WT>IFNAR$^{-/-}$ mice showed lymphopenia, whereas IFNAR$^{-/-}$>WT mice and IFNAR$^{-/-}$>IFNAR$^{-/-}$ negative controls did not (Fig. 3-7B). These data demonstrate that type I IFN stimulation of BM-derived immune cells, but not of radio-resistant cells, including endothelium and stroma, is required for the induction of lymphopenia.

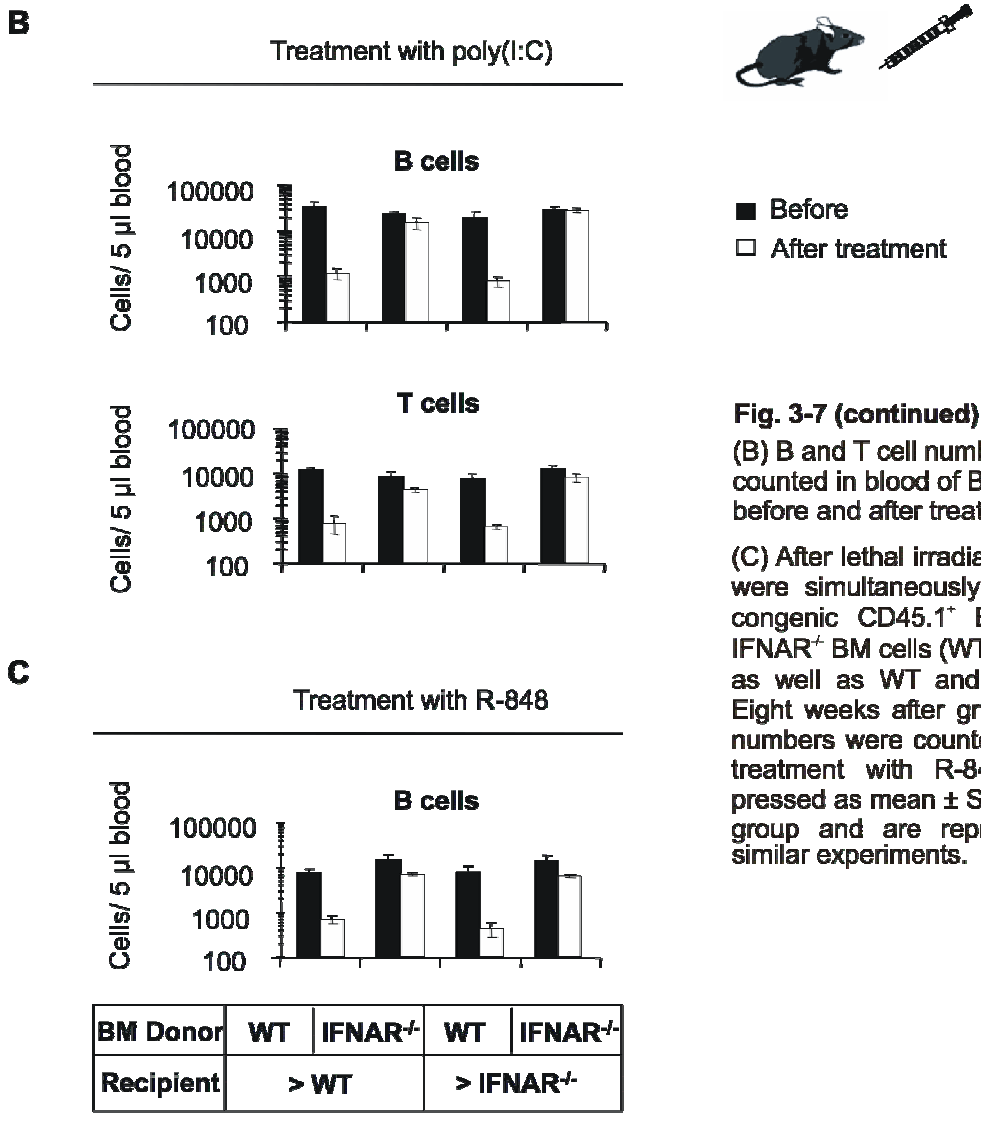


Fig. 3-7 (continued):
(B) B and T cell numbers were counted in blood of BM chimeras before and after treatment with poly(I:C).

(C) After lethal irradiation, recipient mice were simultaneously reconstituted with congenic CD45.1^{+} BL/6 and CD45.2^{+} IFNAR$^{-/-}$ BM cells (WT and IFNAR$^{-/-}$ > WT as well as WT and IFNAR$^{-/-}$>IFNAR$^{-/-}$). Eight weeks after grafting, blood B cell numbers were counted before and after treatment with R-848. Data are expressed as mean ± SD of three mice per group and are representative of two similar experiments.

We next studied the cellular targets for the lymphopenia induced by the TLR7 ligand R-848. For this, we used mixed BM chimeras, which were obtained by simultaneous reconstitution of recipient mice with WT and IFNAR$^{-/-}$ BM ([WT and IFNAR$^{-/-}$>WT] as well as [WT and IFNAR $^{-/-}$>IFNAR$^{-/-}$] mice) (Fig. 3-7C). Reminiscent of the results in poly(I:C)-treated mice, R-848 was able to induce massive B cell lymphopenia of WT immune cells in both WT and IFNAR$^{-/-}$ recipients. IFNAR$^{-/-}$ B cells, however, showed significantly impaired lymphopenia, irrespective of whether the recipients were IFNAR-competent or deficient. In conclusion, R-848 induces B cell lymphopenia by a mechanism dependent on type I IFN stimulation of BM-derived immune cells, but not of endothelia or stroma.

3.2.2 Type I IFN directly stimulates B cells and induces lymphopenia

To address whether type I IFN induces lymphopenia via direct stimulation of lymphocytes or indirectly by activating some immune cell type to secrete relevant factors, we adoptively transferred WT and IFNAR$^{-/-}$ B cells, which had been differentially labelled by TAMRA or CFSE, into WT recipient mice. Under these experimental conditions, all cells except for the adoptively transferred IFNAR$^{-/-}$ B cells (Fig. 3-8, left panel) were sensitive to type I IFN stimulation. After injection with poly(I:C), the endogenous B cells were massively reduced in blood. Similarly, the TAMRA-labelled WT B cells were reduced in absolute numbers. Their frequency, however, remained similar, demonstrating that TAMRA-labelled WT as well as endogenous B cells disappeared efficiently from blood. In contrast, the CSFE-labelled IFNAR$^{-/-}$ B cells were increased in percentages, but remained stable with respect to absolute numbers. Hence, direct type I IFN stimulation is necessary to induce B cell lymphopenia. Reciprocal labelling of the cells (i.e. WT B cells by CFSE and IFNAR$^{-/-}$ by TAMRA) revealed similar results, indicating that the different dyes did not affect the homing properties of transferred B cells.

In IFNAR$^{-/-}$ recipients (Fig. 3-8, right panel), the adoptively transferred WT B lymphocytes were the only cells able to sense type I IFN. Upon poly(I:C) challenge, the IFNAR$^{-/-}$ control cells and the endogenous B cells remained at similar numbers, whereas the WT B cells were reduced with regard to percentages and absolute numbers. Again, lymphopenia showed a pronounced IFNAR dependence in B cells irrespective of the dye that IFNAR-competent or deficient B cells were labelled with. Taken together, these data indicate that direct type I IFN stimulation of B cells is largely sufficient for the induction of B cell lymphopenia.

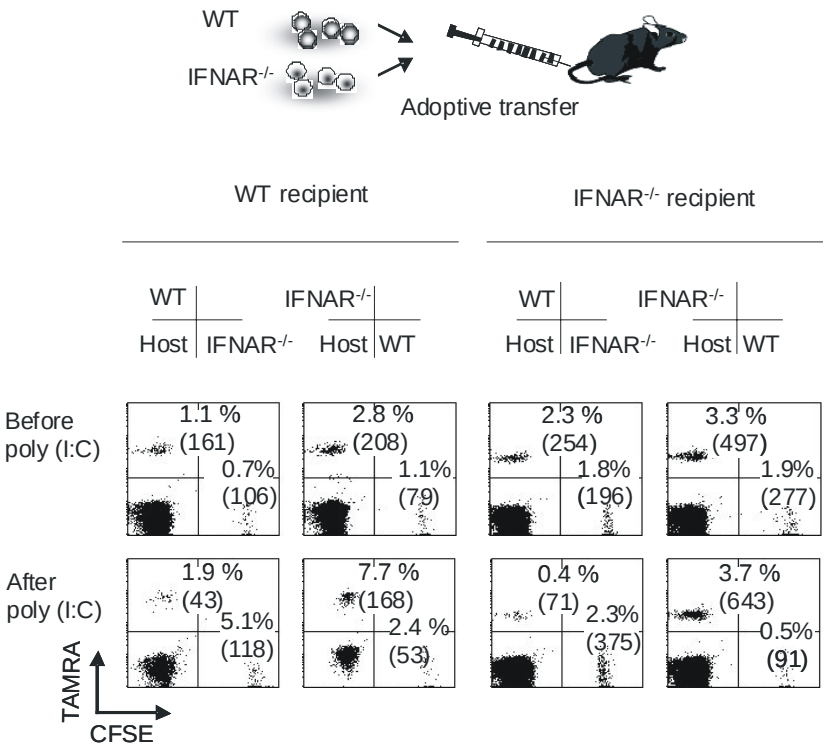


Fig. 3-8: Type I IFN stimulation has a direct effect on B cells.
B cells from WT and IFNAR$^{-/-}$ mice were labelled with TAMRA or CFSE (left panels), or vice versa (right panels) and re-injected into WT or IFNAR$^{-/-}$ recipients. Labelled cells were counted in blood before and after treatment with poly(I:C) and are depicted as percent of total B cells and as absolute numbers in parentheses. Data are representative of one out of three similarly designed experiments.

3.3 Novel genetic mouse models to study the role of direct type I IFN stimulation of lymphocytes

3.3.1 Generation of conditionally gene-targeted mice with a B cell or T cell-specific deletion of the IFNAR

In adoptive transfer experiments, the numbers of injected B cells are always limited when compared to the endogenous B cell pool. Thus, to further study the direct effect of type I IFN stimulation of lymphocytes, Ulrich Kalinke generated mice with a B cell or a T cell-specific IFNAR deletion using a gene-targeting approach with the Cre-loxP strategy[184] (Kalinke et al., manuscript in preparation). To this end, a targeting vector was generated. Besides the exons 7 to 10 of the IFNAR α-chain gene (*ifnar1)*, the targeting vector carried a neomycin resistance (neo) cassette and was flanked at its 5' end by a thymidine kinase (TK) cassette.

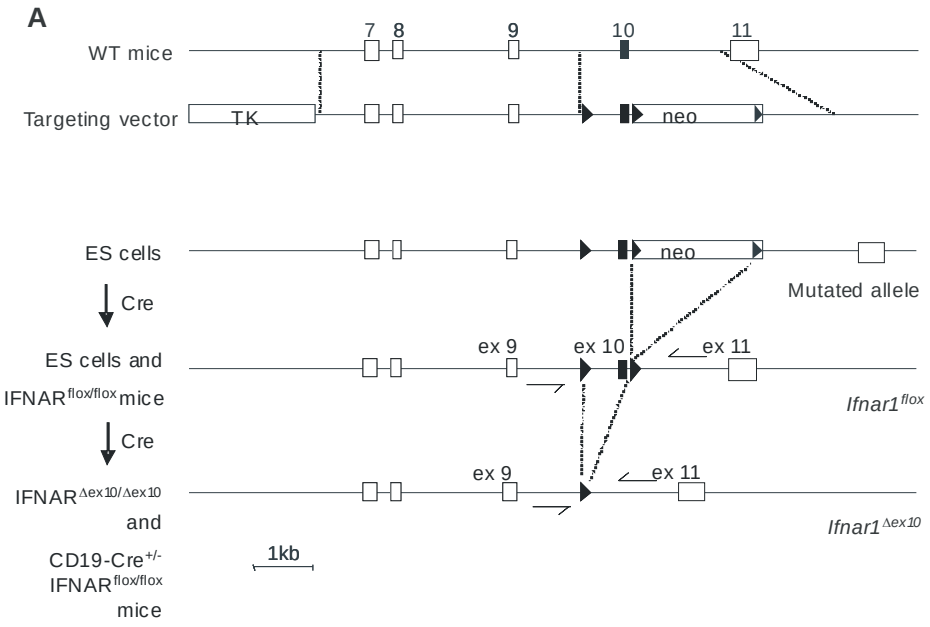


Fig. 3-9: Generation of mice with a B cell-specific IFNAR deletion.
(A) In a targeting vector, exon 10 of *ifnar1* was flanked by loxP sites. After gene-targeting of ES cells, the loxP-flanked neo cassette was removed by transient Cre expression. Upon blastocyst injection, chimeric IFNAR$^{flox/wt}$ mice were obtained. Homozygous IFNAR$^{flox/flox}$ mice were crossed to deleter mice, which express Cre in all tissues, giving rise to mice with an ubiquitous deletion of exon 10 (IFNAR$^{\Delta ex10/\Delta ex10}$), and to CD19-Cre$^{+/-}$ mice to delete IFNAR specifically in B cells. Semi arrows indicate localisation of primers outside the floxed region.

Of note, the exon 10 of *ifnar1* and the neo cassette were flanked by so-called loxP sites, which are bacteriophage-derived 34 bp long asymmetric DNA sequences (Fig. 3-9).

In a next step, IB10 embryonic stem (ES) cells isolated from SV129 mice were electroporated with the targeting vector. By homologous recombination, the mutated sequence integrated into the murine *ifnar1* while the TK cassette was deleted. To select for stably transfected clones, the ES cells were first incubated for one day with gancyclovir, which is metabolised into a toxic product in the presence of TK. Thus, any possible TK-carrying clones with a random integration of the transgene were counter-selected for. Then, the transfected ES cells were also selected in the presence of neomycin. Neomycin-resistant clones were screened by a PCR method for homologous recombination. After Southern blot verification of the homologous gene targeting, the loxP-flanked neo cassette had to be removed since aberrant phenotypes had been observed in mice carrying neo. For this purpose, the gene-targeted ES cell clones were transiently transfected with a vector expressing the enzyme Cre-recombinase (“creates recombination”, Cre). Cre specifically recognises loxP sites and can recombine DNA fragments located in between of two loxP sites. Depending on the identical or opposite orientation of the asymmetric loxP sites, the recombination leads either to deletion or inversion of the respective sequences.

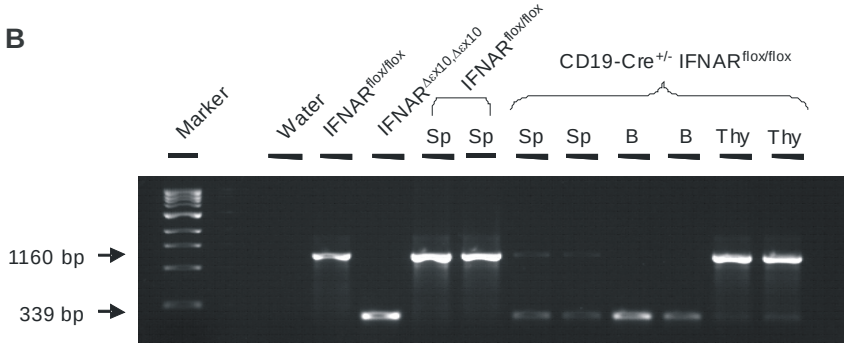


Fig. 3-9 (continued):

(B) B cell-specific IFNAR deletion in CD19-Cre$^{+/-}$ IFNAR$^{flox/flox}$ mice. DNA of tail, spleen, MACS-purified B cells and thymus of the indicated mice was analysed by PCR to detect recombination of exon 10.
Sp: spleen, B: B cells, Thy: thymus.

In the present mouse model, all three loxP sites flanking the neo cassette and exon 10 were oriented in the same direction to allow for removal. Those ES cell clones that had deleted the neo cassette, but still carried the floxed exon 10, were identified by PCR and verified by Southern blot analysis and subsequently microinjected into blastocysts.

Microinjected blastocysts were then transferred into pseudopregnant foster mothers. Chimeric mice were born, which carried IFNAR^{flox} and IFNAR^{wt} alleles and transmitted the IFNAR^{flox} allele to their offspring, giving rise to heterozygous IFNAR$^{flox/wt}$ mice. Next, the IFNAR$^{flox/wt}$ mice, which were on SV129 background, were backcrossed 10-fold to C57BL/6 mice. Finally, the C57BL/6 congenic IFNAR$^{flox/wt}$ mice were intercrossed to obtain homozygous mice with a conditional IFNAR (IFNAR$^{flox/flox}$).

The compatibility of a conditional *ifnar1* allele with a fully functional type I IFN system was confirmed by i.v. infection with VSV, in which IFNAR$^{flox/flox}$ mice turned out to be as resistant as WT controls (Kalinke et al., manuscript in preparation). In a next step, the IFNAR$^{flox/flox}$ mice were crossed to Cre deleter mice,[185] which express Cre in all tissues, to get mice with an ubiquitous deletion of exon 10 (IFNAR$^{\Delta ex10/\Delta ex10}$). The deletion of exon 10, encoding the transmembrane region of IFNAR, induced a frame shift that resulted in a truncated IFNAR α-chain devoid of the transmembrane region and the signalling domain. Thus, the IFNAR was rendered non-functional. Indeed, the IFNAR$^{\Delta ex10/\Delta ex10}$ mice proved to be as sensitive to lethal VSV infection as conventional IFNAR$^{-/-}$ mice (Kalinke et al., manuscript in preparation).

Instead of a ubiquitous deletion, the expression of Cre can also be controlled by a tissue-specific promoter to direct the IFNAR deletion selectively to lymphocyte subsets or other cell types.

To achieve B and T cell-specific IFNAR deletion, after 10-fold backcrossing to the C57BL/6 background, we bred IFNAR$^{flox/flox}$ mice to CD19-Cre$^{+/-174}$ or CD4-Cre$^{+/-175}$ transgenic mice to obtain CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ and CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice. These mice show a B cell or a T cell-specific IFNAR deletion, respectively, since CD19 is a lineage-specific marker for B cells and CD4 is transiently expressed on all thymocytes during the CD4$^+$CD8$^+$ double positive stage and thus directs Cre-mediated IFNAR deletion to T cells, including CD4$^+$ T helper cells and CD8$^+$ cytotoxic T cells.

3.3.2 Analysis of the IFNAR recombination efficiency in CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ and CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice

3.3.2.1 Genetic approach: PCR analysis indicates the quantitative and selective deletion of IFNAR in CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice

We analysed the Cre-mediated recombination efficiency of the conditional IFNAR by molecular biology methods and tested for the selective loss of function. Firstly, a competitive PCR was performed to detect exon 10 of the IFNAR-α chain gene. Primers were chosen which bind outside the flanked region (Fig. 3-9A), so that both the floxed exon 10 and the recombined locus could be recognised, giving rise to a 1160 bp or a 339 bp PCR product, respectively (Fig. 3-9B, preliminary results). Tail DNA from IFNAR$^{flox/flox}$ mice served as negative control and tail DNA from IFNAR$^{\Delta ex10/\Delta ex10}$ mice as positive control for recombination. In spleen of CD19-Cre$^{+/-}$ IFNAR$^{flox/flox}$ mice, partial exon 10 deletion was detectable, whereas MACS-purified splenic B cells showed complete recombination. In thymus, only a very minor signal of exon 10 deletion was found. These results indicate the quantitative, i.e. apparently complete, IFNAR deletion in B cells of CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice.

However, a competitive PCR approach to generate two products of different lengths is generally biased towards the shorter product. Thus, quantitative IFNAR deletion cannot be analysed in detail by competitive PCR. Hence, we further monitored the exon 10 recombination by Southern blot analysis performed in cooperation with Claudia Detje (PEI, in: Prinz et al., manuscript submitted). To screen for any possible IFNAR recombination in non-immune tissues, all organs (excluding spleen, LNs, thymus and BM) were isolated from CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ and

CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice. Since blood-derived B and T cells could give false positive results, these mice were analysed after perfusion with PBS.

DNA was prepared from exsanguinous peripheral organs and MACS-purified splenic B and T cells. This DNA was digested with *Eco*RI and *Hind*III and incubated with a radioactively labelled probe complementary to exon 11 of the IFNAR-α chain gene (data not shown). The Southern blot analysis further demonstrated the selective and quantitative IFNAR deletion in B and T cells of CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ and CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice, respectively.

3.3.2.2 Functional analysis of the B or T cell-specific IFNAR deletion

To verify the inactivation of IFNAR on a functional level, we analysed the expression of the surface markers CD69 and Ly6C, which are upregulated on B and T cells in type I IFN-dependent fashion[152;186;187] (Fig. 3-10A).

As expected, splenic lymphocytes of poly(I:C)-treated BL/6 mice showed a prominent upregulation of CD69, whereas Ly6C was massively induced on T cells. In contrast, B cells showed only a very minor upregulation of Ly6C. In IFNAR$^{-/-}$ mice, however, no upregulation was observed at all. Since poly(I:C) induces many cytokines in addition to type I IFN, these results clearly show that CD69 and Ly6C are induced on lymphocytes by type I IFN, but not by other cytokines.

In poly(I:C)-treated IFNAR$^{flox/flox}$ mice, CD69 and Ly6C expression was increased to a similar extent as in WT mice, thus confirming by a second approach that the mutated *ifnar1* allele did not affect IFNAR functionality. In CD19-Cre$^{+/-}$ mice, where one CD19 allele was replaced by Cre, the pronounced upregulation of CD69 indicated that B cell function was not impaired. Similarly, the strong induction of both markers in poly(I:C)-treated CD4-Cre$^{+/-}$ mice argued that the CD4 transgene, which is randomly integrated in the genome of these mice, did not confer detrimental effects on immune functions regarding the stimulatory capacity of lymphocytes.

Similar to positive controls, T cells of CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ showed upregulation of Ly6C and B cells of CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice exhibited an increased expression of CD69. However, similar to lymphocytes of IFNAR$^{-/-}$ mice, B cells of CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice did not show any induction of CD69 and Ly6C, and T cells of CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice were not able to upregulate Ly6C. These observations reveal that in CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ and CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice, B and T cells, respectively, are unresponsive to type I IFN, confirming the selective and quantitative deletion of the IFNAR.

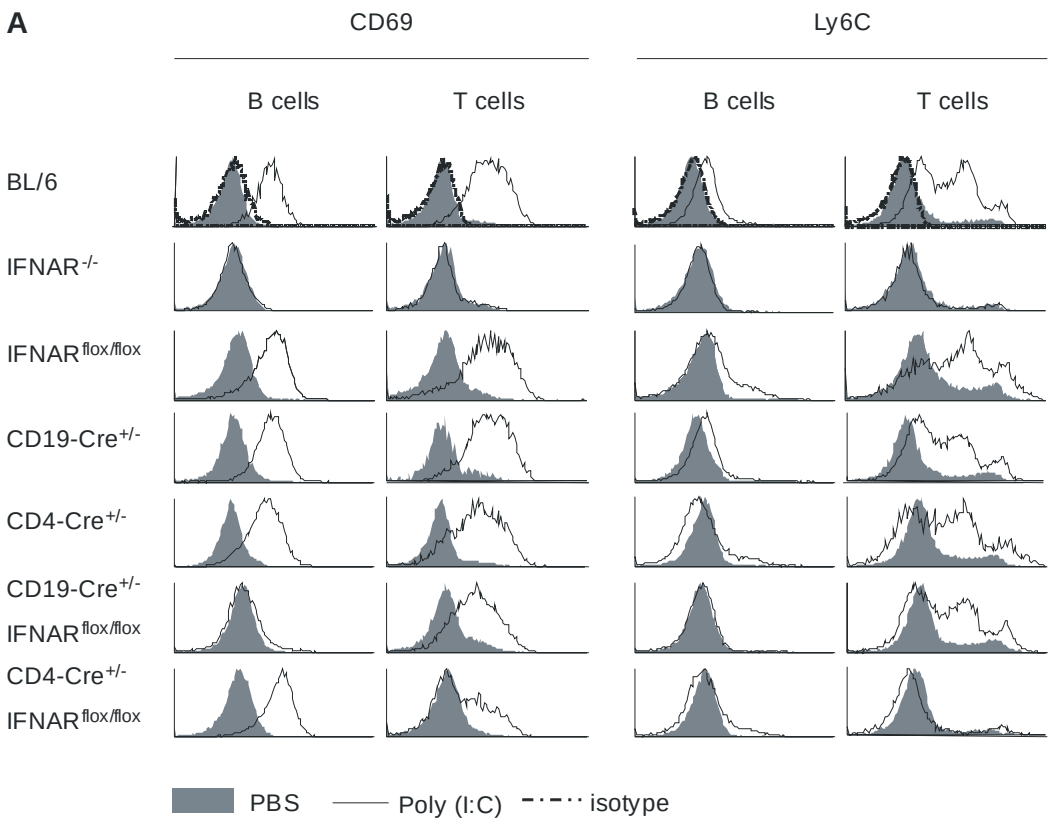
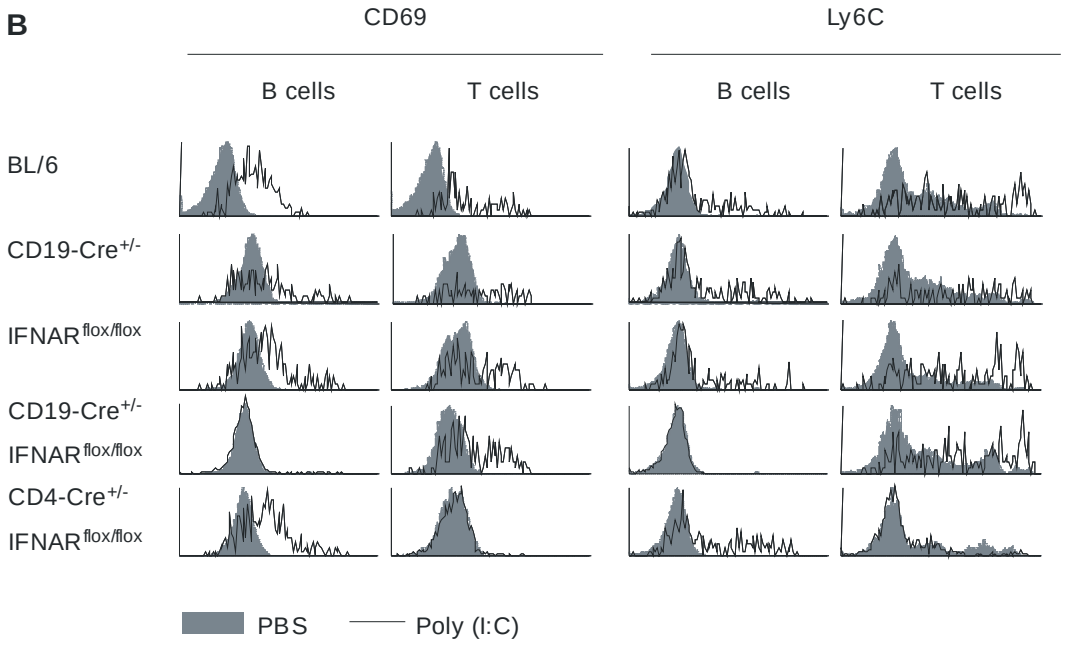


Fig. 3-10: B or T cell-specific type I IFN unresponsiveness in CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ and CD4-Cre$^{+/-}$ IFNAR$^{flox/flox}$ mice, respectively.
Mice were treated with PBS or Poly(I:C), and B and T cells from spleen (A) or blood (B) were analysed by FACS for the type I IFN-dependent markers CD69 and Ly6C. Note the missing upregulation of CD69 on B cells in CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice and of Ly6C on T cells in CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice, indicating quantitative IFNAR inactivation in the respective lymphocyte subsets.
(B) Histogramms are scaled to identical maxima. Representative data out of two experiments are shown.

In blood, similar results were obtained with the few remaining IFNAR-competent lymphocytes (Fig. 3-10B). In contrast, the IFNAR-deficient B and T cells were found at high frequencies in blood of CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ and CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice and did not exhibit any induction of CD69 and Ly6C, thus further endorsing the complete IFNAR inactivation.

Strikingly, the expression of CD69 was partially increased in splenic T cells of CD4-Cre$^{+/-}$ IFNAR$^{flox/flox}$ mice (Fig. 3-10A), demonstrating that CD69 induction on T cells *in vivo* is direct and indirect: CD69 is partially triggered by some type I IFN effects on other cells, but is further mediated via direct type I IFN stimulation of T cells.

In contrast to the T cells isolated from spleen of CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice, the blood-derived T cells (Fig. 3-10B) did not exhibit any upregulation of CD69. Since direct effects of type I IFN cannot be exerted on T cells in these mice, this suggests that the indirect effects of type I IFN, which were observed in spleen, do not act on blood T cells.



3.3.3 Mice with a B or a T cell-specific IFNAR deletion show significantly reduced lymphopenia of B and T cells, respectively

The results of CD69 and Ly6C-staining in blood for the analysis of the IFNAR recombination efficiency (Fig. 3-10B) already revealed normal blood B and T cell counts in poly(I:C)-treated CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ and CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice. Moreover, adoptive transfer studies suggested that type I IFN-unresponsive B cells were largely impaired to undergo lymphopenia.

Indeed, upon poly(I:C) treatment (Fig. 3-11A) and virus infection (data not shown) of CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice, B cells remained in blood, whereas T cells and other IFNAR-competent immune cells disappeared. In CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice, B cells underwent massive lymphopenia, whereas T cell numbers remained overall stable (Fig. 3-11B). In summary, these data confirm that direct type I IFN stimulation of B and T cells is largely sufficient for the induction of lymphopenia.

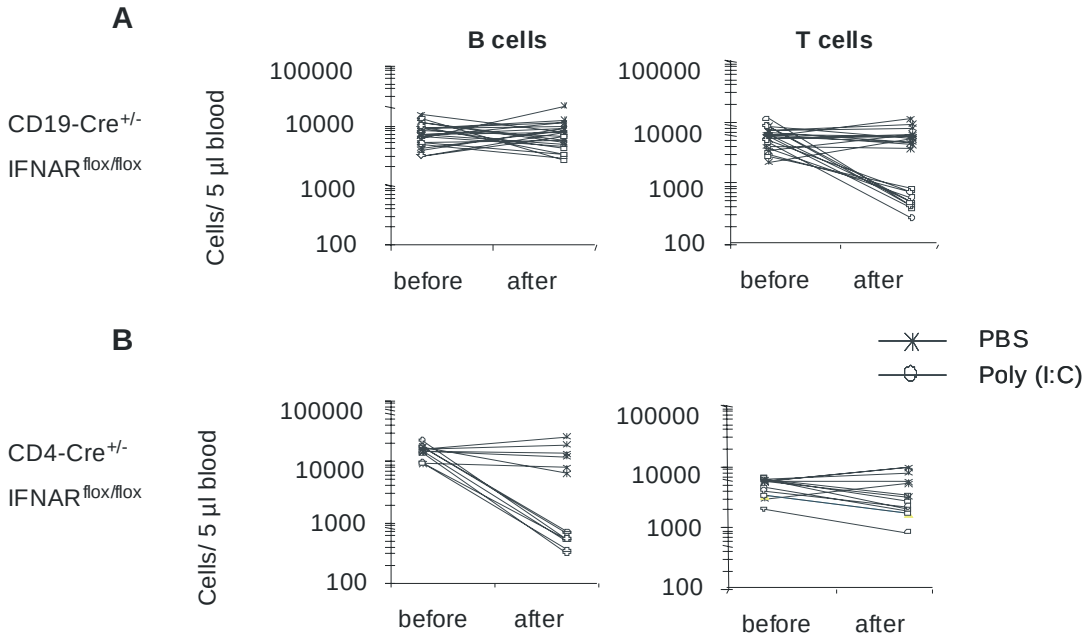


Fig. 3-11: Selective lymphopenia in mice with a B or a T cell-specific IFNAR deletion.
(A) CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ and (B) CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice were injected with poly(I:C) or PBS. Blood lymphocyte counts were determined before and after treatment. Individual results of 25 CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ and 13 CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice are shown.

The IFNAR dependence of lymphopenia following R-848 treatment of IFNAR$^{-/-}$ mice (Fig. 3-3) and BM chimeras (Fig. 3-7C) pointed towards a major role of direct type I IFN stimulation of B and T cells also in this setting.

Upon injection with R-848 (Fig. 3-12), IFNAR-competent B cells in BL/6 and in CD4-Cre$^{+/-}$ IFNAR$^{flox/flox}$ mice disappeared from peripheral blood, whereas the numbers of IFNAR-deficient T cells in CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice were decreased by approximately 50%. In contrast, the numbers of IFNAR-deficient B cells in CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice were only slightly reduced (data not shown). Thus, upon treatment with R-848, B cell lymphopenia is primarily mediated via direct type I IFN stimulation, whereas T cell lymphopenia is only partially dependent of type I IFN and is probably also triggered by some other cytokine(s) or indirect effects of type I IFN.

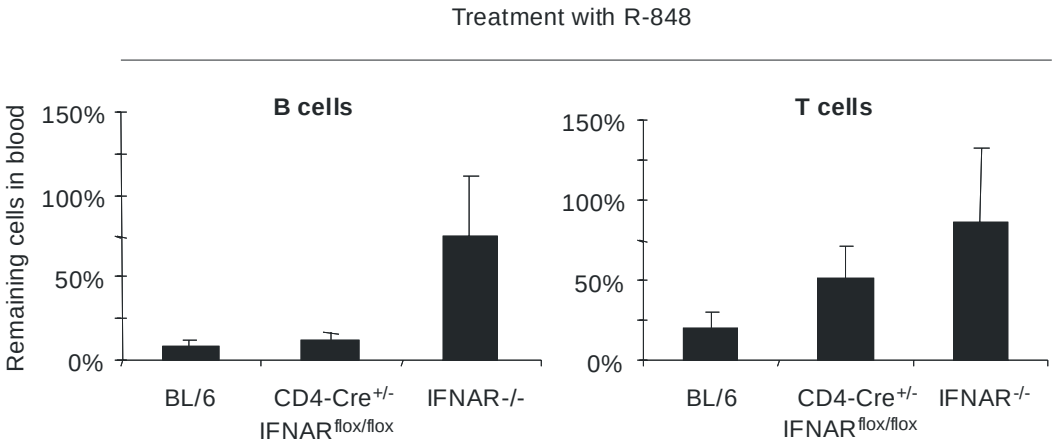


Fig. 3-12: Partial type I IFN dependence of R-848-induced T cell lymphopenia.
Blood T cells of BL/6, CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ and IFNAR$^{-/-}$ mice were counted before and after treatment with R-848. Data are expressed as mean percentages ± SD of remaining cells in blood of three mice per group.

3.4 Homing of lymphopenic B and T cells

3.4.1 Analysis of conditionally targeted mice shows that lymphopenic B cells moderately accumulate in spleen, whereas T cells do not

We considered it likely that virus infection recruited lymphocytes into secondary lymphoid organs (SLOs). In line with this, lymph node (LN) logjam, i.e. blockade of LN output, had already been proposed as the causative mechanism of drastic lymphopenia observed in clinical treatments with the immunosuppressant FTY720 (see also 1.3.2).[27-29]

To investigate the lymphopenic homing induced by type I IFN, we utilised two approaches: on the one hand, the analysis of mice with a B or a T cell-specific IFNAR deletion and on the other, the investigation of adoptively transferred animals. The concept was to study changes in the ratio of lymphopenic to non-lymphopenic cells.

As a first approach, we analysed CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice in which B cells remained in blood after poly(I:C) treatment, while T cells underwent lymphopenia (Fig. 3-11A). Consequently, similar to changes of B:T cell ratios in blood, also in those tissues, where T cells preferentially home to, B:T cells ratios were expected to be affected. A comparative FACS analysis of the lymphocyte redistribution in LNs and spleen of CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice showed that B:T cell ratios remained very similar, indicating that T cells do not preferentially home neither to LNs nor to spleen (Fig. 3-13).

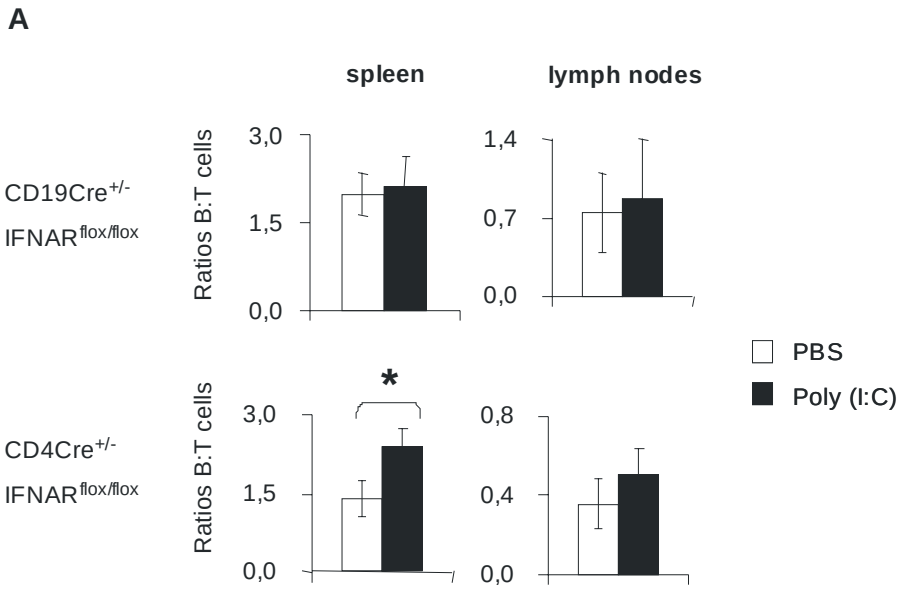
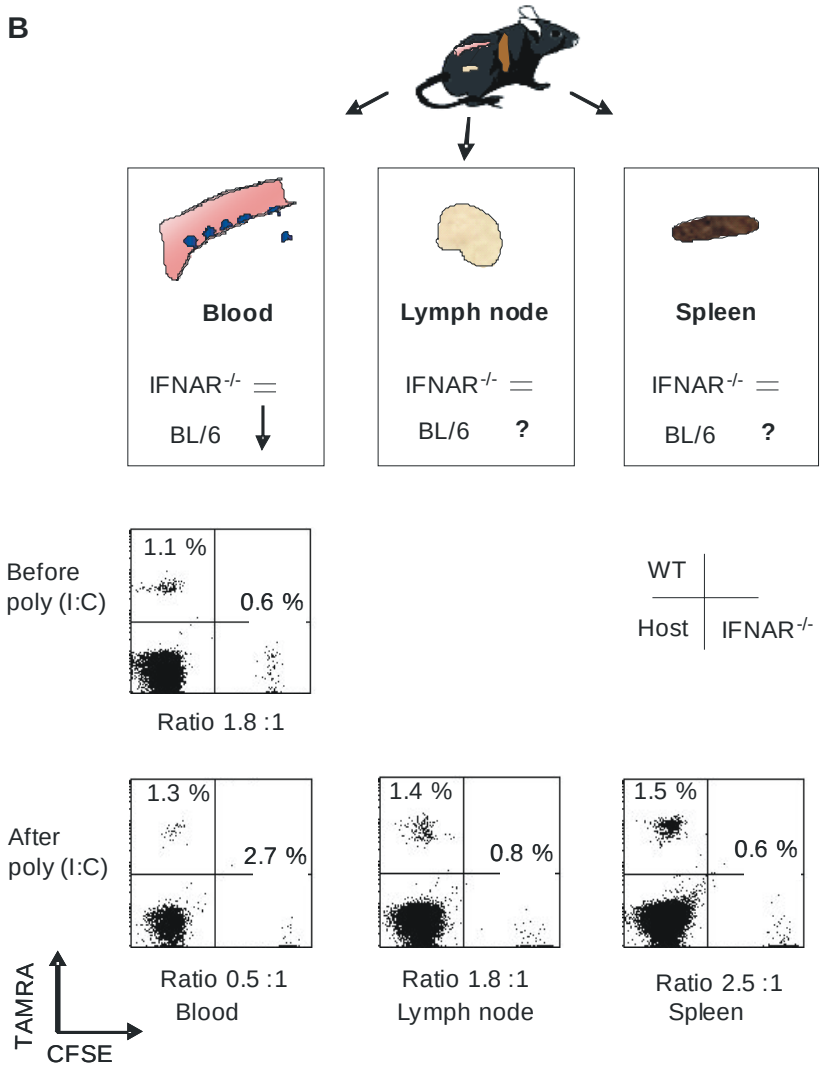


Fig. 3-13: Selective homing during lymphopenia.
(A) CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ and CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice were injected with poly(I:C) or PBS. On day 1, B and T cells from spleen, axillary and inguinal lymph nodes were FACS-analysed. B:T cell ratios are depicted and are expressed as mean ± SD for 25 CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ and 13 CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$mice, already introduced in Fig. 11.
* *P* =0.0126 (PBS versus poly(I:C)).
(B) B cells from WT and IFNAR$^{-/-}$ mice were labelled with TAMRA or CFSE and re-injected into WT recipients. Labelled cells were counted in blood before and after treatment with poly(I:C). Single cell suspensions of spleen and at least three LNs from both sides were analysed by FACS. Percentages and ratios of WT:IFNAR$^{-/-}$ B cells are depicted. Results are representative for one out of three animals of three similar experiments already introduced in Fig. 8.

In CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice, a slight, but statistically significant increase in B:T cell ratios was observed in spleen, but not in LNs, suggesting some preferred homing or retention of B cells in spleen during lymphopenia.

3.4.2 Homing studies using adoptive transfer

In theory, the detection of shifts in B:T cell ratios could be hampered by too low numbers of immigrating lymphocytes as compared to the constitutively abundant B and T cell numbers within SLOs. To overcome this limitation and as a second approach, we transfused mice with differentially labelled WT and IFNAR$^{-/-}$ B cells and analysed SLOs by FACS for ratios of WT:IFNAR$^{-/-}$ cells, the latter serving as a constant reference population (Fig. 3-13B).



As compared to pre-treatment levels, the ratios of WT:IFNAR$^{-/-}$ B cells were reduced upon poly(I:C) treatment in blood, remained constant in LNs, but were slightly increased in spleen, suggesting that WT B cells left the circulation to moderately accumulate in spleen. Similar results were obtained by adoptive transfer of WT splenocytes into PBS or poly(I:C)-treated mice (data not shown). Additionally, laser scan microscopy of non-lymphoid organs (including lung, liver, kidneys, heart, skeletal muscles, ileum, appendix, skin and BM) did not reveal major changes in ratios of differentially labelled cells (data not shown). Thus, only the spleen appears to be a moderately preferred target for B cell homing, whereas other organs are not particularly frequented.

3.5 Search for the molecular mechanism of lymphopenia

3.5.1 Lymphopenia is mainly independent of GPCRs and chemokines

Plenty of receptors and adhesion molecules regulate lymphocyte diapedesis. Recent studies have shown that treatment with FTY720 leads to lymphopenia via downregulation of sphingosine-1 receptor 1 ($S1P_1$), a novel phospholipid receptor.[27-29;188] Conversely, absence of the lymphoid chemokines CCL19 and CCL21[189] or their corresponding receptor CCR7[76] was reported to cause increased lymphocyte counts in blood. Of note, all chemokine and S1P receptors signal through heterotrimeric G proteins, of which the $G_{\alpha i}$ subunit can be inhibited by the toxin of Bordetella pertussis.[22]

Thus, as a first step to find out whether signalling through pertussis toxin (PTX)-sensitive receptors was involved in type I IFN-induced lymphopenia, we adoptively transferred differentially labelled control cells and PTX-treated splenocytes into recipients. After allowing the cells to distribute within the organism for several hours, the mice were injected with PBS or poly(I:C).The effective blockade of GPCR-signalling throughout the experiment was confirmed by laser scan microscopy, showing that PTX-treated cells had not entered LNs and splenic white pulp (Fig. 3-14A), for which chemokine receptor function is essential.

Surprisingly, upon poly(I:C) challenge, control (Fig. 3-14B, top) and PTX-treated lymphocytes (Fig. 3-14B, bottom) were both able to leave peripheral blood, albeit PTX-treated cells disappeared with slightly reduced efficiency. In essence, these data reveal that lymphopenia is mainly independent of GPCRs.

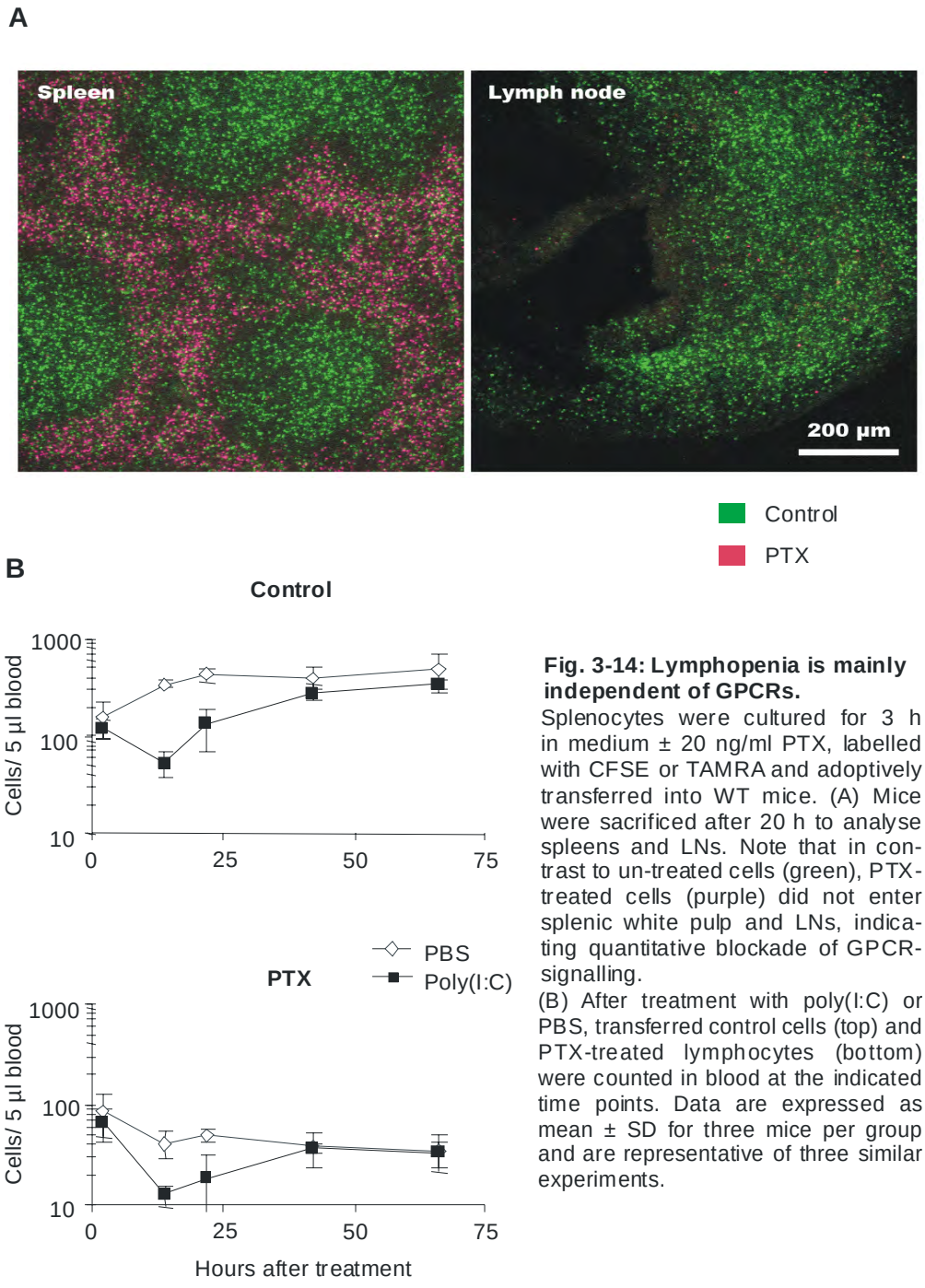


Fig. 3-14: Lymphopenia is mainly independent of GPCRs.
Splenocytes were cultured for 3 h in medium ± 20 ng/ml PTX, labelled with CFSE or TAMRA and adoptively transferred into WT mice. (A) Mice were sacrificed after 20 h to analyse spleens and LNs. Note that in contrast to un-treated cells (green), PTX-treated cells (purple) did not enter splenic white pulp and LNs, indicating quantitative blockade of GPCR-signalling.
(B) After treatment with poly(I:C) or PBS, transferred control cells (top) and PTX-treated lymphocytes (bottom) were counted in blood at the indicated time points. Data are expressed as mean ± SD for three mice per group and are representative of three similar experiments.

3.5.2 Chemotaxis is not modulated in lymphopenia

Homing of B and T cells is crucially controlled by the chemokine receptors CCR7,[76] CXCR4 and CXCR5 and its respective ligands CCL19 / CCL21, CXCL12 and CXCL13.[13;16;20] Since lymphopenia showed a minor dependence on GPCRs, we next studied whether chemotaxis was modulated by type I IFN stimulation and thus contributed to lymphopenia.

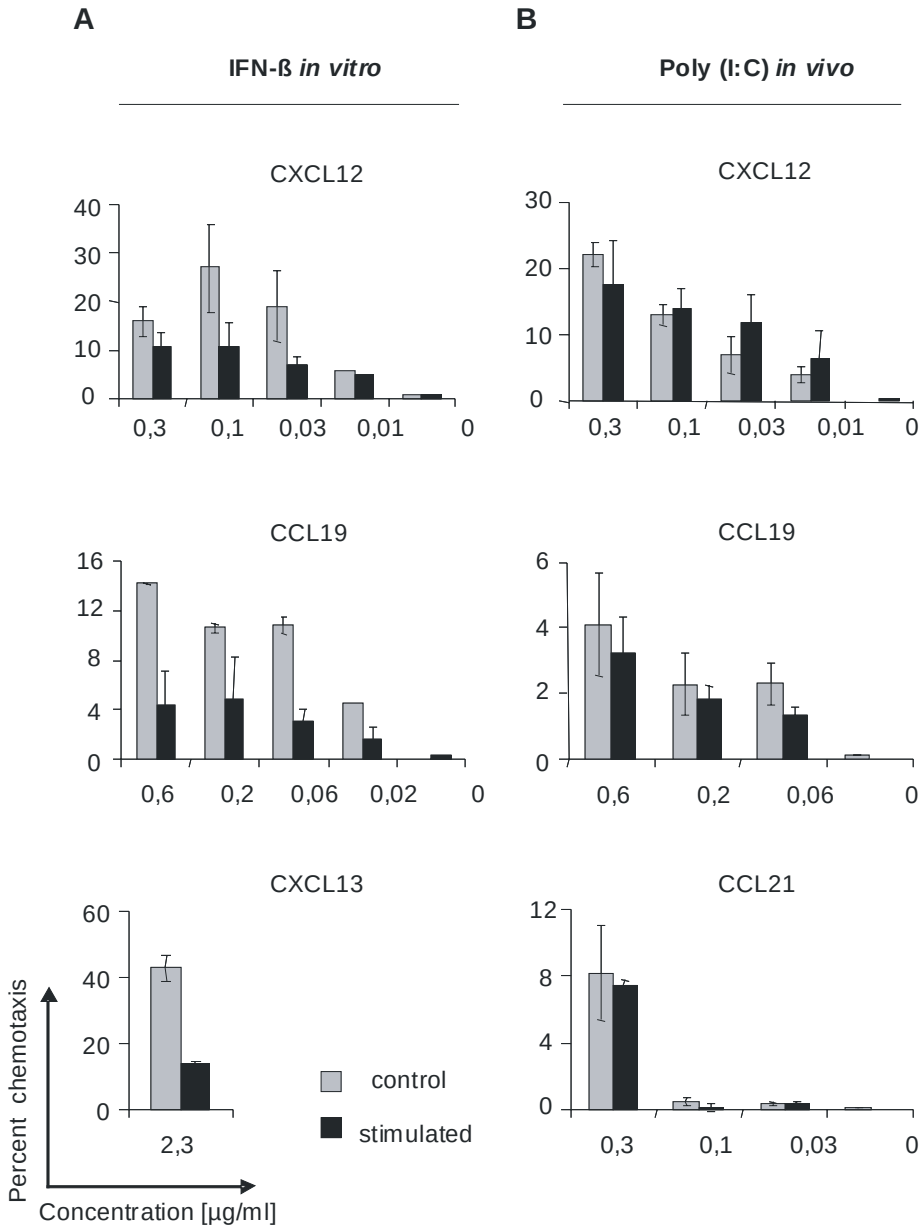


Fig. 3-15: B cell chemotaxis is reduced after IFN-ß culture, but is not affected in poly(I:C)-stimulated mice.
(A) MACS-purified splenic B cells cultured over night ± 10^3 IU IFN-ß or (B) B cells freshly isolated from poly(I:C) or PBS-treated mice were allowed to migrate towards chemokine gradients for 3 h. Migrated cells were counted by FACS. Results are expressed as mean ± SD for triplicates per chemokine concentration and are representative of two to five experiments.

After *in vitro* IFN-ß culture, B cells showed a reduced chemotaxis towards CXCL12, CCL19 and CXCL13 (Fig. 3-15A) in an *in vitro* chemotaxis assay.

However, when B cells were purified from PBS and poly(I:C)-injected mice, similar *ex vivo*-chemotaxis was found towards CXCL12, CCL19 and CCL21 (Fig. 3-15B). Hence, albeit type I IFN stimulation *in vitro* alters chemotaxis, lymphopenia *in vivo* does not rely on modulation of chemotactic migration.

3.5.3 Expression of molecules controlling lymphocyte migration is not regulated during lymphopenia

The finding that lymphopenia was independent of modulation of chemotaxis, was further supported by lymphocyte surface receptor stainings. These revealed that the expression of the chemokine receptors CCR7, CXCR4 and CXCR5, of the integrins CD11a/CD18 and CD29/CD49d as well as of the adhesion molecule CD44 was not affected by poly(I:C) treatment; only L-selectin showed some minor reproducible upregulation (Fig. 3-16A). Apart from the constitutively distinct expression profiles of chemokine receptors, no differences were observed when B and T cells were analysed separately (data not shown). Interestingly, the reduced B cell chemotaxis observed after IFN-ß culture *in vitro* correlated with downregulation of CXCR4, CXCR5 and CCR7 on B cells (Fig. 3-16B).

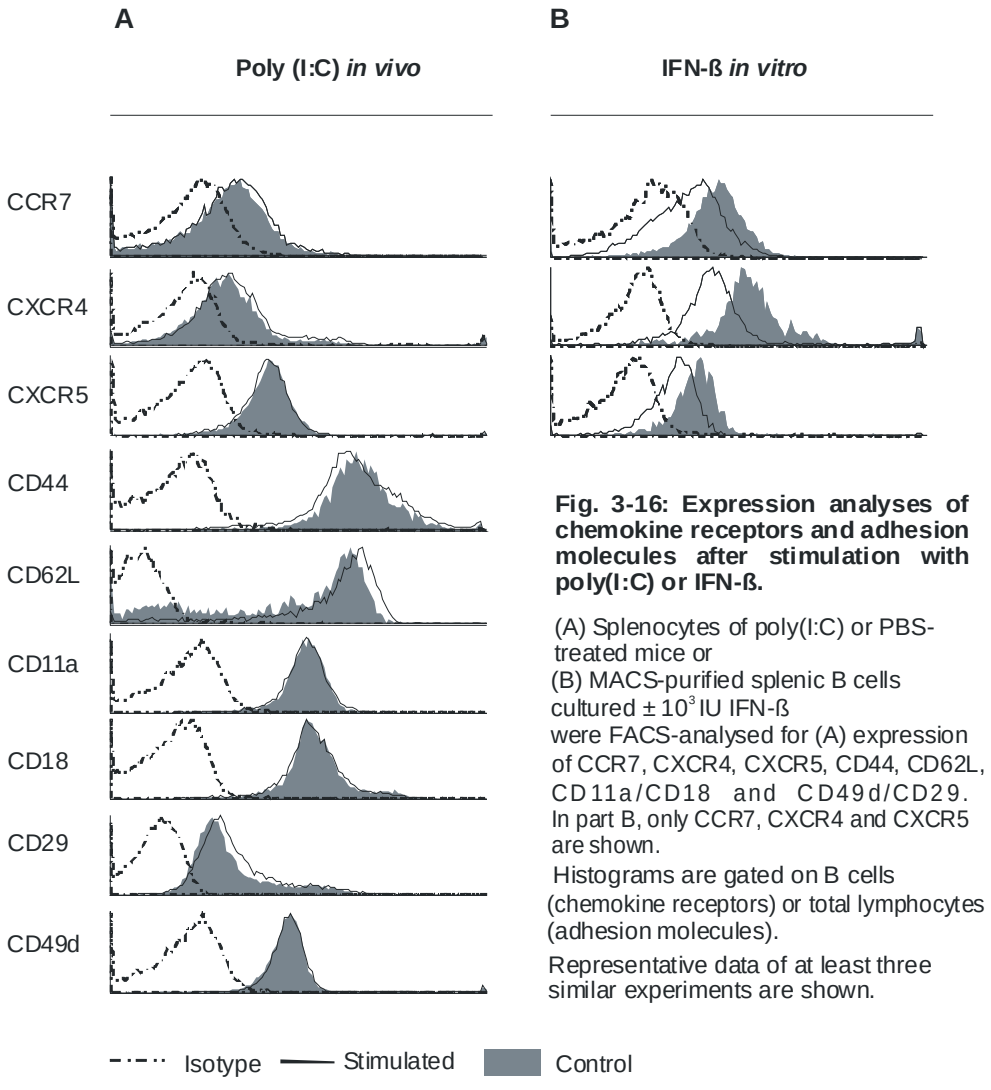


Fig. 3-16: Expression analyses of chemokine receptors and adhesion molecules after stimulation with poly(I:C) or IFN-ß.

(A) Splenocytes of poly(I:C) or PBS-treated mice or
(B) MACS-purified splenic B cells cultured ± 10^3 IU IFN-ß
were FACS-analysed for (A) expression of CCR7, CXCR4, CXCR5, CD44, CD62L, CD11a/CD18 and CD49d/CD29. In part B, only CCR7, CXCR4 and CXCR5 are shown.
Histograms are gated on B cells (chemokine receptors) or total lymphocytes (adhesion molecules).
Representative data of at least three similar experiments are shown.

3.6 Type I IFN stimulation in immune responses

3.6.1 Immunisation with live virus

3.6.1.1 Antibody responses against systemic VSV infection are independent of direct type I IFN stimulation of B cells

Our studies demonstrated that type I IFN exhibited a direct effect on lymphocytes *in vivo* (Kamphuis et al. and Le Bon et al.).[190-192] Thus, we next investigated type I IFN stimulation in the context of immune responses. Besides VSV wild-type virus, we further studied infection with the potent type I IFN inducing mutant VSV-M2 for the reason that any partial type I IFN dependence would be easier recognised in the presence of very high type I IFN amounts as elicited by VSV-M2.

Upon i.v. challenge, BL/6 and CD19-$Cre^{+/-}$IFNAR$^{flox/flox}$ mice were equally resistant to infection with 2 x 10^7 PFU VSV-M2 or VSV and did not develop symptoms of disease (Fig. 3-17 and data not shown).

VSV neutralisation assays showed that the production of VSV-neutralising IgM and IgG in CD19-$Cre^{+/-}$IFNAR$^{flox/flox}$ mice was as highly efficient as in WT controls (Fig. 3-17A). ELISA analysis further revealed a similar pattern of IgG subclasses with a pronounced bias towards IgG_{2b} for both VSV and VSV-M2 (Fig. 3-17B, C and data not shown). Hence, we conclude that type I IFN stimulation of B cells is not critical for B cell responses against systemic VSV infection.

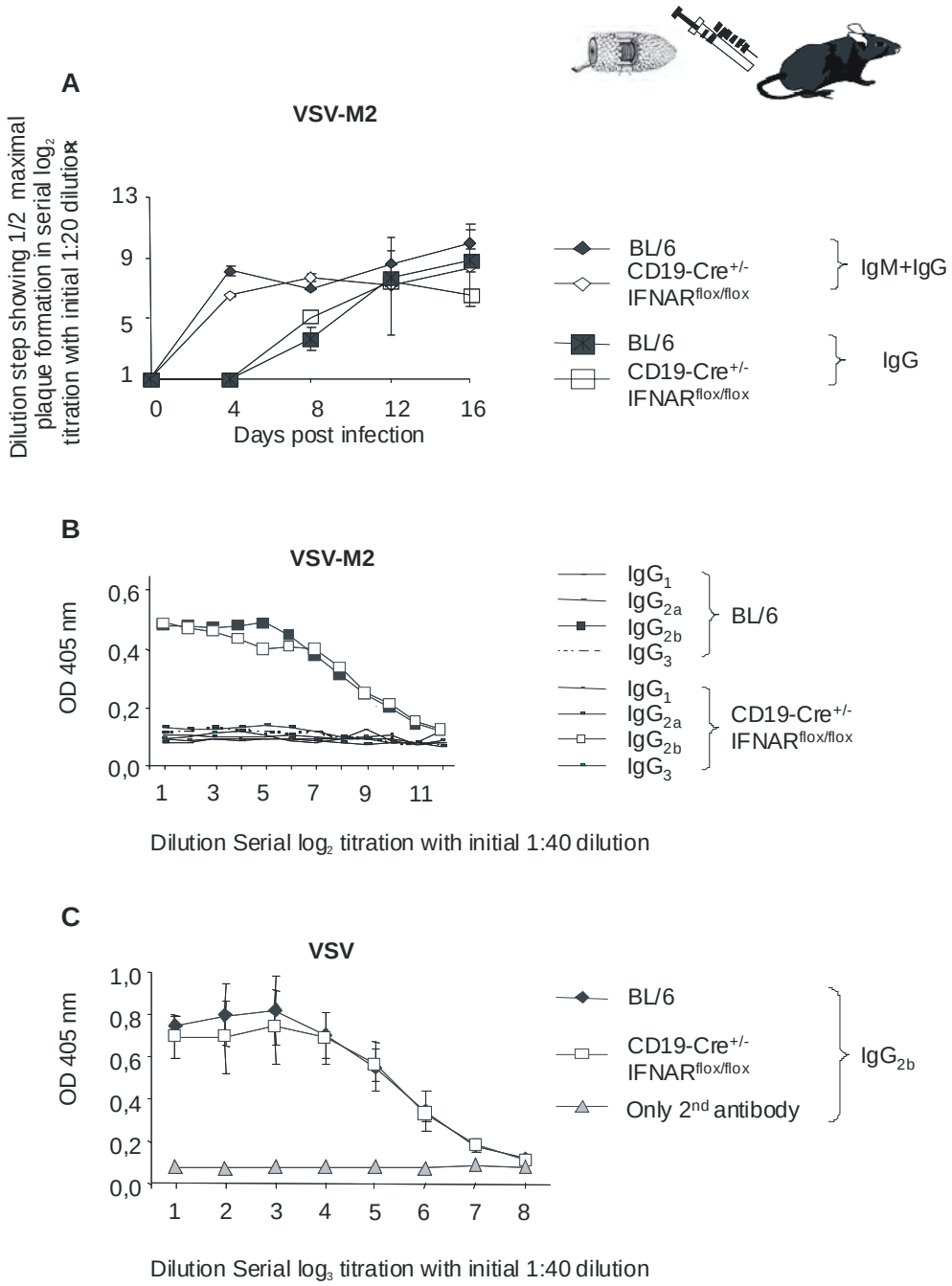


Fig. 3-17: Type I IFN stimulation of B cells is not required for antibody production following intravenous infection with VSV-M2 or VSV.
Mice were infected i.v. with $2x10^7$ PFU VSV-M2 (A, B) or VSV (C) and serum samples were taken. (A) Sera of the indicated time points were tested in an *in vitro* VSV neutralisation assay. Virus-neutralising capacities for total Ig or IgG alone are depicted.
(A, B) VSV-specific ELISA was performed with day 12 sera to detect IgG subclasses (B) or IgG_{2b} alone (C).
Infections were performed twice and results are expressed as mean ± SD for at least two mice per group. (B) Two representative animals out of three are depicted.

3.6.1.2 Type I IFN stimulation of B cells is not critical for immunoglobulin production upon intranasal VSV-M2 infection

Intravenous administration directly targets antigens to the spleen and thus elicits strong immune responses. To study the influence of type I IFN stimulation under conditions of a peripheral infection, which directs the antigens primarily to the draining lymph nodes or mucosa-associated lymphoid tissue, we inoculated mice intranasally (i.n.). Upon i.n. challenge with 10^4 PFU VSV-M2, both CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice and WT controls developed mild symptoms of upper respiratory disease (data not shown) and mounted high IgM and total Ig titres against VSV-M2 on day 5 and day 12, respectively (Fig. 3-18). Thus, humoral responses following i.n. infection with VSV-M2 are not impaired in absence of B cell responsiveness to type I IFN.

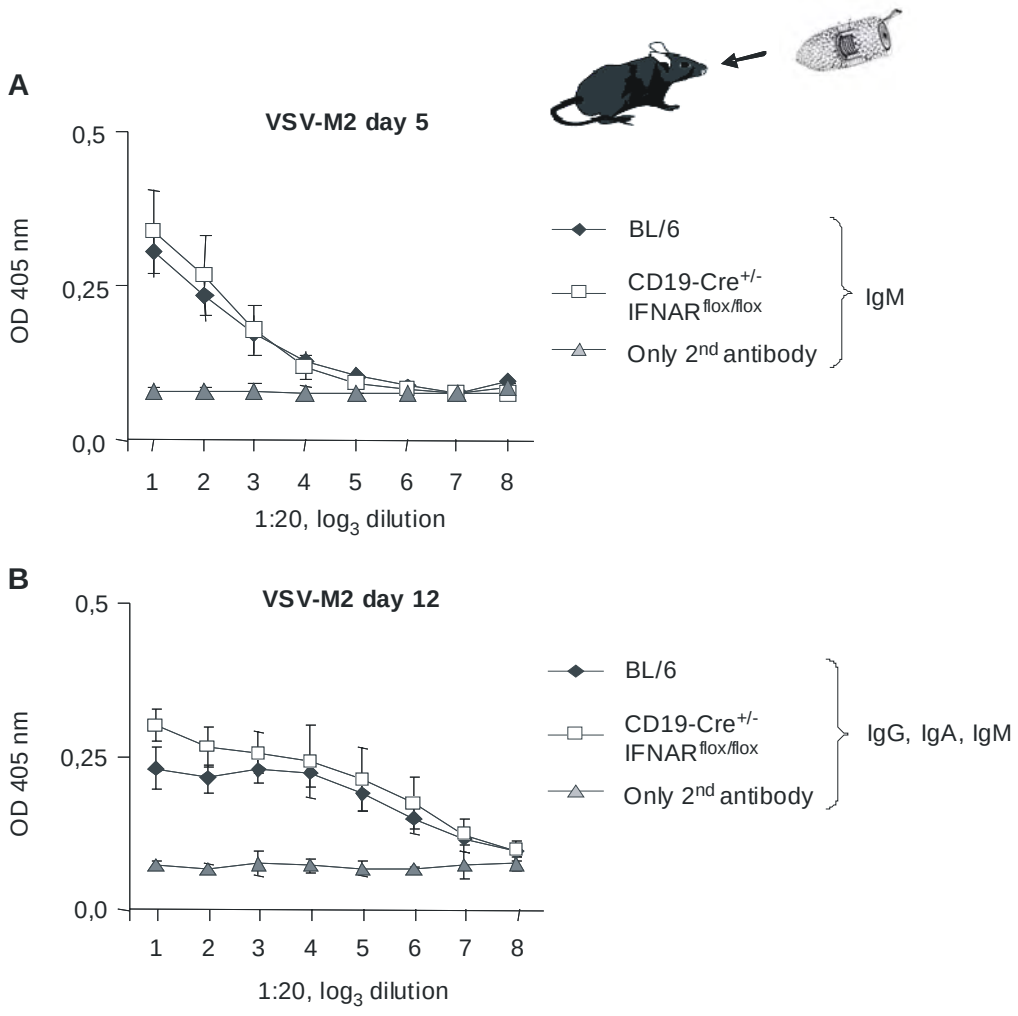


Fig. 3-18: Antibody responses against intransal infection with VSV-M2 are not reduced in absence of direct type I IFN stimulation of B cells.
Mice were infected i.n. with 10^4 PFU VSV-M2 and serum samples were taken. Sera were tested by VSV-specific ELISA to determine IgM on day 5 (A) and IgG, IgA and IgM on day 12 (B). Results are expressed as mean mean ± SD for five mice per group of one infection experiment.

3.6.2 Immunisation with VSV-G-expressing virus-like particles

3.6.2.1 Generation and electron microscopic analysis of virus-like particles expressing VSV-G

Type I IFN stimulation potently enhances humoral immunity towards the soluble protein chicken gamma globulin (CGG) via a direct effect on B cells.[191] On the contrary, we found that after infection with live virus, type I IFN responsiveness of B cells was not critical for the induction of virus-specific and virus-neutralising antibody responses.

To investigate whether i) viral replication, ii) the strong immunogenity of VSV provided by the paracrystalline structure of the viral glycoprotein (G), or iii) pathogen associated molecular patterns (PAMPs) such as the viral single-stranded RNA genome, accounted for this difference, we generated virus-like particles (VLPs) that express the main immunogen VSV-G protein on their surface. These VLPs (VSV-G MLV) are built on the gag (group-specific antigen) backbone of murine leukaemia virus (MLV). They are non-replicative particles devoid of env (envelope proteins) and of any retroviral nucleic acid.[193]

The composition and quality of shock-frozen VSV-G MLV preparations was determined by immuno gold-labelling and subsequent visualisation by electron microscopy (Fig. 3-19). Approximately 30-50% of all particles presented a phenotype of entire virions with an envelope and inner structures, which in the following were referred to as VLPs (Fig. 3-19, lower right).

In contrast, another 30% resembled viral particles, but showed varying structural alterations. Nearly all particles showed VSV-G-staining, though the labelling with α-VSV-G was found to be rather heterogeneous (Fig. 3-19, upper panel). VLPs showed fine fringes of similar surface proteins, as evidenced by methylamine wolframate-contrasting (Fig. 3-19, lower left). Therefore, VSV-G was displayed on the surface in highly organised fashion.

Additionally, the preservation of the immunogen VSV-G epitopes was confirmed by ELISA, which further served as an approximated quantification method of individual VLP preparations (Patricia Bach,[194] data not shown).

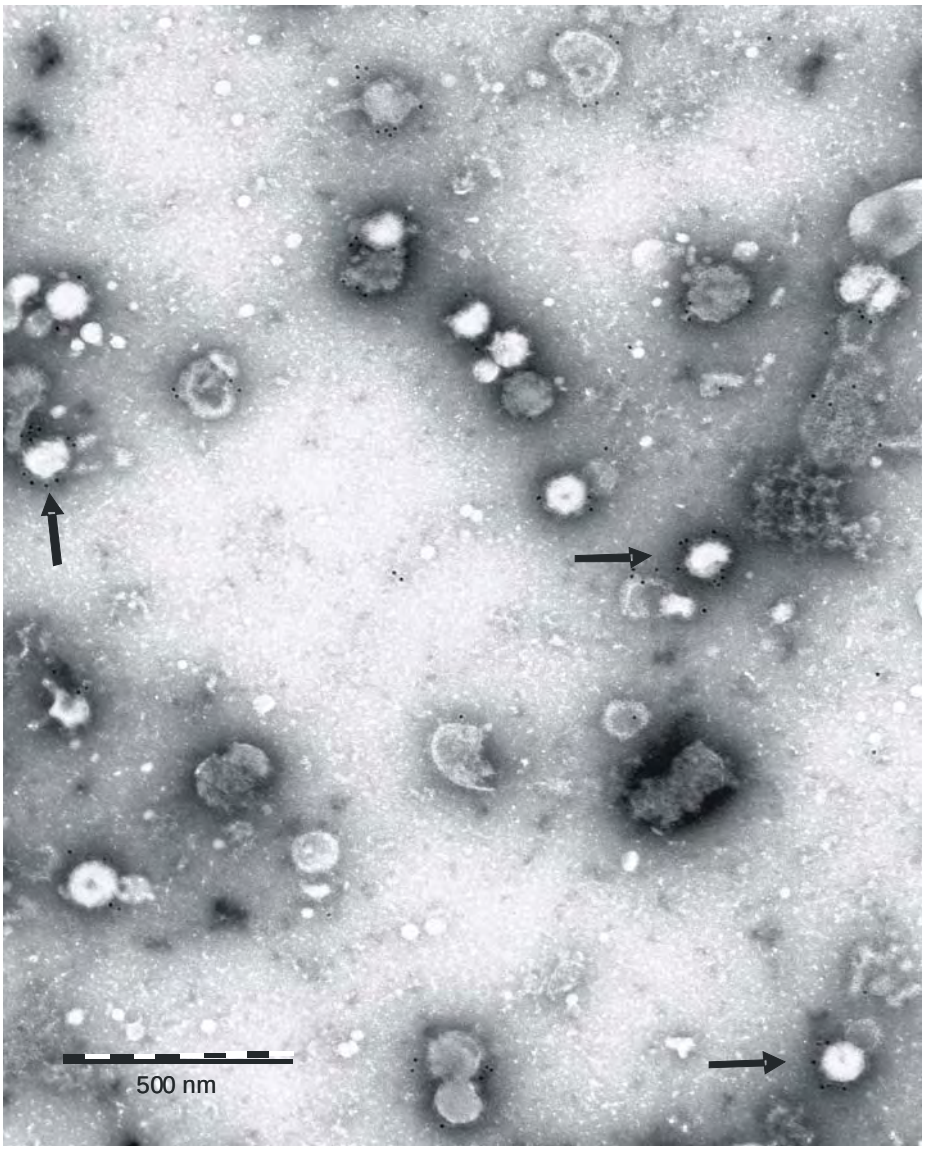
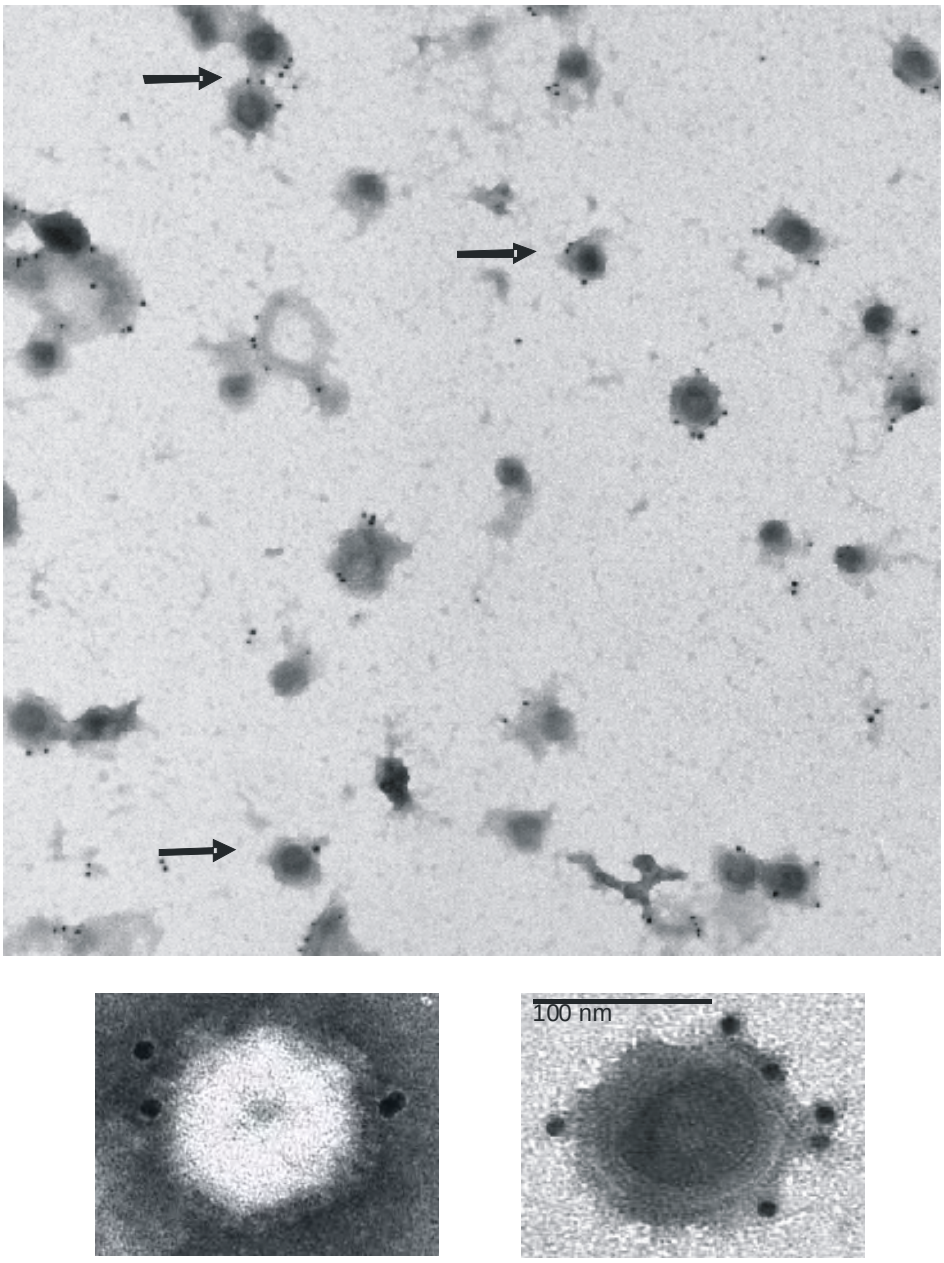


Fig. 3-19: Expression of the immunogen VSV-G on virus-like particles based on the murine leukemia virus.
Vero cells were transfected with vectors expressing VSV-G and the MLV-backbone. Supernatants were harvested twice in 48 h. After filtration, VLPs were purified by centrifugation. Shock-frozen VLPs were stained with gold-labelled α–VSV-G and contrasted with methylamine wolframate (upper left) and uranyl acetate (upper right) and visualised by electron microscopy. Gold-labelling is depicted as black dots (arrows).
Upper row: Overview of the preparation.
Lower row: Detailed section showing virus-like particles in methylamine wolframate (left) and uranyl acetate (right) contrast.



3.6.2.2 Type I IFN responsiveness is necessary for immunoglobulin isotype switch to non-replicative retroviral particles

We then immunised groups of BL/6, CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ and IFNAR$^{-/-}$ mice s.c. with 25 µl of the VSV-G MLV preparation. On day 0, day 1 and day 2, the mice either received s.c. injections of 2 x 10^5 IU IFN-α as adjuvant or were left untreated. In BL/6 mice, CD69 expression on blood B cells was moderately upregulated upon pure VLP injection, but was strongly induced by IFN-α (Fig. 3-20A, preliminary results).

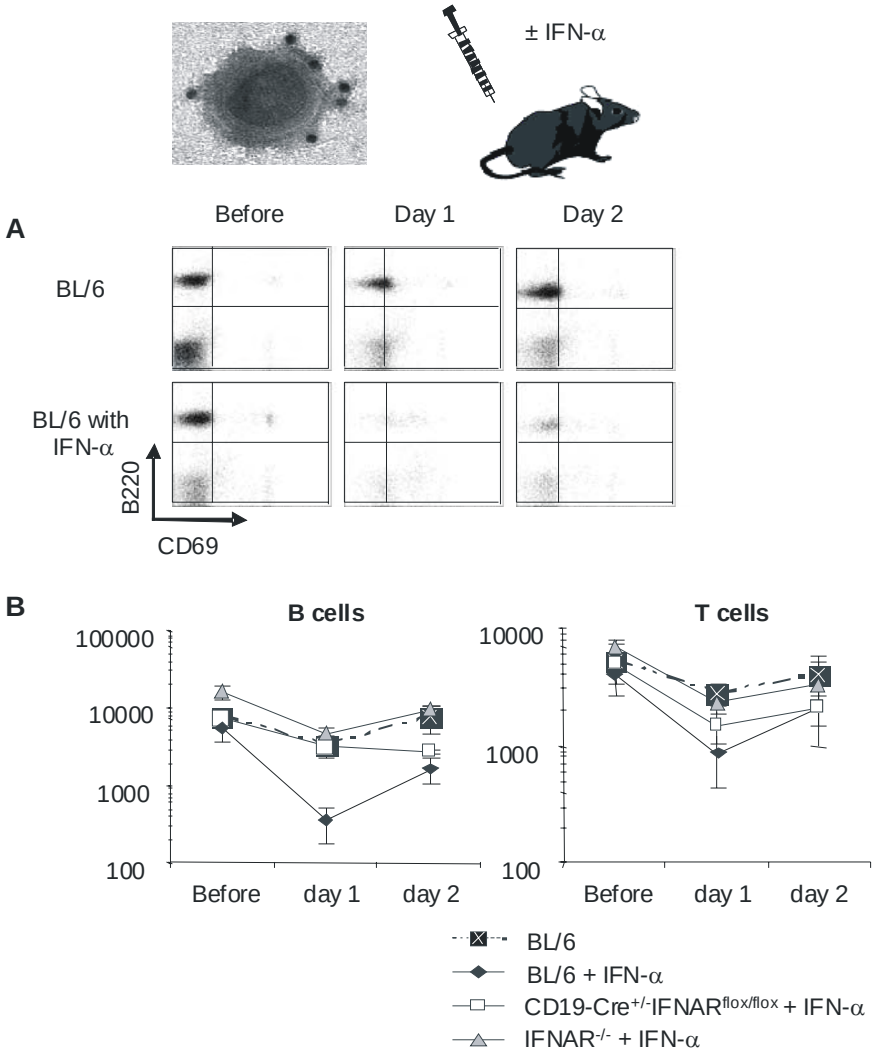
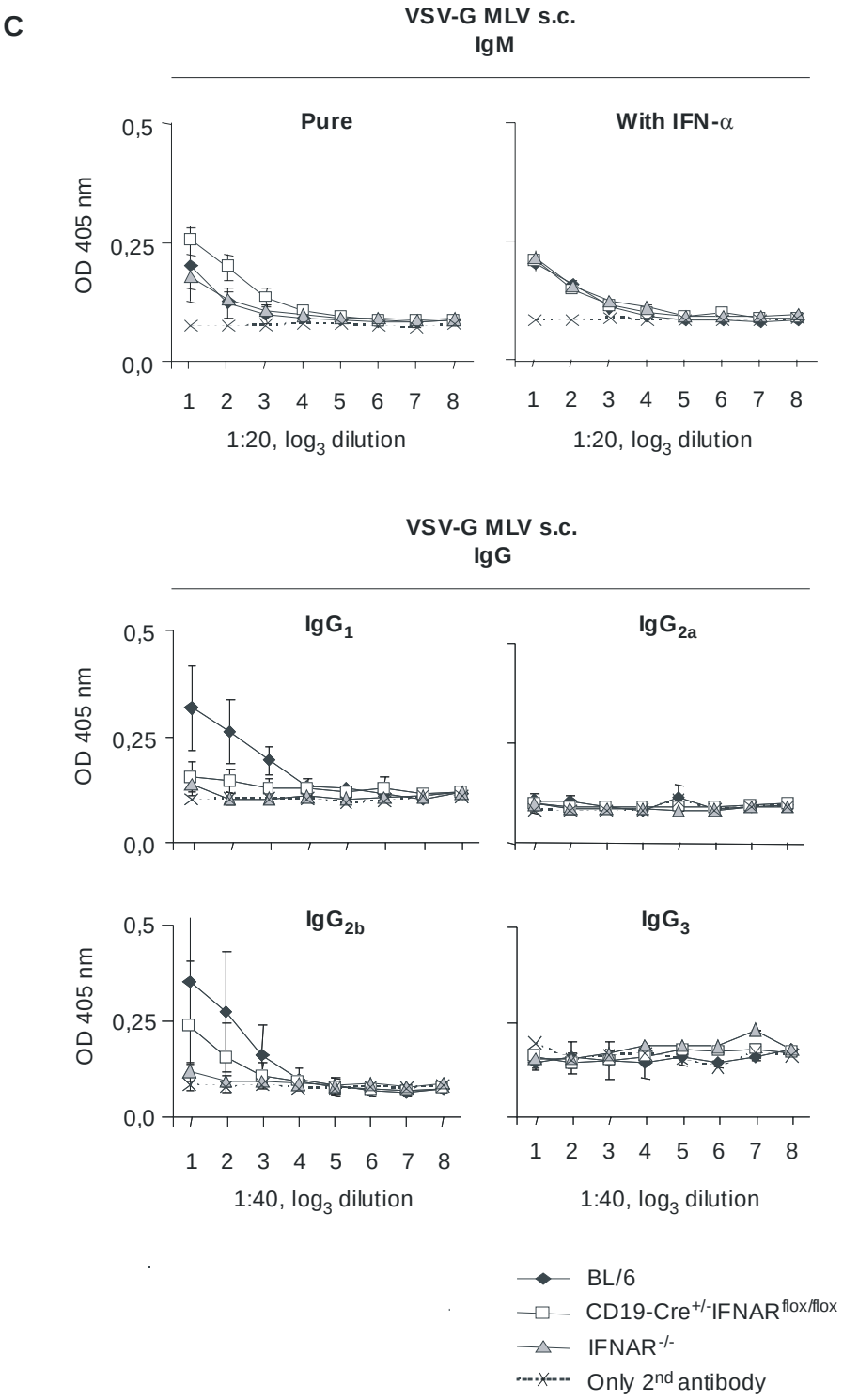


Fig. 3-20: Subcutaneous injection with VSV-G-expressing virus-like particles induces type I IFN-dependent antiviral immune response.
Mice were injected s.c. with 25 µl VSV-G MLV preparation and blood as well as serum samples were taken at different time points.
(A) Peripheral blood lymphocyte counts remain overall stable upon challenge with VSV-G MLV. Blood samples were stained for B220-PE-Cy5 and CD69-FITC and measured by FACS. Since some BL/6 mice from Fig. 3-6 were re-used for the infection, the few highly positive events in the FITC channel were due to former CFSE-labelling.
(B) Numbers of blood B and T cells were counted on the indicated time points.
(C) VSV-G MLV induces immunoglobulin class switch in a type I IFN-dependent way. Day 5 and day 12 sera were analysed for IgM and IgG subclasses, respectively, by VSV-coated ELISA.
Data are expressed as mean ± SD for three mice per group. Immunisation with VSV-G MLV was performed twice.

Blood B cell numbers were slightly reduced on day 1 following administration of VLPs, but decreased massively until day 2 in those mice additionally injected with IFN-α (Fig. 3-20A, B, preliminary results).



B cell counts were moderately reduced in IFN-α-treated IFNAR$^{-/-}$ mice and seemed to decrease to some more extent in CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice (Fig. 3-20B). Similarly, a mild to moderate lymphopenia of T cells was observed in all groups. Taken together, the data indicate that VSV-G MLV induces a mild B and T cell lymphopenia.

Serum analyses revealed similarly low IgM titres on day 5 after immunisation in all groups of mice, irrespective of whether IFN-α had been used as an adjuvant or not (Fig. 3-20C, upper panel).

Thus, the exogenous supply with IFN-α did not enhance the IgM production and furthermore, type I IFN stimulation was not critical for the early humoral IgM response upon s.c. VLP injection.

We next studied the IgG subclasses on day 12 after VLP immunisation (Fig. 3-20C, lower panel) and found moderate levels of IgG_1 and IgG_{2b} in BL/6 mice. The antibody titres varied among individual mice, but were significantly higher than in $IFNAR^{-/-}$ mice, which did not develop IgG responses at all. CD19-$Cre^{+/-}$$IFNAR^{flox/flox}$ mice, however, showed an intermediate IgG_1 and IgG_{2b} production. Similar results were obtained in mice additionally injected with IFN-α (data not shown).

In conclusion, these data show that type I IFN stimulation is necessary to promote isotype switching in response to s.c. immunisation with non-replicative VLPs. Moreover, the results demonstrate that the direct effect of type I IFN on B cells is necessary for IgG production, suggesting additive contributions of type I IFN stimulation of B cells and other cells of the immune system. Finally, since the IgG response in mice immunised only with VLPs was IFNAR-dependent, VLPs seem to induce a source of endogenous type I IFN production.

4 Discussion

Animals and humans respond to viral infections with rapid production of large amounts of type I IFN and an early transient lymphopenia in blood. Lymphopenia has long been used in Medicine and Veterinary Science for diagnostic purposes without understanding the underlying mechanism. In theory, lymphopenia could be related to effects of the virus or to the defence measures undertaken by the host. Since early viral infection is characterised by prominent type I IFN titres, investigation in the 1980s initiated to analyse the role of type I IFN in the induction of lymphopenia. With the then available tools of interferon preparations[105] and anti-interferon antibodies,[108] the studies showed that type I IFN was critically involved in lymphopenia.

Certainly, a long-known major activity of type I IFN is innate antiviral resistance. Growing evidence nowadays further shows that type I IFN also plays important roles in coordinating the immune system. NK cells, DCs and macrophages are stimulated by type I IFN to enhance their specific immune functions. On lymphocytes, experimental studies had demonstrated various effects *in vitro*; however, the direct impact of type I IFN on B and T cells *in vivo* remained elusive.

We hypothesised that type I IFN could directly stimulate lymphocytes to undergo lymphopenia and to promote B and T cell effector functions at later stages of the immune response. To study type I IFN stimulation *in vivo*, we used several genetically-modified mouse models. Conventional $IFNAR^{-/-}$ mice show generic unresponsiveness to type I IFN, whereas the novel CD19-$Cre^{+/-}$$IFNAR^{flox/flox}$ and CD4-$Cre^{+/-}$$IFNAR^{flox/flox}$ mice carry a B or a T cell-specific IFNAR deletion. In contrast to previous studies, these mice allowed to specifically detect the effects of type I IFN, but not of other cytokines. An integral objective of the work was to establish a reliable method to count absolute lymphocyte numbers in limited blood volumes. The development of a new FACS-counting technique based on reference counting beads allowed to directly count antibody-labelled cells in an identical blood sample volume. Thus, precise B and T cell numbers could be determined in a broad range from very low to high numbers and facilitated kinetics analysis at multiple time points.

The first part of the thesis concentrated on the analysis of five major aspects concerning type I IFN-mediated lymphopenia. Experiments were performed to elucidate i) the type I IFN-dependent regulation of blood lymphocyte numbers, ii) the cellular targets of type I IFN, iii) the recombination efficiency of the conditionally gene-targeted mice with a B or a T cell-specific

IFNAR deletion, iv) the homing during lymphopenia and finally v) the molecular mechanism of lymphopenia.

The second focus of the work aimed at the investigation of type I IFN stimulation during immune responses. Three different models of antigens were analysed for the role of type I IFN in the generation of antibody titres. At the PEI, the humoral immunity was studied upon systemic and peripheral infection with live VSV and following peripheral administration of VSV-G-expressing non-replicative VLPs, whereas vaccinations with the soluble protein CGG were performed in collaboration with Agnes Le Bon and David Tough at the Edward-Jenner-Institute in Compton, UK. Furthermore, this collaboration analysed the direct effects of type I IFN on $CD8^+$ T cell cross-priming towards the soluble protein OVA.

4.1 How does type I IFN cause massive lymphopenia?

Viral infection-related lymphopenia has been known in clinical practice for decades and was shown to be dependent on type I IFN action. Currently, lymphopenia has again come into focus since a reduction of blood cell counts has also been observed in treatment with several novel immunomodulatory agents, which were designed for topical and systemic use in transplantation, cancer treatment and immunotherapy. For example, R-848-induced lymphopenia has recently been studied, however, the role of cytokines induced by R-848 and triggering of lymphocytes have not extensively been addressed.[183] Overall, the respective kinetics, mechanism, involvement and distribution of lymphocyte subsets appeared to be rather heterogeneous. Lymphopenia seemed to be a stereotypic reactive pattern rather than a specific reaction.

We studied lymphopenia induced either by infection with VSV or by administration of the TLR ligands poly(I:C) or R-848 and found a maximal reduction of lymphocyte numbers approximately one day p.i., depending on the dose and infection route. Lymphopenia was a reversible phenomenon that did not involve apoptosis since adoptively transferred fluorescently labelled cells reappeared in blood after the cessation of lymphopenia (Fig. 3-6). The use of $IFNAR^{-/-}$ mice allowed addressing the implication of type I IFN in a model independent of previous studies. Upon injection with poly(I:C), lymphocyte numbers remained overall stable, confirming the critical requirement of type I IFN for lymphopenia (Fig. 3-3). Interestingly, administration of R-848 showed similar results, uncovering that type I IFN-signalling was also crucial for mediating lymphopenia to the TLR7 ligand (Fig. 3-3). Treatment with recombinantly produced IFN-α and IFN-β further corroborated that type I IFN was sufficient to induce lymphopenia. Since type I IFN could stimulate lymphocytes directly or indirectly via cytokines

derived from other immune cells, we addressed direct type I IFN stimulation of B cells in adoptive transfer models. In absence of type I IFN responsiveness either on B cells or on all non-B cells (Fig. 3-8), our data showed a direct effect of type I IFN to regulate B cell recirculation. The results strongly suggest that direct stimulation played a necessary major role. However, numbers of adoptively transferred cells were always limited. To quantify the requirements of direct and indirect effects, we studied CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ and CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice, in which all B cells or T cells, respectively, of the animals were IFNAR-deficient.

Taken together, our results highlight a previously unrecognised role of direct type I IFN stimulation of B and T cells as a mechanism to cause lymphopenia. Poly(I:C)-induced lymphopenia showed a pronounced IFNAR dependence in B cells since numbers of IFNAR-deficient B cells remained overall stable after treatment (Fig. 3-8, Fig. 3-11A). IFNAR-deficient T cells disappeared from blood to some extent, demonstrating less requirement of type I IFN stimulation for T cells (Fig. 3-11B). This tendency was also found after injection with R-848 where T cells partially underwent lymphopenia. Hence, R-848-induced T cell lymphopenia is probably also triggered by some additional factor(s) besides type I IFN (Fig. 3-12). Due to their leucopenic potential in clinical trials, TNF-α, IL-12, IL-2 and IFN-γ could contribute to lymphopenia.

Former studies addressed various direct effects of type I IFN on lymphocytes with a focus on B and T cell effector functions at later stages of the immune response.[149;153;154;191] In contrast, here we report an early and systemic direct effect of type I IFN. Lymphopenia follows the kinetics of massive type I IFN responses *in vivo* by several hours and wanes with declining cytokine levels (Fig. 3-1).

Many natural infections, however, take place in the periphery and the initially inoculated pathogen load is often relatively low. Interestingly, upon experimental local administration of low viral doses, lymphopenia occurs delayed when compared to i.v. infection (Fig. 3-2, upper panel). Since the onset of lymphopenia depends on high type I IFN serum titres, the observed delay is probably related to the time required for sufficient viral expansion, before the virus can induce massive type I IFN titres. Since type I IFN is massively produced by pDCs in spleen in response to i.v. VSV infection,[36], it remains elusive whether VSV first has to reach spleen or whether potent type I IFN production can occur locally at the primary site of peripheral infection.

Furthermore, after infection with some viruses such as vaccinia virus, high type I IFN serum titres are not produced, since many pathogens have evolved means to interfere with the production of type I IFN.[133] Viral proteins that can specifically inhibit the induction of type I IFN

represent an emerging field of interest. *In vitro* analyses have identified numerous type I IFN antagonists such as the non-structural protein 1 (NS1) of Influenza B virus and the major structural protein pp65 of human cytomegalovirus.[134;135] Influenza virus largely downregulates the cytokine induction and even hijacks the remnant NFkB activation for its own replication.[138] In contrast, vaccinia virus and hepatitis C virus potently interact with specific steps in the type I IFN induction cascade and hence lead to a nearly complete shut down of the antiviral cytokine response.[136;137] Thus, sufficient type I IFN serum levels to elicit lymphopenia may not be induced. Therefore, prominent lymphopenia might not always be observed in virally infected patients.

In contrast to a complete and selective type I IFN shut down, VSV generically reduces the host protein expression. The interaction of the viral matrix (M) protein with Rae1 and the nucleoporin Nup98 blocks the Rae1/mrnp41 mRNA nuclear export pathway. Thus, VSV diminishes antiviral cytokine responses.[181;182;195] Interestingly, VSV does induce high type I IFN levels and massive lymphopenia (Fig. 3-1, Fig. 3-2 upper panel). However, the virus variant VSV-M2, which lacks the inhibitory properties of the M protein, is indeed much more efficient in triggering cytokines *in vitro*[180] as well as *in vivo* (Zoe Waibler, data not shown). Hence, the observed earlier onset of lymphopenia in VSV-M2-infected mice (Fig. 3-2, lower panel) is probably caused by a more efficient type I IFN production. Critical titres for the induction of lymphopenia were achieved faster than by VSV since VSV-M2 elicited type I IFN even before viral expansion.

4.2 What is the role of endothelia and stromal tissues in lymphopenia?

Endothelial barriers and lymphoid stroma are critically involved in lymphocyte homing. Gunzer et al.[183] previously suggested that R-848 directly stimulated endothelia to acquire a generalised "sticky state" characterised by the increased expression of adhesion molecules. Furthermore, local inflammation alters the molecular lining of vessels by increasing expression of E-selectin, ICAM and VCAM, and type I IFN in particular can induce chemokines and modulate adhesion molecules in human T cell lines[166] and endothelial cells.[167] This led to the hypothesis that endothelia and stroma might play a role in the induction of lymphopenia.

The generation of BM-chimeric mice provided a model to investigate the impact of type I IFN stimulation on endothelia and stroma. For this, we first depleted the immune cells of IFNAR-deficient mice by lethal irradiation. Following reconstitution with IFNAR-competent BM (WT>IFNAR$^{-/-}$), the radiation-resistant cells including endothelia and stroma were unresponsive to type I IFN. Radiation-sensitive immune cells were largely ablated (Fig. 3-7A), and only a

minor population of approximately 5% T cells was found in blood. According to clinical radiotherapy, these T cells are likely to be long-lived memory T cells. The remnant recipient-derived immune cells would disappear within several months; however, chimeric mice would grow too old for use in animal testing.

In WT>IFNAR$^{-/-}$chimeras, B and T cells underwent massive lymphopenia as observed in positive controls (Fig. 3-7B). Hence, type I IFN stimulation of endothelia was not necessary, whereas effects on immune cells were sufficient for the induction of lymphopenia. In contrast, in IFNAR$^{-/-}$>WT chimeras, B and T cells remained overall stable despite type I IFN acted on endothelia and stroma. Therefore, our results reveal that R-848-induced lymphopenia critically depends on type I IFN stimulation of lymphocytes and is independent of IFN-α/β stimulation of endothelium and stroma. The slight decrease in IFNAR$^{-/-}$ T cell counts in poly(I:C)-treated IFNAR$^{-/-}$>WT BM chimeras could reflect either i) lymphopenia of the remnant 5% recipient-derived WT T cells, or ii) a marginal contribution of T cell stimulation by other cytokines induced by PAMPs, or iii) a very minor role of endothelial stimulation by PAMPs, type I IFN and other cytokines.

To investigate possible minor effects of PAMPs, we tested whether the TLR7 ligand R-848 could elicit lymphopenia of IFNAR$^{-/-}$ cells via acting on TLR7-expressing endothelia. Upon R-848 treatment of IFNAR$^{-/-}$>WT BM chimeras, the numbers of IFNAR$^{-/-}$ B cells did not decrease in blood (Fig. 3-7C). Hence, direct stimulation of endothelia by PAMPs is not a limiting step in the induction of lymphopenia.

4.3 Which molecular mechanism leads to lymphopenia?

Leucodiapedesis is controlled by the sequential co-operation of selectins, integrins and G protein-coupled receptors for different chemoattractants. Consequently, numerous molecules might mediate lymphopenia. A crucial approach to investigate the molecular mechanism was to subject donor cells to PTX treatment that inhibited all GPCRs including chemokine receptors and $S1P_1$. Upon adoptive transfer, the PTX-inhibited adoptively transferred B and T cells were unable to enter splenic white pulp and LNs (Fig. 3-14A). Since entry into SLOs depends on chemokine receptor-signalling, the effective GPCR blockade was thus confirmed. Considering the outstanding relevance of chemokine receptors and $S1P_1$ for lymphocyte homing and recirculation, it was striking that the PTX-inhibited adoptively transferred lymphocytes still underwent lymphopenia after treatment with poly(I:C) (Fig. 3-14) or R-848 (performed by our

co-author Tobias Junt, CBR Institute for Biomedical Research, Boston, USA, in: Kamphuis et al.[190]). The extent of lymphopenia, however, was slightly reduced in PTX-treated cells as compared to control lymphocytes. Hence, lymphopenia was mainly independent of GPCRs while signalling through chemokine receptors and $S1P_1$ played a minor, if any, role.

CCR7,[76] CXCR4 and CXCR5[13;16;20] control homeostatic B and T cell homing and are essential for the organisation of SLOs. We tested these chemokine receptors with respect to function and surface expression. *In vivo*-stimulated cells were isolated and *ex vivo* exposed to chemokine gradients in a two-chamber migration plate. This chemotaxis assay setting reflects the *in vivo* migratory properties of B cells. As expected from PTX treatment, the *ex vivo* chemotaxis of B cells was not altered by poly(I:C) stimulation, corroborating the conclusion that chemokines did not play a major role in lymphopenia (Fig. 3-15B). This notion was further supported by surface expression analyses by FACS, which revealed similar chemokine receptor expression on lymphocytes of PBS and poly(I:C)-treated mice (Fig. 3-16A). However, upon *in vitro* stimulation, we observed a reduced B cell chemotaxis (Fig. 3-15A). This was due to a downregulation of chemokine receptor expression, (Fig. 3-16B) as FACS-stainings uncovered. Such contrasting results on type I IFN action are sometimes reported between *in vitro* and *in vivo* analyses. Effects on T cell apoptosis are one example of contrasting data,[141;196;197] to which the aforementioned differential modulation of chemotaxis can be added.

Still, type I IFN-induced modulation of the S1P-$S1P_1$ system could contribute to lymphopenia. Whereas PTX treatment excluded $S1P_1$-signalling to a large extent, our results did not discard downregulation or absence of $S1P_1$ as a possible mechanism. Since ligand-induced internalisation of $S1P_1$[188] or the disruption of S1P gradients by S1P lyase inhibition[25] can lead to lymphopenia, type I IFN stimulation could target $S1P_1$ or the S1P metabolism, either on the level of i) the catabolising enzymes S1P lyase and phosphohydrolases, or ii) the production of S1P by sphingosine kinases. So far, some cytokines and growth factors were found to activate sphingosine kinases.[23] If type I IFN influenced S1P metabolism, the effects would probably be cell type-specific, since S1P-metabolising enzymes are rather ubiquitously expressed, but only direct type I IFN action on lymphocytes is required to induce lymphopenia. For example, type I IFN could trigger B and T cells to produce factors, which in turn act on S1P metabolism, either on lymphocytes or on a systemic level. Downregulation of $S1P_1$ and the inability to respond to S1P might even be a connecting cue between the various agents observed to cause lymphopenia. However, in a microarray analysis carried out in collaboration with Reinhold Förster (Medizinische Hochschule Hannover, Germany), we did not observe a direct effect of type I IFN stimulation on $S1P_1$ mRNA expression levels of IFN-β-stimulated human B cells (data not

shown). Furthermore, $S1P_1$ function could not be addressed since *ex vivo*-isolated B cells did not migrate towards S1P in a chemotaxis assay (data not shown).

Integrins themselves are not affected by PTX, but their function is sensitive to G protein blockade due to a GPCR-mediated change from the constitutively expressed low affinity into the biologically active high affinity conformation.[63;69] In poly(I:C)-treated mice, we did not observe an alteration of lymphocyte integrin expression as compared to control cells (Fig. 3-16A), which is in line with observations that integrin function is frequently regulated via affinity modulation rather than surface expression.[198-201] To address the integrin function experimentally, we performed adhesion assays in collaboration with our co-author Tobias Junt. Poly(I:C)-stimulated B cells exhibited a moderately increased adhesion to the intercellular adhesion molecule-1 (ICAM-1), but not to vascular cell adhesion molecule-1 (VCAM-1) (Kamphuis et al[190]). This increased adhesion probably correlated with enhanced affinity of the $\alpha_L\beta_2$ integrins (CD11a/CD18) and might be related to $S1P_1$, which can amplify integrin activation.[26] Stronger interactions between LFA-1 and ICAM-1 induced by type I IFN could further improve the immunological synapse-formation and thus play a role in enhanced priming of B and T cells. However, since lymphopenia of PTX-treated lymphocytes was only slightly impaired, increased integrin adhesion to endothelially expressed ICAM-1 cannot be the major mechanism of lymphopenia.

Since selectin function is not inhibited by PTX,[63] our data strongly suggest increased rolling as a major mechanism of lymphopenia, consistent with Gunzer´s et al.[183] observations. Enhanced rolling could correlate with the observed minor increase of L-selectin (Fig. 3-16A), with higher lectin affinity or even with a so far unidentified selectin receptor, as described for a novel endothelial L-selectin ligand activity.[80]

Strikingly, we found an upregulation of CD69 following type I IFN stimulation of B cells *in vivo* and *in vitro*. CD69, a member of the C-type lectin-like signalling receptors, is generally known as a very early stimulation marker that is only transiently expressed for several hours on activated lymphocytes, whereas other stimulation markers like CD25 and costimulatory molecules can persist for days or longer. So far, CD69 was identified to play a role in T cell development in thymus, where its high expression on immature thymocytes might mediate stromal retention.[55] On the contrary, thymic egress is dependent on downmodulation of CD69[55] via $S1P_1$-signalling[28;29] in mature thymocytes. Recently, overexpression of CD69 was shown to reduce the recovery of adoptively transferred thymocytes from blood,[55] implicating a so-far overlooked role of CD69 in lymphopenia of B and T cells. Indeed, we observed massive lymphopenia concomitant to CD69 upregulation on lymphocytes. Thus, it will be interesting to

investigate whether CD69 can contribute to tethering and rolling on endothelia or rather mediates adhesion and firm arrest.

Apart from the induction of CD69, T cells further upregulate Ly6C in response to type I IFN stimulation (Fig. 3-10[186]).Thus, the increased expression of Ly6C could furthermore enhance T cell sticking, since Ly6C was suggested to intensify LFA-1-mediated adhesion.[202]

Taken together, lymphopenia seems to be accomplished by the concerted action of several different molecular mechanisms on B and T cells, which altogether lead to increased rolling and adhesion (Fig. 4-1). CD69, Ly6C and selectins most probably contribute to lymphopenia. In contrast, signalling via LFA-1 integrins and $S1P_1$ might play only minor roles, whereas involvement of chemokine receptors is negligible.

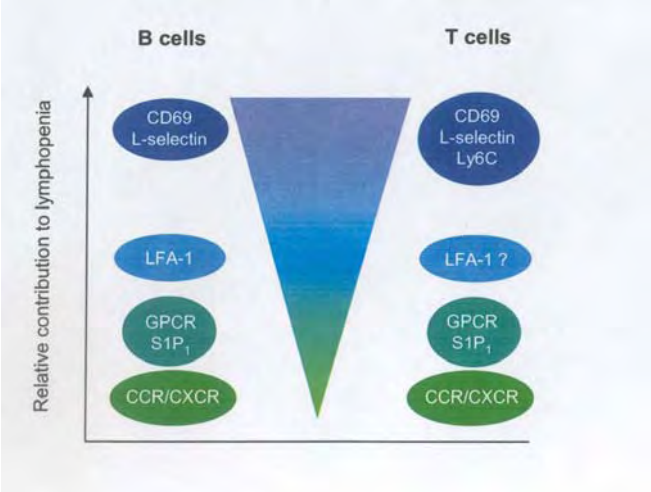


Fig. 4-1: Model of the molecular mechanism of lymphopenia

4.4 How does type I IFN induce CD69 expression in mice with a B or a T cell-specific IFNAR deletion?

In this study, we introduce CD19-$Cre^{+/-}$IFNAR$^{flox/flox}$ and CD4-$Cre^{+/-}$IFNAR$^{flox/flox}$ mice, two novel conditionally gene-targeted mice, that show a B or a T cell-specific IFNAR deletion. We analysed the recombination efficiency, i.e. the quantitative IFNAR deletion in B or T cells, with respect to genetics and loss of function. PCR and Southern Blot were used for genetic analysis. A competitive PCR approach was chosen that simultaneously detected the presence of the larger floxed or the shorter recombinated allel by generating two PCR products of different length. Due to an intrinsic bias towards the shorter recombinated product, the PCR signal intensity did not correlate with the amount of floxed or recombinated DNA. Therefore, splenocytes of CD19-$Cre^{+/-}$IFNAR$^{flox/flox}$ mice yielded a much stronger signal for the floxed allel, although splenocyte

suspensions contain only 50% B cells. Highly purified B cells gave rise to only the shorter product, showing the quantitative IFNAR recombination. However, the competitive PCR did not allow the identification of possible remnant B cells carrying the unrecombinated (floxed) IFNAR. Thymic DNA preparation exhibited a very minor signal of the recombinated allel. Most probably, this represented a natural contamination with very few blood B cells, whose recombinated IFNAR yielded a shorter signal that was favoured in the amplification as compared to the larger floxed signal of thymocytes. Hence, the PCR results point at the selective and quantitative IFNAR recombination in B cells of CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice.

To confirm these results with an independent method that did not show a bias towards the shorter product, we performed a Southern Blot analysis in collaboration with Claudia Detje (data not shown, Kalinke et al., manuscript in preparation). The analysis of perfused CD19-Cre$^{+/-}$ IFNAR$^{flox/flox}$ and CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice avoided the detection of contaminating blood cells. Furthermore, all non-immune organs were screened for a possible IFNAR recombination. The Southern blot analysis further demonstrated the selective and quantitative IFNAR deletion in B cells of CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice and in T cells of CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice.

For functional analysis of the IFNAR inactivation, we monitored the expression of type I IFN-dependent markers on B and T cells. Ly6C is upregulated by type I IFN on T cells[186] and consequently, we did not observe an induction in poly(I:C)-treated IFNAR$^{-/-}$ mice (Fig. 3-10A). On B cells, Ly6C shows only very minor expression that is also dependent on type I IFN (Fig. 3-10A).[187] The analysis of the different gene-targeted mouse lines revealed that B cells of CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice and T cells of CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice were unable to upregulate Ly6C. Notwithstanding, to properly analyse the IFNAR inactivation of B cells, another marker was required that would be massively increased upon IFNAR-signalling. Therefore, we studied CD69 that is known to be expressed in type I IFN-dependent manner.[152]

Since poly(I:C) treatment did not elicit CD69 induction in IFNAR$^{-/-}$ mice, we confirmed that CD69 expression on lymphocytes is regulated by type I IFN stimulation (Fig. 3-10A). On B cells, where the direct effects of type I IFN stimulation are sufficient to induce CD69 *in vivo* and *in vitro* (Kamphuis et al.[190], Sun et al.[152]), we observed a broad upregulation under all experimental conditions tested. The study of the different mouse lines showed that only the B cells of CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice could not upregulate CD69, thus confirming the complete B cell-specific IFNAR deletion in these mice.

On T cells, however, the regulation of CD69 expression seems to be complex. *In vitro*, CD69 upregulation is observed upon IFN-β stimulation of total splenocytes, but not of MACS-purified T cells (Ulrich Kalinke, data not shown).

Therefore, CD69 is induced on T cells exclusively by indirect effects of type I IFN. Splenocytes can release several mediators in response to type I IFN stimulation. IL-15 is most likely one key player to upregulate CD69.[145]

In vivo, we found CD69 induction following viral infection and poly(I:C) challenge. All control mouse lines analysed upregulated CD69 massively on splenic T cells. As the results of Ly6C expression showed, T cells of CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice were unresponsive to type I IFN.

Surprisingly, however, splenic T cells of CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice exhibited only a partial CD69 upregulation (Fig. 3-10A). We did not expect any reduction of CD69 expression on T cells of CD4-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice since *in vitro* studies demonstrated that indirect effects of type I IFN were sufficient for CD69 induction. Thus, apparently, the indirect effects of type I IFN regulate CD69 expression, whereas direct stimulation of T cells can further enhance, but not induce CD69.

In contrast, when we analysed T cells derived from blood of the same mice (Fig. 3-10B), we did not find any CD69 induction. Hence, indirect type I IFN stimulation does not seem to reach blood T cells. This might be due to local release of type I IFN mediators, which exhibit their effects only in paracrine fashion within the spleen.

4.5 Where do lymphopenic B and T cells home to?

Treatment with R-848 was recently shown to direct lymphocytes to SLOs, liver and lung,[183] whereas FTY720 is classically known to sequester lymphocytes in LNs, although in this context, the role of spleen and homing of B cells seem to vary between studies.[27-29] Nevertheless, in adoptively transferred or conditionally IFNAR-targeted mice treated with poly(I:C), we found some preferred B cell, but not T cell, accumulation in spleen (Fig. 3-13). These findings are corroborated by the prominent lymphopenia in absence of GPCR-signalling, indicating that lymphoid organs are indeed no essential homing targets during lymphopenia. Similarly, in an older study, Gresser et al.[105] analysed homing of chromium-labelled lymphocytes upon IFN-induced lymphopenia, but did not find any increase of radioactivity in SLOs. Since recently Sugito et al.[203] demonstrated that FTY720 was able to induce lymphopenia in splenectomised aly/aly mice, which are devoid of any SLOs, also in case of FTY720 the classical model of LN logjam does not suffice to provide an explanation for the experimental observations. Hence, the current concept of initial $S1P_1$-mediated lymphocyte sequestration with lymphopenia being merely a consequence of inhibited lymphocyte supply to blood, should be revised.

Furthermore, considering evidence for enhanced rolling and unaffected *ex vivo* chemotaxis, lymphocytes do probably not migrate into tissues, but remain attached to the endothelium and become part of the marginal pool. In conclusion, the vasculature probably represents the main homing target during lymphopenia.

Notwithstanding, previous reports pointed out that the distribution of morphologically identified leucocytes within spleen was altered upon poly(I:C) challenge,[108;115] without directly addressing the lymphocyte subsets involved. In collaboration with Tobias Junt, we could show a massive B and T cell depletion from splenic red pulp during poly(I:C)-induced lymphopenia and a concomitant purgation of marginal zone B cells (Kamphuis et al.[190]). During lymphopenia, it is probable that recruitment of lymphocytes into B and T cell zones prepares for lymphocyte priming.

Note added in proof:

After the completion of this dissertation, a paper was published by Jason Cyster´s group (Shiow et al.) that investigated lymphopenia in blood and lymph by a completely independent approach.[204] Shiow et al. found that IFN-α/ß largely inhibits lymphocyte egress from LNs via a partially lymphocyte-intrinsic effect that downregulates $S1P_1$. They report a reduced *ex vivo* S1P chemotaxis of lymphopenic T cells and also of B cells, but only when isolated and furthermore cultured *in vitro* for several hours. In absence of CD69, lymphopenia was greatly diminished. Only partial $S1P_1$ downmodulation occurred on $CD69^{-/-}$ cells as compared to WT cells that downregulated $S1P_1$ completely. In several approaches, Shiow et al. finally showed a selective protein/protein interaction between CD69 and the S1P receptor $S1P_1$ being responsible for the negative regulation of GPCR surface expression.

These findings are in accordance with the data presented in this dissertation and complement our study with respect to the molecular mechanism. Shiow et al. studied a possible role of absence of $S1P_1$, whereas our approach with PTX treatment analysed the possible involvement of GPCR-signalling. However, due to i) lack of reliable $S1P_1$ antibodies, ii) absence of *ex vivo* B cell chemotaxis towards S1P (data not shown) and iii) similar $S1P_1$ mRNA expression levels (data not shown), we did not further focus on $S1P_1$ function. Shiow et al. hypothesise that CD69 protein interactions could represent a novel mechanism of GPCR downregulation. However, since they found this interaction to be selective, it does not provide an explanation for our reduced chemokine receptor expression after *in vitro* IFN-ß stimulation. In one experiment they show a decreased *ex vivo* T cell chemotaxis towards CXCL12 (SDF-1), however they did not further comment on these data.

Interestingly, Shiow et al. report a partial lymphopenia in absence of CD69, however, without further addressing possible other mechanisms which might account for residual effects. Therefore, in line with our proposed complementary molecular model, the absence of $S1P_1$ is one important and necessary, but not fully sufficient mechanism to induce lymphopenia. In this regard, Sugito´s observation of FTY720-induced lymphopenia in absence of SLOs further corroborates the existence of additional mechanisms, which are not related to $S1P_1$-mediated lymphocyte logjam in SLOs.

Besides, Jason Cyster´s group did neither further quantify the requirements of direct type I IFN stimulation of lymphocytes nor analyse the possible involvement of endothelia. Moreover, they did not comment on published evidence for enhanced rolling.

In conclusion, their study provides one novel molecular mechanism of lymphopenia and thus further extends our concept of different cellular and molecular contributions that inter-relate the various types of lymphopenia.

4.6 What is the role of type I IFN stimulation in immune responses?

Lymphopenia is the consequence of a rather short-term stimulation (for several hours) during early immune responses when high type I IFN titres are found in serum. However, once systemic levels decrease, local type I IFN production continues to exert effects on lymphocytes, probably for days, depending on the type of immune reaction. Interestingly, in absence of detectable type I IFN levels in serum, peripheral blood lymphocytes still exhibit type I IFN-stimulated gene expression profiles.[205;206]

Type I IFN stimulation could shape immune responses via early and/or late effects. To discriminate between the influence of early systemic lymphopenia and late peripheral stimulation in SLOs, a model would be required that allowed to selectively abrogate type I IFN responsiveness at early or later time points. Adoptive transfer of $IFNAR^{-/-}$ cells or analysis of CD19-$Cre^{+/-}$$IFNAR^{flox/flox}$ and CD4-$Cre^{+/-}$$IFNAR^{flox/flox}$ mice, however, are not appropriate to address this issue experimentally, since in these animals the cell-specific type I IFN responsiveness is abolished throughout the time course of an infection.

In theory, the IFNAR deletion could be induced (or, in a new genetic approach, reconstituted) at a desired time point (when lymphopenic cells have returned to recirculate) by breeding $IFNAR^{flox/flox}$ mice to mice that controlled Cre expression under an inducible tamoxifen-sensitive

promoter. Cre recombination and IFNAR deletion could then be induced by feeding the animals with tamoxifen.

However, the complete IFNAR inactivation would not be achieved within few days. Therefore, the currently available gene-targeted mouse models do not allow for identifying the relative contributions of early lymphopenia and localised type I IFN stimulation of lymphocytes at later time points.

Critical roles of type I IFN stimulation in T cell immunity were recently shown in a model of cross-priming to ovalbumin (OVA)[192] and in infection with lymphocytic choriomeningitis virus (LCMV).[154] In collaboration with the group of David Tough, we could demonstrate that besides the previously observed stimulation of DCs,[153] IFN-α directly prolonged the proliferation and expansion of antigen-specific $CD8^+$ T cells to allow for cross-priming to OVA.[192]

Similarly, Kolumam et al. found that type I IFN stimulation of T cells was critical for the generation of cytotoxic T lymphocyte (CTL) responses towards LCMV by promoting the survival of the proliferating antigen-specific $CD8^+$ T cells.

4.6.1 Does type I IFN influence B cell responses?

Several vaccination studies performed by Proietti et al., Le Bon et al. and others already pointed towards a role of type I IFN in the induction of antigen-specific humoral immunity. In particular, B cell responses towards an influenza subunit vaccine[156] and against the soluble protein chicken gamma globulin (CGG)[149;191] were dependent on type I IFN stimulation. In case of CGG immunisation, DCs were first identified as targets for type I IFN. Only recently, in a collaborative approach with the group of David Tough, Edward Jenner Institute for Vaccine Research, Compton, UK, we found that type I IFN also exerts direct effects on B cells as well as on T cells in order to elicit anti-CGG responses.[191]

In contrast to the initially mentioned work of Le Bon et al., the group of Rolf Zinkernagel and Michel Aguet assessed the role of type I IFN stimulation in humoral immunity against live viruses. Their model of LCMV infection normally elicits potent CTL responses one week post infection, but rather moderate neutralising antibody titres. When $IFNAR^{-/-}$ mice were challenged with 1 - 3 x 10^2 PFU LCMV, CTL induction was not detectable and virus persisted in several organs.[139] At the same time, immunoglobulin production remained normal with respect to total IgG titres and the subclass spectrum,[207] demonstrating that antibody responses towards a replicative virus can be induced in absence of type I IFN stimulation.

Since survival of VSV-infected mice is conferred by the concerted action of type I IFN in the early phase and by neutralising antibodies from day 3 onwards, VSV provides an appropriate model to study the impact of type I IFN on B cell responses. In line with the results obtained with LCMV, here we show that CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice develop normal Ig responses and are fully protected following systemic i.v. or peripheral i.n. infection with VSV, suggesting that type I IFN responsiveness of B cells is not critical to combat infections with live viruses (Fig. 3-17, Fig. 3-18).

The distinct requirements of type I IFN stimulation for vaccination with CGG and infection with live viruses are probably related to four major aspects regarding the different immunogenities of the respective antigens:

Firstly, soluble proteins such as CGG do not provide any danger signals and therefore do not elicit immune responses themselves. In contrast, VSV contains ssRNA, which is recognised as a danger signal by TLR7.[208]

Secondly, replication itself can provide stimulatory signals to infected cells. For example, IFN-α responses to VSV can only be induced in DCs upon infection with live virus, but not with ultraviolet (UV)-inactivated virus.[129] Upon VSV replication, dsRNA is generated, which can trigger TLR3 and the RNA helicases RIG-I, PKR and mda5.[126;127;209] In response to VSV, RIG-I and probably also others receptors induce a variety of host cytokines such as type I IFN, TNF-α, IL-12, IL-6 and IL-10, depending on the cell type targeted.

Thirdly, several reports indicated that, depending on the activation state and species,[210] B cells can express TLR1,[211] TLR3,[212] TLR4,[212;213] TLR5,[213] TLR7[214] and TLR9,[211] and could thus be directly stimulated by the respective PAMPs such as diacyl lipopeptides, dsRNA, lipopolysaccharide (LPS), flagellin, ssRNA and CpG-containing DNA motifs. Importantly, additional direct stimulation by TLRs can be necessary for efficient B cell responses.[213] Hence, it is conceivable that both the direct B cell activation by viral ssRNA and dsRNA and the indirect effects of cytokines induced by PAMPs and viral replication, could compensate for the lack of direct type I IFN stimulation of B cells in CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice. Indeed, if we consider that many viruses have developed means to interfere with the induction of type I IFN,[133-137;180] it seems likely that some kind of functional redundancy may have evolved in order to reliably achieve the efficient protection against viral infections.

Fourthly, the highly repetitive organisation of the immunogen VSV-G protein on the surface of VSV further accounts for the strong immunogenity of the virus. VSV-G is densely packed at a

distance of 5-10 nm with only its tips appearing on the virion´s surface. Thus, VSV leads to B cell receptor (BCR) cross-linking[169] that provides sufficiently strong activation to induce IgM secretion in absence of T cell help. Consequently, VSV constitutes a T cell-independent (TI) antigen. IgG responses towards VSV-G, however, require T cell help.[169;215]

Since *in vitro*, type I IFN-stimulated B cells show an increased sensitivity to limited IgM receptor ligation,[158;159] type I IFN stimulation could lower the threshold for B cell induction during immune responses. This might be relevant to the observed strong Ig responses in VSV-infected CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice since the highly repetitive VSV-G TI antigen already provides optimal conditions for BCR cross-linking, in contrast to the T cell-dependent (TD) antigen CGG. Thus, detecting possible requirements for type I IFN stimulation of B cells could be hampered by using a TI antigen, which overcomes a possible need for type I IFN.

Generally, TI antigens are classified into two groups by their random or repetitive structure and their ability to elicit a polyclonal or a specific B cell proliferation (Tab. 4-1).

Tab. 4-1: Classification of VSV as a T cell-independent antigen

Antigen	TI-1	TI-2	VSV
Structure	Randomly organised	Highly repetitive	Highly repetitive VSV-G
B cell proliferation	Polyclonal mitogen	Specific	Specific
IgM response in nude or CD4$^+$-depleted wt mice	Yes	Yes	Yes
IgM response in CD4$^+$-depleted XID mice	Yes	No	Yes
Classical example	LPS	Polysaccharides, flagellin, Ficoll[96]	
Classification			TI-1, subtype: specific B cell activators (Zinkernagel´s group,[169;216] No specification by Paul´s textbook[8] TI-2 in this dissertation

The independence of T cell help is experimentally determined in two different mouse lines: in $CD4^+$-depleted WT mice or athymic nude mice that have only few mature T cells, and furthermore in $CD4^+$-depleted X chromosome-linked immunodeficiency (XID) mice. XID mice carry a missense mutation in the gene encoding for the intracellular tyrosine kinase Btk that participates in BCR signal transduction. Therefore, XID B cells exhibit an activation defect, which facilitates the identification of any residual requirements for T cell help during IgM responses. According to the classification used by Zinkernagel´s group, TI-1 antigens are completely independent of T cell help and thus can be elicited in XID mice, whereas TI-2 antigens require residual help from T cells or NK cells.[217-219]Consequently, Zinkernagel classifies VSV as a TI-1 antigen and defines a TI-1 subgroup of specific B cell activators.

However, Paul remarks[8]: "It should be noted that there are circumstances in which antibody responses can be seen in XID mice to antigens that are by other criteria TI-2 antigens, so at this point there is no absolute empirical criterion to separate TI-1 from TI-2 antigens." Furthermore, it can be added that in the last years specific receptors for the classical examples of TI antigens, namely TLR4 for LPS and TLR5 for flagellin, have been identified. In face of emerging roles of these molecules in innate, but also adaptive immunity,[213] TI antigens should be classified into TLR-mediated and TLR-independent antigens. In conclusion, due to its characteristics, we refer to VSV as a TI-2 antigen.

4.6.2 Can type I IFN promote humoral immune responses to virus-like particles?

The generation of non-replicative virus-like particles expressing the VSV-G protein enabled us to study antibody responses to the immunogen determinant of VSV in absence of PAMPs and replication. As EM analysis revealed, the envelope of the VLPs was composed of similar structural elements forming a fine fringe. Since the particles were generated in absence of retroviral env, only cellular proteins and VSV-G could constitute the surface. According to the regular appearance, VSV-G seems to cover the VLPs in a highly organised fashion. Therefore, VSV-G MLV does probably also induce BCR cross-linking and thus might constitute a TI antigen, similar to inactivated VSV, but in contrast to CGG (Tab. 4-2).

Without changing the model, VLPs furthermore allowed the analysis of anti-VSV-G responses in $IFNAR^{-/-}$ mice. In these mice, antibody responses are impossible to study with live VSV since the animals would quickly succumb to infection. The efficient generation of antibody responses was reported for i.v. administration of retroviral particles[193] and vaccination with icosahedral Qβ phage VLPs.[97]

Tab. 4-2: Overview of the antigens used in the vaccination studies discussed in the text

Agent	VSV	VSV-G MLV	CGG
Structure	Highly repetitive VSV-G protein in envelope, paracrystalline organisation	Highly organised VSV-G protein in envelope	Soluble
Immunogenity	High (due to PAMPs and BCR cross-linking[169])	Intermediate	None
Antigen type	TI-2 antigen (see table 4.1)	Not determined, most probably TI-2	TD
Type I IFN stimulation	Not critical for humoral response	Necessary for Ig switch; not critical for IgM	Critical for IgM and switch

Following s.c. injection without adjuvants, VSV-G MLV induced considerable antibody titres. This further demonstrated the immunostimulatory capacity of VLPs, which might be related to the highly organised expression of VSV-G on the surface (Fig. 3-19).

Using a model for non-productive viral infection, here we report a previously unrecognised role of type I IFN responsiveness for isotype switching towards VLPs (Fig. 3-20C, lower panel). Unlike for the induction of IgM titres obtained after vaccination with CCG,[149;191] type I IFN stimulation was not required for the induction of the early IgM response to the retroparticles (Fig. 3-20C, upper panel). Hence, for IgM production, the immunogenity of VSV-G MLV was functionally redundant with the adjuvant activity of type I IFN (Bach, Kamphuis et al.[194]). It remains elusive whether the VLP immunogenity relates to increased stimulation of APCs or to local induction of other cytokines, which might act either directly or indirectly on B cells or other immune cells.

Most conspicuously, however, the IFNAR dependence of the anti-VSV-G MLV antibody response clearly indicated an endogenous type I IFN induction. Since VLPs were described to be generally devoid of any viral nucleic acid,[193] it will be of particular interest to determine whether some randomly included cellular mRNAs or other components might trigger host innate receptors. The mild lymphopenia and concomitant CD69 upregulation observed in blood of VLP-vaccinated animals (Fig. 3-20A,B) suggested the stimulation with locally effective quantities of type I IFN.

On systemic level, however, type I IFN titres did not reach critical amounts to induce massive lymphopenia. Notwithstanding, the mild reduction of IFNAR$^{-/-}$ lymphocyte numbers hints at the induction of other cytokines, too.

It was remarkable that the exogenous administration of IFN-α could not further increase antibody levels (Fig. 3-20C, upper panel, preliminary data and data not shown), suggesting that locally available quantities were already saturating for the type I IFN-dependent stimulation of immune cells. Nevertheless, the anti-VLP antibody response was still relatively low as compared to strong anti-viral immunity, which might be attributed to the s.c. application route and the lack of further activation by other mechanisms.

In line with our recent collaborative findings upon vaccination with CGG,[191] the direct type I IFN stimulation of B cells seemed to be necessary for the IgG switch in response to VSV-G MLV (Fig. 3-20C, lower panel). Since Ig titres were reduced in CD19-Cre$^{+/-}$IFNAR$^{flox/flox}$ mice, but not completely absent as in IFNAR$^{-/-}$ mice, the data further indicate that type I IFN responsiveness was required on other cells than B cells, as well (Bach, Kamphuis et al.[194]). Among these, DCs and T lymphocytes are likely candidates since they are critically involved in type I IFN-mediated enhancement of anti-CGG responses.[149;191]

4.6.3 How could type I IFN induce isotype switching to virus-like particles?

Isotype switching occurs within the unique microenvironment of germinal centres (GCs) that form within follicles of SLOs.[92;220] GCs provide networks of follicular dendritic cells (FDCs), which enable the T cell-driven B cell differentiation into plasma cells. Undergoing rapid expansion, B cells switch their isotype and undergo affinity maturation by somatic hypermutation. Throughout the GC reaction, B cells remain in close contact and keep activated by antigen-presenting FDCs and T cells via costimulatory molecules and cytokines.

Possible mechanisms, by which type I IFN induces isotype switching, could affect B cell-priming and the GC reaction through upregulation of the costimulatory molecules CD40-CD40L, OX40-OX40L, CD80 or CD86-CD28 on B and T lymphocytes, respectively.[221-223] Alternatively, the immunological synapse-formation could further be improved by stronger interactions between LFA-1 and ICAM-1 (Kamphuis et al.[190]). Furthermore, type I IFN stimulation induces the recruitment of lymphocytes into B and T cell zones (performed in collaboration with Tobias Junt, in: Kamphuis et al.[190]) and could thus enhance the isotype switching.

Interestingly, IgG production to viral glycoproteins was observed in absence of T cell CD40 ligand (CD40L) stimulation, pointing towards a crucial role of other stimulatory molecules.[218;224] On DCs, it was shown that type I IFN increases the expression of "B lymphocyte stimulator protein" (BlyS, also called BAFF) and of APRIL,[93] "a proliferation-inducing ligand", which are critical for Ig class switching in absence of CD40L stimulation.[94] Possibly, type I IFN could also increase the expression of the receptors for BlyS and APRIL on the B cell level.[225] Numerous studies furthermore demonstrated that type I IFN stimulation promotes DC maturation, as characterised by upregulation of costimulatory and adhesion molecules, which allows for potent antigen presentation.[226-229]

Results stemming from *in vitro* investigations suggest that type I IFN acts directly on B cells to induce the differentiation into plasma blasts, whereas IL-6 is further needed for the development of plasma cells.[161] On naïve B cells, type I IFN leads to BCR internalisation and enhances proliferation as well as Ca^{2+} influx following limited BCR ligation.[158] Finally, type I IFN stimulation enhances B cell growth *in vitro*[163] and could hence rescue B cells from apoptosis *in vivo*, an effect which was recently identified on $CD8^+$ T cells as a crucial mechanism to promote the generation of cytotoxic T lymphocytes.[154]

We are currently investigating antibody responses in mice with single or combined tissue-specific IFNAR deletions in order to elucidate the complementary actions of type I IFN stimulation on different immune cell types. Using the more immunogen i.v. administration route, anti-VSV-G MLV titres turned out to be consistent within treatment groups and confirm the previously observed effects (data not shown, see: Bach, Kamphuis et al.[194]). Additionally, the vaccination with UV-inactivated VSV, live VSV and the VSV variant VSV-M2 will allow dissecting the mechanism of antibody production with respect to viral replication and type I IFN stimulation under limiting infectious dose conditions.

In this study, we analysed the effects of type I IFN stimulation of lymphocytes. However, *in vitro* studies suggested that type I IFN responsiveness of other non-immune tissues might be critical to survival, as well. Among these, the sites of primary viral replication including keratinocytes and gastrointestinal epithelia, or other organs, which usually do not get infected in immunocompetent mice, such as the central nervous system (CNS), will be of particular interest to study. Hence, breeding $IFNAR^{flox/flox}$ mice to mice, which express Cre in non-immune tissues, will provide novel tools to investigate the course of viral spreading and the role of type I IFN signalling in important target tissues for possible viral replication.

4.7 What is the clinical relevance of type I IFN stimulation?

4.7.1 Which adverse effects can be caused by type I IFN and lymphopenia?

Type I IFN can exert clinically relevant effects on the organism. These effects can be dissected into systemic and localised actions, whose impact depends on the short or long duration of the cytokine stimulation. In addition to alterations of the immune system, type I IFN treatment can cause metabolic (and even psychical) disturbances that affect the gastrointestinal, nerval, cardiovascular, motoric and reproductive system or the skin. Most frequently however, type I IFN treatment goes along with an influenza-like complex of symptoms, local reactions at the injection site and lymphopenia.[230]

As our results show, high systemic type I IFN levels directly stimulate lymphocytes and cause a prominent blood lymphopenia. Besides lymphocytes, type I IFN also targets natural killer cells, DCs, macrophages and granulocytes, leading to an overall stimulation of the innate and adaptive immune system in SLOs. Thus, a long-lasting presence of high systemic type I IFN levels can induce over-activation and have detrimental effects on the immune balance. Indeed, a continuous type I IFN stimulation has been identified as a crucial pathogenic mechanism to cause autoimmune diseases such as lupus erythematosus systemicus.[231;232] In line with this, the induction of lupus-like diseases represents a serious complication of chronic type I IFN treatment in humans. In dogs and cats, however, autoimmune disorders have not been observed so far[112] (Virbagen Omega® package insert), which might be related to the shorter treatment and lifespan of the diseased animals or to less clinical experience with feline IFN-ω.

In order to prevent a harmful over-activation, cellular inhibitory mechanisms set on early during viral infections to downregulate the massive type I IFN production. Consequently, blood lymphocyte numbers normalise again. Therefore, the naturally occurring lymphopenia is a short-lasting effect, which itself usually does not compromise immunity. There are only two reports on reduced immune reactions: In case of contact hypersensitivity, lymphopenia was shown to induce transient immune incompetence;[183] since in this model, the immigration of T cells into antigen-containing peripheral tissues has to occur within a narrow time window in order to elicit the localised ear swelling of infiltrating $CD4^+$ and $CD8^+$ T cells. Secondly, following intracerebral inoculation of vaccinia virus, the reiterated administration of poly(I:C) decreased T cell involvement in viral meningitis.[233]

However, clinically relevant immune suppression with opportunistic infections may rather be a consequence of sustained lymphopenia induced by prolonged exposure to type I IFN, as reported in IFN-α/β-treated human patients. Thus, immune-suppressive systemic effects of type I IFN may appear in blood together with an autoaggressive over-stimulation of immune cells in peripheral organs. However, in viral infections such as equine influenza, the frequently diagnosed bacterial super-infections may not primarily be dependent on the immune suppression caused by short-lasting lymphopenia, but on viral immune evasion.

In contrast to the aforementioned examples, immune suppression caused by long-lasting lymphopenia can also be a desired effect. Following transplantation, the systemic treatment with FTY720 reduces infiltration of lymphocytes into the graft. Thus, FTY720 defers rejection, but in contrast to classical immune suppressants, it still allows the generation of immune responses in SLOs.

Local type I IFN stimulation seems to be critical for the immune response to cancer. For years, type I IFN has been approved for treatment of several neoplasias without understanding the mechanisms involved. In the meanwhile, various experimental mouse models have provided growing evidence that local stimulation of both immune and stromal cells can be essential for controlling or rejecting established or expanding tumors.[234] Among other mechanisms of action, type I IFN stimulation can skew the immune response towards a Th1 phenotype[235] and control tumor-supplying neovascularisation.[236]

4.7.2 Does type I IFN influence the vaccination efficiency?

Systemic and peripheral VSV infection experiments showed that type I IFN stimulation of B cells is not critically required to induce protective antiviral immunoglobulin responses. In peripheral vaccination with non-replicative VLPs, however, the type I IFN stimulation of immune cells, including B cells, played an essential role in inducing IgG titres. Finally, challenge with mere protein did not elicit any humoral response in absence of type I IFN stimulation. Thus, peripheral effects of type I IFN can be relevant to vaccination protocol design. For example, the efficacy of attenuated live PPRSV vaccines was tried to augment by induction of IFN-α; however, viremia and antibody titres remained unaffected, suggesting that the effects of type I IFN were compensated by stimulation through PAMPs or by viral replication.[237] On the other hand, the endogenously induced type I IFN was shown to be responsible for the activity of important adjuvants such as TLR agonists and complete Freund´s adjuvant.[146;149]

Taken together, the efficacy of the generally poor immunogen subunit vaccines, which are composed of only few pathogen-derived proteins, may well be enhanced by administrating the vaccines together with natural or long-lived pegylated interferons[156;238] or by enhancing the endogenous type I IFN production. This can be accomplished by adding type I IFN-inducing adjuvants to the vaccine.[146;149] In veterinary surgery, the concomitant administration of *parapox ovis* virus-based paramunity inducers, i.e. unspecific stimulators of immunity such as Baypamun®/Zylexis®, can furthermore elicit type I IFN production. The current approval of type I IFN for therapy in animals and humans would hereby facilitate the design and feasibility of field studies and large scale clinical trials with different vaccines. However, depending on the costs of recombinantly produced type I IFN and the current market value when applied in immunotherapy, type I IFN may possibly not have the economic potential for wide-spread use in immune prophylaxis.

Therefore, the generation of virus-like particles, which contain one or several components of the current subunit vaccines and which elicit the production of a spectrum of cytokines, could provide a promising tool to improve vaccination efficacy. This is of particular interest since in the last years a change occurred in political and social opinion on epizootics control measures, which revalued and generally favoured the use of vaccines for both prophylaxis and prevention of further disease spreading.

Nevertheless, the growing demand for efficient vaccines is not restricted to the currently emerging and recurrent communicable and notifiable diseases such as influenza, classical swine fever and foot-and-mouth-disease. It does also apply to viral diseases such as equine herpesvirus infections, against which only poor immunity can be induced so far.[239] Since a recombinant VLP-based vaccine would contain only part of the antigens of a specific pathogen, it could serve as a negative marker vaccine, allowing distinguishing vaccinated animals from naturally infected counterparts by their induced antibody spectrum. This applicability as a marker vaccine represents an important prerequisite for a possible use in epizootics control though it does not eliminate the residual risk of viral persistence in the vaccinated animal. Finally, the zoonotic potential and the unpredictable evolution of the highly pathogenic avian influenza virus H5N1 is only one current example for a possible need of large quantities of vaccines. To satisfy such a large scale demand, highly efficient vaccines will be needed which induce reliable immunity with relatively little antigen content.

Therefore, our data suggest that recombinant virus-like particles expressing influenza antigens would be better immunogens than the currently produced subunit vaccines. It will be of particular interest to investigate whether the required amount of antigen per dose can be reduced and whether such recombinant vaccines can be rendered further efficacious by addition of adjuvants.

Certainly, for a possible use as licenced vaccines, any recombinant influenza antigen-expressing VLPs first would have to fulfil the requirements of efficacy and safety. In this regard, since 11 equine[240] and 17 human[241] influenza vaccines based on other technologies are already licenced, pharmaceutical companies would only launch a new production if the VLP vaccination results were outstanding. In a cost-intensive priority trial to compare with existing vaccines, VLPs would have to prove protection of all patient age groups and to efficiently reduce viral shedding of vaccinated individuals after infection with influenza. With respect to safety, special inactivation steps would have to be adopted to exclude any possible contamination with intrinsic viruses of the production cell line. Furthermore, the potential oncogenic risk of inserting cell line-derived DNA into the patient´s genome would have to be assessed (Michael Pfleiderer, PEI, personal communication). Considering the current market situation, VLPs first require further research before a possible commercial application could be approached.

In conclusion, our data show that type I IFN causes lymphopenia through direct stimulation of B and T cells. We propose a molecular mechanism of lymphopenia that is largely independent of G protein-coupled receptors, while adding further hints to a possible adhesive function of CD69 on lymphocytes. The data presented here contribute to elucidate the potential use of type I IFN as a natural vaccine adjuvant. Our results extend the concept of lymphopenia and provide new insights into how type I IFN essentially links the innate and adaptive immune system in naturally occurring infections and clinical treatments.

5 Abstract

5.1 Summary

Early viral infection elicits potent type I IFN responses and is often associated with lymphopenia, a transient reduction of blood lymphocyte counts, long before the onset of humoral and cellular immunity. We have investigated the direct effect of type I IFN stimulation of lymphocytes on early lymphopenia and on the generation of antibody responses at later stages of infection.

Lymphopenia induced by infection with vesicular stomatitis virus (VSV) or treatment with the Toll-like receptor agonists poly(I:C) and R-848 was critically dependent on type I interferon receptor (IFNAR)-signalling. Using bone marrow-chimeric mice, radio-resistant cells, such as stroma and endothelium, could be excluded as type I interferon targets for the induction of lymphopenia. Instead, adoptive transfer experiments and studies in conditionally gene-targeted mice with a B or T cell-specific IFNAR deletion demonstrated that type I IFN exerted a direct effect on lymphocytes that was necessary and largely sufficient to induce lymphopenia. The investigation of the molecular mechanism revealed that lymphopenia was mainly independent of G protein-coupled receptors (GPCRs) and chemokines. Homing studies performed by FACS and laser scan microscopy showed that B cells, but not T cells, partially accumulated in spleen, but not in other organs.

Furthermore, we found that neutralising antibody responses following intravenous or intranasal infection with live VSV were not affected by type I IFN responsiveness of B cells. However, vaccination with recombinant virus-like particles (VLPs) containing the glycoprotein of VSV (VSV-G) induced IFNAR-independent IgM responses, whereas the immunoglobulin (Ig) switch to IgG was fully dependent on type I IFN responsiveness and partially dependent on the IFNAR-signalling in B cells. Thus, live VSV expressing repetitive VSV-G determinants in a highly ordered paracrystalline manner did not require type I IFN stimulation on B cells to induce neutralising IgM and IgG responses. In contrast, replication-deficient VLPs displaying VSV-G in a well-organised fashion induced IFNAR-independent IgM, whereas the switch to IgG was IFNAR-dependent.

In conclusion, these observations identify new effects of type I IFN stimulation of lymphocytes that profoundly affect lymphocyte redistribution and promote Ig switch towards less immunogenic antigens such as VLPs.

5.2 Zusammenfassung

Die Frühphase von Virusinfektionen ist gekennzeichnet durch starke Typ I Interferon (Typ I IFN)-Antworten und geht häufig vor Einsetzen der humoralen und zellulären Immunität mit einer transienten Verringerung der Lymphozytenzahlen im Blut, einer sogenannten Lymphopenie, einher. Gegenstand der vorliegenden Dissertation ist die Untersuchung des direkten Effekts, den Typ I IFN-Stimulation von Lymphozyten auf die frühe Lymphopenie sowie auf die Entstehung von Antikörperantworten in späteren Infektionsstadien ausübt.

Nach Infektion mit dem Virus der Stomatitis vesicularis (VSV) oder Behandlung mit poly(I:C) und R-848, zwei Agonisten für Toll-ähnliche Rezeptoren, war die Lymphopenie von Signalvermittlung durch den Typ I IFN-Rezeptor (IFNAR) abhängig. Mit Hilfe knochenmarksrekonstituierter Mäuse konnten strahlungsresistente Zellen wie Stroma und Endothel als Zielgewebe für Typ I IFN-Stimulation zur Auslösung von Lymphopenie ausgeschlossen werden. Anstelle dessen zeigten Experimente mit adoptivem Transfer sowie die Untersuchung gewebespezifisch genveränderter Mäuse mit B- oder T-Zell-spezifischer Ausschaltung des IFNAR, dass Typ I IFN einen direkten Effekt auf Lymphozyten ausübt, der zur Induktion von Lymphopenie notwendig und auch größtenteils ausreichend war. Die Untersuchung des molekularen Mechanismus ergab, dass Lymphopenie hauptsächlich unbeeinflußt von G-Protein-gekoppelten Rezeptoren (GPCR) und Chemokinen abläuft. Weiterhin zeigte sich, dass B-Zellen, nicht aber T-Zellen, partiell in der Milz, nicht aber in anderen Organen akkumulierten.

Nach intravenöser und intranasaler Infektion mit VSV waren neutralisierende Antikörperantworten nicht von Typ I IFN-Stimulation der B-Zellen abhängig. Hingegen induzierte die Impfung mit rekombinanten virusähnlichen Partikeln (VLPs), die das VSV-Glykoprotein (VSV-G) enthalten, IFNAR-unabhängige IgM-Antworten, wohingegen der Immunglobulin (Ig)-Klassenwechsel zu IgG eine vollständige Abhängigkeit von Typ I IFN-Wirkungen zeigte, die partiell auf Typ I IFN-Stimulation von B-Zellen beruhten. Insofern benötigte VSV-Lebendvirus mit repetitiven VSV-G-Determinanten in hochstrukturierter parakristalliner Form keine Typ I IFN-Stimulation von B-Zellen zur Induktion neutralisierender IgM- und IgG-Antworten. Im Gegensatz dazu riefen replikationsdefiziente VLPs mit regelmäßig angeordnetem VSV-G eine IFNAR-unabhängige IgM-Produktion hervor, während der Wechsel zu IgG IFNAR-abhängig war.

Zusammengenommen zeigen die Daten neuartige Wirkungen der Typ I IFN-Stimulation von Lymphozyten, welche die Lymphozytenverteilung tiefgreifend beeinflussen und den Ig-Klassenwechsel gegenüber weniger immunogenen Antigenen wie VLPs ermöglichen.

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Acknowledgements

The present dissertation was realised as an external PhD thesis in association with Prof. Dr. Heinz-Jürgen Thiel at the Institut für Virologie of the Justus-Liebig-Universität Gießen. The PhD was carried out at the Division of Immunology of the PAUL-EHRLICH-INSTITUT in Langen, Germany under the supervision of Dr. Ulrich Kalinke and Prof. Dr. Johannes Löwer. This work was supported by the European Community (contract QLK2-CT-2001-02103), Deutsche Forschungsgemeinschaft (SFB432, B15) and by Volkswagen Stiftung.

There are many people whom I would like to thank for their contribution to realise my PhD:

First of all, I am very thankful to Prof. Dr. H.-J. Thiel for supervising my external dissertation performed at the Paul-Ehrlich-Institut. You provided me an atmosphere of confidence to carry out my research in a field largely independent from your own focus. I appreciated very much that you supported me and were always interested to follow the progress of my studies and of the dissertation itself.

I am particularly grateful to Prof. Dr. J. Löwer for being my PhD supervisor and mentor at the PEI. Despite your definitively busy schedule, you always made it possible to keep in contact and discuss my studies regularly. Your questions helped me to reflect my work and to acquire a broader view of Immunology and Medicine. You were a good mentor, also with respect to my future plans!

My very special and profound thank goes to my immediate PhD supervisor Dr. Ulrich Kalinke, who not only offered me an excellent research topic to investigate a novel conditional mouse model, which perfectly fitted to my Veterinary expertise and interests, but who also taught me effective working and organisational skills as well as a really good presentation style. In our atmosphere of mutual reliability, I could progressively grow into the responsibilities of research and writing scientific papers. I will always remember our exciting discussions, spontaneous last-minute (!) attendances of university lectures, cloister-meetings in Walberberg and Maastricht and our fascinating EU project partner meetings in Erlangen, Langen, Oslo and Milan… (*indimenticabile!*) Last, but not least, *mille grazie* for your help with my applications and for your continuous motivation!

Certainly, I thank the whole lab team of the Division of Immunology at the Paul-Ehrlich-Institut without whom my PhD could have not been realised; especially Patricia Bach, for our collaboration in the VLP vaccination studies, blood sampling and plenty of funny moments; Dorothea Kreuz, Jana Überschär and Susanne Fricke for optimal mouse breeding and screening; Sven Flindt, for your idea of the counting beads; Susanne Röderstein, for our collaboration in generating and monitoring BM-chimeric mice, for many enthusiastic discussions and your creativity in Photoshop…; Claudia Detje, for your co-operation in Southern blot screening and your opera-solos; Zoe Waibler, for measuring serum IFN-α levels and Sabine Falk, Linda Sender, Cathy Srokowsky, Lydia Wald, Mara Perkic, Thomas Hinz and Martin König, for helping in many moments! When I was exhausted, you helped me to laugh and recover; my special thanks go to the "Doktorandenzimmer"!

I am also very grateful to Wilfried Dreher and Klaus Boller for assisting me in LSM, EM and Corel Draw and to Christian Buchholz for providing the VSV-G displaying vector!
I furthermore thank our EU project partners Agnes Le Bon and David Tough for providing IFN-α and Sanjiv Luther for having sent the CCL19-Fc fusion protein.
Jana Mehlhase and Marco Pietrella, for your funny company at late hours of research, many moments of sharing, … e per essere andati a mangiare un gelato qualche volte…!

Vicky Fachinger – I think, you saw me holding a pipette for nearly the first time, then in Tübingen… You always encouraged me in my interest in Immunology – swine, mice, humans, whatsoever!!! I am happy and thankful that now "your little trainee" has handed in her PhD thesis and that you critically red my manuscript!

My special thanks go to James Buchanan for his excellent native speaker language corrections of my international application! Last, but not least, thanks to Peter Münch for kindly offering help to format the dissertation – it´s really amazing where all those trouble arise from…!

Sudhir for everything.

Judith, Clemens, Angel and Pater Heinrich Watzka for always being available in London, Madrid, Nürnberg or wherever when I needed help, support, assistance, motivation, orientation or understanding…

My parents for their continuous and reliable support at any hour any day of the year… for cooking and baking tons of cakes and meals, driving, embassy visits, phone calls and prayers…

Vielen Dank!

Thank you!

Maltiox!

Mille grazie!

¡Muchas gracias!

édition scientifique
VVB LAUFERSWEILER VERLAG

VVB LAUFERSWEILER VERLAG
STAUFENBERGRING 15
D-35396 GIESSEN

Tel: 0641-5599888 Fax: -5599890
redaktion@doktorverlag.de
www.doktorverlag.de

ISBN 3-8359-5178-5

9 783835 951785