Mass spectrometric identification of Varicella-Zoster Virus (VZV) proteins recognized by human T cells

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1 Abstract

Primary varicella-zoster virus (VZV) infection during childhood leads to varicella commonly known as chickenpox. After primary infection has occurred VZV establishes latency in the host. During subsequent lifetime the virus can cause reactivated infection clinically known as herpes zoster or shingles. In immunodeficient patients' dissemination of the virus can lead to life-threatening disease. Withdrawal of acyclovir drug prophylaxis puts allogeneic hematopoietic stem-cell transplantation (HSCT) patients at increased risk for herpes zoster as long as VZV-specific cellular immunity is impaired. Although an efficient live attenuated VZV vaccine for zoster prophylaxis exists, it is not approved in immunocompromised patients due to safety reasons. Knowledge of immunogenic VZV proteins would allow designing a noninfectious nonhazardous subunit vaccine suitable for patients with immunodeficiencies. The objective of this study was to identify T cell defined virus proteins of a VZV-infected Vero cell extract that we have recently described as a reliable antigen format for interferon-gamma (IFN-γ) enzyme-linked immunosorbent spot (ELISpot) assays (Distler et al. 2008). We first separated the VZV-infected/-uninfected Vero cell extracts by size filtration and reverse-phase high performance liquid chromatography (RP-HPLC). The collected fractions were screened for VZV reactivity with peripheral blood mononuclear cells (PBMCs) of VZV-seropositive healthy individuals in the sensitive IFN-γ ELISpot assay. Using this strategy, we successfully identified bioactive fractions that contained immunogenic VZV material. VZV immune reactivity was mediated by $CD4^+$ memory T lymphocytes (T cells) of VZV-seropositive healthy individuals as demonstrated in experiments with HLA blockade antibodies and T cell subpopulations already published by Distler et al. We next analyzed the bioactive fractions with electrospray ionization mass spectrometry (ESI-MS) techniques and identified the sequences of three VZV-derived proteins: glycoprotein E (gE); glycoprotein B (gB), and immediate early protein 62 (IE62). Complementary DNA of these identified proteins was used to generate *in vitro* transcribed RNA for effective expression in PBMCs by electroporation. We thereby established a reliable and convenient IFN-γ ELISPOT approach to screen PBMCs of healthy donors and HSCT patients for T cell reactivity to single full-length VZV proteins. Application in 10 VZV-seropositive healthy donors demonstrated much stronger recognition of glycoproteins gE and gB compared to IE62. In addition, monitoring experiments with *ex vivo* PBMCs of 3 allo-HSCT patients detected strongly increased $CD4^+$ T cell responses to gE and gB for several weeks to months after zoster onset, while IE62 reactivity remained moderate. Overall our results show for the first time that VZV glycoproteins gE and gB are major targets of the post-transplant anti-zoster $CD4^+$ T cell response. The screening approach introduced herein may help to select VZV proteins recognized by memory $CD4^+$ T cells for inclusion in a subunit vaccine, which can be safely used for zoster prophylaxis in immunocompromised HSCT patients.

2 Immunological background

2.1 The Immune System

The human immune system can be defined as an overall coordination of the biological mechanisms involved in the integrity and protection of the host from malignancy and infectious agents such as viruses. The human immune system needs to distinguish pathogens and malignant tissue from the organism own healthy cells and tissue in order to function properly. The typical human immune system consists of many types of proteins, cells, organs, and tissues that interact in an elaborate and dynamic network. The human immune system can be divided into two major parts, termed innate and adaptive (acquired, specific) immune system in which the latter creates immunological memory. The innate immune response is very rapid, but sometimes leads to tissue damage because of its missing specificity, whereas the adaptive immune response is very specific but needs days or sometimes weeks to develop. Immunological memory, created from a primary response to a specific pathogen, provides an enhanced response to secondary encounters with that same, specific pathogen. Combining both immune responses leads to an efficient elimination of pathogens, like viruses. Disorders in the immune system can result in disease, including autoimmune diseases, inflammatory diseases and cancer (Delves and Roitt 2000; O'Byrne and Dalgleish 2001; Parkin and Cohen 2001).

2.1.1 Innate Immunity

The front line of the host response to pathogens is made of internal and external epithelia, which provides a physical barrier between the internal milieu and the external world. The importance of the physical barrier in protection is obvious when the barrier is breached caused by wounds, burns and loss of the integrity of the body's epithelia. Additionally, most epithelial surfaces are associated with a normal flora of nonpathogenic bacteria that compete with pathogenic microorganisms and some also produce antimicrobial substances (Risso 2000). After entering tissue many pathogens are recognized by the mononuclear phagocytes, or macrophages, that reside in these tissues. In addition circulating neutrophils can recognize, ingest and destroy many pathogens as well. Both cell types together respond to pathogens in a generic way, but unlike the adaptive immune system, it does not confer long-lasting or protective immunity to the host. Another very important effect of activated macrophages is the release of cytokines, chemokines, and other important molecules that set up a state of inflammation activating the acute phase response in the tissue and bring effector cells of the innate immune system and plasma proteins to site of infection (Svanborg et al. 1999). In addition, viral pathogens are recognized by the cells in which they replicate leading to the production of the antiviral effector molecules called interferon-alpha (IFN-α) and interferon-beta (IFN-β) that serve to inhibit viral replication and to activate natural killer (NK) cells,

which in turn can distinguish infected from uninfected cells. Activated NK cells serve to contain virus infection while the adaptive immune response is generating antigen-specific cytotoxic T lymphocytes (CTLs) that can clear the infection. Patients deficient in NK cells prove to be highly susceptible to early phases of herpes infection (Janeway 2005). Monocytes/macrophages, dendritic cells (DCs), NK cells, and NKT cells recognize invaded pathogens by their cell surface receptors that can discriminate between “self” and “non-self”. These effector cells recognize constitutive and conserved products of microbial metabolism known as pathogen-associated molecular patterns (PAMPs), e.g., viral proteins, lipopolysaccharides (LPS), pedtidoglycan (PGN), unmethylated CpG DNA, or double-stranded viral RNA, via a variety of so-called pattern recognition receptors (PRRs), which include toll-like receptors (TLRs), scavenger receptors, NK cell receptors, killer cell immunoglobulin-like receptors (KIRs) and mannose-binding receptors (Gough and Gordon 2000; Apostolopoulos and McKenzie 2001; Heine and Lien 2003). Ligation of many of these cell surface receptors by pathogens leads to phagocytosis of the pathogen followed by its death inside the phagocytes. Furthermore, phagocytes produce a variety of other toxic compounds, like nitric oxide (NO), the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) that help to kill the engulfed microorganisms. Additionally to the cellular innate immune response plasma proteins accumulate at the site of infection, including the complement components provide circulating or humoral innate immunity. Cytokines, chemokines, phagocytic cells, and NK cells are also involved in the adaptive immune response, which uses variable receptors to target specific pathogen antigens. Finally, complement is a system of more than 30 proteins in the plasma and on cell surfaces, constituting more than 15% of the globular fraction of plasma (Walport 2001). Complement proteins are organized into a hierarchy of proteolytic cascades that start with the identification of pathogenic surfaces and lead to the generation of potent pro-inflammatory mediators (e.g., anaphylatoxins), opsonization (coating) of the pathogenic surface through various complement components (e.g., C3b), and targeted lysis of the pathogenic surface through the assembly of membrane-penetrating pores known as the membrane attack complex (MAC).

2.1.2 Adaptive immunity

Innate immunity, by itself, may not be sufficient to protect a host against an invading pathogen or to prevent disease from occurring. The innate immune system is not specific in its response and reacts equally well to a variety of organisms, whereas the adaptive immune system is antigen-specific and reacts only with the organism that induced the response. In addition the adaptive immune system exhibits immunological memory. It "remembers" that it has encountered an invading organism (antigen) and reacts more rapidly on subsequent exposure to the same organism. The adaptive defense consists of antibodies and lymphocytes, often called the humoral response and the cell-mediated response. The adaptive immune

system is comprised of B and T cells that express receptors with remarkable diversity tailored to recognize aspects of particular pathogens, or antigens. B cells, which are derived from the bone marrow, become the cells that produce antibodies. T cells, which mature in the thymus, differentiate into cells that either participate in lymphocyte maturation, or kill virus-infected cells. Both humoral and cell-mediated responses are essential for antiviral defense. The contribution of each varies, depending on the virus and the host. Antibodies generally bind to virus particles in the blood and at mucosal surfaces, thereby blocking the spread of infection. Binding of antibody inactivates viruses and microbial toxins (such as tetanus toxin or diphtheria toxin) by blocking their ability to bind to receptors on host cells. Antibody binding also marks invading pathogens for destruction, mainly by making it easier for phagocytic cells of the innate immune system to ingest them. In contrast, T cells recognize and kill infected cells. The first adaptive response against a virus – called the primary response – often takes days to mature. In contrast, a memory response develops within hours of infection. Memory is maintained by a subset of B and T cells called memory cells which survive for years in the body. Memory cells remain ready to respond rapidly and efficiently to a subsequent encounter with a pathogen. This so-called secondary response is often stronger than the primary response to infection. Consequently, childhood infections protect adults, and immunity conferred by vaccination can last for years. Vaccines protect us against infection because of immune memory.

B and T cells express on their surface, receptor proteins known as B cell receptor (BCR) or T cell receptor (TCR) which determine antigenic specificity. Each B or T cell expresses a unique BCR or TCR (a heterodimer of two proteins, either $\alpha\beta$ or $\gamma\delta$); the total number of molecules that they can recognize is very large as the numbers of different combinations possible are large. This concept that each lymphocyte expresses a unique cell surface receptor for antigen recognition is important as it allows only selected cells containing the appropriate receptors to proliferate and mount responses during an immune reaction. The formation of antigen-specific B and T cell receptor occurs early in the development and is accomplished for both lymphocyte types by a similar mechanism of random rearrangement and splicing together of multiple DNA segments, coding for the antigen-binding region of the receptor (complementarity-determining regions, CDRs). This recombination of so called variable (V), diversity (D), joining (J) and constant (C) regions of coding genes for B and T cell receptors are performed by enzymes collectively called VDJ recombinase. Clonal diversity, leads to a repertoire of over 10^8 T cell receptors and 10^{10} antibody specifities. The repertoire of B cell receptors is even more increased by somatic hypermutation.

B and T cell receptors recognize antigens by completely different mechanisms (Grawunder et al. 1998). The TCR binds linear peptides out of 8 to 10 amino acids (Major histocompatibility complex (MHC) class I) or at least 13 amino acids long (MHC class II) that have

been produced by intracellular processing of antigen-presenting cells (APCs), whereas antibodies recognize the conformational structure of an epitope without the need of processing.

2.1.2.1 Antigen presentation and processing

Antigen processing and presentation are processes that occur within a cell that result in fragmentation (proteolysis) of proteins, association of the fragments with MHC molecules, and expression of the peptide-MHC molecules at the cell surface where they can be recognized by the T cell receptor on a T cell. Exogenous antigens, derived from extracellular pathogens are usually displayed on MHC class II molecules, which activate $CD4^+$ helper T cells (Th), whereas endogenous antigens, such as viral or tumor proteins are typically displayed on MHC class I molecules, and activate $CD8^+$ cytotoxic T cells.

MHC class I pathway. All nucleated cells express class I MHC. Endogenous proteins, e.g. viral, tumor, bacterial, or defective cellular proteins are fragmented in the cytosol by proteasomes (a complex of proteins having proteolytic activity) or by other proteases. The resulting fragments are then transported across the membrane of the endoplasmic reticulum (ER) by transporter proteins named the transporter in antigen processing (TAP). The N-terminus is further trimmed by aminopeptidases within the ER to a peptide length of eight to ten amino acid residues. Parallel the synthesis and assembly of MHC class I heavy chain and β_2 microglobulin occurs in the endoplasmic reticulum. Trimmed peptides are then loaded onto MHC class I molecules by the peptide loading complex. After dissociation from the peptide loading complex, MHC class I peptide complex is transported through the Golgi apparatus to the cell surface (Janeway 2005).

MHC class II pathway. Whereas all nucleated cells express class I MHC, only a limited group of cells express class II MHC, which includes macrophages, DCs and B cells, named professional APCs. The expression of class II MHC molecules is either constitutive or inducible, especially by IFN-γ in the case of macrophages. Exogenous proteins taken in by endocytosis or in the case of B cells by internalization of surface immunoglobulin-bound proteins are fragmented by proteases in an endosome, which subsequently fuse with MHC class II-containing acidic lysosomes, leading to protein degradation. The α and β chains of MHC class II, along with an invariant chain, are synthesized, assembled in the endoplasmic reticulum, and transported through the Golgi and trans-Golgi apparatus to reach the endosome, where the invariant chain is digested, and the peptide fragments from the exogenous protein are able to associate with the class II MHC molecules, which are finally transported to the cell surface (Janeway 2005).

2.1.2.2 T lymphocyte activation via APCs

The three main types of APCs are DCs, macrophages and B cells, although other cells, that express class II MHC molecules, (e.g., thymic epithelial cells) can act as APCs in some cases. DCs, which are found in skin and other tissues, ingest antigens by pinocytosis and migrate, via chemotactic signals, to the T cell enriched lymph nodes and spleen. During migration, DCs undergo a process of maturation in which they lose most of their ability to engulf other pathogens and develop an ability to communicate with T cells. In the lymph nodes and spleen they are found predominantly in the T cell areas. DCs are the most effective APCs and can present antigens to naïve T cells. Furthermore, they can present internalized antigens in association with either class I or class II MHC molecules (cross presentation), although the predominant pathway for internalized antigen is the class II pathway.

Naïve lymphocytes that have not previously encountered antigen migrate to peripheral lymphoid organs (lymph nodes), the sites where immune responses start. The primary immune response leads to the clonal expansion of antigen-specific T cells, generating both effector and memory T cells. Effector T cells are activated lymphocytes capable of performing the functions required to eliminate pathogens. Memory T cells are long-lived, functionally silent cells, which mount rapid responses to antigen challenge, so called recall or secondary responses (Delves and Roitt 2000; Parkin and Cohen 2001). At least two types of memory cells have been defined in humans based on their functional and migratory properties. T central-memory ($T_{(CM)}$) cells are found predominantly in lymphoid organs and cannot be immediately activated, whereas T effector-memory ($T_{(EM)}$) cells are found predominantly in peripheral tissue and sites of inflammation and exhibit rapid effector function (Klebanoff et al. 2006). The current model of T cell activation requires two signals. The first signal is specific, requiring T cell receptor recognition and binding to MHC/Antigen presented by an antigen-presenting cell (Chapter 2.1.2.1). The second signal termed co-stimulus is nonspecific, resulting from the binding of B7 ligand on the APC with its receptor, cluster of differentiation 28 (CD28), on the T cell. If both signals are provided, the T cell will proliferate and secrete cytokines. Cytotoxic T lymphocyte antigen 4 (CTLA4, CD152), another receptor for B7 ligand that is up-regulated following T cell activation, can deliver an inhibitory signal, down-regulating T cell proliferation. The B7 family of ligands has mainly two family members, B7.1 (CD80) and B7.2 (CD86). They both bind to CD28 and CTLA4, but they differ in their binding affinity, structure, and temporal expression. Activated, effector and memory T cells provide B cell help in the lymph nodes and traffic to sites of infection where they secrete anti-microbial cytokines and kill infected cells (Carreno and Collins 2002; Greenwald et al. 2005).

2.1.2.3 Effector mechanisms of T cells

Two major types of effector T cells are classified regarding to their co-receptors, known as $CD4^+$ T cells, the mainly cytokine secreting helper T cells, and $CD8^+$ T cells mainly CTLs. Naïve $CD4^+$ helper T cells (Th0) may differentiate into subsets of effector cells, Th1 and Th2 helper T cells, that produce distinct sets of cytokines and therefore show distinct effector functions. The Th1 response is characterized by the production of IFN-γ, which activates macrophages to kill intracellular bacteria, and induces B cells to produce opsonizing antibodies. Another important cytokine of Th1 cells is interleukin-2 (IL-2), which together with IFN-γ induces T cell proliferation of $CD8^+$ T cells and has an autocrine effect on $CD4^+$ T cells. The Th2 response is characterized by the release of IL-4, IL-5, IL-6, and IL-10 which results in the activation of B cells to make neutralizing (killing) antibodies (Janeway 2005). Generally, Th1 responses are more effective against intracellular pathogens (viruses and bacteria that are inside host cells), while Th2 responses are more effective against extracellular bacteria, parasites and toxins (Delves and Roitt 2000; Parkin and Cohen 2001). A third type of $CD4^+$ T cells can differentiate into Th17 functional cells due to TGF-β and IL-6 stimulation. These cells secrete IL-17, which also induces pro-inflammatory cytokines and tips the balance towards inflammatory immune responses (Bettelli et al. 2007). Finally, regulatory T cells (T_{regs}), limit and suppress the immune system, and may control aberrant immune responses to self-antigens; an important mechanism in controlling the development of autoimmune diseases (Schwartz 2005).

CTLs are a sub-group of T cells which induce the death of cells that are infected with viruses (and other pathogens), or are otherwise damaged or dysfunctional. CTLs release perforin and granulysin cytotoxins which form pores in the target cell's plasma membrane, allowing ions and water to flow into the infected cell, and causing it to burst or lysis. Furthermore CTLs release granzyme B, a serine protease that enters cells via pores to induce apoptosis. Another mechanism of CTL killing is mediated by binding of Fas ligand (FasL, CD95L) on the CTL to the Fas receptor (FasR, CD95), which is expressed on many target cell types and induces apoptosis of the target cell by activation of caspases. Cytokines produced by $CD8^+$ T cells upon antigen encounter include IFN-γ, tumor necrosis factor alpha (TNF-α) and lymphotoxin, which function to activate macrophages and induce inflammation. Memory $CD8^+$ T cells must be able to increase their numbers rapidly and exert potent effector functions when the antigen is re-encountered and they need the ability for self-renewal in order to persist for long periods, a state termed homeostatic proliferation. These properties are provided by $CD4^+$ T cells, which help directly by cytokine secretion, as well as indirectly by activating APCs and B cells via CD40L/CD40 stimulation, leading to an efficient priming of naïve $CD8^+$ T cells (Barry and Bleackley 2002).

2.2 Varicella-Zoster Virus (VZV)

VZV belongs to one of the major virus family known as *Herpesviridae*. This DNA virus family is subdivided into three subfamilies called *Alpha-, Beta-,* and *Gammaherpesvirinae*. The most prominent family members that are known to cause disease in humans are listed in Table 1. The family name is derived from the Greek word ερπειν (herpein), which means “to creep”, describing the development of the vesicular rashes referring to the latent, recurring infections recognized as herpes simplex and zoster. VZV is most closely related to herpes simplex virus (HSV) type I and type II, sharing much genome homology (Davison and Scott 1986). The VZV virion consists of DNA packaged in an icosahedral nucleocapsid that is surrounded by tegument proteins and enclosed in a lipid membrane envelope shown in Figure 1. The VZV virions are pleomorphic and 150-200 nm in diameter. Its lipid envelope encloses the nucleocapsid of 162 capsomers arranged in a hexagonal form. VZV is highly cell-associated making it difficult to raise sufficient quantities of cell free virus for molecular analysis. The linear, double stranded DNA genome consists of approximately 125 kilobase (kb) pairs and is thus the smallest of all herpesviruses studied (Mueller et al. 2008). Sequencing of the VZV Dumas strain reveals a genome of approximately 69 open reading frames (ORFs), (Davison and Scott 1986).

Alphaherpesvirus gene transcription is classified into three distinct kinetic groups. The first genes to be expressed are the immediate early (IE) genes, which regulate the transcription of early (E) and late (L) virus genes (Arvin 1996). VZV IE proteins with gene regulation activities include the products of open reading frames 4,10,61,62 and 63. These proteins can be detected 4-6 hours post-infection (Cohrs et al. 1996). The product of ORF 62, the IE62 protein, is the initial transactivating protein and the major tegument component (Eisfeld et al. 2006). The tegument proteins prepare the cell to produce virus and separate from the nucleocapsid during transportation of virus particles to the nucleus. IE transcripts are translated in the cytoplasm and the IE (regulatory) proteins are then transported back into the nucleus where they induce the early gene expression and downregulate further IE gene transcripts. The onset of early gene transcription precedes virus DNA replication. Viral replication and the involved proteins like the viral DNA polymerase, viral thymidine kinase, and protein kinases are important targets for drug design and immunotherapy (Chapter 2.5). The L genes encode the major virus structural components including viral glycoproteins and nucleocapsid proteins, which are important targets for the host immune system (Cohen 1996; Cohen et al. 1999; Mueller et al. 2008).

The known envelope glycoproteins (gB, gC, gE, gH, gI, gK, gL) correspond with those in HSV, with the exception that there is no equivalent of HSV gD. The VZV envelope glycoproteins mediate viral attachment and cell entry, and their expression on cell membranes promotes cell fusion, permitting cell-to-cell spread of the virus (Abendroth and Arvin 2001). The gE protein (ORF68) is most abundantly produced in VZV-infected cells; it is noncova-

lently linked to gI and has been shown to bind the Fc fragment of immunoglobulin G (IgG), (Berarducci et al. 2009). The cellular receptor for gE has been recently shown to be insulin-degrading enzyme (IDE), (Li et al. 2006). The gB and gE proteins are targets of neutralizing antibodies and probably play a role in virus entry (Haumont et al. 1997). Most of the glycoproteins are transported to the trans-Golgi network, where the post translational processing occurs before expression on the cell surface. During viral latency products of ORFs 4, 21, 29, 62 and 63 are produced and are present in the cytoplasm as well as the nucleus. The VZV gene 63 transcript is the most prevalent and abundant detected during latency (Kinchington et al. 1994; Cohen et al. 2005).

VZV is an exclusively human, highly neurotropic, highly temperature sensitive alphaherpesvirus, with inactivation occurring at 56° to 60°C and it is not infectious if the virion envelope is disrupted.

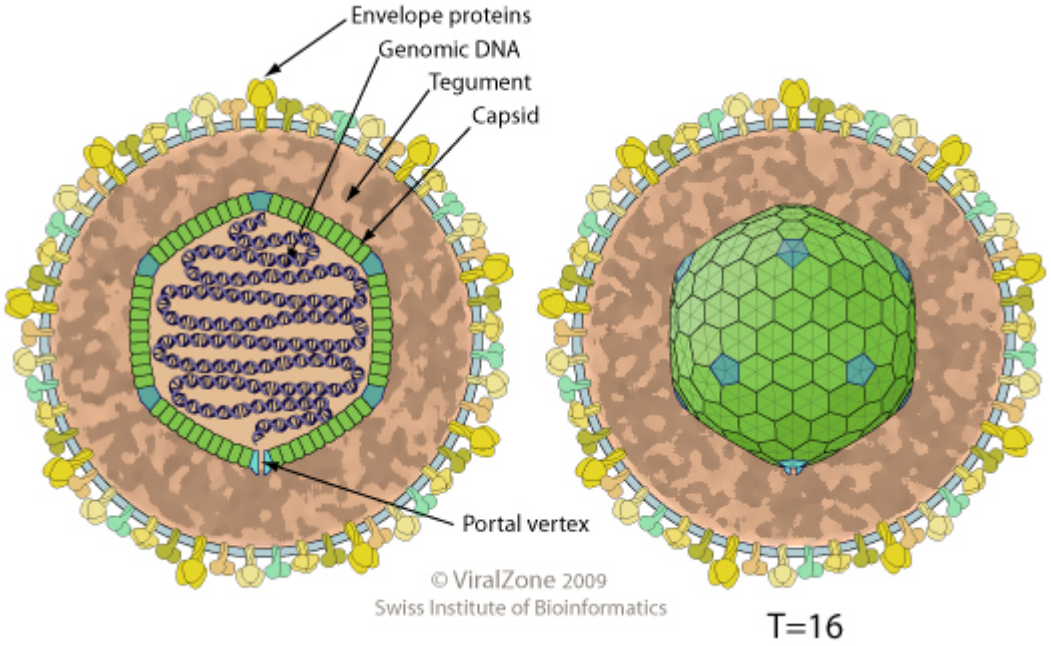


Figure 1: Varicella-zoster virus. The herpesvirus nucleocapsid contains one linear, double stranded DNA genome packaged in concentric spherical shells. The tegument resides between the nucleocapsid and the host-derived lipid envelope. Viral glycoproteins associated with the envelope build the virion surface. The nucleus shows the icosahedral (T=16) symmetry (adapted and modified from Swiss Institute of Bioinformatics).

Type	Synonym	Sub-family	Primary target cell	Pathophysiology	Site of Latency	Means of Spread
HHV-1	Herpes simplex virus-1 (HSV-1)	α	Mucoepithelial	Oral and/or genital herpes (predominantly orofacial)	Neuron	Close contact (sexual transmitted disease)
HHV-2	Herpes simplex virus-2 (HSV-2)	α	Mucoepithelial	Oral and/or genital herpes (predominantly genital)	Neuron	Close contact
HHV-3	Varicella zoster virus (VZV)	α	Mucoepithelial	Chickenpox and shingles	Neuron	Respiratory and close contact
HHV-4	Epstein-Barr virus (EBV)	γ	B cells and epithelial cells	Infectious mononucleosis, Burkitt's lymphoma, CNS lymphoma in AIDS patients, etc.	B cell	Close contact, transfusions, tissue transplant, and congenital
HHV-5	Cytomegalovirus (CMV)	β	Monocytes, lymphocytes, granulocytes and epithelial cells	Infectious mononucleosis-like syndrome, retinitis, etc.	Monocyte, granulocyte and lymphocyte	Saliva
HHV-6	Roseolovirus	β	T cells	Sixth disease (roseola infantum or exanthem subitum)	T cell	Respiratory and close contact
HHV-7	Roseolovirus	β	T cells	Sixth disease (roseola infantum or exanthem subitum)	T cell	?
HHV-8	Kaposi's sarcoma associated virus (KSHV)	γ	Lymphocytes and other cells	Kaposi's sarcoma, primary effusion lymphoma, some types of multicentric Castleman's disease	B cell	Close contact (sexual), saliva

Table 1: Human Herpesvirus classification (HHV). Adapted and modified from Ryan KJ; Ray CG; Sherris Medical Microbiology (4th edition)

2.3 Epidemiology, clinical manifestation and pathogenesis of VZV

2.3.1 Primary infection (Varicella, Chickenpox)

VZV is highly infectious, thus the transmission occurs by simply coughing or breathing due to respiratory droplets or airborne particles or by direct contact with skin lesions from infected individuals. Humans are the only natural host for the virus, although there is a report of a gorilla in captivity contracting varicella from a handler with lesions (Myers et al. 1987). Varicella consultations and hospitalization occur mainly in children under 15 with the highest rate in 0-to 4-year-olds (Guess et al. 1986). Primary infection in adults is usually more severe and may be accompanied by interstitial pneumonia (Choo et al. 1995). In most temperate climates, more than 90% of people are infected before adolescence in contrast to tropical climates where VZV infection occurs later in life and adults are more susceptible than children (Lolekha et al. 2001). Varicella has a peak incidence in the late winter and in the spring and epidemics tends to occur every 2 to 5 years (Paul and Thiel 1996; Wharton 1996).

In primary viremic phase the virus disseminates to regional lymph nodes, that permits the transport of the virus to the liver or other cells of the mononuclear phagocyte system. The secondary viremia is documented late in the incubation period and results in the transport of infectious virus to skin sites. Skin lesions appear on the trunk, face, scalp, and extremities, with the greatest concentration on the trunk (Ozaki et al. 1986; Koropchak et al. 1989). Cell associated viremia provides the virus access to epidermal cells, and replication in these cells causes the typically varicella rash. The average time from virus exposure to development of the rash is approximately 14 days (Preblud et al. 1984). Fever, malaise, headache, and loss of appetite are common during the secondary viremic phase of the illness just before and after the appearance of the rash. The traditional two-phase model of viremia in which initial viral replication in the respiratory lymph nodes seeds secondary sites of replication in the liver, spleen and other organs has now been challenged by one that describes T cell targeting of VZV to the skin, followed by innate and adaptive immune responses to the virus. The infected person becomes infectious roughly 48 hours before the onset of vesicle eruption and remains infectious for 4-5 days after vesicle eruption (Brunell 1989). At this time point infectious VZV can be recovered from PBMCs by in situ hybridization or polymerase chain reaction (PCR), (Koropchak et al. 1991). Both, lymphocytes and monocytes/macrophages cell types may harbor infectious virus. Viremia during primary VZV infection is cell associated, but it has been difficult to identify the PBMC subpopulation that becomes infected because of the low frequency of positive cells (Fenner 1948; Grose 1981; Ku et al. 2005; Breuer and Whitley 2007). Recovery from the primary infection results in most people in lifelong immunity to exogenous infection, although re-infection has been described in children and pregnant woman with low antibody titers (Hall et al. 2002). A sub-clinical re-infection is even more common in seropositive individuals showing significant rises in the level of specific antibodies

and boosting of cell-mediated immunity (Arvin et al. 1983). Age, immune status and pregnancy have been described as risk factors for severity of varicella.

In immunocompromised individuals primary VZV infection has the potential to cause disseminated infection of the lungs, the central nervous system, and other organs if the host response is inadequate to terminate cell-associated viremia. Immunocompromised children are at risk for severe varicella with prolonged new lesion formation and many cutaneous lesions (Feldman 1986). Progressive infection with visceral dissemination results in pneumonia, hepatitis, encephalitis, and disseminated intravascular coagulopathy (Leung et al. 2000). VZV may also occur as primary infection in patients following allogeneic HSCT, causing severe disease manifestation (Chapter 2.3.4).

2.3.2 Latency

The alphaherpesviruses, such as HSV-1, HSV-2 and VZV, have evolved genetically defined and effective strategies to infect, persist latently within, and reactivate from neuronal tissue shown in Figure 2. VZV infection of cells in the dorsal root ganglia is probably a consequence of all primary VZV infections. Cell-free virus in the skin is required to infect nerve endings, allowing spread to the neurons. T cell-mediated VZV transfer may also be implicated in the transfer of virus to the dorsal root ganglia during the establishment of latency. Analysis of ganglia recovered from autopsies of fatal varicella cases shows that both neurons and satellite cells are virus positive and, in many cases, there are positive satellite cells surrounding a neuron that appears virus negative (Croen et al. 1988). The trigeminal and thoracic ganglia are most frequently infected. Once VZV has infected human neural tissues, the virus has a choice of undergoing productive replication or switching to a latent infection. Less is known about the mechanism how VZV remains latent and what triggers reactivation. As described previously viral transcripts and protein products of ORFs 4, 21, 29, 62 and 63 are produced during latency and presented in the cytoplasm as well as the nucleus (Cheatham et al. 1956; Mahalingam et al. 1993). The evolutionary advantage of becoming integrated is that a viral genome is certain to be retained and replicated every time the host cell genome is replicated and another advantage is the ability to avoid host immune clearance so that they can persist in humans for life (Chapter 2.4).

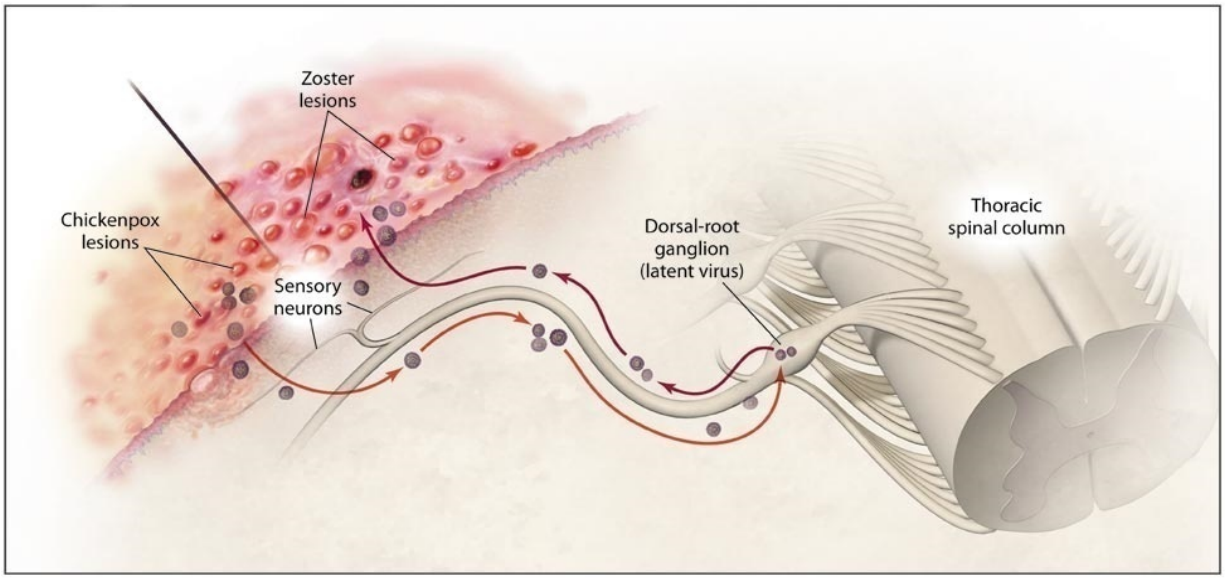


Figure 2: Establishment of VZV latency in sensory-nerve ganglia. After primary VZV infection (chickenpox), viral latency is established in the dorsal-root ganglia, and herpes zoster occurs with subsequent reactivation of the virus (Kimberlin and Whitley 2007).

2.3.3 Reactivation (Herpes Zoster, Shingles)

VZV reactivation is closely associated with the immune status of the host. Age related waning of VZV-specific T cell-mediated immunity correlates directly with the higher incidence of reactivation and herpes zoster in the elderly. The age at which the sharpest increase in zoster occurs is around 50 to 60 years (Hope-Simpson 1965). Unlike varicella, which occurs primarily in the spring, there is no seasonal predilection for zoster. An important epidemiological feature of zoster is the increased frequency and complications of zoster in patients with suppressed cell-mediated immunity compared to immunocompetent individuals. Immune deficits seen in cancer patients and transplant recipients (Chapter 2.3.4), and ultimately in patients with acquired immune deficiency syndrome (AIDS) contribute to the higher incidence of reactivation as well (Poulsen et al. 1996; Gershon et al. 1997). Immunosuppressive treatment including radiation therapy, cancer chemotherapeutic agents, corticosteroids, post transplant immunosuppressants and agents for immune mediated diseases are thought to increase the risk of zoster (Rusthoven et al. 1988).

During VZV reactivation in neuronal cells, viral proteins relocate from the cytoplasm to the nucleus in a process involving proteasome degradation, implicating this pathway in the regulation of VZV reactivation. The full repertoire of viral genes is expressed, new virions are assembled, and the virus probably reaches the skin by transport along sensory neural axons and is detected within neurons and satellite cells during reactivation (Abendroth and Arvin 2001). Viral reactivation causes a localized, pruritic rash in the dermatomal distribution of one or more adjacent sensory nerves. The dermatomal rash is preceded or accompanied by acute neuritis with intense local pain and hyperesthesia. A few patients experience this acute segmental neuralgia without developing a cutaneous eruption, a syndrome called zoster sine

herpete (Gilden et al. 1992). The failure of the host defense mechanism to contain the virus in the ganglia after such prolonged periods of time is not understood. Inadequacy of cell-mediated immunity, as mention above, is likely to be of critical importance since not only the elderly but patients with Hodgkin lymphoma, HIV and similar diseases are more likely to experience zoster (Zolezzi et al. 1990; Gershon et al. 1997). Subclinical reactivation of latent VZV has been documented in immunocompromised patients by the detection of periodic increases in humoral and cell-mediated immunity to VZV and by the detection of VZV DNA by PCR in PBMCs (Arvin et al. 1983). The antigenic stimulations provided by these subclinical reactivations and exogenous reinfection helps to maintain the immunity to VZV that was initiated by childhood varicella and to slow the decline in that immunity that occurs over time (Schunemann et al. 1998). When the immunity falls below a critical level, reactivated virus can no longer be controlled (Gershon et al. 1984).

2.3.4 VZV reactivation after allogeneic/autologous HSCT

VZV disease occurs very frequently in patients after autologous and allogeneic HSCT with an incidence of up to 25-50% (Han et al. 1994; Kawasaki et al. 1996; Koc et al. 2000). It is usually caused by the reactivation of endogenous viruses from latency rather than by *de novo* exposures. Most disease episodes occur 2 to 10 months after transplantation. Post-transplant disease mostly presents as classical herpes zoster. It typically occurs localized as a dermatomal rash that is preceded by pain and paresthesia in the involved dermatome (Arvin 2000). Delayed or insufficient host response results in an average time for cessation of new zoster lesion formation of 8 days, compared with 3 to 5 days in the normal host. A small but significant proportion of the patients show disseminated VZV infection of visceral organs and the central nervous system, occurring few days before the characteristic skin rash becomes evident. Because virus-specific T cell immunity is essential to prevent and control virus reactivation, the risk for VZV disease is highest after allogeneic HSCT, particularly upon procedures that include T cell depleting agents during conditioning therapy (Locksley et al. 1985; Arvin 2000). In addition to T cell depletion, further risk factors are older age at HSCT, radiation in the pre-transplant conditioning therapy, ongoing acute and chronic graft-versus host disease, residual malignant disease, and continuous immunosuppressive treatment contribute to the lost functionality of VZV-specific T cell immunity (Meyers et al. 1980; Wilson et al. 1992; Han et al. 1994). Some HSCT patients may also develop a generalized vesicular exanthema that resembles varicella. The latter indicates uncontrolled spread of VZV due to the lack of efficient antiviral T cell immunity and is associated with a high mortality rate (Han et al. 1994; Koc et al. 2000). When disease manifestations have resolved, second episodes of VZV reactivation occur rarely and are mostly a result of persistent risk factors. The nucleoside analogues acyclovir, valacyclovir, and famcyclovir effectively reduce VZV replication by interfering with the viral thymidine kinase activity. The drugs have been shown to be effective

for the treatment of recurrent VZV infection in immunocompetent as well as immunodeficient individuals (Shepp et al. 1986; Beutner et al. 1995; Tyring et al. 1995). Several groups have also demonstrated that long-term suppressive prophylaxis with acyclovir is a sufficient means to decrease the incidence of VZV reactivation after HCST. (Ljungman et al. 1986; Thomson et al. 2005; Boeckh et al. 2006; Erard et al. 2007). However, antiviral prophylaxis is unable to mediate complete protection from zoster manifestation, which can still appear as breakthrough infections. While some studies have described enhanced rates of VZV disease after cessation of long-term prophylactic medication (Steer et al. 2000; Thomson et al. 2005), other investigators did not observe such a rebound effect (Erard et al. 2007). A clear disadvantage of long-term antiviral prophylaxis with nucleoside analogues is that side effects are more common in HSCT recipients than in other populations, mainly owing to extensive comedication and frequent transplantation-related organ damage. Another disadvantage is that low dose medication used for prophylaxis may also facilitate the selection of VZV strains that are thymidine kinase-negative and therefore resistant (Reusser et al. 1996). Humoral immunity seems to be less important in the host response to VZV than cell-mediated immunity which was demonstrated in healthy subjects and immunocompromised patients (Arvin et al. 1986).

2.4 VZV immunity

2.4.1 Host response to primary VZV infection

The first host response during primary VZV infection is mediated by the innate immune system through antiviral cytokines and NK cell activation. These primary mechanisms are likely to be important at the mucosal infection site of inoculation to limit the initial spread of VZV within the host and to trigger the adaptive, VZV-specific immunity. Activated NK cells are a major source of IFN-γ production which finally increases the clonal expansion of antigen-specific T cells. In addition, the secretion of granulysin by NK cells may enhance the early destruction of VZV infected cells and IFN-α secretion by monocytes as well as NK cells is relevant for the limitation of the viral spread (Arvin et al. 1986; Pena and Krensky 1997). Viral replication during the incubation period of primary VZV infection usually does not elicit humoral immunity, but low titers of IgM and IgG antibodies may be detected when the exanthema appears. Significant antibody production is usually measured within three days after the onset of symptoms. However, later on the adaptive immune response to primary VZV infection includes the induction of B cells producing VZV IgG, IgA, and IgM antibodies directed against many VZV proteins such as the nucleocapsid proteins as well as the late glycoproteins (Schmidt and Gallo 1984). VZV-specific antibodies neutralize the virus, with or without complement, and lyse infected cells through antibody-dependent cellular cytotoxicity (ADCC), (Ihara et al. 1984). Although active antibody responses may have limited impact on

the outcome of primary VZV infection, passively acquired VZV-specific IgG antibodies can interfere with the initial stages of VZV replication *in vivo* and can transplacentally protect infants from developing primary VZV infection (Chapter 2.6) or modify the severity of varicella during the first six month of life (Zaia et al. 1983; Miller et al. 1989). IgM antibodies decline within a few months, but IgG antibodies to many viral proteins persists for years.

Nevertheless, the evidence suggests that VZV-specific antibody responses are less important than cell-mediated immunity for restricting VZV replication and spread during acute infection (Arvin et al. 1986; Camitta et al. 1994). Both, MHC class I-restricted $CD8^+$ T cells and MHC class II-restricted $CD4^+$ T cells are sensitized to VZV antigens during primary VZV infection because of processing and presentation of viral proteins on professional APCs like DCs, B cells or macrophages. The early T cell proliferative response to VZV antigens is mediated by VZV-specific $CD4^+$ T cells and is accompanied by the release of Th1-type cytokines, including IL-2 and IFN-γ, which in particular enhances the rapid clonal expansion of VZV-specific $CD4^+$ and $CD8^+$ T cells. The glycoproteins gE, gB, gC and gH as well as the IE62 and IE63 proteins have been identified as targets of both $CD4^+$ and $CD8^+$ T cells (Arvin 2008). The glycoproteins are expected to be important targets of the initial host response because they are virion envelope components and are expressed on the surfaces of VZV infected cells. Although the classic cytotoxic T cell (CTL) response is mediated by $CD8^+$ T cells that recognize antigenic peptides in the context of MHC class I molecules, VZV-specific CTL exhibit MHC class II as well as MHC class I-restricted killing of infected cells (Hayward et al. 1989; Huang et al. 1992; Sharp et al. 1992). The analysis of the VZV protein targets of $CD4^+$ and $CD8^+$ CTLs indicate that diverse MHC class I and class II phenotypes develop T cells that can recognize the same major viral proteins, although the specific amino acid sequences that are processed for class I and class II presentation can be expected to be different (Jenkins et al. 1999). Interestingly, also VZV like HSV has evolved mechanisms to interfere with MHC class I and MHC class II-mediated antigen presentation (Abendroth and Arvin 1999; Abendroth et al. 2000; Abendroth and Arvin 2001; Eisfeld et al. 2007).

2.4.2 Host response during latency and reactivation

The memory T cell response against VZV is considered to be most relevant for the control of viral reactivation. VZV-specific memory immunity consists on the one hand of antibody persistence, especially IgG and IgA as well as on the other hand of VZV-specific $CD4^+$ and $CD8^+$ T cells. The function of VZV-specific IgG antibodies is primarily the neutralization of VZV infection at sites of inoculation on reexposure to the virus by contact with individuals who suffer from varicella or herpes zoster. Subjects who have recovered from a primary VZV infection evolve T cells which recognize gE, gB, gC ,gH, the IE62 protein and IE63 (Arvin et al. 1986; Sadzot-Delvaux et al. 1998; Jenkins et al. 1999; Arvin 2008). It seems likely that VZV immunity during latency is based on the repertoire that is established during primary

infection and is modulated or expanded by antigens that reach antigen-presenting cells during latency for example by subclinical reinfection (Chapter 2.3.3). Individuals vary in the rate at which responses to individual VZV proteins develop. Extensive studies by Ann Arvin and co-workers document the persistence of VZV-specific $CD4^+$ and $CD8^+$ T cells for 20 years following a primary infection (Arvin 1996). Interestingly, $CD4^+$ T cells seem to play a central role in the control of VZV reactivation and lack of these cells after HSCT may correlate with the risk of severe disease (Distler et al. 2008). Decline of cellular immunity as a consequence of immunosenescence or immunosuppression is considered as the key determinant for symptomatic VZV reactivation.

2.4.3 Immune evasion mechanisms

VZV encodes viral gene products that mediate the down-regulation of MHC class I expression on VZV-infected T cells, an effect which may facilitate the transport of VZV to cutaneus sites of replication during the incubation period, without evoking adaptive immune responses (Abendroth and Arvin 1999; Eisfeld et al. 2007). Since MHC class I molecules present peptides to $CD8^+$ T cells, interference with their transport to the cell surface makes the infected cells' expression invisible to this component of the antiviral immune response. Additionally, VZV-infected cells resist the up-regulation of MHC class II expression that is triggered by IFN-γ (Abendroth et al. 2000). Although the virus has mechanisms for immune evasion, the innate and adaptive immune response act to terminate VZV replication a few days after the appearance of the cutaneus lesions in the otherwise healthy host.

2.5 Antiviral therapy

2.5.1 Treatment of varicella

In healthy individuals the illness is generally mild and complications are rare. Typically no treatment is required apart perhaps from soothing lotions for itching and antibiotics if there is any question of secondary infection. However, varicella in immunocompromised patients is more severe and consequently its management must be very different in these patients from that of healthy individuals. Although many immunocompromised patients cope with varicella in general, considerations should always be given to the possibility of administering antiviral drugs to these patients prophylactically. Acyclovir (Zovirax®) was the first virostatic agent which was introduced more than 20 years ago. This antiviral molecule is still the treatment of choice, however in the near past it was modified because of the reduced bioavailability after oral application (Snoeck et al. 1999). Now, prodrugs like valacyclovir (Valtrex®) and famcyclovir (Famvir®), the oral prodrug of pencyclovir are available.

2.5.2 Treatment of herpes zoster

Herpes zoster occurring in an immunocompetent healthy individual, although unpleasant, is usually not of any concern. The main difficulty in the management of these patients is treating the postherpetic neuralgia pain, particularly when this is prolonged and intractable. The aims in immunocompetent healthy individuals are, firstly to reduce the severity and duration of the acute symptoms, secondly, particularly in the elderly to reduce likelihood and duration of complications such as chronic neuralgia and ocular damage (Mustafa et al. 2009; Sampathkumar et al. 2009). These benefits are also of some importance in the immunocompromised patients but in these individuals the prime consideration is to prevent the morbidity and mortality associated with visceral dissemination of VZV. Since the manifestations of acute herpes zoster are caused by replication of VZV, inhibition of such replication reduces disease severity. There is a number of orally active nucleoside analogs that are clinically effective in the treatment of herpes zoster in immunocompetent patients. Currently available data indicate that oral acyclovir, famcyclovir and valacyclovir each provide similar clinical efficacy for the treatment of herpes zoster in the intact host (Wood et al. 1998; Mustafa et al. 2009). In immunocompromised patients with highest risk for localized or disseminated herpes zoster acyclovir is currently the drug of choice (Breuer and Whitley 2007). Ljungman et al. have also demonstrated in a randomized trial that acyclovir can inhibit VZV-specific T cell reconstitution by preventing subclinical viremia (Ljungman et al. 1986). This observation is consistent with two further reports showing that viremia without progression to herpes zoster may be already sufficient to stimulate VZV-specific T cell proliferation in a significant proportion of allogeneic HSCT recipients (Ljungman et al. 1986; Wilson et al. 1992). However, a more recent randomized study performed by Boeckh et al. did not confirm an inhibitory effect of acyclovir for the development of antiviral T cell immunity (Boeckh et al. 2006). Vice versa our group has observed that avoiding antiviral prophylaxis with nucleoside analogues does not necessarily favor the recovery of VZV-specific T cell immunity in patients undergoing T cell depleted allogeneic HSCT (Distler et al. 2008).

2.6 Prevention

2.6.1 Active Immunization against varicella and herpes zoster

Although individual cases of varicella may be prevented or modified by varicella-zoster immunoglobulin (VZIG) or with antiviral drugs, control of varicella-zoster in the population can only be achieved by widespread vaccination. Active immunization also has the advantage in individual at risk patients by offering long term protection. A varicella vaccine based on the attenuated Oka strain of VZV is available since 1974, when it was first developed in Japan (Takahashi; 1974). The original vaccine was derived from VZV isolated from vesicles of a three-year-old child with typical varicella and was attenuated by serial passage

in guinea-pig cells and human embryo lung cells. Live attenuated varicella vaccines (Varivax®, 1995 and Zostavax®, 2006) produced from the Oka virus strain are commercially available and are licensed for the use in immunocompetent individuals (Jones 2002). Varicella vaccines have demonstrated efficacy in healthy, naive children to prevent primary infection with VZV (Vazquez et al. 2001). They were also found to reduce the incidence and severity of herpes zoster in patients independent of age (Hardy et al. 1991; Oxman et al. 2005). There is clear evidence from immunomonitoring studies that live-attenuated VZV vaccines are capable of enhancing antiviral $CD4^+$ and $CD8^+$ T cell immunity in immunocompetent individuals (Diaz et al. 1989; Frey et al. 2003; Levin et al. 2008). However, VZV vaccines can also trigger VZV reactivation by vaccine-induced immunomodulation, which may lead to herpes zoster or zoster sine herpete in single cases (Patel and Ortin 2005). In addition, the vaccine virus can establish latency by itself and reactivation may occur thereafter. Because the Oka virus strain retains susceptibility to nucleoside analogues (Shiraki et al. 1993), vaccine-related herpes zoster can be safely treated with acyclovir. Live attenuated varicella vaccines elicit both humoral and cells mediated immune responses and are contraindicated in pregnancy and in immunocompromised individuals, because they contain infectious virus, which can reactivate and establish latency in the dorsal root ganglia (Hardy et al. 1991).

2.6.2 Vaccine strategies in immunocompromised individuals after HSCT

Immunization of HSCT patients against VZV is a significant challenge, because reactivated VZV infection occurs during the first months after transplantation when T cell immunity is still heavily compromised. Several groups have demonstrated that VZV-specific T cell reconstitution often does not recur without clinical reactivation of the endogenous virus, which usually induces a T cell response and re-establishment of latency in the dorsal root ganglia (Meyers et al. 1980; Locksley et al. 1985; Ljungman et al. 1986; Distler et al. 2008). This observation suggests that a vaccination approach, as a substitute for natural re-sensitization by virus reactivation, may help to accelerate the reconstitution of VZV-specific T cell immunity in the early post-transplant period. However, live vaccines are contraindicated in HSCT recipients because they contain infectious virus. The vaccinated virus can establish latency in the dorsal root ganglia by itself and reactivation may occur thereafter, which may lead to herpes zoster or zoster sine herpete (Patel and Ortin 2005). Thus, they should be restricted to patients that have recovered competence of cell-mediated immunity, which is usually a long period of time (i.e., >1-2 years) after zoster manifestations have already occurred in the post-transplant period (Sauerbrei et al. 1997).

Two prospective randomized studies performed by Ann Arvin's group have established the safety and feasibility of a heat-inactivated formulation of an Oka-derived varicella vaccine in HSCT patients. The inactivated vaccine was immunogenic when three doses were given and led to a reduced clinical severity and a significantly decreased risk of herpes zos-

ter (Gershon et al. 1997; Redman et al. 1997; Hata et al. 2002). In a subsequent trial in lymphoma patients receiving autologous HSCT, the same group showed that the inactivated varicella vaccine injected before transplantation and three times during 90 days thereafter significantly decreases the risk of zoster (Hata et al. 2002). The protection correlated with the reconstitution of $CD4^+$ T cell immunity against VZV. These data indicate that post-transplant vaccination with inactivated VZV vaccine is safe and able to accelerate the reconstitution of VZV-specific memory T cells that have persisted during the pre-transplant conditioning regimen. Additionally clinical efficacy appears to require the boosting of antiviral memory T cells in autologous HSCT recipients before transplantation, which also improves the control of viral reactivation during immunosuppression. However, no formal recommendation for use of the inactivated varicella vaccine can be given at this time because it remains investigational. Nevertheless, the vaccine holds promise for decreasing the zoster incidence in autologous HSCT recipients within the first year after transplantation.

The translation of this finding to the allogeneic HSCT setting may require immunizing the donor prior to the isolation of either stem cell or donor lymphocyte products. Such a procedure would lead to the adoptive transfer of VZV-specific cellular immunity from the donor to the patient upon transplantation (Kato et al. 1990). Earlier studies in children with leukemia receiving a VZV vaccine have raised concerns that donor vaccination with live virus carries the potential risk of disseminated virus infection in immunodeficient hosts (Hardy et al. 1991). However, Leung et al. recently showed the feasibility and safety of this strategy by vaccinating sibling donors subcutaneously with a live-attenuated VZV vaccine (Leung et al. 2007). Although transplant recipients had a lower, albeit non-significant, risk of zoster compared with historical controls, the lack of a randomized control group does not allow the conclusion that this strategy can be regarded as safe and efficient in allogeneic HSCT patients. After adoptive transfer, sustaining the VZV-specific T cell response by applying repeated vaccine doses to the transplant recipient is likely to be necessary to preserve clinical efficacy against VZV reactivation. The application of a heat-inactivated VZV vaccine was followed by the restoration of antiviral $CD4^+$ T cells in peripheral blood of HSCT recipients (Hata et al. 2002). The enhanced $CD4^+$ immunity may provide helper functions necessary to expand VZV-specific $CD8^+$ CTL populations that appear essential to keep the virus in the latency stage. However, human $CD4^+$ T cells also function effectively as antiviral CTLs against many viruses, including VZV (Arvin et al. 1991). Nevertheless, accumulating evidence from immunobiology studies on other pathogens suggest that optimal VZV vaccines should be capable of directly stimulating VZV-specific $CD4^+$ and $CD8^+$ T cells.

2.7 Motivation and aim of this work

VZV reactivation in VZV seropositive patients after allogeneic HSCT can lead to a life threatening disease due to the severe immunodeficiency in these individuals. Although an efficient live-attenuated Oka strain VZV vaccine for zoster prophylaxis exists, it is by now not approved in immunocompromised or immunodeficient patients due to safety reasons. The therapy of choice is currently based on high affinity antiviral drugs, which block VZV-specific polymerase leading to an inefficient replication of VZV. These antiviral drugs may interfere with the sufficient recovery of VZV-specific humoral and cellular immune responses because the latter needs natural re-sensitization with virus antigens during viremia. Thus after drug treatment is stopped viral reactivation can once again occur (Hackanson et al. 2005; Boeckh et al. 2006). The objective of this study was to identify T cell-defined virus proteins of a VZV-infected VERO cell extract that we have recently described as a reliable antigen format to detect *ex vivo* T cell responses against VZV by an IFN-γ ELISpot assay (Distler et al. 2008). Immunological screening of VZV cell extract preparations was planned to be performed with *ex vivo* PBMCs without prior *in vitro* stimulation and manipulation to consider the *in vivo* hierarchy of T cell mediated immunity against VZV antigens. RP-HPLC and ESI-MS were the methods of choice to identify VZV-specific proteins in VZV cell extract, and in vitro transcribed (IVT)-RNA of the identified immunodominant proteins should be used for *in vitro* screening with PBMCs of healthy donors and patients after allogeneic HSCT. Ultimately, the knowledge of immunogenic VZV proteins would allow designing a noninfectious nonhazardous subunit vaccine suitable for patients with immunodeficiencies, including those undergoing HSCT.

3 Materials and Methods

3.1 Protein biochemistry

3.1.1 VZV-infected/-uninfected Vero cell extract

The WHO developed a master cell bank for Vero cells, a continuous cell line established from the kidneys of an African green monkeys, which are highly susceptible to infection with a multitude of different viruses (Barrett et al. 2009). VZV-infected and -uninfected (control) Vero cell extracts as antigen sources were purchased from tebu-bio (Offenbach, D). The protein concentration of the purchased VZV-infected/-uninfected Vero cell extracts varied in between 0.4 to 0.7 mg/ml. VZV-infected Vero cell extracts has been specifically tested for residual infectivity and no infectious virus was detected. The extract was titered in Vero cells over a 15-day period with endpoint determination by cytopathic effect (CPE). This product was treated using methods of inactivation that have been validated *in vitro* for complete inactivation of high-titered herpesviruses.

3.1.2 High performance liquid chromatography (HPLC)

3.1.2.1 Principle

High performance liquid chromatography is a form of column chromatography used frequently in biochemistry and analytical chemistry to separate, identify, and quantify compounds based on their idiosyncratic polarities and interactions with the column's stationary phase. HPLC utilizes different types of stationary phase (typically, hydrophobic saturated carbon chains), a pump that moves the mobile phase(s) and the analyte through the column, and an extremely sensitive detector that provides a characteristic retention time for the analyte. The detector may also provide other characteristic information (i.e. UV/Vis spectroscopic data or conductivity data).

3.1.2.2 Reverse-phase chromatography (RP-HPLC)

RP-HPLC was performed with an Ettan LC system equipped with a Jupiter 5 µm, C_5, 300 Å, (250x2.0 mm) column or alternatively with a Jupiter 4 µm, C_{12}, Proteo 90 Å, (250x2.0 mm) column and a 50 µl PEEKSIL-sample loop. The aqueous mobile phase (mobile phase A) was H_2O with 0.1% trifluoroacetic acid (TFA). The organic mobile phase (mobile phase B) was 0.1% TFA in acetonitrile (ACN). Samples (50 µl injection) were filtrated (0.2 µm) and subsequently loaded onto the column in direct injection mode with 5% mobile phase B for 10 min at 150 µl/min. Peptides were eluted from the column with a gradient from 5-25% mobile phase B over 10 min at 150 µl/min followed by a second gradient from 25-65% mobile phase

B over 40 min, finally followed by a 10 min gradient from 65-90% mobile phase B. The column was immediately re-equilibrated at initial conditions (5% mobile phase B) for 10 min. The components of the analyte mixture (VZV-infected Vero cell extract, 0.2 µm filtered) passed over stationary-phase particles bearing pores large enough for them to enter, where interactions with the hydrophobic surface removed them from the flowing mobile-phase (gradient modus) stream. The strength and nature of the interaction between the sample particles and the stationary phase depended on both hydrophobic interactions and polar interactions. As the concentration of organic solvent in the eluent has increased, it reached a critical value for each analyte which desorbed it from the hydrophobic stationary-phase surface and allowed it to elute from the column in the flowing mobile phase. Since this elution depended on the precise distribution of hydrophobic residues in each species, each analyte eluted from the column at a characteristic time, and the resulting peak can be used to confirm its identity and quantify it. The 150 µl fractions were collected in 96 well plates and for bioassays, like the IFN-γ ELISpot assay, half of the fractions were evaporated and subsequently reconstituted in PBS in contrast to the fractions used for MS-analysis, which were not reconstituted in PBS, but evaporated for tryptic digestion (Chapter 3.1.3.2.). Fractions were stored at -80°C.

1.) Eluents i.e.

a.) H_2O, 0.1% TFA

b.) ACN, 0.1% TFA

2.) Pumps

3.) Mixer

4.) Injector

5.) Column (RPC, GFC…etc)

6.) UV-Detector

7.) Conductivity Detector

8.) Fraction Collector

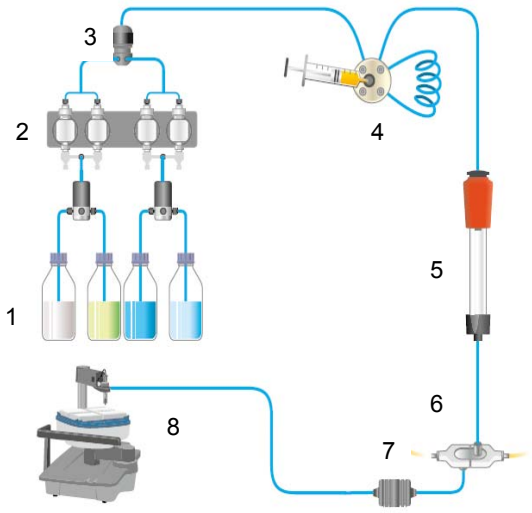


Figure 3: Flow scheme of an Ettan LC system. (Adapted and modified from GE Healthcare)

0.1% TFA in HPLC grade acetonitrile	Carl Roth GmbH (Karlsruhe, D)
0.1% TFA in HPLC grade water	Carl Roth GmbH (Karlsruhe, D)
Column: Jupiter 4 µm, C_{12}, Proteo 90 Å, (250x2.0 mm)	Phenomenex, Inc (Aschaffenburg, D)
Column: Jupiter 5 µm, C_5, 300 Å, (250x2.0 mm)	Phenomenex, Inc. (Aschaffenburg, D)
Ettan LC system	GE Healthcare (Munich, D)
Filter FP 30 (0.45 µm; 0.2 µm)	Carl Roth GmbH (Karlsruhe, D)
HPLC-MS grade water	Mallinckrodt Baker (Griesheim, D)
Lo bind tubes (1.5 ml; 0.5 ml)	Eppendorf AG(Hamburg, D)
Maximum recovery tips (1-200 µl)	Abimed GmbH (Langenfeld, D)
Software: UNICORN 4.11 (Build 211)	GE Healthcare (Munich, D)

Table 2: HPLC-materials

3.1.3 Electrospray ionization mass spectrometry (ESI-MS)

3.1.3.1 Principle

Electrospray ionization is the ion source of choice to couple liquid chromatography with mass spectrometry. The analysis can be performed “online”, by feeding the liquid eluting from the LC column directly to an electrospray, or “offline”, by collecting fractions to be later analyzed in a classical nanoelectrospray-mass spectrometry setup. In electrospray, a liquid is passing through a nozzle. The plume of droplets is generated by electrically charging the liquid to a very high voltage. The charged liquid in the nozzle becomes unstable as it is forced to hold more and more charge. Soon the liquid reaches a critical point, at which it can hold no more electrical charge and at the tip of the nozzle it blows apart into a cloud of tiny, highly charged droplets. The emitted ions are sampled by a sampling skimmer cone and are then accelerated into the mass analyzer for subsequent analysis of molecular mass and measurement of ion intensity. To obtain structural information, the precursor ions of interest can be mass selected and further fragmented in a collision cell. The fragment ions can then be mass analyzed by a second mass analyzer of a tandem mass spectrometer system. The mass spectrum is a graphical display of the relative abundance of ion signals against the mass-to-charge (m/z) ratios. It is a common practice that the highest signal is taken as 100% abundance and all the other signals are expressed as a percentage of this (Kebarle and Verkerk 2009)

3.1.3.2 Protein digest preparation

Bioactive RP-HPLC fractions were evaporated and ultimately solubilized in 25 mM ammonium bicarbonate containing 0.1% Rapigest making proteins more susceptible to enzymatic cleavage. Proteins were reduced by adding 5 mM dithiothreitol (DTT; 45 min, 56°C) and free cysteines alkylated with 15 mM iodoacetamide (1 h in the dark, 25°C), (Kramer-Albers et al. 2007). Porcine sequencing grade trypsin (0.2 µg) was added and the samples were incubated overnight at 37°C. After digestion, RapiGest was hydrolyzed by adding 10 mM HCl (10 min, 37°C) and the resulting precipitate was removed by centrifugation (13.000xg, 15 min, 4°C) and the supernatant was transferred into an autosampler vial for peptide analysis via LC-MS (Kramer-Albers et al. 2007).

3.1.3.3 UPLC configuration (RP-UPLC)

Capillary LC of tryptic peptides was performed with a Waters NanoAcquity UPLC system equipped with a 75 µm x 150 mm Bridged Ethyl Hybrid (BEH) C18 RPRP column and a 2.6 µl PEEKSIL-sample loop (Kramer-Albers et al. 2007). The aqueous mobile phase (mobile phase A) was H_2O with 0.1% formic acid. The organic mobile phase (mobile phase B) was 0.1% formic acid in ACN. Samples (2.6 µl injection) were loaded onto the column in direct injection mode with 3% mobile phase B for 15 min at 400 nl/min, followed by an additional 10 min wash (3% B) for 10 min at 300 nl/min. Peptide were eluted from the column with a gradient from 3-35% mobile phase B over 90 min at 300 nl/min followed by a 20 min rinse of 80% mobile phase B. The column was immediately re-equilibrated at initial conditions (3% mobile phase B) for 20 min. [Glu1]fibrinopeptide was used as lockmass at 300 fmol/µl. Lockmass solution was delivered from the auxiliary pump of the nanoAcquity system at 400 nl/min to the reference sprayer of the NanoLockSpray™ source. Samples were analyzed in triplicate.

3.1.3.4 Mass spectrometer configuration

MS analysis of tryptic peptides was performed by Dr. Stefan Tenzer at the Institute of Immunology in Mainz using a Waters Q-TOF Premier API system, operated in V-mode with typical resolving power of at least 10000. All analyses were performed using positive mode ESI using a NanoLockSpray source. The lock mass chanel was sampled every 30 s. The mass spectrometer was calibrated with a [Glu1]fibrinopeptide solution (300 fmol/µl) delivered through the reference sprayer of the NanoLockSpray source. Accurate mass LC-MS data were collecting in an altering, low energy (MS) and elevated energy (MSE) mode of acquisition. The spectral acquisition time in each mode was 1 s with a 0.05 s interscan delay. In low energy MS mode, data were collected at constant collision energy of 3 eV. In MSE

mode, collision energy was ramped from 16 to 32 eV during each 1 s data collection cycle. One cycle of MS and MSE data was acquired every 2.1 s. The radio frequency applied to the quadrupol mass analyzer was adjusted such that ions from m/z 300 to 1500 were efficiently transmitted, ensuring that any ions observed in LC-MSE data less than m/z 300 were known to arise from dissociations in the collision cell.

3.1.3.5 Data processing and protein identification

The continuum LC-MSE data were processed and searched using the IDENTITYE-Algorithm of ProteinLynx Global Server (PLGS) version 2.3 (Weinzierl et al. 2008). The resulting peptide and protein identifications were evaluated by the software using statistical models similar to those described by Skilling et al. (Skilling et al. 2004). Protein identifications were assigned by searching the Uni-ProtKB/Swiss-Prot Protein Knowledgebase (http://expasy.org/sprot/) for macaca mulatta (349 entries), cercopithecus aethiops (181 entries), pongo pygmaeus (239 entries), homo sapiens (20405 entries), pan troglodytes (688 entries), pan paniscus (122 entries), gorilla gorilla (279 entries), papio papio (190 entries), varicella-zoster virus (Dumas strain, 69 entries), supplemented with known possible contaminants (porcine trypsin). Maximum mass deviation was set to 15 ppm for precursor ions and 30 ppm for fragment ions. For valid protein identification, the following criteria had to be met: at least two peptides detected with together at least seven fragments. The false-positive rate for protein identification was set to 1% based on search of a 5x randomized database

[Glu^1]fibrinopeptide	Sigma-Aldrich Chemie GmbH (Munich, D)
Acetonitrile (HPLC grade)	Carl Roth GmbH (Karlsruhe, D)
Ammonium bicarbonate	Sigma-Aldrich Chemie GmbH (Munich, D)
BEH C18 RPRP column (75 µm x 150 mm)	SGE Europe LTD (Darmstadt, D)
Dithiothreitol (DTT)	Sigma-Aldrich Chemie GmbH (Munich, D)
Formic acid (HPLC grade)	Carl Roth GmbH (Karlsruhe, D)
H_2O (HPLC grade)	Carl Roth GmbH (Karlsruhe, D)
HCl	Sigma-Aldrich Chemie GmbH (Munich, D)
Iodoacetamide	Sigma-Aldrich Chemie GmbH (Munich, D)
NanoAcquity UPLC system	SGE Europe LTD (Darmstadt, D)
PEEKSIL-sample loop (2.6 µl)	SGE Europe LTD (Darmstadt, D)
Q-TOF Premier API system	SGE Europe LTD (Darmstadt, D)
RapiGest SF Surfactant	Waters GmbH (Eschborn, D)
Trypsin	Promega GmbH (Mannheim, D)

Table 3: Materials for protein digestion, UPLC and MS analysis

3.2 Cell culture

3.2.1 Primary cells of patients and healthy donors

Informed consent was obtained from all participants in accordance with the Declaration of Helsinki. The study was approved by the local Ethics Committee. PBMCs were separated from heparinized blood samples (Chapter 3.2.3), collected at different time points after VZV reactivation in patients after allogeneic HSCT for treatment of hematological malignancies. Serum was taken for immunological detection of VZV-specific IgG (quantitative) and IgM (qualitative) antibodies. Patients showing viral reactivation after allogeneic HSCT are listed in Table 4. For establishing the immunological methods and for primary screening experiments leukapheresis products and buffy coats of healthy individuals (Center for Blood Transfusion, Mainz) were used. PBMCs were isolated according to the same standard protocol (Chapter 3.2.3 and 3.2.4).

Patients (P)	Age	Gender	Diagnosis	VZV reactivation (days after allogeneic HSCT)
P1	46	f	Anaplastic large cell T-NHL	+ 365
P2	59	m	AML after MDS	+ 107
P3	67	m	AML M2	+ 139
P4	54	m	CLL	+ 464
P5	70	m	AML	+ 885

Table 4: Primary cells of patients. Listed are age, gender, diagnosis, and time point of viral reactivation in patients after undergoing allogeneic HSCT.

AIM V medium	Gibco/Invitrogen GmbH (Darmstadt, D)
AIM V medium for T cell culture	AIM V + 10% human serum (HS)
DC medium	AIM V + 1% human serum (HS)
Dimethyle sulfoxide (DMSO)	Merck KGaA (Darmstadt, D)
Ficoll lymphocyte separation medium LSM1077	PAA Laboratories (Pasching, A)
Freezing medium	X-VIVO 15 8% human albumin 10 U/ml heparin (Liquemin® 5000 U/500 µl) (10% DMSO added prior to use)
Heparin (Liquemin® N5000)	Roche GmbH(Grenzach-Wyhlen, D)
Human albumin (20% Octalbumin®)	Octapharma GmbH (Langenfeld, D)
Human serum (HS)	Isolated after informed consent from blood of healthy donors; pooled, heat-inactivated, sterile filtrated (0.22 µm), and stored at -80°C until use.

Phosphate-buffered saline (PBS),+ $CaCl_2$, $MgCl_2$	Gibco/Invitrogen GmbH (Darmstadt, D)
Phytohemagglutinine (PHA)	Murex Biotech LTD (Dartford Kent, UK)
Trypane Blue (solution for cell counting) Trypane Blue (stock solution)	75 ml stock solution + 25 ml NaCl [150 mM] Trypane Blue 2.0 g ad 1 l H_2O Merck KGaA (Darmstadt, D)
X-VIVO 15 medium	Biowhittaker (Viersen, B)

Table 5: Cell culture materials and media. Culture of eukaryotic cells was performed according to standard procedures under sterile conditions. All cells were cultivated at 37°C and 5% CO_2. All cell culture media were stored at 4°C

3.2.2 Cytokines

Cytokine	Manufacturer	Stock concentration	Final concentration
IL-2 (Proleukin™)	Chiron Behring (Marburg, D)	$2.5x10^4$ IU/ml	10 IU/ml
GM-CSF (Granulocyte monocyte colony stimulating factor)	Sandoz Biopharmaceuticals (Milano, I)	1000 IU/µl	800 IU/ml (DC d1) 1600 IU/ml (DC d3)
IL-4	Strathmann Biotec (Hannover, D)	500 IU/µl	1000 IU/ml
PGE_2 (prostaglandin 2)	Sigma-Aldrich Chemie GmbH (Munich, D)	1 µg/µl	1 µg/ml
IL-1β	Pharmingen (San Diego, USA)	10 ng/µl	10 ng/ml
TNF-α (Tumor necrosis factor-α)	R&D Systems (Minneapolis, USA)	10 ng/µl	10 ng/ml
IL-6	Strathmann Biotec (Hannover, D)	1000 IU/µl	1000 IU/ml

Table 6: Cytokines used for cultivation of primary cells. Manufacturer, stock concentration and final concentration are listed. Cytokines were dissolved in AIM-V medium and stored at -80°C or -20°C.

3.2.3 Isolation of PBMCs from whole blood samples

Sterile Leucosep® tubes filled with 15 ml Ficoll lymphocyte separation medium were shortly centrifuged (30 s at 1000xg and RT). The separation medium was then located below the porous barrier. 15-30 ml of the whole blood sample were gently pipetted on top. Ficoll tubes were centrifuged without brake for 20 min at 1000xg. The lower density white blood mononuclear cell (MNC) ring was harvested and transferred into new 50 ml tubes, by combining two rings into one tube. The PBMC enriched cell fraction was washed by adjusting to 50 ml with ice-cold PBS and centrifugation at 500xg for 10 min. After discarding the supernatants, cell pellets were resuspended in ice-cold PBS, pooled into one 50 ml tube and again spun down (250xg, 5 min). The supernatant was discarded and PBMCs were resuspended in ice-cold PBS and counted.

3.2.4 Isolation of PBMCs from leukapheresis products

For the cryo-preservation of leukapheresis material of healthy individuals, the content of a leukapheresis bag was first transferred into sterile tubes by using a perfusor syringe. The high number of white blood cells was diluted with PBS (1:5) and the isolation of MNC was proceeded using the above protocol (Chapter 3.2.3) with the exception that the Ficoll density centrifugation was done in 50 ml tubes without the porous barrier, because of the high number of MNC.

3.2.5 Generation of DCs from PBMCs

For the generation of dendritic cells from PBMCs, blood monocytes were first isolated by plastic adherence. For that purpose, PBMCs were plated in 6 well plates at a density of $3x10^7$ cells per well in 3 ml of complete culture medium (AIM V 1% HS) and incubated at 37°C and 5% CO_2 for 1 h. After a microscopic control of adherence, the non-adherent fraction was removed and 3 ml of fresh, warm complete medium were added (day 0). The non-adherent fractions were centrifuged and plated once more in a new 6 well plate for re-adherence. The non-adherent fraction from these ‘replate' dishes was discarded after 1 h adherence. All adherent fractions were cultured until day 1, then culture medium was taken off carefully so that loosely adherent cells were not removed, and new culture medium containing GM-CSF (800 U/ml final concentration) and IL-4 (1000 U/ml final concentration) was added. Cytokines were added again on day 3 in 3 ml fresh medium (containing 1600 U/ml GM-CSF and 1000 U/ml IL-4) per well. On day 5 all cells were harvested, counted and replated in fresh complete medium (containing cytokines in the same dosage as described above) in 24 well plates at a density of $5x10^5$ cells/well in 1 ml medium. Alternatively, the immature d5 DCs (iDCs) were cryo-persevered. Immature DCs were further cultivated for

another 48 hours in the presence of the maturation cytokines IL-1β, IL-6, TNF-α and PGE_2 (final concentrations are listed in Table 6) to generate mature DCs (mDCs). After 48 hours incubation the maturation can be monitored by FACS analysis for expression of the cell surface marker CD80, CD83, CD86, and HLA-DR.

3.2.6 Freezing and thawing of cells

For cryo-preservation in liquid nitrogen, cells were frozen in portions of 2-5x10^6 (T cells and DCs) or 10-100x10^6 (PBMCs) in 1 ml of freezing medium containing 10% DMSO. Cryo tubes were transferred in cryo boxes and stored over night at -80°C, before they were placed into the nitrogen tank. For thawing of cells, cryo tubes were shortly put into the water bath (37°C) until frozen cells began to thaw. The content of the tube was transferred with fresh RPMI medium in a cell culture tube. In order to remove DMSO, cells were centrifuged, thawing medium was discarded, and cells were counted (Neubauer chamber) and seeded in fresh medium.

3.3 Immunological methods

3.3.1 Magnetic cell separation (MACS)

3.3.1.1 Principle

The MACS™ technology (Miltenyi Biotec, Bergisch-Gladbach) is based on the use of magnetic MicroBeads that are coupled to monoclonal antibodies, which bind to a specific cell surface antigen on the target cell population. By using a MACS column placed in a permanent magnetic field (MACS Separator), the magnetically labeled target cells are retained. Unlabelled cells are washed out by rinsing the column with MACS-buffer. The labelled cell fraction is obtained by removing the column from the magnet.

3.3.1.2 Isolation of $CD8^+$/$CD4^+$T cells from healthy donor and patient PBMCs

The isolation procedure was performed according to the standard protocol by Miltenyi Biotec. $CD8^+$ isolation was started with approx. 2x10^8 PBMCs per one LS column or a lower amount. After determining the cell number, PBMCs were washed in MACS buffer and the cell suspension was centrifuged at 400xg for 5 minutes. PBMCs were resuspended in 80 µl of MACS buffer per 10^7 total cells and 20 µl of CD8 MicroBeads per 10^7 total cells were added. The cell suspension was mixed and incubated for 15 min at 4°C. PBMCs were washed by

adding 1-2 ml of MACS buffer per 10^7 cells and centrifuged at 400xg for 5 minutes. PBMCs were resuspended in 500 µl of MACS buffer and applied on the pre-wetted LS/MS column, which was placed in a magnetic field. The column type used for $CD4^+$ and for $CD8^+$ isolation depended on the cell number. The column was washed three times with 500 µl (MS column) or 3 ml (LS column) MACS buffer and the unlabeled cells that passed through the column were collected. The magnetically labeled CD8 positive fraction retained within the column. After removing the column out of the magnetic field the appropriate amount of MACS buffer was pipetted onto the column (MS column, 1 ml and LS column, 5 ml). Microbead-labeled cells were immediately flushed out by firmly pushing the plunger into the column. The isolation steps were performed at 4°C (on ice). Cell recovery was determined by counting each separated fraction. Isolation of $CD4^+$ T cells was performed using the eluated CD8 negative fraction and CD4 MicroBeads instead of CD8 MicroBeads. Purity of each fraction was controlled by flow cytometry.

Albumine, bovine (BSA)	Carl Roth GmbH (Karlsruhe, D)
CD4 MicroBeads, human	Miltenyi Biotec # 130-045-101
CD8 MicroBeads, human	Miltenyi Biotec # 130-045-201
Ethylendiamine-tetra-acetic acid (EDTA)	Sigma-Aldrich Chemie GmbH (Munich, D)
LS columns	Miltenyi Biotec # 120-000-475
MACS buffer	PBS 1x (-$CaCl_2$, -$MgCl_2$) 0.5% BSA, 2 mM EDTA Sterile filtrated (0.22 µm), stored at 4°C
MACS Multistand	Miltenyi Biotec # 130-042-303
MidiMACS Separator	Miltenyi Biotec # 130-042-302
MiniMACS Separator	Miltenyi Biotec # 130-042-102
MS columns	Miltenyi Biotec # 120-000-472
Preseparation filter	Miltenyi Biotec # 120-002-220

Table 7: Materials for magnetic cell separation

3.3.2 Flow cytometry

3.3.2.1 Principle

Flow cytometry (FACS, flurescence-activated cell sorting) is a method used for the phenotypic analysis or sorting of a cell population. A flow cytometer can detect fluorescence on individual cells in a suspension and thereby determine the number of cells expressing the molecule to which a fluorescent probe binds, as well as the expression level of a certain molecule on a cell population. Cells are first stained by incubating the cell suspension with a fluorescently labeled antibody specific for a surface antigen of a cell population. The amount of probe bound by each cell in the population is measured by passing the cells one at a time

through a fluorimeter with a laser-generated incident beam. The incident laser beam is of a designated wavelength, and the light that emerges from the sample is analyzed for forward and sideward scatter as well as fluorescent light or one or more wavelengths that depend on the fluorochrome labels attached to the antibodies. The forward and side light-scattering properties of measured cells reflect cell size and internal complexity, respectively. The relative amount of a particular cell surface antigen on different cell populations can be compared by staining each population with the same probe and determining the amount of fluorescent emitted.

3.3.2.2 Monoclonal antibodies used for flow cytometry

For immunophenotyping of cell populations by flow cytometry, the following mouse antihuman monoclonal antibodies (mAbs) were used: All antibodies were stored at 4°C and handled under sterile conditions.

Antibody specificity	Fluorochrome conjugation	Manufacturer
Isotype control mouse IgG1	FITC/PE, APC	Immunotech/Coulter (Marseille, F)
CD3	FITC, APC	Immunotech/Coulter (Marseille, F)
CD4	FITC/PE	Immunotech/Coulter (Marseille, F)
CD8	FITC/PE	Immunotech/Coulter (Marseille, F)
CD16	FITC	Immunotech/Coulter (Marseille, F)
CD56	PE	Immunotech/Coulter (Marseille, F)

Table 8: FACS antibodies used for the detection of cell surface antigens. Fluorochrome-conjugations: fluorescein isothiocyanate (FITC), phycoerythrin (PE) and allophycocyanin (APC).

Antibody	Specificity	Isotype	Reference
NB110-57255	VZV glycoprotein E	mouse IgG1	Olson et al, 1997
NB110-57257	VZV glycoprotein B	mouse IgG1	Jacquet et al, 1998
NB110-57261	VZV immediate early protein 62	mouse IgG1	Kinchington et al, 1998

Table 9: VZV-specific monoclonal antibodies. These monoclonal antibodies (Novus Biologicals, Inc., Littleton, USA) were used for indirect immunofluorescent staining in combination with FITC-labeled goat-anti-mouse (gam) IgG (Immunotech, Marseille, F).

3.3.2.3 Immunofluorescent staining

For direct immunofluorescent staining, $5x10^4$ to $2x10^5$ cells were washed with FACS buffer and incubated for 15 min at 4°C with 2-5 µl FITC-, PE-, or APC-conjugated monoclonal antibodies specific for indicated CD antigens (Table 8) in a total volume of approx. 20 µl. After washing off unbound antibody, stained cells were resuspended in 0.3 ml fixation solution. Flow cytometry analysis was performed using the flow cytometer BD FACSCanto II. After gating of viable lymphocytes, 10.000 events were measured and analyzed applying BD FACS DIVA software. Additional analysis was performed using the FlowJo software (Tree Star, Inc.)

3.3.2.4 Intracellular VZV protein staining

For intracellular VZV protein staining $5x10^5$ to $1x10^6$ cells were fixed and permeabilized for 20 min at 4°C using the BD Cytofix/Cytoperm™ Kit. Afterwards cells were washed two times in BD Perm/Wash™ buffer and stained with the VZV protein-specific primary antibody (Table 9) for 30 min at 4°C. Cells were again washed two times in BD Perm/Wash™ buffer and stained with the secondary FITC-labeled goat-anti-mouse IgG (1:200 in PBS). After incubation for 30 min at 4°C cells were again washed two times in BD Perm/Wash buffer. Stained cells were diluted in FACS buffer and monitored by Flow cytometry. Alternatively, cells were resuspended in 300 µl AIM V medium and transferred to the chamber slides for confocal laser scanning microscopy (cLSM) experiment (Chapter 3.3.5).

Albumine, bovine (BSA)	Carl Roth GmbH (Karlsruhe, D)
BD Cytofix/Cytoperm™ Kit	BD Bioscience (Heidelberg, D)
FACS buffer	PBS 1x (-$CaCl_2$, $MgCl_2$) 0,1% BSA Sterile filtrated (0.22 µm), stored at 4°C
FACS fixation solution	1% PFA in FACS buffer
Flow Cytometer	BD FACSCanto II, BD Biosciences (Heidelberg, D)
Paraformaldehyde (PFA, 37% solution)	Merck KGaA (Darmstadt, D)
Software	FlowJo Tree Star, Inc. (Ashland, USA)
Software	BD FACS DIVA, BD Biosciences (Heidelberg, D)

Table 10: Materials and equipment for flow cytometry analysis

3.3.3 IFN-γ ELISpot (Enzyme-Linked Immunosorbent Spot) assay

3.3.3.1 Principle

IFN-γ is one of the cytokines that is released from T cells following antigen contact. The principle of the ELISpot assay (Figure 4) is the capture and colorimetric detection of a cytokine released by individual T cells, accomplished by a sequential antibody-binding and enzyme-substrate reaction. First, a monoclonal antibody (the primary antibody) directed against one cytokine-epitope binds with the constant region of its heavy chain to a nitrocellulose- or nylon-membrane in 96-well ELISpot plates. Lymphocytes are co-incubated in those wells together with their target cells, and release cytokines, when antigen contact takes place. The cytokine molecules are bound by the variable region of the primary antibody. After cells have been washed from the membrane, a second mAb is added that recognizes a different epitope of the cytokine. This secondary antibody is coupled to biotin, which can on its part be bound by avidin. Avidin in turn is coupled to an enzyme (horseradish peroxidase or alkaline phosphate) that converts in the last step its substrate in a colour reaction, thereby marking the place of cytokine-release by an individual T cell as a couloured spot. The number of spots can be counted and quantifies thereby the reactivity or frequency of single antigen-specific T cells.

3.3.3.2 IFN-γ ELISpot assay for VZV reactivity screening

ELISpot plates were coated with the anti-IFN-γ primary mAb (10 µg/ml in PBS, 60 µl/well) overnight at 4°C. Unbound primary mAb was washed from the membranes with PBS, and free binding spaces on the membrane were blocked by incubation with serum-containing medium. PBMCs at 0.2-2.0x10^6/well were seeded in AIM V medium with 10% human serum in ELISpot plates and 25-50 µg/well VZV infected cell extract were added to a total volume of 100-200 µl/well. For the determination of the HLA-restriction elements by blocking experiments, target cells were incubated with saturating concentrations of HLA blocking mAbs for 30 min before seeding PBMCs in the ELISpot plates. The following antibodies were used: W6/32, an anti-HLA-class-I IgG2a, GAP-A3, an anti-HLA-A03 IgG2a, and HB55, an anti-HLA-DR IgG2a (Dorrschuck et al. 2004). Stimulation of PBMCs with the mitogenic agent phytohemagglutinin (PHA) served as a positive control for IFN-γ spot production. After a culture period of 40 h at 37°C, plates were washed with PBS containing 0.05% Tween20 in order to remove the cells, and the secondary, biotinylated anti-IFN-γ antibody was added (2 µg/ml in PBS/0.5% BSA, 60 µl/well) and incubated at 37°C for 2 h. After washing again with PBS/0.05% Tween20, plates were incubated with the avidin/peroxidase-complex (100 µl/well) for 1 h at room temperature in the dark. The avidin/peroxidase-complex solution was prepared 30 min in advance by mixing 10 ml of PBS/0.1% Tween20 with one drop of

reagents A and B from the Vectastain® Elite Kit (Vector Laboratories). Plates were again washed (3x with PBS/0.05% Tween20, 3x with PBS), and as the final step, the AEC (3-amino-9-ethylcarbazole)-substrate solution (100 µl/well) was added. The substrate solution was activated by the addition of 25 µl 30% H_2O_2 per 50 ml AEC solution. The color reaction was stopped after 10-15 min by rinsing the plates thoroughly with tap water. After the membranes had dried, IFN-γ spots were automatically visualized and counted using an Axioplan 2 microscope combined with the computer-assisted image system KS ELISpot 4.9 (Herr et al. 1997). Results represent means of duplicate or triplicate wells. The frequency of VZV-reactive T cells was calculated based on spot-forming cell (SFC) counts either per ml peripheral blood or per plated PBMCs. The PBMC count was assesed by measuring the percentage of total lymphocytes and monocytes in white blood cell differential analysis.

Acetate buffer	615.8 ml H_2O 1.8 g Na-acetate 9.2 ml Acetic acid (100%)
Acetic acid	Carl Roth GmbH (Karlsruhe, D)
AEC (3-amino-9-ethylcarbazole) tablets	Sigma-Aldrich Chemie GmbH (Munich, D)
Albumine, bovine (BSA)	Carl Roth GmbH (Karlsruhe, D)
Anti-hIFN-γ-antibody #1-D1K (mouse IgG)	Mabtech AB (Nacka, S)
Anti-hIFN-γ-antibody #7-B6-1 (mouse IgG), biotinylated	Mabtech AB (Nacka, S)
Axioplan 2 microscope	Carl Zeiss Vision (Hallbergmoos, D)
Buffer for secondary mAB	PBS/0.5% BSA
Computer-based imaging system and Software KS ELISpot 4.9	Carl Zeiss Vision (Hallbergmoos, D)
Ethanol 35% (v/v)	Merck KGaA (Darmstadt, D)
H_2O_2 30% solution (w/w)	Sigma-Aldrich Chemie GmbH (Munich, D)
Multiscreen HTSTM IP 96 well filtration system	Millipore GmbH (Eschborn,D)
N,N-Dimethylformamide	Sigma-Aldrich Chemie GmbH (Munich, D)
Na-acetate	Sigma-Aldrich Chemie GmbH (Munich, D)
PBS	PBS Instamned® Powder (9.55 g/l), Biochrom (Berlin,D)
Tween20	Merck KGaA (Darmstadt, D)
Vecstatin® Elite Kit (reagents A + B)	Vector Laboratories (Burlingame, USA)
Wash buffer	PBS/0.05% Tween 20

Table 11: Materials and equipment for IFN-γ ELISpot experiments

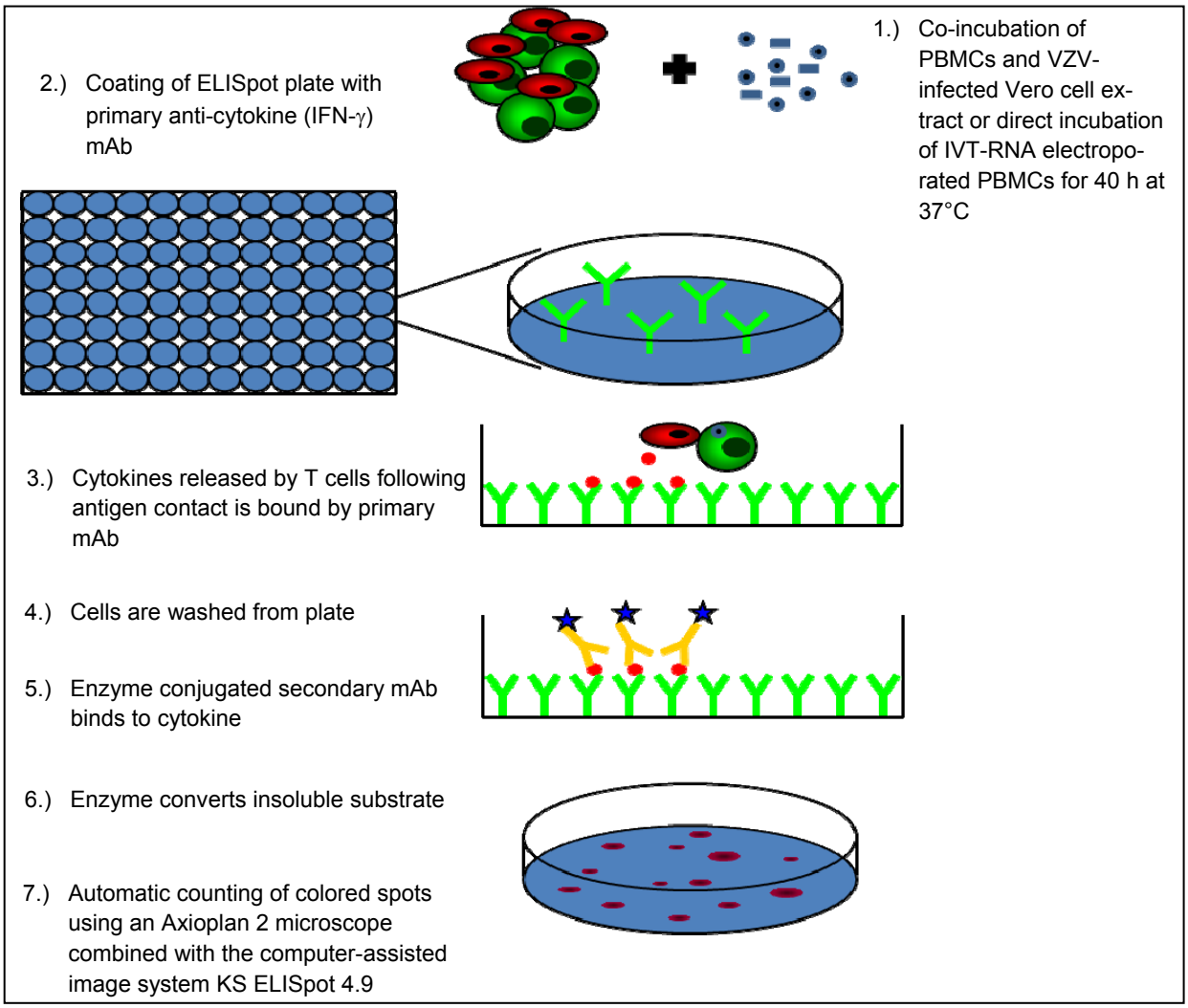


Figure 4: Principle of ELISpot assay.

3.3.4 Western blot

3.3.4.1 Principle

Western blot is an analytical technique used to detect specific proteins in a given sample of cell extract. It uses gel electrophoresis to separate native or denaturated proteins by the length of the polypeptide (denaturating conditions) or by the 3-D structure of the protein (native/non-denaturating conditions). The proteins are then transferred to a membrane, typically nitrocellulose or polyvinylidene fluoride (PVDF) where they are detected using antibodies specific for the target protein.

3.3.4.2 Detection of VZV-specific protein expression by Western blot

SDS-polyacrylamide electrophoresis was performed according to Laemmli (Laemmli 1970). The electroporated PBMCs were centrifuged and resuspended in Laemmli buffer (10^6 PBMCs in 10 µl). Solubilized cell proteins were separated on a gel containing 12% (w/v)

acrylamide. Protein separation was conducted at 600 V, 30 mA per gel for 4 h. Afterwards immunoblotting was performed according to Towbin et al., using Bio-Rad pure nitrocellulose transfer membranes (0.45 µm), which were reacted with specific primary antibodies (Towbin et al. 1979). Unspecific binding to the transfer membrane was blocked with 5% (w/v) skim milk suspended in PBS buffer. Immunoblotting was conducted at 600 V, 400 mA for 1.45 h. Immunoreactive bands were visualized by a peroxidase linked polyclonal rabbit anti-mouse secondary antibody and the electrochemiluminescence (ECL) Western blotting detection system. ECL-stained blots were exposed to X-ray films for 60 s or longer and the developed films were edited using Corel Draw 8.0. All primary antibodies used are listed in Table 9.

Amersham ECL Plus™ Western Blotting Detection Reagents	GE Healthcare Europe GmbH (München, D)
Blotting-buffer contains	
Tris base (25 mM)	Merck KGaA (Darmstadt, D)
Glycerol (192 mM)	AppliChem (Darmstadt, D)
Methanol (10%)	Merck KGaA (Darmstadt, D)
Chromatography-paper 3mm	Fisher, Scientific GmbH (Schwerte, D)
Electrophoresis Unit SE600 Series	Hoefer, Inc. (Holliston, MA, USA)
Laemmli buffer 2x contains	
Tris base (125 mM)	Merck KGaA (Darmstadt, D)
β-mercaptoethanol (2%)	Merck KGaA (Darmstadt, D)
Glycerol (10%)	AppliChem (Darmstadt, D)
EDTA, Titriplex® II (1 mM)	Merck KGaA (Darmstadt, D)
Bromphenol blue (0.005%)	Sigma-Aldrich Chemie GmbH (Munich, D)
PBS (1x) contains	Biochrom AG, (Berlin, D)
Tween20 (0.1%)	Merck KGaA (Darmstadt, D)
PeqGOLD Protein Marker IV	PEQLAB Biotechnologie GmbH (Erlangen,D)
Polyclonal Rabbit Anti-Mouse Immunoglobulins/HRP	Dako (Glostrup, DK)
PVDF transfer membrane	PerkinElmer (Waltham, MA, USA)
Running buffer 5x (pH 8.3) contains	
Tris base (25 mM)	Merck KGaA (Darmstadt, D)
Glycerol (192 mM)	AppliChem (Darmstadt, D)
SDS (0.1%)	Merck KGaA (Darmstadt, D)
Separating-gel (12%) contains	
Tris/HCl pH 8.8 (1.5 M)	Merck KGaA (Darmstadt, D)
Acrylamide/bis-acrylamide (30%)	Merck KGaA (Darmstadt, D)
SDS (10%)	Merck KGaA (Darmstadt, D)
APS (10%)	Sigma-Aldrich Chemie GmbH (Munich, D)
TEMED (15 µl)	Sigma-Aldrich Chemie GmbH (Munich, D)
ad 25 ml dd. Millipore water	
Stacking-gel (4%) contains	
Tris/HCl pH 6.8 (1.0 M)	Merck KGaA (Darmstadt, D)
Acrylamide/bis-acrylamide (30%)	Merck KGaA (Darmstadt, D)

SDS (10%) APS (10%) TEMED (15 µl) ad 12,5 ml dd. Millipore water	Merck KGaA (Darmstadt, D) Sigma-Aldrich Chemie GmbH (Munich, D) Sigma-Aldrich Chemie GmbH (Munich, D)

Table 12: Materials and equipment for the Western blot

3.3.5 Confocal laser scanning microscopy (cLSM)

3.3.5.1 Principle

CLSM is based on a conventional microscope which is equipped with a laser light source, the laser scanning head and an automatic focusing stage, connected to a monitor and PC. In cLSM, the laser is moved across the sample in a raster (x-y) pattern, and by moving the focus vertically (z), multiple slices can be used to build up a full three-dimensional image. It provides the capacity for direct, noninvasive, serial optical sectioning of intact, thick, living specimens with a minimum of sample preparation as well as a marginal improvement in lateral resolution. Biological samples are often treated with fluorescent dyes to make selected objects visible.

3.3.5.2 Identification of VZV-specific proteins by cLSM

CLSM analysis of VZV-specific protein expression in DCs was performed by Dr. Dennis Strand, 1st Department of Medicine in Mainz. Intracellular localization of VZV-specific proteins was confirmed with a Zeiss LSM 510-UV confocal laser scanning microscope equipped with Zeiss LSM Image Examiner software. For cLSM analysis of mDCs, cells were matured as described in the protocol (Chapter 3.2.5). On day 7, mDCs were electroporated (Chapter 3.4.2) with VZV-specific mRNA encoding for the desired protein and incubated in 6-well culture plates for 4 hours. Mature, electroporated DCs were stained with the primary VZV-specific antibody for 30 min at 4°C and counterstained with a secondary FITC-conjugated mAb for 30 min at 4°C. Nuclear staining was performed parallel by incubating the cells with 200 nM Hoechst 33342 for 30 min at 4° (Chapter 3.3.2). Afterwards 10^5 cells in 300 µl medium per well were transferred into Lab-Tek 8-well chamber slide. The imaging was done after the DCs were settled on the chamber bottom.

Hoechst 33342	Invitrogen GmbH (Darmstadt, D)
Lab-Tek™ 8- well chamber slides	Fisher, Scientific GmbH (Schwerte, D)
Zeiss LSM 510-UV	Carl Zeiss GmbH (Jena, D)
Zeiss LSM Image Examiner Software (Version 3.2.0.115)	Carl Zeiss GmbH (Jena, D)

Table 13 Materials and equipment for cLSM

3.4 Molecular biology

3.4.1 *In vitro* transcription of VZV-specific cDNA into RNA

For *in vitro* transcriptions pcDNA™3.1-gE, pcDNA™3.1-gB and pcDNA™3.1-IE62 vectors kindly provided by Prof. Ann Arvin, (Stanford University, MI, USA) were linearized by XbaI restriction, purified with standard phenol/chloroform extraction and ethanol precipitation, and used as DNA templates (Heiser et al. 2000). XbaI recognized a specific sequence of nucleotides and produce a double-stranded cut at the restriction site of the vector DNA. Generally, 1 µl of enzyme is added to 1 µg of purified DNA in a final volume of 50 µl of the appropriate 1x NEBuffer followed by incubation over night at the recommended temperature according to the manufacturer´s instructions. Afterwards DNA fragments resolved in aqueous solution were purified by phenol/chloroform (50%/50%; v/v) extraction, which is based on phase separation by centrifugation (30 s, 15000xg) resulting in an upper aqueous phase including the nucleic acid sample and a lower organic phase containing protein contaminations. Finally DNA is precipitated in a solution containing 0.3 M sodium acetate pH 5.2 and ice-cold abs. ethanol by overnight incubation. The precipitated nucleic acid is separated from the rest of the solution by centrifugation (60 min, 15000xg). The nucleic acid pellet is washed in ice-cold 70% ethanol and after a second centrifugation step ethanol is removed, and the nucleic acid pellet is allowed to dry before being resuspended in clean aqueous buffer. Subsequently, *in vitro* transcription was performed with the T7 RNA polymerase using the mMESSAGE mMACHINE T7 Ultra kit according to the manufacturer´s instructions. After enzymatic DNaseI digestion to remove the template DNA and subsequent enzymatic polyadenylation, the IVT RNA was purified using the RNeasy Mini Kit and verified by agarose gel (1.2%) electropheresis.

3.4.2 RNA electroporation of PBMCs

Unstimulated PBMCs (alternatively: DCs) were thawn, counted and washed twice with OptiMEM without phenol red and resuspended in OptiMEM at a concentration of 2.5-5 x 10^7/ml Electroporation was performed in a 4 mm cuvette with the GenePulser Xcell system applying a square wave pulse of 350 V, 12 ms, to 2.5-10 x 10^6 cells mixed with 20 µg IVT RNA in a total volume of 200 µl OptiMEM medium. Immediately after electroporation cells were transferred to a 6 well plate and resuspended with 3 ml fresh AIM V medium per well containing 10% HS without any further supplementation. Electroporated PBMCs/DCs were cultured for 4h at 37°C. Afterwards transfected PBMCs (DCs) were used for further experiments.

3 M sodium acetate, pH 5.5	Ambion (Darmstadt, D)
Agarose (for routine use)	Sigma-Aldrich Chemie GmbH (Munich, D)
CCD-cameras - monochrome FK-7512-IQ	Pieper (Schwerte, D)
Chloroform	Sigma-Aldrich Chemie GmbH (Munich, D)
Diethyl pyrocarbonate	Sigma-Aldrich Chemie GmbH (Munich, D)
Electroporation Cuvettes (4mm)	Peqlab (Erlangen, D)
Ethanol	Carl Roth GmbH (Karlsruhe, D)
Ethidium bromide solution	Sigma-Aldrich Chemie GmbH (Munich, D)
Formaldehyde solution 36,5%	Sigma-Aldrich Chemie GmbH (Munich, D)
GenePulser Xcell sytem	Bio-Rad (Munich, D)
mMESSAGE mMACHINE® T7 ULTRA Kit	Ambion (Darmstadt, D)
MOPS-EDTA-Sodium Acetate Buffer	Sigma-Aldrich Chemie GmbH (Munich, D)
NEBuffer 4	NEB GmbH (Frankfurt am Main, D)
Opti-MEM® I Reduced-Serum Medium (1x), liquid	Invitrogen/Gibco (Darmstadt, D)
Phenol-chloroform-isoamyl alcohol mixture	Sigma-Aldrich Chemie GmbH (Munich, D)
RNeasy Mini Kit	Qiagen (Hilden, D)
ssRNA ladder (500-9000 bases)	NEB GmbH (Frankfurt am Main, D)
Thermomixer 5436	Eppendorf AG(Hamburg, D)
UV Transilluminator UST-30M-8PC, 312 nm	Biostep (Jahnsdorf, D)
Wide Mini-Sub Cell GT Cell	Bio-Rad (Munich, D)
XbaI	NEB GmbH (Frankfurt am Main, D)

Table 14: Materials and equipment for molecular biology

3.5 Further materials

Aluminium foil	Carl Roth GmbH (Karlsruhe, D)
Autoclave	KSG Sterilisatoren GmbH (Olching, D)
Cell culture tube-racks	VWR (Darmstadt, D)
Centrifuge (Megafuge 1.0R)	Hereaus (Langenselbold, D)
CO2-Incubator	Binder (Tuttlingen, D)
Cryo tube-racks	VWR (Darmstadt, D)
Examination gloves	Semperet (Wien, A)
FACS tube-racks	VWR (Darmstadt, D)
Ice machine	Ziegra (Isernhagen, D)
Laboratory water purification system	Elga Bergfelde (Celle, D)
Minishaker MS2	IKA (Staufen, D)
Nitrogen cryo bank	Air liquide DMC (Marne-la-Vallée, F)
Nitrogen reservoir tank	Taylor Wharton XL-180, Tec Lab (Königstein, D)
Phase contrast microscope	Axiovert 25, Carl Zeiss AG (Jena, D)
Pipette device	Pipetus® Hirschmann Laborgeräte (Eberstadt, D)
Pipettes: 1-10 µl	Eppendorf AG(Hamburg, D)

20-200 µl, 200-1000 µl Multichannel 5-50 µl, 25-200 µl	Gilson (Villiers-le-Bel, F) Dunn Labortechnik GmbH (Asbach, D)
Precision scale	Precisa, PAG Oerlikan AG (Zürich, CH)
Refrigerator, Freezers (+4°C, -20°C, -80°C)	Hereaus, Kendro (Langenselbold, D)
Sterile work bench	NuAire (Plymouth, MN, USA)
Water bath	GFL (Burgwedel, D)
Water-jet vacuum pump	Brand (Wertheim, D)

Table 15: Laboratory equipment

Cell culture flasks (30 cm^2, 80 cm^2, 175 cm^2)	Greiner (Nürtingen, D)
Cell culture tubes (15 ml, 50 ml)	Greiner (Nürtingen, D)
Cryo boxes	Nalgene® Freezing Containers, Nunc (Wiesbaden, D)
Cryo tubes (1,8 ml)	Nunc (Wiesbaden, D)
FACS tubes	Sarstedt, (Nürnbrecht, D)
Ficoll separation tubes	Leucosep tubes, Greiner (Nürtingen, D)
Microtiter plates (96-well flat-, round-, V-bottom)	Greiner (Nürtingen, D)
Perfusor syringes (50 ml)	Braun (Melsungen, D)
Petri dishes (9 cm, 13 cm)	Greiner (Nürtingen, D)
Pipette single use (1/2/5/10/25/50 ml)	Greiner (Nürtingen, D)
Pipette tips (0.5-10 µl, 1-200 µl, 200-1000 µl)	TipOne Pipette Tips, Graduated Filter Tips, Starlab GmbH (Ahrensburg, D)
Reaction tubes (1.5 ml)	Eppendorf AG(Hamburg, D)
Sterile filter (0.22 µm, 0.45 µm)	Stertop®, Millipore (Eschborn, D)
Syringes (10 ml, 20 ml)	Braun (Melsungen, D)
Tissue culture plates (6-/24-/48-/96-well)	Greiner (Nürtingen, D)

Table 16: Plastic material

Beaker	Schott (Mainz, D)
Cell counting chamber (Fuchs-Rosenthal)	Schreck (Hofheim, D)
Duran glas flasks	Schott (Mainz, D)

Table 17: Glass material

4 Results and Discussion

4.1 Experimental procedure

Screening with PBMCs of immunocompetent healthy individuals who showed high and specific reactivity to the VZV-infected Vero cell extract in IFN-γ ELISpot assays

VZV-infected Vero cell extract filtration (0.2 µm) and separation of the highly immunogenic filtrate by RP-HPLC

Immunological screening of the separated fractions with earlier identified VZV-reactive PBMCs of immunocompetent healthy individuals by IFN-γ ELISpot assay

Tryptic digestion of the bioreactive fractions and mass spectrometric analysis of VZV-specific protein fragments by ESI-MS/MS

Identification of the corresponding VZV proteins by data bank analysis

Plasmids encoding for the identified VZV proteins were kindly provided by Prof. Ann Arvin, (Stanford University, MI, USA)

IVT RNA generation of the described plasmids

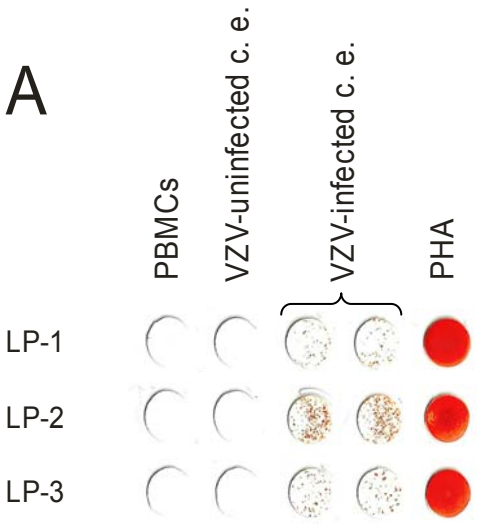
Establishing an electroporation protocol to transfect VZV-specific IVT RNA in PBMCs

Screening of VZV-specific IVT RNA transfected healthy donor PBMCs to identify the *in vivo* hierarchy and recognition pattern of VZV-reactive T cells.

Screening of VZV-specific IVT RNA transfected PBMCs of patients after allogeneic HSCT to identify the *in vivo* hierarchy and recognition pattern of VZV-reactive T cells in those immunocompromised patients.

4.2 VZV-specific cellular immunity in healthy individuals

In the starting experiments we screened *ex vivo* PBMCs of leukapheresis products from healthy individuals (Figure 5) on VZV reactivity in a previously established IFN-γ ELISpot assay (Distler et al. 2008) using commercially available VZV-infected/-uninfected Vero cell extracts as antigen sources.



B

PBMCs	VZV-uninfected c. e.	VZV-infected c. e.		PHA
2	1	184	210	>1000
2	3	406	397	>1000
1	0	111	114	>1000

Figure 5: VZV reactivity of healthy donor PBMCs. PBMCs of 3 leukapheresis products (LP-1 to LP-3) of 3 different healthy virus carriers were screened for VZV reactivity in the IFN-γ ELISpot assay. Each well contained 0.5 Mio PBMCs. VZV-infected/-uninfected Vero cell extracts had a final concentration of 25 µg/ml. Phytohemagglutinin (PHA) served as nonspecific positive control. PBMCs alone, and the VZV-uninfected Vero cell extract were used as negative controls. After 40 h of incubation IFN-γ spots were visualized **(A)** and measured by a computer-controlled ELISpot reader and quantified **(B)** by the KS ELISpot software 4.9. The high spot number induced with PHA was not exactly quantified by the ELISpot reader and therefore estimated.

We chose VZV-infected/-uninfected Vero cell extract concentrations of 25 µg/ml in the screening experiments because we observed maximum reactivity using this concentration, which was already shown by Distler et al in 2008. Previous investigators have already used a whole VZV cell extract as antigen format containing a variety of VZV-derived glycoproteins, regulatory, structural, and nonstructural proteins to allow for highly sensitive and specific quantitative determination of VZV-specific T cells, including immunity post-immunization (Sauerbrei and Wutzler 2006). Furthermore, viral gene transcription and the relative expression profile of VZV ORFs during lytic infection of African green monkey cells (VERO cells) is well described by Cohrs et al (Cohrs et al. 2003). In line with these previous data the VZV-infected Vero cell extract enriched for viral proteins served herein as a reliable antigen format determining VZV-specific cellular immunity using PBMCs of healthy individuals shown in Figure 5. Additionally, the VZV-infected Vero cell extract as protein source was sufficient for the identification of immunodominant VZV proteins by mass spectrometry shown in Figure 8.

The repertoire of VZV-specific T cells during viral reactivation in PBMCs of healthy virus carriers and immunocompromised patients is mainly made up of memory T cells, generated after primary VZV infection (Arvin et al. 1986). Memory T cells, the main mediators of cytokine production after viral reactivation and antigen recognition via MHC molecules, produce cytokines like IFN-γ, interleukin-2 (IL-2), and tumor necrosis factor alpha (TNF-α), (Guidotti and Chisari 2000). Therefore, we selected IFN-γ as a reliable cytokine which is critical for innate and adaptive immunity against viral and intracellular bacterial infections and for tumor control. IFN-γ is produced predominantly by NK and NKT cells as part of the innate immune response, and by $CD4^+$ and $CD8^+$ cytotoxic T lymphocyte effector T cells once antigen-specific immunity has developed (Malmgaard 2004). *In vivo* cytokines modulate the inflammatory response, expanding and recruiting antigen-specific CTLs to the site of viral infection or reactivation. Cytokines made by $CD4^+$ T cells are also required to induce B cells to produce antibodies of the IgG, IgM and IgA subclasses that can bind to VZV proteins or mediate antibody-dependent cellular cytotoxicity (ADCC). In the above and in the following ELISpot experiments phytohemagglutinin (PHA) served as positive control. PHA is found in highest concentrations in uncooked kidney beans and served herein as mitogenic compound to trigger cell division in T cells, which finally led to nonspecific IFN-γ production. PBMCs of healthy individuals without any background reactivity were selected for further experiments. Background reactivity is defined as antigen-nonspecific IFN-γ production detected with unstimulated PBMCs or with PBMC wells containing the VZV-uninfected Vero cell extract.

The level of VZV-specific IFN-γ spots and consequently the frequency of VZV-specific T cells detected by incubating PBMCs of healthy donors with the VZV-infected Vero cell extract in IFN-γ ELISpot assays varied between the donors, because of reasons like age, and previous histories of subclinical or clinical VZV reactivations. The age dependent disappearance of the T cell memory response is very obvious and has been reported by several researchers studying VZV epidemiology and pathogenesis (Arvin 1996; Wharton 1996; Leung et al. 2000). Subclinical re-infections and sporadic reactivations of latent virus (Chapter 2.3) boosting VZV-specific immunity without any signs of clinical manifestation are important factors for refreshing and improving VZV-specific immunity (Arvin et al. 1983; Ljungman et al. 1986). PBMCs of selected healthy individuals with strong VZV-reactivity (Figure 5) were used for VZV-specific fraction screening and for establishing a reliable RNA electroporation protocol in the second part of this thesis.

4.2.1 MHC blockade experiments

In blocking experiments using HLA-class-I (W6/32) and HLA-class-DR (HB-55) monoclonal antibodies and additionally in $CD4^+$/$CD8^+$ subpopulation experiments (data not shown) we found that antiviral $CD4^+$ memory T cells were the main IFN-γ producers in PBMCs of healthy donors as well as in PBMCs of patients after allogeneic HSCT (Distler et al. 2008). In detail the results showed that PBMC reactivity was only blocked by anti-HLA-DR (i.e. HLA-class-II), but not HLA-class-I antibodies. These findings suggested that the endosomal uptake of the VZV-infected Vero cell extract and the lysosomal degradation of proteins to peptides were mainly mediated by the MHC class II pathway (anti-HLA-DR blockade) and consequently the presentation was privileged to $CD4^+$ T cells (Sadzot-Delvaux et al. 1997; Jones et al. 2006). Previously some research groups have described and characterized VZV-specific memory $CD4^+$ T cell epitopes and additionally have analyzed the expression of phenotypic markers on virus-specific memory T cells in several publications (Huang et al. 1992; Vossen et al. 2004; Jones et al. 2007; Malavige et al. 2008). $CD8^+$ T cell responses are paramount in immunity to respiratory viruses, but they may be much less important for VZV-specific immunity. Comparable findings were also documented by Asanuma et al., who showed that VZV-specific $CD4^+$ T cells but not $CD8^+$ T cells could be detected using stimulation with viral lysate but that both human cytomegalovirus (HCMV)-specific $CD8^+$ and $CD4^+$ T cells were readily detectable (Asanuma et al. 2000).

HCMV persistence and replication in cells of the monocyte/macrophage lineage may require more active immune surveillance than it is required to contain VZV and HSV within cells of the sensory ganglia. Additionally a lot of mechanisms are currently known in herpesviruses, which downregulate the MHC class I expression on virus-infected cells due to a blockade of the MHC class I pathway (Cohen 1998; Abendroth and Arvin 2001; Eisfeld et al. 2007). VZV-specific proteins block important enzymes during protein degradation in the infected cell and prevent thereby the presentation of viral proteins to VZV-specific $CD4^+$ and $CD8^+$ T cells. Finally, decreased stimulation and proliferation of $CD8^+$ T cells might lead to anergy and apoptosis of antigen-specific $CD8^+$ T cells.

However, significant VZV reactivity caused by $CD8^+$ T cells was observed in allogeneic HSCT patients directly after viral reactivation published by Distler et al. These findings have demonstrated that even though the VZV-infected Vero cell extract might be a suboptimal antigen format to detect VZV-specific $CD8^+$ T cells in PBMCs of healthy donors, its use within the IFN-γ ELISpot assays proved sufficient to visualize antiviral $CD8^+$ T cell responses early after zoster onset. Furthermore, *ex vivo* stimulated and expanded VZV-specific $CD8^+$ T cells were generated by Frey et al (Frey et al. 2003). Later on van der Heiden et al. (van der Heiden et al. 2009) have demonstrated that VZV-specific $CD8^+$ T cells are detectable without prior *in vitro* stimulation in patients with VZV reactivation following T cell depleted allogeneic HSCT. In these experiments potential HLA-A2 peptides were tested for their binding charac-

teristics and afterwards selected peptides were used to generate VZV-specific pentamers, which led to the first documented class-I epitope of VZV. In all following experiments we did not use $CD4^+$ or $CD8^+$ subpopulations. The focus in this study is on *ex vivo* PBMCs without any prior stimulation to maintain the *in vivo* hierarchy of VZV-specific T cells.

4.3 VZV reactivation in patients after allogeneic HSCT

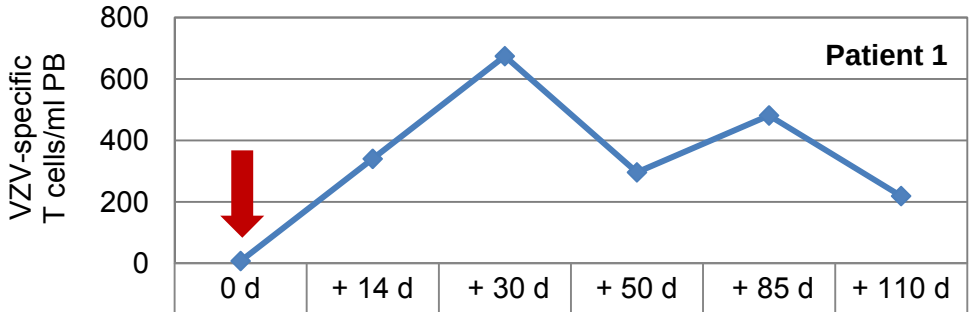
We started collecting blood samples (30 ml heparinized blood) from patients after allogeneic HSCT directly at clinical occurrence of VZV reactivation and constantly continued collecting blood samples for several months after zoster onset. VZV reactivation in allogeneic HSCT patients was clinically diagnosed due to the typical dermatomal rash and laboratory confirmation evaluated by PCR analysis of blood samples in the Institute of Virology in Mainz (Chapter 2.3.3). The antiviral medication was given directly at clinical onset and discontinuation of antiviral medication was performed as soon as two consecutive PCR results were negative. Prophylaxis with oral acyclovir or valacyclovir is currently recommended for seropositive allogeneic HSCT patients, for 1 year, or longer in the presence of GVHD and immunosuppressive therapy. Several studies are available, where the administration of long term acyclovir for 6 months post allogeneic HSCT is described (Chapter 2.5.2). They demonstrated that viral reactivation was prevented during acyclovir administration, but occurred frequently after discontinuation of prophylaxis (Ljungman et al. 1986; Perren et al. 1988). PCR is the most sensitive method for detecting VZV DNA in clinical samples and has markedly improved the diagnosis of VZV infection (Koropchak et al. 1991; Kalpoe et al. 2006). Quantitative monitoring of circulating VZV DNA in HSCT patients is also useful for assessing the response to treatment of visceral VZV infections without skin manifestations (de Jong et al. 2001; Ishizaki et al. 2003).

Two of three PCR results of the screened blood samples from allogeneic HSCT patients were positive in the beginning of viral reactivation and became negative during antiviral treatment. Some patients, like patient 3 were continuously VZV PCR positive, which led to a prolongation of antiviral treatment until two consecutively negative PCR results. In patient 1 the typical dermatomal rash had indicated the viral reactivation and antiviral treatment was given, although the PCR results were negative. Medication was stopped until the negative PCR result was validated two weeks later. Certainly, VZV is detected in dermatomal lesions for a longer period of time, but studying the lesions on reactivated virus is not established as a clinical routine in immunocompromised patients after allogeneic HSCT. Much more important is the exclusion of viral generalization via blood stream, detected in blood samples, to avoid organ manifestation, which might lead to a severe progression of VZV reactivation in patients after allogeneic HSCT.

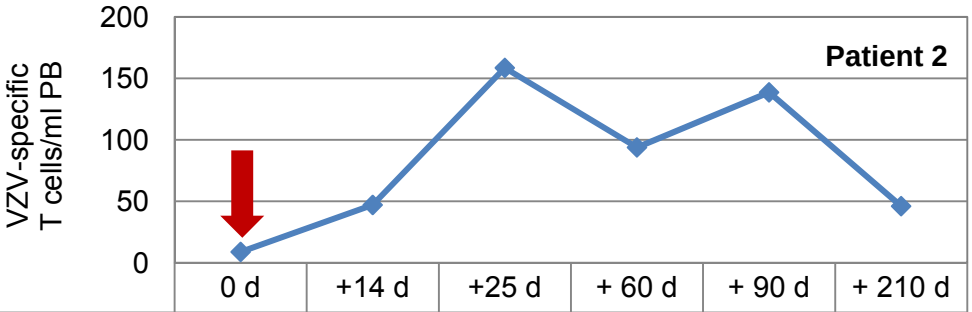
T cell mediated VZV-specific immunity was screened in Ficoll-isolated PBMCs of the collected blood samples and corresponding sera were examined for VZV-specific IgG and IgM antibodies. Posttransplantational recovery of VZV specific T cell immunity was very well demonstrated in all patients participating in this study and documented in further patients after allogeneic HSCT (Distler et al. 2008). The number of circulating antiviral T cells measured by IFN-γ ELISpot assay was extremely low up to zero directly before zoster onset had occurred. VZV-specific T cell counts were increased dramatically within 2-4 month after zoster manifestation and remained at significant levels for several months (Figure 6). Most of the allogeneic HSCT patients have developed zoster in the first year after undergoing bone marrow transplantation or beyond this time if undergoing prolonged immunosuppressive therapy with T cell depleting agents or having chronic GVHD. The very low number of circulating VZV-specific memory T cells in these patients appears as the main reason for insufficient protection in the period directly after allogeneic HSCT till viral reactivation, which then finally results in herpes zoster. The frequency of VZV-specific T cells at the time point of maximum IFN-γ production ranged from 0,010% (103 spots/1Mio PBMCs) to 0,045% (453 spots/1Mio PBMCs) in screened PBMCs of allogeneic HSCT patients. These frequencies of VZV-specific reactive T cells are comparable to frequencies of approximately 0.04% of VZV-specific memory T cells in PBMCs of healthy individuals (Hayward and Herberger 1987; Arvin and Gershon 1996). In comparison, HSV-specific T cells are being measured at nearly equal frequencies to VZV-specific T cells, and CMV-specific T cells are reported to be 10 fold higher (Asanuma et al. 2000).

In contrast to the significant increase of VZV-specific T cells in all patients screened upon zoster onset, the humoral immunity composed of VZV-specific IgG and IgM antibody levels varied during zoster manifestation. In patient 1 VZV-specific IgG levels showed a strong increase during zoster manifestation, and in patient 2 and 3 VZV-specific IgG levels did not alter significantly. VZV-specific IgM responses were generated in the acute phase of the disease and were detected in sera of all three HSCT patients. However, VZV-specific IgM antibodies were not detected in several other patients after allogeneic HSCT. These data were already published by Distler et al (Distler et al. 2008). In summary, the humoral immune response alone is not sufficient to cope with the reactivated virus in every patient and for that reason VZV-reactive T cells appear as the main mediators of the VZV-specific effector immune response after viral reactivation. Moreover, patients with X-linked agammaglobulinemia whose VZV-specific cell-mediated immunity responses are relatively intact are not at increased risk of herpes zoster (Oxman et al. 2005; Levin et al. 2008). Therefore, it is clear that the level of cell-mediated immunity to VZV determines the risk and severity of herpes zoster and PHN (Chapter 2.3.3), whereas antibodies to VZV appear to play a less significant role.

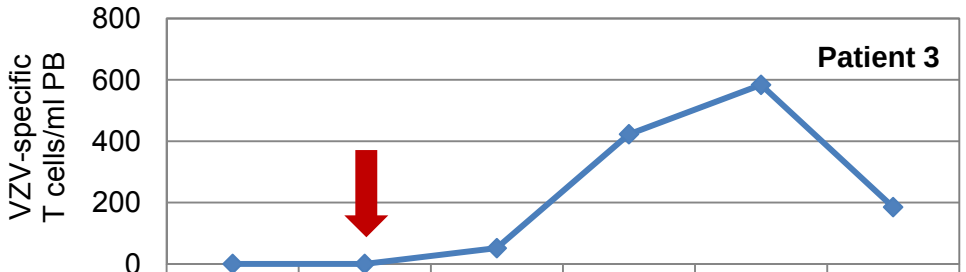
Patients who did not develop symptomatic VZV reactivation during the first year after undergoing the same allogeneic HSCT protocol (Meyer et al. 2007) were well characterized by Distler et al (Distler et al. 2008). These patients had shown no signs of VZV-specific cellular immunity measured by IFN-γ ELISpot assay and thus they were at high risk for developing herpes zoster later on. In addition, symptomatic VZV reactivation was required to boost antiviral T cells in patients after allogeneic HSCT. These findings suggest that a vaccination approach, as a substitute for natural resenzitation by virus reactivation, may help to accelerate the reconstitution of VZV-specific T cell immunity in allogeneic HSCT recipients (Redman et al. 1997; Hata et al. 2002). Therefore, the aim of this study was to use the VZV-infected Vero cell extract as an antigen source for the following experiments to identify immunodominant VZV proteins by RP-chromatography and quantitative mass spectrometry.



	0 d	+ 14 d	+ 30 d	+ 50 d	+ 85 d	+ 110 d
Frequency/ml PB	7,5	340	674	296	481	219
IgG (mIU/ml)	700	2000	2000	2000	2000	2000
IgM	-	+	+++	+	-	-
PCR	-	-	-	-	-	-



	0 d	+14 d	+25 d	+ 60 d	+ 90 d	+ 210 d
Frequency/ml PB	9	47	159	94	139	46
IgG (mIU/ml)	650	1100	700	1100	940	875
IgM	-	+	-	-	-	-
PCR	+	-	-	-	-	-



	-20 d	0 d	+ 30 d	+ 50 d	+ 85 d	+ 125 d
Frequency/ml PB	0	0	51	423	584	185
IgG (mIU/ml)	240	120	490	510	440	500
IgM	-	+++	++	++	+	-
PCR	-	+	+	+	+	+

Figure 6: Detection of VZV-specific T cells during clinical VZV reactivation in patients after allogeneic HSCT. PBMC samples were screened for VZV-specific T cells/ml peripheral blood (PB) in the IFN-γ ELISpot assay with 0.5 Mio PBMCs/well (blue line). Same time points were screened on humoral immunity as well, determining VZV-specific IgG (pos. > 100 IU/ml; max. 2000 IU/ml) and IgM (qualitative) antibodies. Finally each time point was screened using VZV-specific primers by PCR analysis. The clinical onset referred to as zoster onset is highlighted with red arrows. After 40 h of incubation IFN-γ spots were visualized and measured by a computer-controlled ELISpot reader and quantified by the KS ELISpot software 4.9.

4.4 HPLC separation of the VZV-infected Vero cell extract

In the first experiments we separated the VZV-infected Vero cell extract by gel filtration. This method is based on protein separation according to molecular weight and protein size. The viral cell extract was separated under standard conditions (PBS), denaturizing conditions (8 M urea), or using detergents like CHAPS. Separated fractions were subsequently analyzed for reactivity in PBMCs by IFN-γ ELISpot assay. However, VZV-specific reactivity was spread all over the eluted fractions (data not shown). These results led us to the conclusion that the complexity of the sample was not adequate to allow for sufficient fractionation by gel filtration as chromatographically method and we decided to switch over to reverse phase (RP) chromatography. For RP chromatography we first filtrated (0.2 µm) VZV-infected and -uninfected cell extracts to exclude cell debris from the sample prior to HPLC injection. Afterwards filtrate and retentate were screened for VZV reactivity in PBMCs by the IFN-γ ELISpot assay (Figure 7). The result demonstrated that most of the reactivity was present in the filtrate of the VZV-infected Vero cell extract (100 fold more reactivity) and less in the retentate. There was no reactivity detectable in the VZV-uninfected control cell extract, as expected. Consequently, the filtrate of the VZV-infected Vero cell extract was separated by RP chromatography using a C-12 column with a pore diameter of 90 Å suitable for the separation of larger peptides and smaller proteins in the range of 1-50 kDa. Collected fractions were concentrated by speed-vac and subsequently reconstituted in PBS for screening by IFN-γ ELISpot assay using 0.5 Mio PBMCs of healthy individuals (LP-1 to LP-3) per well.

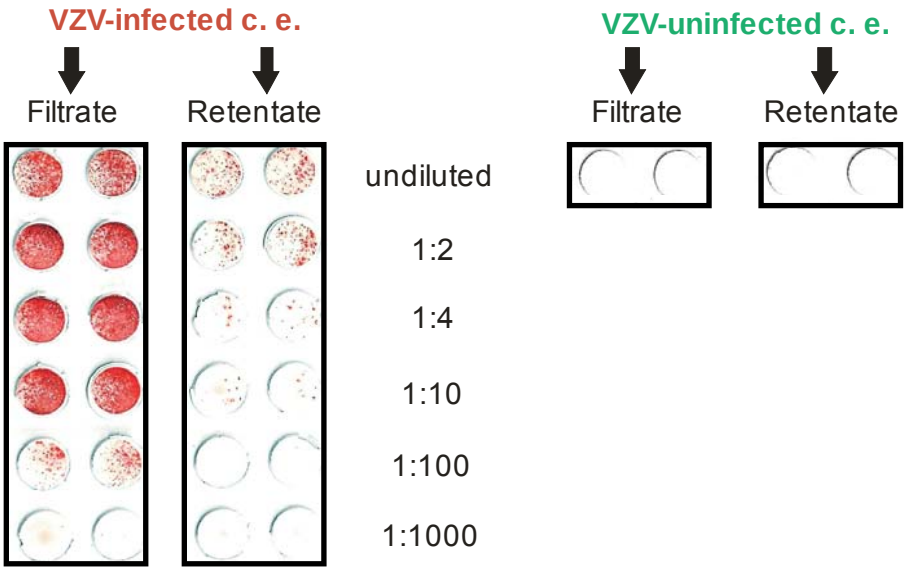


Figure 7: Superior VZV-specific reactivity in 0.2 µm filtrate compared to retentate. VZV-infected/-uninfected Vero cell extracts were initially filtrated (0.2 µm). Filtrate and retentate of both cell extracts were subsequently titrated in PBS and screened on VZV reactivity in a 40 h IFN-γ ELISpot assay using 0.5 Mio PBMCs per well. PBMCs of LP-2 were used for the titration experiment.

Fractions A1-G9 did not show any reactivity in the separation range for hydrophilic peptides and low molecular weight compounds (data not shown). However, clear reactivity above background (< 5 spots) was found in fractions with high acetonitrile concentration (G10-H1) illustrated in green indicating the high hydrophobicity and/or size of the eluted proteins of the VZV-infected Vero cell extract (Figure 8).

The separating conditions were improved in several HPLC separation experiments until we reached a satisfying peak resolution and fractionation interval. High molecular weight material was certainly not efficiently separated on the C-12 column that is suitable for peptides and smaller proteins, but the resolution profile of the reactive proteins was not an important factor for the subsequent identification of immunodominant VZV-specific proteins by mass spectrometry shown in Figure 9. Instead, high molecular weight proteins in the range of 50 to 300 kDa can be more efficiently separated by a C-4 or C-5 column with a pore diameter of 300 A. We expected VZV protein sizes predominantly in the range of 1 to 250 kDa, because the molecular mass of 50 of the known 71 VZV proteins is smaller than 50 kDa (Human herpesvirus 3 strain Dumas).

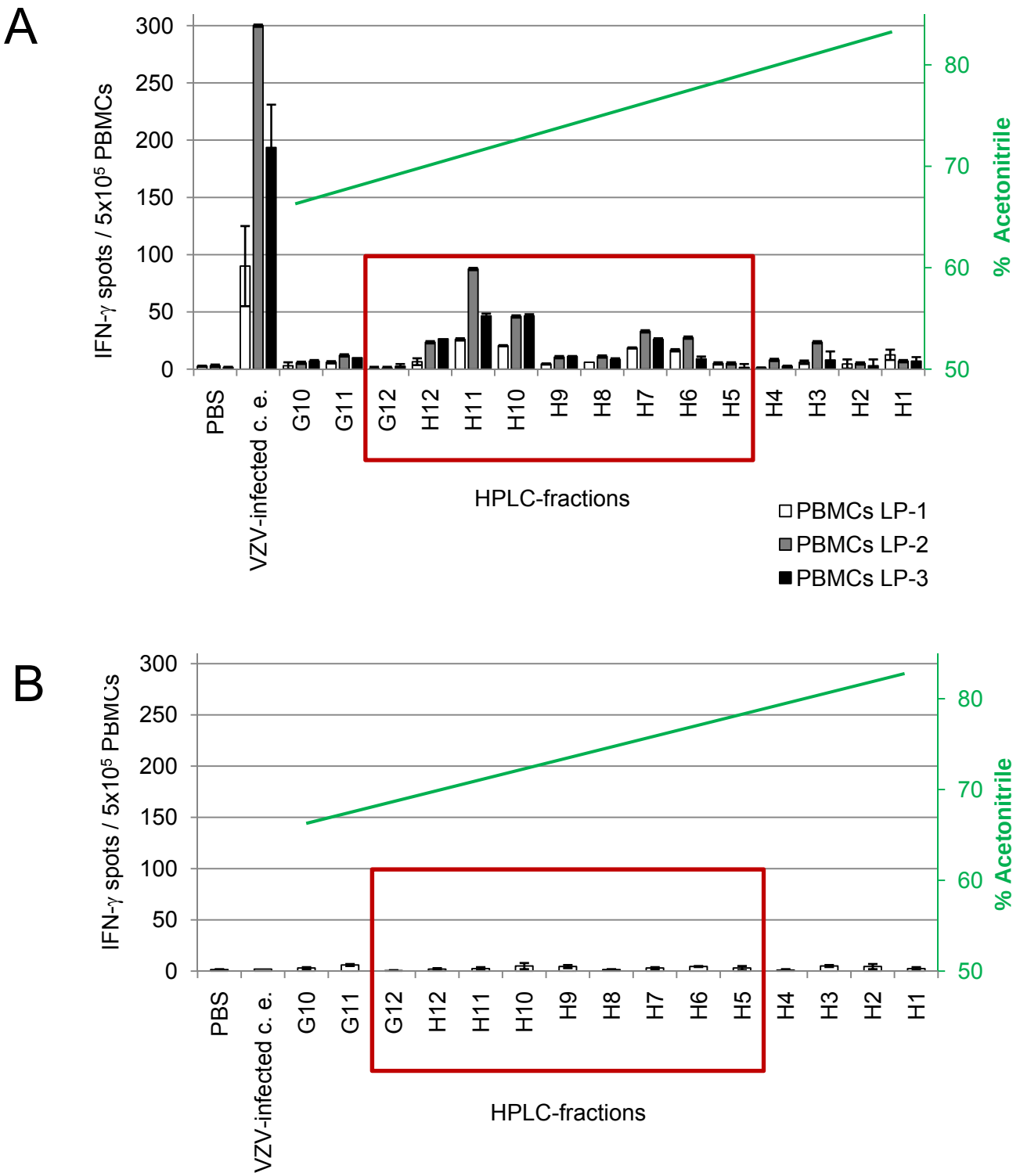


Figure 8: VZV fraction screening after RP-HPLC separation. In **Figure 8A** HPLC fractions (A1-H1) separated by RP chromatography were screened for IFN-γ ELISpot reactivity with PBMCs of three healthy individuals (LP-1 to LP-3). In fractions A1-F12 no IFN-γ ELISpot reactivity was detected and therefore data from these fractions were not shown in this figure. Each well contained 0.5 Mio PBMCs. PBS served as negative control and the un-separated VZV-infected Vero cell extract as positive control. Additionally the percental acetonitrile concentration is shown to visualize the hydrophobicity of the eluated VZV-specific proteins (green). Fractions selected for MS peptide sequencing are highlighted in the red square. In **Figure 8B** the corresponding spontaneous release of IFN-γ in fractions G10-H1 is demonstrated. Fractions were incubated under the same conditions without PBMCs. After 40 h of incubation IFN-γ spots were visualized and measured by a computer-controlled ELISpot reader and quantified by the KS ELISpot software 4.9.

4.5 Mass spectrometric analysis of VZV-reactive fractions

VZV-infected cell extract fractions with bioactivity in ELISpot assay (G12-H5) were analyzed by quantitative mass spectrometry after tryptic digestion and nano-HPLC separation of fraction content. During tryptic digestion, all proteins included in the VZV-reactive fractions were cleaved mainly at the carboxyl side of the amino acids lysine or arginine, except they are followed by proline. Cleaved peptide fragments were subsequently once again separated by reverse phase chromatography and automatically injected in the mass spectrometer. Peptide fragments found in these fractions by ESI-MS were compared to data basis containing entire VZV proteins (ROD strain) and proteins of different monkey cell lines (UniProtKB/Swiss-Prot Protein Knowledgebase, Chapter 3.1.3.5).These monkey cell lines were chosen due to the close genetic relation to the African green monkey cell line, which was used as host cell line for viral infection and replication and is by now not fully sequenced. VZV specific protein fragments found in the sequenced fractions are shown in Table 18. Vimentin was added in each VZV-reactive fraction as internal control and the digested Vimentin peptides were also detected by ESI-MS shown in Figure 10. We detected several peptides derived from three different VZV proteins in these fractions. VZV glycoprotein E (69,9 kDa) and intermediate early protein 62 (139,9 kDa) are well described in the literature as proteins containing T-cell epitopes in contrast to VZV glycoprotein B (105,3 kDa), until now lacking evidence to play an important role as a target of the VZV-specific immune response (Arvin et al. 1986; Arvin et al. 1991; Bergen et al. 1991; Sharp et al. 1992; Fowler et al. 1995; Lowry et al. 1997).

VZV protein	Mass (kDa)	Peptide MH+ (Da)	Identified peptide sequence	Score
IE62	139,9	1034,57	(R)SLETVSLGTK(L)	4075
IE62	139,9	1635,75	(R)SHAPDPIEDDSPVEK(K)	2086
IE62	139,9	1974,03	(R)EHEIVSIPSVSGLQPEPR(T)	2886
IE62	139,9	1309,66	(R)KAYCTPETIAR(L)	870
VGLB	105,3	1526,83	(R)VPIPVSEITDTIDK(F)	7427
VGLB	105,3	1409,69	(R)DTGLLDYSEIQR(R)	3022
VGLB	105,3	840,49	(R)ALLEPAAR(N)	1939
VGLB	105,3	1677,81	(R)TGTSVNCIIEEVEAR(S)	1490
VGLE	69,9	1223,59	(K)EDQLAEISYR(F)	6728
VGLE	69,9	1493,83	(K)EITPVNPGTSPLLR(Y)	3037

Table 18: VZV-specific protein fragments detected by ESI-MS. Sequenced VZV-specific peptides in the single amino acid letter code with the highest scores are listed. Peptide masses of the detected fragments and the masses of the corresponding proteins are shown. The score is calculated by ProteinLynx Global Server (Waters, Eschborn, D) and scores > 200 are categorized as results < 0.1% false positive. The mass spectrum of the highlighted VZV-specific protein fragment (light grey) is shown in Figure 9.

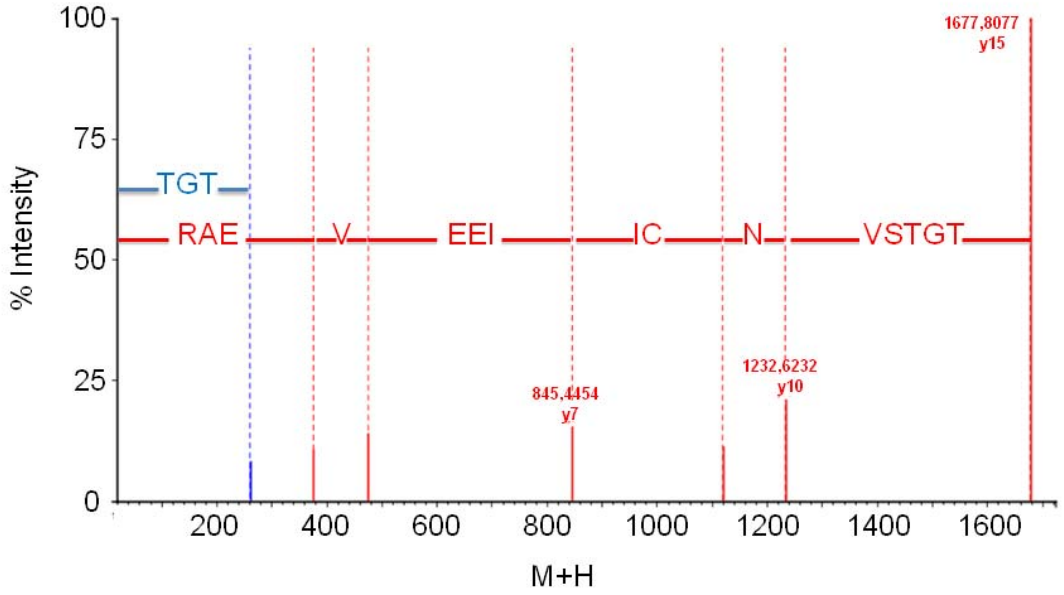


Figure 9: Mass spectrum of the VZV-specific gB protein fragment (R)TGTSVNCIIEEVEAR(S) detected in fraction H6 and H7. The mass spectrum shows the detected protonated molecular ions (M+H) of the identified peptide which is part of VZV gB. The amino acids are shown in the single letter code. The intensity of the detected protonated molecular ions is given in percental of maximum intensity measured. The molecular mass (Da) and the C-terminal fragment number (y) of the detected peptide fragments with the highest intensity are shown.

It has already been shown that the main effectors recognizing these two immunodominant VZV proteins are $CD4^+$ T cells, like we observed in our experiments using the VZV-infected Vero cell extract. However, some research groups discovered $CD8^+$ T cell responses to IE62 after *in vitro* stimulation of $CD8^+$ T cells with peptide bound pentamers (Frey et al. 2003; van der Heiden et al. 2009). Arvin et al. have described the induction of both, $CD4^+$ and $CD8^+$ VZV-specific T cells by VZV infection which target the IE62 protein to a similar extent as the major VZV glycoprotein, gE (Arvin et al. 1986; Arvin et al. 1991; Bergen et al. 1991). Functionally, IE62 is similar to HSV-1 ICP4 in that it is a potent transcriptional activator. IE62 can stimulate the transcription of all VZV genes and also repress its own transcription (Disney et al. 1990; Perera et al. 1992; Moriuchi et al. 1994). IE62 binds to specific DNA sequences similar to those sequences that bind HSV-1 ICP4, although there is a wide divergence from a consensus sequence motif.

VZV gE is one of the most immunogenic proteins and is a major target for the cellular and humoral immunity (Arvin et al. 1986). Neutralizing antibodies are well described and recognize distinct domains of gE (Wu and Forghani 1997). VZV gE recycles from the surface of the cytoplasmic membrane by internalization (Zhu et al. 1996). It is phosphorylated on multiple sites and the cytoplasmic tail of gE is target for several protein kinases (Grose et al. 1989). VZV gE is noncovalently linked to glycoprotein I, like in other herpesviruses and results in co-precipitation of gI with the help of gE-specific monoclonal antibodies (Vafai et al. 1989; Yao et al. 1993). It is not known from the literature whether gB induces a cellular VZV-specific immune response, but it is obvious that most of the glycoproteins are expected to be targets of the initial host immune response, because they are virion envelope proteins and expressed in high numbers on the viral surface (Cohrs et al. 2003). Furthermore, it has been demonstrated that gB, like gE is a target for neutralizing antibodies (Massaer et al. 1993). Additionally, gB is a dominant target and has been extensively studied in HCMV infections. A neutralizing antibody response as well as $CD4^+$ T cell response was found in several studies regarding HCMV (Marshall et al. 1992; Beninga et al. 1995; Hopkins et al. 1996). The gB proteins are the most highly conserved proteins upon alphaherpesviruses and it is likely that VZV gB, like its HSV-1 and HCMV homologues, is probably important for adsorption and fusion of the viral membrane to the cell membrane (Pereira 1994; Britt and Mach 1996).

The IFN-γ ELISpot profile and the corresponding detected proteins in fractions G12-H5 are shown as overlay in Figure 10. To make clear which are the immunodominant proteins and therefore main mediators of cellular VZV-specific immunity we decided to focus on every single detected VZV protein, except for glycoprotein I, which was detected only in fraction G6. There was not any VZV-specific IFN-γ ELISpot reactivity detectable in this fraction using PBMCs of 3 healthy donors (LP-1 to LP-3) shown in Figure 5. This observation suggested that glycoprotein I is not an immunodominant VZV protein.

We primarily focused on the other three VZV proteins (i.e. gE, gB and IE62) in the upcoming experiments. The IFN-γ ELISpot profile of fractions G12-H5 was closely related to the MS fragment detection pattern of gE. This finding led us to conclude that gE is probably one of the main compounds of the VZV-infected Vero cell extract, which was documented by the strong Western blot reactivity of anti-gE antibodies within the VZV-infected Vero cell extract shown in Figure 13. The detection pattern of IE62 fragments was very similar to the detection pattern of gE fragments with the exception that IE62 fragments, like gB fragments were not detected in fractions H10, H9 and H8, where high levels of IFN-γ reactivity with PBMCs of 3 healthy donors (LP-1 to LP-3) were measured. This observation indicated the recognition of gE by VZV-reactive T cells and probably the immunodominance of this VZV-specific protein. The ranking and importance of gE, gB and IE62 in the hierarchy of immunodominant VZV proteins had to be explored in the following experiments.

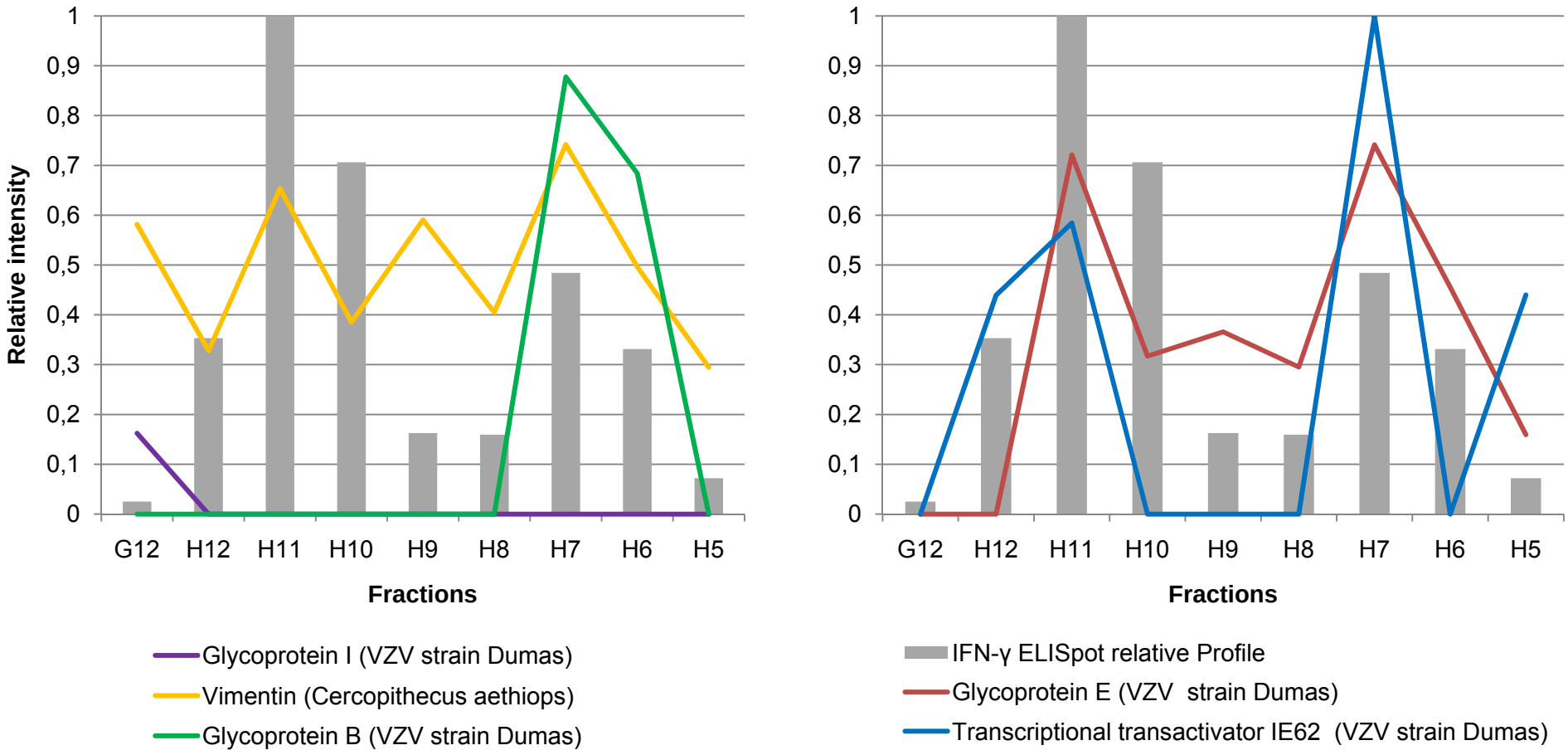


Figure 10: Overlay of the abundance of the identified VZV-specific proteins in quantitative ESI-MS and the corresponding profile of the IFN-γ ELISpot reactivity. The detected VZV proteins are shown in different colors (purple, green, red, and blue). VZV proteins are divided on two graphs with the same IFN-γ ELISpot profile (grey bars) as background scheme to simplify the overview. The IFN-γ ELISpot profile is demonstrated as mean IFN-γ reactivity of LP-1 to LP-3 (mean spot numbers per $5x10^5$ PBMCs) shown in Figure 8. Vimentin (yellow) was added in each fraction as internal standard.

4.6 Transfection of VZV-specific RNA in PBMCs

4.6.1 RNA purity control by agarose gel electrophoresis

In the following experiments we analyzed PBMCs of healthy individuals transfected by electroporation with RNA encoding for the earlier MS-identified VZV proteins. PcDNA™3.1 plasmids were kindly provided by Prof. Ann Arvin, Stanford University, USA. PcDNA™3.1 is a 5.4 kb vector designed for high-level stable and transient expression in mammalian hosts. PCR fragments of full length gE (1869 bp), gB (2793 bp), and IE62 (3930 bp) were cloned into the Invitrogen vector pcDNA™3.1, which puts the ORFs under the control of the CMV promoter (Kinchington et al. 2000; Berarducci et al. 2009; Oliver et al. 2009). We started our work with the linearization of the plasmids using the restriction enzyme XbaI and subsequently in vitro transcribed (IVT) the cDNA with the T7 RNA polymerase. IVT RNA was polyadenylated, increasing the RNA stability and purified by phenol/chloroform extraction. The length and purity of native and polyadenylated RNA was proven by RNA gel electrophoresis in comparison to a marker RNA ladder, shown in Figure 11.

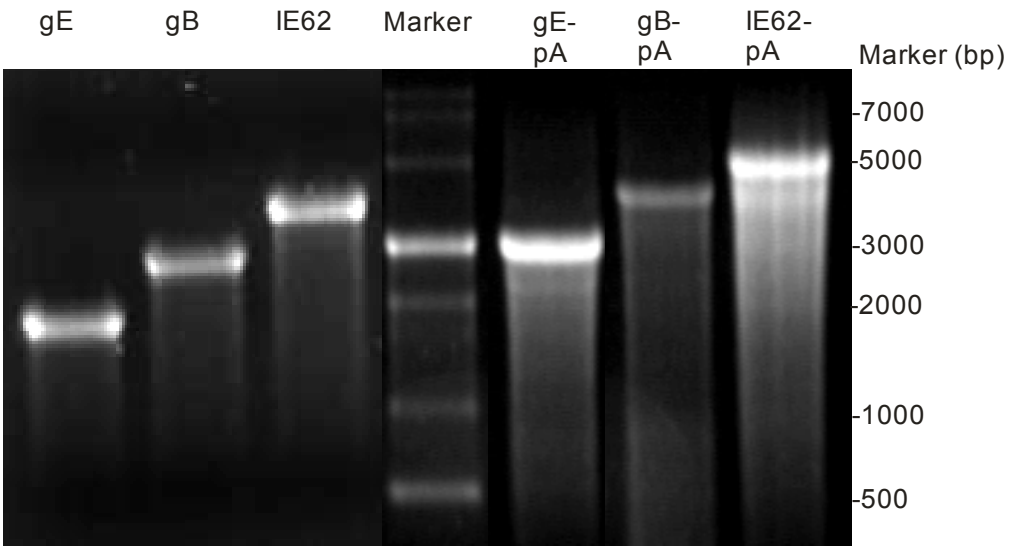


Figure 11: RNA gel electrophoresis to visualize length and purity of the IVT RNAs encoding VZV proteins. The plasmids provided by Prof. Arvin were linearized and in vitro transcribed into mRNA and subsequently polyadenylated (pA) to increase the RNA stability. Polyadenylated and native RNA were compared to a marker RNA ladder (500-7000 bp) after separation on agarose gel (1.2%) electrophoresis. The RNA was visualized in an UV-transilluminator.

Native and polyadenylated RNAs of gE, gB, and IE62 were separated by agarose gel electrophoresis. Both, native and polyadenylated IVT RNAs of IE62, gB and gE were reliably fractionated by gel electrophoresis and the corresponding bands were detected at the expected RNA marker length. In the process of polyadenylation at the 3´ terminus of the IVT RNA, the latter was efficiently tailed with at least 150 adenines (up to 1000 adenines) regarding the manufacturer instructions. Finally, all three polyadenylated RNAs could be used for further experiments. The concentration of purified polyadenylated RNA was measured photometrically. RNA was frozen in an amount of 20 µg RNA per tube.

4.6.2 Transfection parameters

Transfection of mRNA by electroporation is a safe, highly efficient and clinically applicable method for antigen expression (Van Driessche et al. 2009). Most of the reported transfection methods require *in vitro* stimulation of autologous APCs, like monocyte-derived DCs prepared from PBMCs. Furthermore, the use of DCs in immunomonitoring assays, like the IFN-γ ELISpot assay depends on the availability of a large number of APCs, which leads apriori to a time-consuming and costly *in vitro* culture from precursor cells isolated from a significant amount of blood samples, since the fraction of DC precursors is only 0.1-0.5% of human PBMCs (Cavanagh and Von Andrian 2002). Beside this, monocyte-derived DCs also initiate primary responses which might result in overestimation of the target-specific *in vitro* detected recall response (Knight et al. 2002). Alternatively, it was recently demonstrated that mRNA could be efficiently electroporated into PBMCs and mRNA-transfected PBMCs were able to induce antigen-specific CTLs *in vitro* (Schaft et al. 2006; Hiura et al. 2007).

The starting transfection experiments were used to adjust electroporation conditions and parameters, like the electrical pulse (duration, voltage and interval). Electric field strength and pulse duration are key parameters to maximize transfection efficiency and maintain cell viability (Rols and Teissie 1998). Charged molecules like RNA would never passively diffuse across the hydrophobic lipid bilayer core of PBMCs or DCs. Therefore the electrical pulse generated by a square wave opens up a pore which acts as a conductive pathway through the lipid bilayer (Rols 2006). Every cell type requires slightly different electroporation conditions that must be identified experimentally. In the developed standard protocol for PBMCs (Chapter 3.4.2) we applied a single electrical pulse of 350 V for 12 ms (Van Camp et al.). In addition to the electrical parameters, buffer composition influences transfection efficiency and cell viability as well (Heiser 2000). Electroporation is also influenced by cell condition and density as well as nucleic acid concentration and type. The optimal mRNA amount for all following experiments is the most important parameter and was defined as 20 µg RNA (Figure 12). Larger RNA amounts of 30 µg showed fewer IFN-γ spots especially during transfection of IE62 and gE, which appeared to be a consequence of RNA saturation ef-

fects. RNA saturation might lead to high competition among the transfected RNA and the endogenous pool of mRNAs for the intracellular machinery that processes RNA. As a consequence, transfected APCs might become less efficient stimulators due to the overdriven cellular machinery and the inefficient cellular signaling. On the other hand this saturation effect may allow the nonspecific background signal to increase due to the enhanced cellular stress. Nonspecific IFN-γ production was slightly observed in the highest RNA concentration (30 µg) as confirmed by GFP transfection. To minimize IFN-γ background spot production, cytokines, like IL-2 or IL-7 were not added to PBMCs. All the work was performed using unstimulated, both fresh and cryopreserved ex vivo PBMCs. GFP expression level and efficiency was monitored by FACS analysis. The gate was set on CD14 positive monocytes as APCs and the GFP expression was measured by autofluorescence of GFP in the FITC channel. GFP expression correlated with the increased amount of GFP-RNA used for electroporation, as expected. The expression level of 77% GFP in GFP-transfected PBMCs using 20 µg GFP-RNA proved that the developed protocol was sufficiently functional to transcribe transfected IVT RNA into the related protein. Thus electroporation conditions and experimental set up was fixed for all subsequent experiments with the most important parameter being 20 µg IVT RNA for transfection.

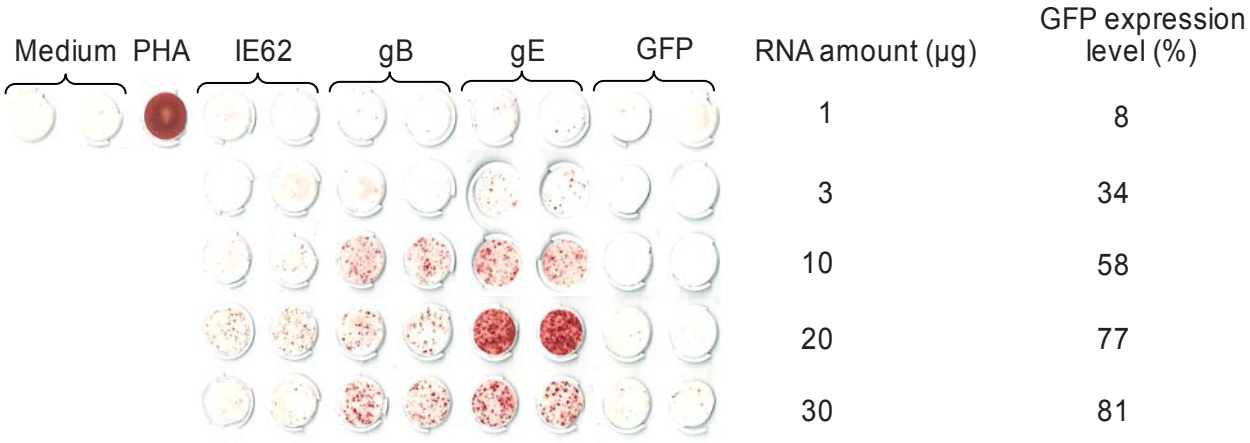


Figure 12: Optimization of the RNA amount for the following transfection experiments. Electroporation was performed under standard conditions (350 V, 12 ms) with $1x10^7$ PBMCs of LP-3 per electroporated RNA species. IFN-γ ELISpot assay was then conducted with $1,5x10^6$ electroporated PBMCs of LP-3 seeded per well. IE62, gB, gE and GFP RNAs were used in 5 different concentrations (1, 3, 10, 20 and 30 µg) for the transfection experiment. GFP RNA served as positive transfection control and the expression of the related GFP protein was monitored by FACS analysis 4 h after transfection. GFP expression level is shown in percentage of GFP positive CD14 positive monocytes. PHA served as nonspecific positive control. After 40 h of incubation IFN-γ spots were visualized. Data are representative of experiments in 3 healthy VZV-immune donors (LP-1 to LP-3).

4.6.3 VZV protein expression controlled by Western blot analysis

VZV protein expression in PBMCs was evaluated by commercially available VZV protein-specific antibodies. Antibodies were first screened for their affinity to commercially available VZV-infected fibroblasts fixed on object slides by immunofluorescence microscopy (data not shown). All three antibodies were capable of binding to their target proteins in VZV-infected fibroblasts. Antibodies directed to gE showed a much higher immunofluorescence signal, which might be the result of an enhanced antibody binding affinity to the target protein or higher expression levels of gE in VZV-infected fibroblasts. VZV-specific protein expression in PBMCs of healthy donors by IVT RNA electroporation was first tested in a time course experiment by Western blot analysis shown in Figure 13.

Protein detection by Western blot is a very sensitive, fast and simple method. VZV-RNA encoding the three MS-identified VZV proteins were transfected in PBMCs by electroporation and PBMCs were cultured for 0-12 h. The incubation period was stopped at distinct time points and transfected PBMCs were prepared for Western blot analysis. VZV-specific antibodies were incubated with the transfected and denaturated PBMCs and Western blot analysis was performed using the standard protocol. However, only antibodies directed against gE bound to their target protein with maximum reactivity after 4 h incubation post transfection. The molecular weight of the unglycosylated form of VZV gE is approx. 70 kDa and the corresponding band was clearly visible in the time course experiment between 1h to 4 h after RNA transfection. The glycosylated form of gE expressed in mammalian cells has a molecular mass of approx. 98 kDa (Olson et al. 1997). The latter was also clearly visible at this molecular mass in between 2 h to 4 h post transfection shown in Figure 13. Reactivity of anti-gE antibodies to the corresponding protein decreased after 8 h of pre-incubation prior to sample preparation for Western blot analysis. This result suggested that the process of enzymatic degradation in the cytoplasm of transfected PBMCs started 4 h post transfection. Consequently the presentation of enzymatically degraded VZV peptides by MHC molecules was supposed to start approximately 8 h to 10 h post transfection. The other two antibodies did not show any sign of reactivity to the transfected VZV proteins by Western blot analysis. This might be the result of three dimensional alterations of the expressed VZV proteins due to the important denaturation process by homogenizing transfected PBMCs prior to gel electrophoresis. However, these two VZV protein-specific antibodies (anti-gB and anti-IE62) were inappropriate for Western blot experiments under denaturating conditions, although it was recommended by the manufacturer. This unsatisfactory result led us to the next experiments where we screened VZV protein transfected DCs and PBMCs with the commercially available VZV-specific antibodies by laser scanning microscopy (LSM).

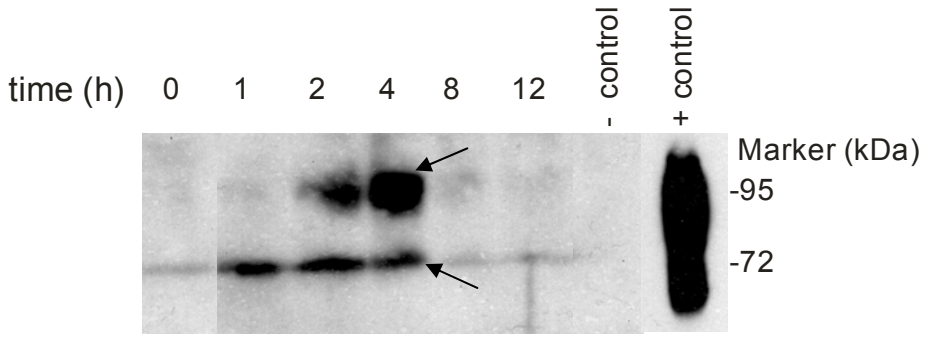


Figure 13: VZV glycoprotein E detection in a time course experiment by Western blot. VZV gE transfected (20 µg RNA) healthy donor PBMCs (LP-1) were cultured in AIM V medium + 10 % HS after electroporation. 1 Mio PBMCs were taken out of the culture at every time point (1, 2, 4, 8, 12 h) and the cell pellet was resuspended and denaturated (10 min 95°C) in Laemmli buffer. $2x10^5$ transfected PBMCs per lane were used for Western blot analysis. Shown are the horseradish peroxidase-reacted nitrocellulose membranes of gels containing 12% (w/v) acrylamide. Arrows represent the unglycosylated (~70 kDa) and glycosylated (~95kDa) forms of VZV gE. The secondary antibody was used as negative control.

4.6.4 VZV protein expression in DCs examined by LSM analysis

In LSM experiments VZV protein-transfected DCs were again screened with each VZV protein specific antibody. DCs were prepared from PBMCs following the standard protocol (Chapter 3.2.5). LSM experiments were performed with fixed VZV-RNA transfected DCs without pre-denaturating procedures, which conserved the three dimensional structure of the VZV proteins. In contrast to the Western blot experiments all antibodies demonstrated binding ability to their corresponding VZV proteins. This data confirmed the binding ability of the VZV protein-specific antibodies in the earlier immunofluorescence experiments (not shown).

IE62 was uniquely detected in the nucleus of the VZV-transfected DCs (Figure 14) and the expression level was quite moderate. The two glycoproteins were detected in the cytoplasm as well as on the cell membrane of the VZV-infected DCs, as expected (Arvin 1996; Maresova et al. 2005). The expression level of the two glycoproteins was much higher compared to the expression level of the nuclear protein IE62. The low expression level of IE62 could be explained by the protein structure and localization of the nuclear active conformation and cytoplasmic inactive conformation of IE62 (Shiraki and Hyman 1987; Forghani et al. 1990; Baudoux et al. 1995; Kinchington and Turse 1998; Kinchington et al. 2000; Lynch et al. 2002; Eisfeld et al. 2006). IE62-specific antibodies are unable to bind to cytoplasmic IE62 due to a hidden antibody binding motif in the inactive cytosolic conformation of this protein. IE62, a regulatory protein acts as transcription factor in the nucleus of the infected cell and enters the nucleus using a single classical arginine/lysine-rich nuclear localization signal (NLS) mapping to amino acids 677 to 685 (Kinchington and Turse 1998; Eisfeld et al. 2006).

Nuclear active IE62 could be bound by anti-IE62 antibodies due to the altered conformation, which is caused by the transcriptional activity and was afterwards detected by LSM analysis in DCs. At later stage of viral replication IE62 accumulates predominantly in the cytoplasm of the infected cell, which is mediated by ORF66 protein kinase. The IE62 accumulation in the cytoplasm leads to protein degradation and epitope presentation generating IE62-specific T cells and establishing a T cell memory response at later stage of infection (Lowry et al. 1997; Frey et al. 2003). This memory T cell response might be refreshed during viral persistence in the trigeminal and dorsal root ganglia, because IE62 was found to be expressed during latency (Mahalingam et al. 1993). In contrast to the inadequate functionality of two anti-VZV antibodies (anti-gB and anti-IE62) in Western blot experiments, all three antibodies were functional in LSM experiments.

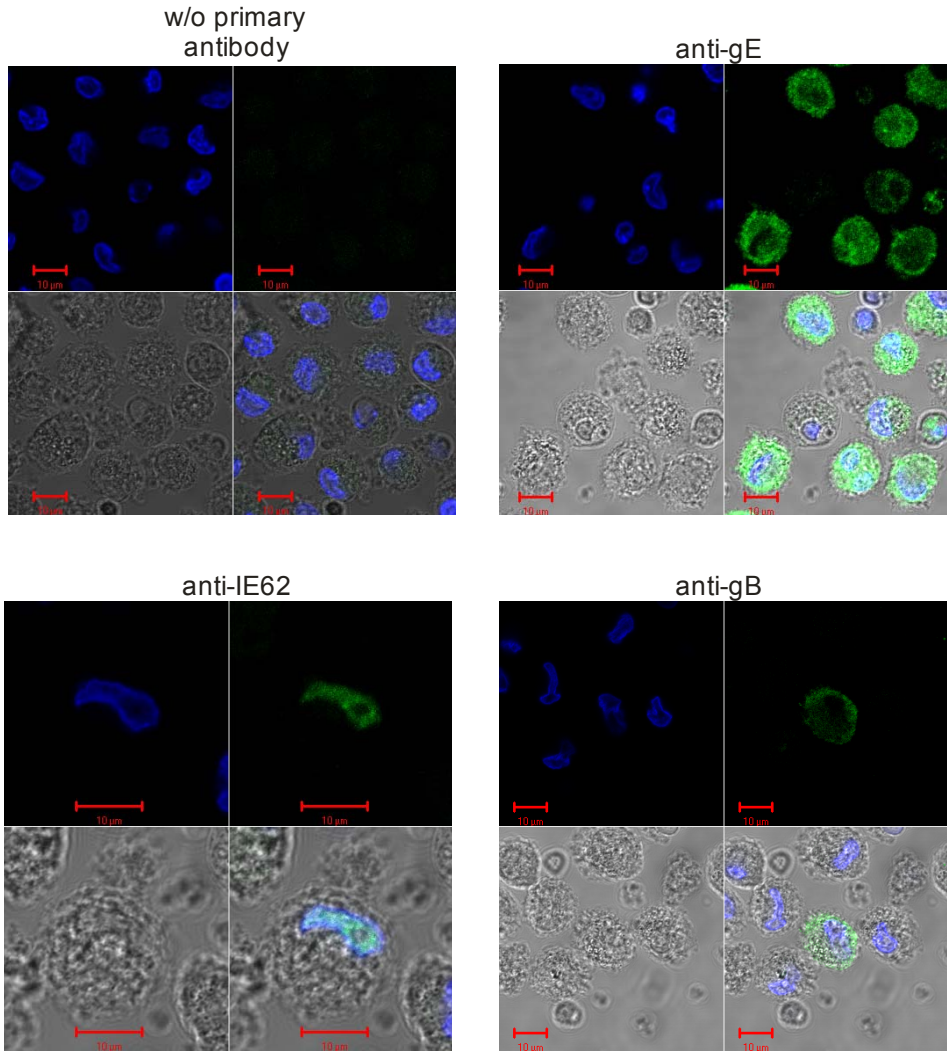


Figure 14: VZV protein expression examined by LSM analysis. VZV proteins were stained 4 hours post transfection in fixed mature DCs by intracellular staining with VZV protein-specific primary antibodies (anti-IE62, anti-gE and anti-gB) and a gam-FITC-labeled secondary (light green) antibody. Hoechst 33342 served as nuclear staining dye (blue) in LSM analysis. The scale is shown in 10 µm (red).

4.6.5 T cell reactivity to entire VZV proteins in PBMCs of 10 healthy individuals

PBMCs of 10 healthy donors were electroporated with IVT RNA of the three identified VZV proteins under above described standard conditions. In these experiments VZV-infected Vero cell extract served as positive control and transfection without RNA (mock) as negative control. Electroporated PBMCs were cultured for 4 hours post transfection to allow them to express the desired proteins without any interruption. Afterwards transfected PBMCs were counted and viable cells were seeded with 2 Mio PBMCs per well in the IFN-γ ELISpot assay. The latter was developed after 40 hours of incubation and the IFN-γ spots were automatically counted using the computer-controlled ELISpot reader. The negative control transfected without VZV-specific RNA showed only a few spots that was defined as unspecific background reactivity. Most of the reactivity was measured with PBMCs transfected with the two glycoproteins (gB: median 54 spots, range: 10-250 spots per 10^6 PBMCs; gE: median 45 spots, range: 8-250 spots per 10^6 PBMCs) and less reactivity was found in PBMCs transfected with IE62 (median 25 spots, range: 0-114 spots per 10^6 PBMCs).

The recognition pattern of the two glycoproteins and IE62 varied considerably from donor to donor. For example, PBMCs of single healthy individuals, like HD-9, showed very strong glycoprotein reactivity whereas PBMCs of HD-6 showed much weaker glycoprotein reactivity. The earlier detected low expression level of IE62 in DCs and PBMCs (Figure 14) might be a reason for lower spot numbers using PBMCs transfected with IE62 RNA in nearly all 10 healthy individuals. On the other hand PBMCs of HD-4 showed very strong IE62 reactivity, which suggested that the expression of IE62 in PBMCs was sufficient, but the number of IE62-reactive T cells in most of the screened healthy individuals were markedly lower compared to gB- and gE-reactive T cells. We also observed strong reactivity to the VZV infected cell extract which served as positive control in the IFN-γ ELISpot assay and which presumably contains the entire viral proteome including the MS-detected immunogenic proteins. Total spot numbers induced by the cell extract summed up that of the two glycoproteins and IE62. We believe that the comparably low concentration of VZV-encoded proteins present in the Vero cell lysate as well as inter protein competition issues has resulted in suboptimal capability to stimulate every single VZV-specific T cell precursor. Altogether the two VZV glycoproteins gB and gE are recognized most efficient with PBMCs of 10 healthy individuals. In the following experiments PBMCs of patients after allogeneic HSCT were screened for T cell reactivity to VZV proteins upon clinical VZV reactivation.

15A

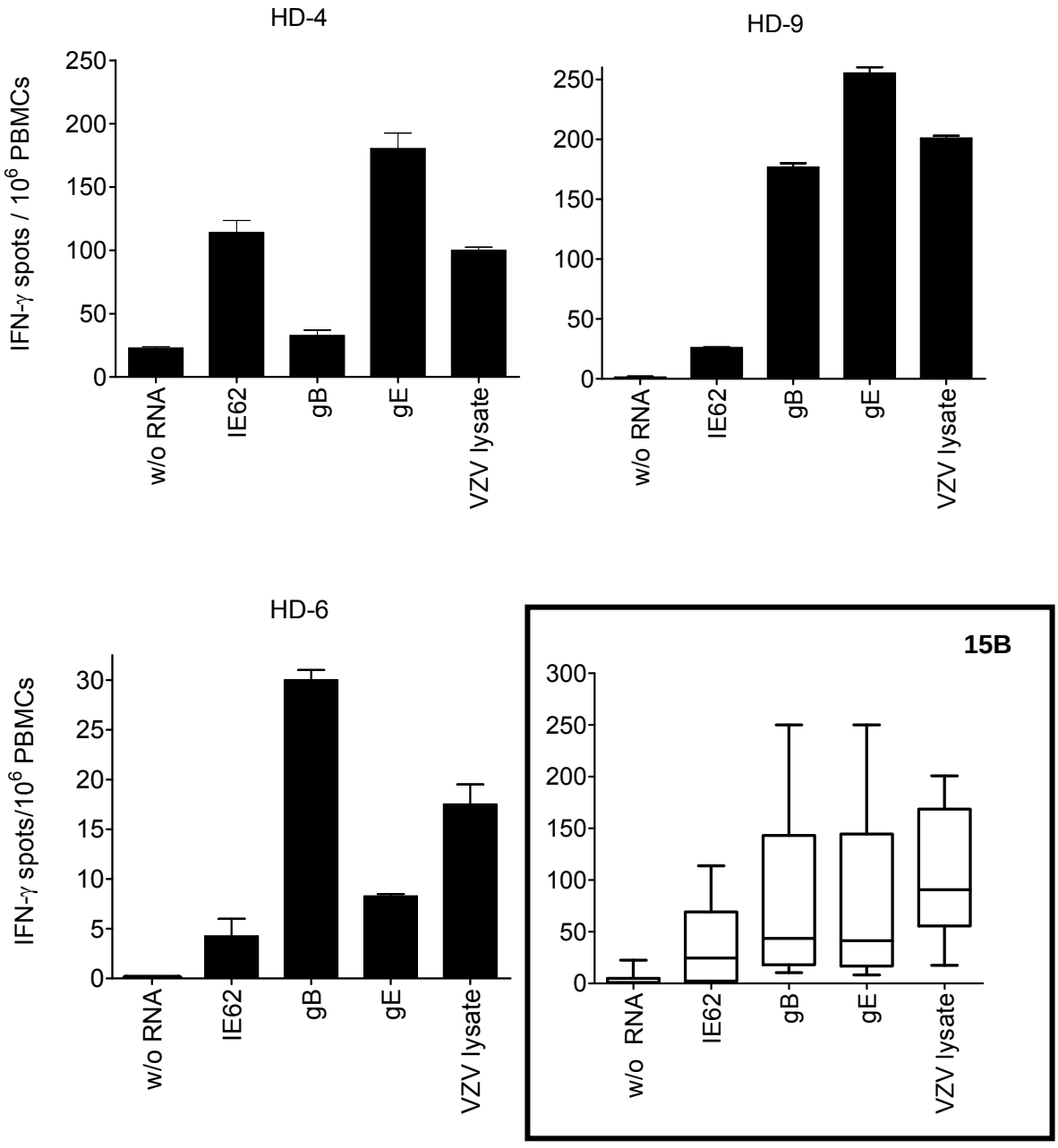


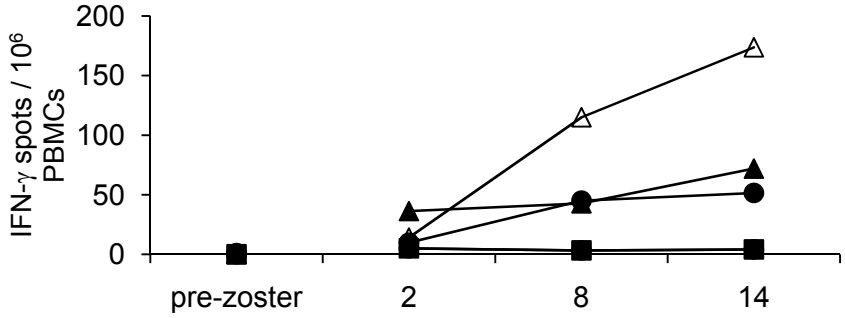
Figure 15: Screening for VZV proteins-specific T cells in PBMCs of 10 healthy donors by IFN-γ ELISpot assay. PBMCs of 10 healthy (m=8; w=2; age in between 28-59 years) donors were transfected with 20 µg IVT RNA encoding for IE62, gB, gE. Transfection without RNA (mock control) served as negative control. VZV-infected Vero cell extract (VZV lysate) incubated with non electroporated PBMCs served as positive control. PBMCs were seeded four hours post transfection with 2 Mio. PBMCs per well in the IFN-γ ELISpot assay. The reactivity profiles of 3 of the 10 screened healthy donors (HD-4, HD-6 and HD-9) are shown in 15A. The reactivity profile combining all 10 screened healthy donors is depicted in a box-and-whisker diagram in 15B.

4.6.6 T cell reactivity to entire VZV proteins in PBMCs of allogeneic HSCT patients after zoster

We followed the course of the frequency of T cells specific for entire VZV proteins gE, gB, and IE62 at different time points prior to herpes zoster and after clinical VZV reactivation in patients after allogeneic HSCT. Vigorous expansions of T cells recognizing VZV gB and gE were observed after zoster onset, while IE62 reactivity was negative or at very low level. In patients analyzed during the first 4 weeks after the onset of zoster, median IFN-γ spot numbers per 10^6 PBMC to glycoproteins B (55, 0-177) and E (32, 8-83) exceeded that to IE62 (2, 0-5), respectively (Figure 17). The reasons for the lack of detectable IE62-specific T cells in our zoster patients remain unclear at this point. Notably, T-cell responses to any of these 3 VZV proteins were not detected in PBMCs obtained from these patients before zoster occurred (Figure 16). Additionally, we observed that the frequency of circulating gE-specific T cells exceeded that of gB during the second month after zoster (Figure 17B). However, VZV cell extract reactivity detected in all patients was increased during viral reactivation and suggested that VZV reactivity became broader and VZV-specific T cells with various specificities could be found in the screened pool of PBMCs. Anti-HLA blocking assays suggested that early glycoprotein reactivity after zoster was mediated by both $CD4^+$ and $CD8^+$ T cells (data not shown). A considerable number of glycoproteins and immediate early (IE) proteins of VZV have already been identified as a source of $CD4^+$ and $CD8^+$ T cell antigens (Arvin 2008). Amongst those, gE, IE62, and IE63 were defined as being immunodominant (Weinberg and Levin; Arvin et al. 1991; Arvin 1992; Huang et al. 1992; Sadzot-Delvaux et al. 1998). Furthermore we screened the sera of patient 4 and 5 for the humoral VZV-specific immunity. The humoral VZV-specific immunity of patients 1, 2 and 3 was already shown and described in Figure 6. In the serum of patient 4 and 5 we detected IgM antibodies immediately after viral reactivation has occurred, but the IgG antibody level did not changed significantly. These data confirmed the former results and the minor role of the humoral immune response in contrast to the significant role of VZV-specific T cell immunity during viral reactivation shown in Figure 6 and Figure 16. The humoral immune response to VZV is obviously not a reliable marker for proving viral reactivation in contrast to the sensitive detection of VZV-specific T cells after viral reactivation has occurred. Overall we could show that the two glycoproteins (gE and gB) were recognized most efficiently upon zoster infection and the kinetic time course for both glycoproteins is very different in screened patient PBMCs. IE62 seemed to play a minor role as antigenic target in T cell immunity of screened patient PBMCs.

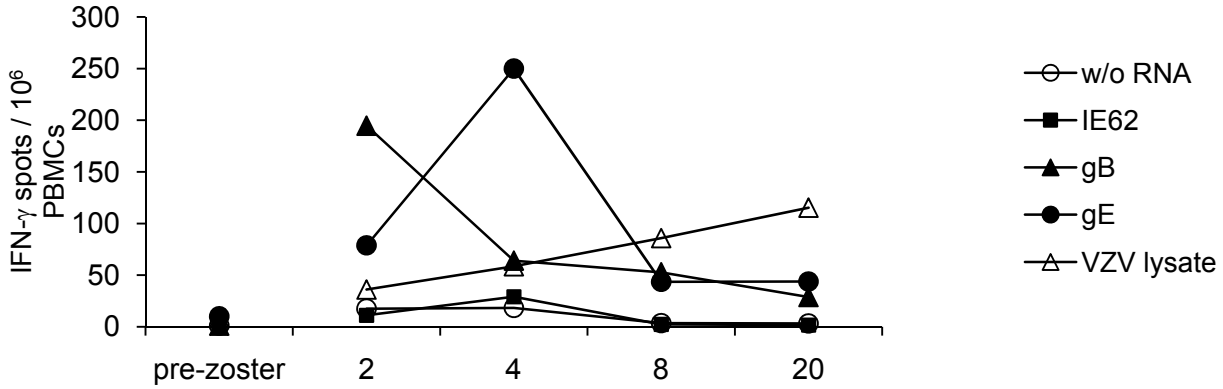
IgG	250	250	500	500
IgM	-	90	50	15
PCR	-	+	+	+

patient 3



IgG	n.d.	950	1400	1250	1000
IgM	n.d.	15	10	< 5	< 5
PCR	-	+	-	-	-

patient 4



IgG	600	100	175
IgM	< 5	< 5	< 5
PCR	-	+	-

patient 5

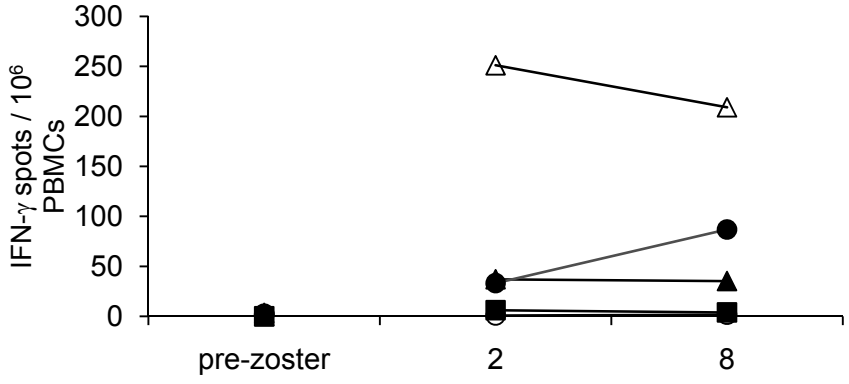


Figure 16: Expansion of VZV glycoprotein B and E specific T cells during posttransplant herpes zoster. Patients 1 to 3 developed zoster at 225, 614, and 935 days after reduced-intensity allogeneic HSCT. Conditioning therapy included lympho-depleting antibody alemtuzumab in patients 1 and 3, but not in patient 2. Diagrams show the frequencies of VZV-reactive T cells in leukapheresis-derived PBMC of patients prior to zoster and at indicated time points after zoster. Results were obtained by IFN-γ ELISPOT assay using IVT-RNA of VZV proteins IE62, gB, and gE, as well as VZV-infected cell lysate, as described in Fig. 15. In control experiments, pre-zoster PBMC samples showed strong T-cell reactivity to an inactivated whole influenza virus preparation and to phytohemagglutinin, indicating that these PBMC were able to respond to antigens (data not shown). Diagrams include lab data on VZV-specific IgM (U/mL) and IgG (mIU/mL), as well as on VZV-PCR.

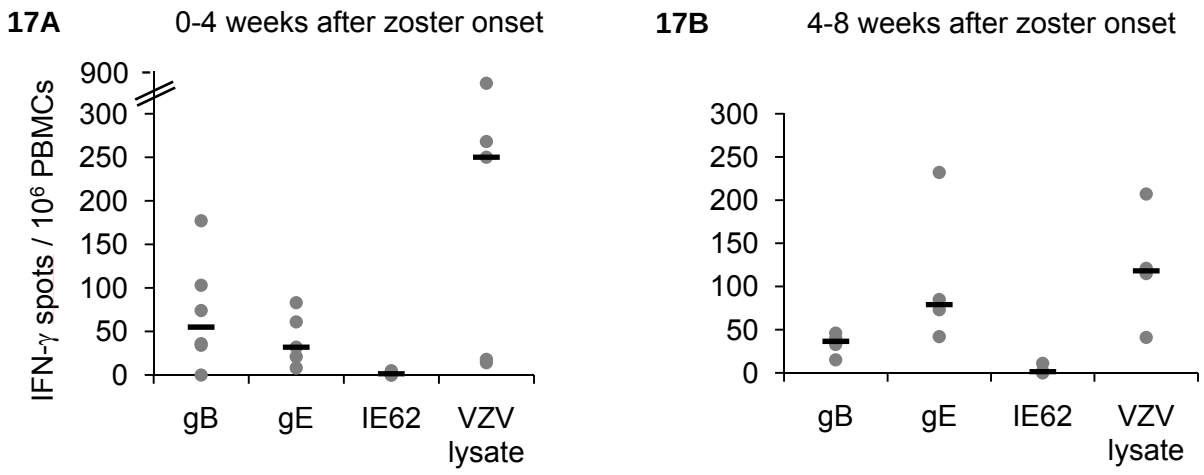


Figure 17: Post zoster immune responses to the MS-identified VZV proteins. Shown is the IFN-γ ELISPOT reactivity to single VZV proteins (gB, gE, IE62) and to entire VZV-infected cell lysate in PBMC of allogeneic HSCT patients during the first (A) and second (B) month after zoster onset (median d +401 (225-935)). Medians are indicated as bars. Patients (n=6) were at a median age of 60 (51-69) y. Three of them received alemtuzumab during conditioning therapy.

5 Summary and Conclusion

The topic of this thesis addresses the clinical problem of herpes zoster in VZV seropositive patients after allogeneic and autologous hematopoietic stem-cell transplantation (HSCT). The incidence of zoster in these patients is up to 25-50%. Most disease episodes occur 2-10 months after transplantation (Han et al. 1994; Koc et al. 2000). Post-transplant disease mostly presents as classical herpes zoster. However, a small but significant proportion of the patients with strong immunosuppressive treatment show disseminated VZV infection of visceral organs and the central nervous system (CNS), which is associated with a high mortality rate. The cellular immune response in the immunocompetent host mainly consisting of VZV-specific memory T cells is essential to prevent and control virus reactivation. In immunocompromised or immunodeficient patients, however, cellular immunity cannot compete with the reactivated virus adequately, since the functionality of virus-specific T cell immunity is heavily compromised in these patients (Meyers et al. 1980; Wilson et al. 1992). Immunocompromised patients are regularly treated with nucleoside analogues like Aciclovir, Valaciclovir and Famciclovir which effectively reduce VZV replication by interfering with the viral thymidine kinase activity. However, antiviral prophylaxis is unable to mediate complete protection from zoster manifestation, which can still appear as breakthrough infections. A clear disadvantage of long-term antiviral prophylaxis with nucleoside analogues is that side effects are more common in HSCT recipients than in other populations, mainly owing to extensive co-medication and frequent transplantation-related organ damage. In addition, the success of antiviral prophylaxis may be compromised by lack of patient compliance or by renal dysfunction that is rather common after transplantation and may require the discontinuation of prophylactic medication due to nephrotoxic side effects. Another disadvantage is that low dose medication used for prophylaxis may also facilitate the selection of VZV strains that are thymidine kinase-negative and therefore resistant (Reusser et al. 1996). Active immunization of HSCT patients against VZV is a significant challenge, because reactivated VZV infection occurs during the first months after transplantation when T cell immunity is still heavily compromised. Several groups have demonstrated that VZV-specific T cell reconstitution often recurs with clinical reactivation of the endogenous virus, which usually induces a T cell response and reestablishment of latency in the dorsal root ganglia (Meyers et al. 1980; Locksley et al. 1985; Ljungman et al. 1986; Distler et al. 2008). This observation suggests that a vaccination approach, as a substitute for natural re-sensitization by VZV reactivation, may help to accelerate the reconstitution of VZV-specific T cell immunity in the early post-transplant period. Long-term prophylaxis by vaccination aims at the renewal of VZV-specific T cells resulting in a repertoire of memory T cells that can compete with the viral reactivation and contribute to a decreased severity of the reactivated virus. However, live attenuated vaccines are not approved in HSCT recipients because they may still contain infectious virus. A candidate vaccine based on a heat-inactivated whole virus preparation of VZV has already

demonstrated clinical and immunologic activity in 2 pilot trials in the setting of HSCT (Redman et al. 1997; Hata et al. 2002). As an alternative approach, a VZV glycoprotein E (gE) subunit vaccine has been developed and is currently being tested in a non-HSCT pilot study (Leroux-Roels G VP, Abstract, 2010) The protection correlated with the reconstitution of $CD4^+$ T cell immunity against VZV. These data indicate that post-transplant vaccination with inactivated VZV vaccine is safe and able to accelerate the reconstitution of VZV-specific memory T cells that have persisted during the pre-transplant conditioning regimen. The enhanced $CD4^+$ T cell immunity may provide helper functions necessary to expand VZV-specific $CD8^+$ cytotoxic T lymphocyte (CTL) populations that appear essential to keep the virus in the latency stage. However, human $CD4^+$ T cells may also function effectively as antiviral CTLs against many viruses, including VZV (Arvin et al. 1991). Nevertheless an optimal VZV vaccine should be capable of directly stimulating VZV-specific $CD4^+$ and $CD8^+$ T cells.

In this doctoral thesis we studied cryo-preserved peripheral blood mononuclear cells (PBMCs) of VZV-seropositive healthy individuals and patients after allogeneic HSCT on their recognition of VZV proteins. In order to match the *in vivo* situation as closely as possible and avoid in vitro bias, we established a novel screening approach. We thought to identify immunodominant VZV proteins, which might be used in a subunit vaccine as a substitute for the above mentioned currently existing inactivated whole virus VZV vaccine in immunocompromised HSCT patients. In the first set of experiments we analyzed VZV-immune healthy donors for T cell reactivity to the commercially available VZV-infected Vero cell extract to guarantee sensitive and strong VZV reactivity in the already established interferon-γ enzyme-linked immunosorbent spot (IFN-γ ELISpot) assay (Distler et al. 2008). We selected PBMCs with the strongest detected T cell response to the VZV-infected Vero cell extract for the following HPLC fraction screening experiments (Figure 5). Initially PBMCs showing a strong T cell response were used for HLA blocking and T cell subpopulation experiments, where we observed mainly a $CD4^+$ T cell response to the VZV-infected Vero cell extract (data not shown). These findings were already documented in other publications, but seemed to be unusual for intracellular pathogens, like viruses, which usually mediate a $CD8^+$ T cell response (Vossen et al. 2004; Jones et al. 2006; Distler et al. 2008; Jones et al. 2009). The immunodominance of $CD4^+$ T cells in VZV latency and during VZV reactivation is associated with the decreased expression of major histocompatibility complex (MHC) class I compared to MHC class II in the neuronal ganglia (Gogate et al. 1996). This deficiency of MHC class I molecules may lead to an insufficient presentation of viral epitopes and to a decreased stimulation of VZV-specific $CD8^+$ T cells. Referring to this, VZV and herpes simplex virus (HSV) antigen-specific $CD4^+$ T cells were detected without prior *in vitro* stimulation of PBMCs in the peripheral blood of healthy individuals using intracellular staining of either IFN-γ or tumor necrosis factor-α (TNF-α) (Asanuma et al. 2000). In contrast, VZV-specific $CD8^+$ T cells had

to be enriched by *in vitro* stimulation of PBMCs before detection with VZV-specific pentamers containing a VZV-encoded CD8 epitope (van der Heiden et al. 2009). Together, VZV-specific $CD4^+$ T cells dominate the host T cell response of immunocompetent and immunocompromised subjects and form the basis of VZV-specific T cell immunity, which is reconstituted by subclinical reactivation and reinfection. Besides the immunodominant $CD4^+$ T cell response during viral reactivation in patients after allogeneic HSCT we observed that immediately after transplantation a VZV-specific T cell response was not detectable (Figure 6). Only when clinically manifested viral reactivation has occurred a strong increase of VZV-reactive T cells was observed (Distler et al. 2008). Furthermore we could demonstrate that VZV-specific humoral immunity quantified by VZV-specific antibodies of IgG and IgM type played a minor role in the control of the reactivation of the endogenous virus (Arvin et al. 1986).

In the following experiments we filtrated (0.2 µm) the commercially available VZV-infected Vero cell extract and separated the filtrate by RP-HPLC (Figure 7). The collected HPLC fractions containing the separated VZV proteins were screened by IFN-γ ELISpot assay using the VZV-reactive PBMCs of the three healthy individuals (Figure 8). All VZV-reactive PBMCs recognized the same HPLC fractions in the IFN-γ ELISpot assay. Afterwards these immunoreactive fractions were examined for the presence of VZV proteins by mass spectrometry (ESI-MS) and all detected proteins were sequenced and compared with the known VZV proteome by data bank analysis (Figure 9 and Figure 10). Three VZV proteins were identified in the bioactive HPLC fractions known as glycoprotein E, glycoprotein B, and immediate early protein 62. In previous work substantial data has been published with regard to VZV glycoprotein E and IE62 (Arvin et al. 1986; Arvin et al. 1991; Bergen et al. 1991; Lowry et al. 1997), which described these proteins as immunogenic and several T cell epitopes have already been defined (Frey et al. 2003; Jones et al. 2009; van der Heiden et al. 2009). VZV gB was described as an immunogenic VZV protein in only a few publications and seemed to play a minor role in the hierarchy of immunodominant VZV proteins (Arvin 2008). All VZV-encoded T cell epitopes known so far were identified by antigen-specific memory T cells of healthy individuals which persist in the circulation and in primary lymphoid organs for whole life time. However, patients after allogeneic HSCT frequently lack sufficient numbers of VZV-reactive memory T cells in the peripheral blood, because of the conditioning therapy prior to bone marrow transplantation and the posttransplant immunosuppressive therapy (Meyers et al. 1980; Wilson et al. 1992; Han et al. 1994). Consequently the intention of a newly designed VZV subunit vaccine should be the reconstitution of a protective T cell immunity from limited persisting VZV-reactive memory T cells in immunocompromised allogeneic HSCT patients.

In cooperation with Prof. Ann Arvin (Stanford University, Stanford, CA, USA) who successfully worked for decades in the VZV research area we obtained plasmids encoding for the three identified VZV proteins. First, cDNAs encoding for the three identified VZV pro-

teins were evaluated by PCR analysis using suitable primers to check the identity and integrity of the cDNAs. Afterwards we *in vitro* transcribed (IVT) the validated cDNAs into RNAs and improved RNA stability by polyadenylation. RNAs were checked on purity and integrity in comparison to marker RNA by gel electrophoresis (Figure 11). All three IVT-RNAs showed bands at the expected size in both native and polyadenylated forms. We developed a standard protocol for the transfection experiments in which all experimental conditions, like pulse duration, buffer composition, RNA amount and culturing duration are optimized to obtain effective RNA transfection of PBMCs and dendritic cells (DCs) (Figure 12). IVT-RNAs were then transfected in DCs of healthy individuals and the protein translation was evaluated by Western blot and confocal LSM analysis.

The results gained by Western blot analysis showed that the commercially obtained VZV-specific antibodies directed against gB, and IE62 were not suitable for Western blot analysis as incorrectly declared by the manufacturer (Figure 13). Only antibodies directed against VZV gE bound to the gE-transfected DCs at the expected molecular weight in the Western blot (Olson et al. 1997). In the following experiments we examined the expression of the transfected proteins in DCs by confocal laser scanning microscopy (LSM) analysis (Figure 14) and found that all three VZV-specific antibodies bound to their corresponding VZV protein proving their functionality. In these experiments we observed that the expression of both VZV glycoproteins was exclusively detected in the cytoplasm as well as on the cell membrane of the transfected DCs. Contrary to this, IE62 was exclusively detected in the nucleus of the transfected cell due to a hidden antibody binding motif in the inactive cytosolic conformation of this protein. Nevertheless, IE62 as well as both glycoproteins gB and gE were available in the cytoplasm of the transfected cells, which finally led to enzymatic degradation and presentation of viral peptides via MHC.

In the following experiments PBMCs of 10 healthy virus-immune individuals were transfected with IVT-RNA encoding for the three identified VZV proteins and the protein-specific T cell reactivity was measured by IFN-γ ELISpot assay (Figure 15). Virtually all 10 healthy individuals showed significantly increased IFN-γ production when stimulated with both glycoproteins, gB and gE. VZV-specific T cells of just two healthy individuals recognized IE62 in a similar extent or even stronger compared to the immune response to one or both glycoproteins. This immunodominance of gE and gB was confirmed in the next experiments using PBMCs of immunocompromised patients after allogeneic HSCT (Figure 16).

We observed that herpes zoster occurring after allogeneic HSCT appears to predominantly stimulate a memory T cell response to viral glycoproteins gB and gE, because both glycoproteins are anchored in a great excess in the viral cell membrane and are additionally synthesized and presented in a great amount inside the infected cells (Haumont et al. 1997; Kutinova et al. 2001; Maresova et al. 2005). A vaccine approach containing these two glycoproteins would simulate the early stage of endogenous VZV reactivation effectively. Both

VZV glycoproteins, gB and gE are therefore candidates for a viral subunit vaccine suitable for immunocompromised patients. In a currently ongoing study in the United States and Canada, glycoprotein E is already tested as a component for a VZV subunit vaccine (Leroux-Roels G VP, Abstract, 2010).

The herein described approach has considerable advantages compared to many other methods to identify viral T cell antigens. It allows for the rapid detection of naturally expressed candidate antigens from a relatively low (<1 mg) initial amount of virus-infected cell lysate material. Furthermore, the use of PBMCs from VZV-seropositive donors as the preferred screening population in a sensitive IFN-γ ELISpot assay avoids *in vitro* culturing of T cells. The latter is both time-consuming and prone to bias due to possible selective expansion of distinct T cell specificities under the chosen culture conditions. In addition, our approach is flexible in many directions. First, screening with PBMC from patients with active herpes zoster may be suitable to unravel additional T-cell antigens of VZV that could play an important role during the acute phase of the disease. Moreover, the virus antigen source used herein was prepared from Vero cells after one week of VZV infection. Modification of the infection period with regard to time and addition of drugs interfering with virus metabolism could most likely change the composition and concentration of expressed virus proteins and might lead to the identification of additional known (e.g. glycoprotein I, IE63), (Arvin 2008) or even novel T-cell antigens of VZV. It is well conceivable that the system can be easily extended towards other viruses that grow strictly cell-associated (as VZV) and in which T-cell antigens are completely or partly undefined. Considering that B-cell immunity is unsuitable to prevent zoster after allogeneic HSCT (Arvin 2000), stimulation of VZV-specific T cells will be a major goal in developing an efficient vaccine for HSCT patients. Monitoring such T-cell responses on the basis of individual VZV proteins in vaccinees could be an important application for the described assay system. In prospective trials it may help to optimize vaccine strategies and to define a threshold frequency of circulating VZV antigen-specific T cells that correlates with protection from zoster disease and may ultimately allow for safe discontinuation of antiviral drug prophylaxis. The work presented herein was very recently summarized in a manuscript that is currently submitted for publication (Patrick Kleemann, Eva Distler, Eva M. Wagner, Simone Thomas, Sebastian Klobuch, Steffi Aue, Elke Schnürer, Hansjörg Schild, Matthias Theobald, Bodo Plachter, Stefan Tenzer, Ralf G. Meyer, and Wolfgang Herr; Varicella-zoster virus glycoproteins B and E are major targets of $CD4^+$ and $CD8^+$ T cells reconstituting during zoster after allogeneic transplantation).

6 Zusammenfassung und Fazit

Die vorliegende Dissertation beschäftigte sich mit der Reaktivierung des in den Spinalganglien persistierenden Varicella Zoster Virus aus dem Latenzstadium bei Patienten nach allogener oder autologer hämatopoetischer Stammzelltransplantation (HSZT). Die Wahrscheinlichkeit einer VZV-Reaktivierung in dieser Patientengruppe liegt zwischen 25% und 50% und tritt meist in einem Zeitraum von 2-10 Monaten nach der HSZT auf (Han et al. 1994; Koc et al. 2000). Eine VZV-Reaktivierung bei Patienten nach HSZT entspricht dem klinischen Bild eines klassischen Herpes Zoster. Dieser lokal begrenzte Hautausschlag wird begleitet von Schmerzen und Missempfindungen in den befallenen Dermatomen. Bei einem geringen, aber trotzdem signifikanten Anteil dieser Patientengruppe mit starker Immunsuppression kann es zu schwereren Komplikationen bis hin zu einer disseminierten Virusmanifestation mit generalisiertem Organbefall und Befall des ZNS mit erhöhter Mortalitätsrate kommen. In diesen Fällen ist das körpereigene zelluläre Immunsystem, mit den in immunkompetenten Individuen vorhandenen virus-spezifischen T-Zellen, essentiell um eine antivirale Immunantwort auszulösen. Eine Virusreaktivierung in den stark immunkompromittierten Patienten kann jedoch nicht mehr adäquat verhindert oder kontrolliert werden, da das virusspezifische zelluläre Immunsystem durch die HSZT und durch die Konditionierungstherapie derart in Mitleidenschaft gezogen wurde, dass die virus-reaktiven T-Zellen funktionsuntüchtig (anerg) bzw. völlig zerstört wurden (Meyers et al. 1980; Wilson et al. 1992). Diese stark immunsupprimierten Patienten werden derzeitig mit den Nukleosidanaloga Aciclovir, Valaciclovir oder Famciclovir prophylaktisch behandelt. Diese Virostatika hemmen die virale Thymidinkinaseaktivität und unterbinden somit die Virusreplikation. Eine Langzeitprophylaxe mit Nukleosidanaloga bietet jedoch keinen vollständigen Schutz vor einer VZV-Reaktivierung und einer Zoster Manifestation. Ein weiterer klarer Nachteil dieser antiviralen Langzeitprophylaxe mit Nukleosidanaloga bei Patienten nach HSZT ist auch, dass Nebenwirkungen häufiger auftreten als in anderen Patientengruppen, hauptsächlich aufgrund der umfangreichen immunsuppressiven Komedikation und der transplantationsbegleitenden Organschädigungen. Eine zusätzliche Limitation der antiviralen Langzeitprophylaxe mit Nukleosidanaloga ist, dass die Selektion von VZV-Stämmen, welche Thymidinkinase-negativ sind und somit resistent, gefördert wird (Reusser et al. 1996). Eine aktive Immunisierung von Patienten nach HSZT gegen VZV ist eine besondere Herausforderung, da eine VZV-Reaktivierung in den ersten Monaten nach der HSZT auftritt und die T-Zellimmunität in dieser Zeit noch stark beeinträchtigt ist. Einige Gruppen haben gezeigt, dass eine Rekonstitution der VZV-spezifischen T-Zellantwort meistens nur auftritt, wenn es zu einer klinischen Reaktivierung des endogenen Virus kommt, welche eine T-Zellantwort induziert und zu einem erneuten Zurückdrängen der Viren in die neuronalen Ganglien führt (Meyers et al. 1980; Locksley et al. 1985; Ljungman et al. 1986; Distler et al. 2008). Diese Beobachtungen deuten an, dass eine aktive Immunisierung als Ersatz für eine natürliche Sensibilisierung durch die virale

Reaktivierung helfen könnte, die Rekonstitution der VZV-spezifischen T-Zellimmunität in einer frühen Phase nach der HSZT zu beschleunigen. Eine Langzeitwirkung durch die Auffrischung oder Erneuerung der körpereigenen VZV-spezifischen Immunantwort würde zur Bildung von T-Gedächtniszellen und T-Effektorzellen führen, die den Verlauf einer VZV-Reaktivierung in dieser Patientengruppe mildern oder die VZV-Reaktivierung ganz verhindern können. Die für immunkompetente Personen verfügbaren VZV-Impfstoffe sind jedoch aufgrund des zu hohen Risikos, dass in ihnen noch infektiöser Virus enthalten sein könnte, bei immunkompromittierten Patienten kontraindiziert und wurden deswegen bisher für Patienten nach HSZT nicht zugelassen. In anderen Studien wurden HSZT-Patienten mit einem hitzeinaktivierten VZV geimpft (Redman et al. 1997; Hata et al. 2002). Die beobachtete Schutzwirkung korrelierte mit der Rekonstitution der VZV-spezifischen $CD4^+$ T-Zellimmunität. Diese Studien belegen, dass eine Impfung mit einem hitzeinaktiviertem Impfstoff nach HSZT sicher ist, und dass diese Impfung zu einer Rekonstitution der antiviralen T-Gedächtniszellen führt. Die verstärkte $CD4^+$ T-Zellantwort könnte notwendig sein, um VZV-spezifische $CD8^+$ T-Zellen zu expandieren, welche das Virus im Latenzstadium halten. Nichtsdestotrotz können humane $CD4^+$ T-Zellen effektiv als antivirale zytotoxische T-Zellen gegen viele verschiedene Viren einschließlich VZV fungieren (Arvin et al. 1991). Eine optimale Impfung sollte jedoch beides, sowohl VZV-spezifische $CD4^+$ als auch $CD8^+$ T-Zellen stimulieren.

In der vorliegenden Dissertation habe ich immundominante VZV Proteine identifiziert, die zukünftig in einer sogenannten Subunitvakzine als Alternative zu den bereits erwähnten Impfstoffen bei immunkomprimierten Patienten eingesetzt werden könnten. In Vorversuchen haben wir geeignete VZV-reaktive gesunde Spender mit Hilfe eines kommerziell erhältlichen VZV-infizierten Vero Zelllysats gesucht. Wir detektierten eine sehr gute VZV-Reaktivität in dem bereits für VZV-spezifische T-Zellantworten etablierten sensitiven immunologischen Testsystem, dem IFN-γ ELISpot Assay (Distler et al. 2008). Es wurden mononukleäre Zellen des peripheren Blutes (PBMCs) von drei gesunden Spendern mit einer sehr guten T-Zellantwort auf das VZV-infizierte Zelllysat für das spätere Screening der HPLC Fraktionen ausgewählt (Abbildung 5). Zunächst wurden die PBMCs der drei gesunden Spender in Subpopulations- und Blockadeexperimenten eingesetzt (Daten nicht gezeigt), (Distler et al. 2008). Dabei stellte sich heraus, dass die detektierte T-Zellantwort auf das VZV-infizierte Vero Zelllysat hauptsächlich von $CD4^+$ T-Zellen vermittelt wurde. Dieses Ergebnis wurde auch in einigen anderen Publikationen bereits beschrieben, ist jedoch konträr zu der allgemein beschriebenen $CD8^+$ getriggerten T-Zellantwort von virusinfizierten Zellen (Vossen et al. 2004; Jones et al. 2006; Distler et al. 2008; Jones et al. 2009). Die Immundominanz der $CD4^+$ T-Zellen im viralen Latenzstadium hängt unter anderem mit der allgemein verringerten Expression von MHC Klasse I gegenüber MHC Klasse II in den neuronalen Ganglien zusammen (Gogate et al. 1996). Dieses Defizit an MHC Klasse I Molekülen führt zu einer unzureichenden Präsentation von viralen CD8 Epitopen und damit zu einer verringerten Stimula-

tion der virus-reaktiven $CD8^+$ T-Zellen. Dieses führt letztendlich zum Abschalten oder sogar zur Apoptose der VZV-spezifischen $CD8^+$ T-Zellen. In einer diesbezüglichen Studie konnten diese Ergebnisse bestätigt werden und ausschließlich $CD4^+$ T-Zellantworten gegen VZV und HSV ohne Vorstimulation im peripheren Blut der Probanden mittels einer intrazellulären Zytokinfärbung von IFN-γ oder TNF-α nachgewiesen werden (Asanuma et al. 2000). Hingegen war eine geeignete Vorstimulation der PBMCs *in vitro* erforderlich, um VZV-spezifische $CD8^+$ T Zellen nachzuweisen (van der Heiden et al. 2009). VZV-spezifische $CD4^+$ T-Zellen dominieren somit die T-Zellantwort in immunkompetenten als auch in immunkompromittierten Individuen und werden durch gelegentliche subklinische Reaktivierungen bzw. unerkannte Reinfektionen stimuliert und bilden somit die Basis der VZV-spezifische Immunantwort. Neben der immundominanten $CD4^+$ T-Zellantwort im Zuge der VZV-Reaktivierung konnte bei Patienten nach allogener HSZT festgestellt werden, dass unmittelbar nach der Transplantation bis zum Zeitpunkt der endogenen Virusreaktivierung keine VZV-spezifische zelluläre Immunität mit der von uns gewählten Methode detektierbar ist (Abbildung 6). Zum Zeitpunkt der Reaktivierung konnte gezeigt werden, dass es zu einem immensen Anstieg der VZV-reaktiven T-Zellen kam (Distler et al. 2008). Desweiteren konnte bestätigt werden, dass die VZV-spezifische humorale Immunität, quantifiziert über VZV-spezifische Antikörperbestimmungen von IgG und IgM, nur eine untergeordnete Rolle in der Kontrolle der endogenen VZV-Reaktivierung spielt (Arvin et al. 1986).

In den folgenden Experimenten haben wir das kommerziell erhältliche VZV-infizierte Vero Zelllysat zuerst größenfiltriert (0.2 µm) und anschließend das Filtrat über die RP-Chromatographie aufgetrennt (Abbildung 7). Die gesammelten Fraktionen mit den separierten VZV Proteinen haben wir unmittelbar im IFN-γ ELISpot Assay mit den VZV-reaktiven PBMCs der gesunden Spender getestet (Abbildung 8). Alle VZV-reaktiven Spender PBMCs erkannten die gleichen HPLC Fraktionen im IFN-γ ELISpot Assay. Diese immunreaktiven Fraktionen wurden anschließend mittels Massenspektrometrie (ESI-MS) auf die Gegenwart von VZV Proteinen durch Sequenzierung trypsinisierter Proteinfragmente durchmustert (Abbildung 9). Eine Datenbankanalyse des bekannten VZV Proteoms identifizierte drei VZV Proteine in den reaktiven Fraktionen, unter anderem die beiden VZV Glykoproteine gE und gB und das IE62 Protein (Abbildung 10). VZV gE und IE62 sind in der Literatur hinlänglich als immundominante VZV Proteine beschrieben (Arvin et al. 1986; Arvin et al. 1991; Bergen et al. 1991; Lowry et al. 1997), und einige T-Zellepitope wurden bereits identifiziert (Frey et al. 2003; Jones et al. 2009; van der Heiden et al. 2009). VZV gB wird zwar ebenfalls als immunogenes VZV Protein beschrieben, scheint aber eine untergeordnete Rolle in der Hierarchie der immundominanten VZV Proteine zu spielen (Arvin 2008). Alle bisher bekannten T-Zellepitope, die vom VZV abgeleitet wurden, sind mit Hilfe von antigen-spezifischen T-Gedächtniszellen gesunder Probanden identifiziert worden. Die Patienten besitzen nach der allogenen HSZT jedoch kaum noch VZV-reaktive T-Gedächtniszellen im peripheren Blut auf-

grund der vorangegangenen Transplantation und der begleitenden Therapie mit Immunsuppressiva (Meyers et al. 1980; Wilson et al. 1992; Han et al. 1994). In Folge dessen liegt der Sinn und Zweck einer sogenannten Subunitvakzine in der Auffrischung der noch persistierenden T-Gedächtniszellen und in der Etablierung einer neuen Immunantwort bei immunkomprimierten Patienten nach allogener HSZT.

In einer Kooperation mit Prof. Ann Arvin (Stanford University, CA, USA), die auf dem Gebiet der VZV Forschung jahrzehntelang erfolgreich gearbeitet hat, erhielten wir freundlicherweise die drei cDNAs der identifizierten VZV Proteinen. In den folgenden Experimenten sequenzierten wir zuerst alle drei cDNAs mit den entsprechenden Primern, um die Identität und Vollständigkeit der verschiedenen cDNAs zu prüfen. Anschließend haben wir alle drei geprüften cDNAs in IVT-RNAs transkribiert und polyadenyliert, um die Stabilität der RNAs zu verbessern. Danach analysierten wir die drei IVT-RNAs in einer RNA-Gelelektrophorese auf Reinheit und Vollständigkeit im Vergleich zu einer Marker RNA (Abbildung 11). Alle drei IVT-RNAs zeigten, sowohl in der nativen als auch in der polyadenylierten Form, das richtige Laufverhalten in der Gelelektrophorese und die RNA Banden waren bei den zu erwartenden Größenordnungen zu finden. Danach wurde ein Protokoll für die Transfektion der VZV-spezifischen IVT-RNAs in dendritische Zellen und PBMCs etabliert, um die idealen Elektroporationsbedingungen, wie z.B. Pulsdauer, Pufferzusammensetzung, RNA Menge (Abbildung 12) und Kulturbedingungen zu ermitteln. Die drei IVT-RNAs wurden zunächst in dendritische Zellen von gesunden Spendern transfiziert und die Transfektion mittels Western Blot und anschließend mit konfokalen LSM untersucht.

Das Western Blot Ergebnis (Abbildung 13) zeigte, dass 2 der 3 Antikörper nicht für Western Blot Analysen geeignet waren, wie vom Hersteller angegeben, und nur einer der drei bestellten Antikörper überhaupt sein spezifisches Antigen (Glykoprotein E) bei dem zu erwartenden Molekulargewicht detektierte (Olson et al. 1997). In den darauffolgenden Experimenten haben wir die Expression der transfizierten VZV Proteine in DCs mittels konfokalem LSM untersucht (Abbildung 14). Alle drei VZV-spezifischen Antikörper haben in diesen Experimenten an das jeweilige VZV Protein gebunden und somit ihre Funktionalität unter Beweis gestellt. In diesen Experimenten stellte sich heraus, dass die Expression der beiden Glykoproteine im Zytoplasma bzw. auf der Zellmembran der transfizierten dendritischen Zellen und nicht im Zellkern zu beobachten war. IE62 (aktive Konformation) war jedoch ausschließlich im Zellkern als nukleäres Protein durch den anti-IE62 Antikörper zu detektieren. IE62 (inaktive Konformation) und die beiden Glykoproteine waren also in den transfizierten Zellen vorhanden, sodass die enzymatische Degradierung sowie die anschließende Präsentation der viralen Peptide über den MHC der transfizierten Zellen stattfinden konnte.

In den darauffolgenden Experimenten wurden PBMCs von 10 gesunden Spendern mit den IVT-RNAs der drei identifizierten VZV Proteine transfiziert und die proteinspezifische Reaktivität im IFN-γ ELISpot Assay gemessen (Abbildung 15). Die Mehrzahl der transfizier-

ten PBMCs der 10 gesunden Spender zeigte eine deutlich stärkere IFN-γ Produktion und damit eine stärkere VZV-spezifische Immunantwort für die beiden VZV-Glykoproteine gB und gE. Nur VZV-spezifische T-Zellen von zwei gesunden Spendern erkannten IE62 ähnlich stark oder besser als die beiden oder einer der beiden Glykoproteine und führten somit zu einer erhöhten oder ähnlich starken IFN-γ Produktion. Eine Verfälschung der Ergebnisse durch eine vermeintlich zu geringe zytoplasmatische Expression von IE62 ist damit eher unwahrscheinlich. Die Immundominanz dieser beiden Glykoproteine, gB und gE, wurde auch in den Experimenten mit PBMCs von immunkomprimierten Patienten nach allogener HSZT bestätigt (Abbildung 16,17).

Wir konnten dabei zeigen, dass der nach allogener HSCT gehäuft auftretende Herpes Zoster zu einer verstärkten T-Gedächtniszellantwort gegen die VZV-Glykoproteine gE und gB (und nicht gegen IE62) führt. Beide Glykoproteine sind in der Zellmembran infizierter Zellen verankert und werden im Überschuss von der infizierten Zelle synthetisiert und dem T-Zell-System präsentiert (Haumont et al. 1997; Kutinova et al. 2001; Maresova et al. 2005). Eine Impfung mit diesen beiden Proteinen würde somit die frühe Phase einer endogenen VZV Reaktivierung simulieren. Diese beiden VZV Glykoproteine sind somit erstklassige Kandidaten für eine Subunitvakzine, die bei immunkomprimierten Patienten unbedenklich eingesetzt werden könnte. In einer momentan in Kanada und USA laufenden klinischen Studie wird bereits das VZV Glykoprotein E als Hauptkomponente eines VZV Impfstoffs bei älteren Patienten unabhängig von einer HSCT getestet (Leroux-Roels G VP, Abstrakt, 2010). Die hier vorgestellte analytische Methodik zur Identifizierung von viralen T-Zellantigenen hat einige Vorzüge im Vergleich zu anderen Methoden. In der schnellen biochemischen Analytik wird nur eine sehr geringe Menge des viralen Zellmaterials benötigt (<1 mg), um die natürlich prozessierten viralen Proteine zu identifizieren. Desweiteren wird durch den direkten Einsatz der ex vivo PBMCs im IFN-γ ELISpot Testsystem die zeitaufwendige und teure Kultivierung von T-Zellen vermieden. Die aufwendige T-Zell Kultivierung verbirgt außerdem die Gefahr einer unerwünschten selektiven Expansion einzelner T-Zellspezifitäten. Außerdem ist die beschriebene analytische Methode sehr flexibel anwendbar. Zur Identifizierung von neuen T-Zellantigenen können PBMCs von Patienten während der viralen Reaktivierung und auch der Latenzphase getestet werden. Weiterhin könnte durch die Variation der Infektionsdauer der VERO Zellen mit VZV oder durch die Zugabe von viralen Stoffwechselinhibitoren die Zusammensetzung und Konzentration der exprimierten viralen Antigene verändert werden. Diese Veränderung könnte ebenfalls zur Identifikation bereits bekannter aber auch neuer virus-spezifischer T-Zellantigene führen. Diese analytische Methodik könnte auch leicht auf andere Viren, bei denen noch keine oder nur wenige Antigene bekannt sind, übertragen werden. Im Hinblick darauf, dass die B-Zellimmunität eine VZV-Reaktivierung bei Patienten nach HSZT nicht verhindern kann (Arvin 2000), nimmt die Stimulation von VZV-spezifischen T-Zellen in der Entwicklung einer effizienten Vakzine eine übergeordnet wichtige Rolle ein.

Die Messung und Quantifizierung dieser T-Zellantworten auf der Basis unterschiedlicher VZV Proteine, welche potentielle Vakzinen beinhalten können, wäre eine wichtige Anwendung dieser analytischen Methodik. In zukünftigen Studien könnte diese Verfahren helfen, Vakzinierungsstrategien zu optimieren und einen Schwellenwert für VZV-spezifische T-Zellen zu definieren, der direkt mit dem Schutz vor viralen Reaktivierungen korreliert. Somit könnte letztlich das sichere Ausschleichen der antiviralen Medikation erleichtert werden. Die hier vorgelegte Arbeit haben wir in einem Manuskript zusammengefasst, welches gegenwärtig zur Publikation eingereicht ist (Patrick Kleemann, Eva Distler, Eva M. Wagner, Simone Thomas, Sebastian Klobuch, Steffi Aue, Elke Schnürer, Hansjörg Schild, Matthias Theobald, Bodo Plachter, Stefan Tenzer, Ralf G. Meyer, and Wolfgang Herr; Varicella-zoster virus glycoproteins B and E are major targets of $CD4^+$ and $CD8^+$ T cells reconstituting during zoster after allogeneic transplantation).

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8 Abbreviations

ADCC	Antibody-dependent cellular immunity
AIDS	Acquired Immune Deficiency Syndrome
APC	Antigen-presenting cell
APC	Allophycocyanin
APS	Ammonium persulfate
Approx.	Approximately
BSA	Bovine serum albumine
CD	Cluster of differentiation
cDNA	Complementary DNA
CMV	Cytomegalovirus
CNS	Central nervous system
CpG	Cytosine-phosphodiester-guanine
CTL	Cytotoxic T lymphocyte
CVS	Congenital varicella syndrome
DC	Dendritic cell
DLI	Donor lymphocyte infusion
DNA	Desoxyribonucleic acid
E	Early
EBV	Epstein Barr-Virus
EDTA	Ethyendiamine-tetra-acetic acid
ELISpot	Enzyme-linked Immunosorbent Spot assay
FACS	Fluorescence activated cell sorting
FcR	Fc receptor
FCS	Fetal calf serum
FITC	Fluorescein-iso-thio-cyanat

GM-CSF	Granulocyte/macrophage-colony stimulating factor
GVHD	Graft-versus-host disease
GVL	Graft-versus-leukemia
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPLC	High performance liquid chromatography
HS	Human serum
HSCT	Hematopoietic stem cell transplantation
HSV	Herpes simplex virus
HZO	Herpes zoster ophthalmicus
IDE	Insulin degrading enzyme
IE	Immediate early
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IU	International unit
Kb	Kilo base
KSHV	Kaposi’s sarcoma associated virus
L	Late
mAb	Monoclonal antibody
MACS	Magnetic activated cell sorting
MEM	Minimal Essential Medium
MHC	Major histocompatibility medium
MOPS	4-Morpholinepropanesulfonic acid
MS	Mass spectrometry
NK	Natural killer
NSAIDS	Nonsteroidal anti-inflammatory drugs
ORF	Open reading frame

PAMP	Pathogen associated molecular pattern
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PHA	Phytohemagglutinin
PHN	Postherpetic neuralgia
RNA	Ribonucleic acid
Rpm	Rounds per minute
SDS	Sodium dodecyl sulfate
TAP	Transporter in antigen processing
TCR	T cell receptor
TEMED	Tetramethylethylenediamine
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TK	Thymidine kinase
Tris	Tris(hydroxymethyl)aminomethane
VZIG	Varicella-Zoster Immune Globulin
VZV	Varicella-Zoster Virus
WHO	World Health Organization