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„Misdirected antibody responses against non-neutralizing epitopes on human Cytomegalovirus“

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## ■ Abstract English

Human Cytomegalovirus (CMV) is a widely distributed member of the Herpesviridiae family. Whereas the virus only seldom causes medical troubles in healthy human individuals, CMV infection in immunocompromised patients can cause a life-threatening disease. Also for unborn children an infection can be risky.
Although developing mechanisms for treating and preventing CMV infections have been of high priority for scientists all across the world, still no effective strategies have been found as CMV has developed a series of highly efficient mechanisms to evade its host's immune system.

In our research we found that CMV lysate treatment with denaturing agents leads to the exposure of additional epitopes, which do not seem to be present on the native antigen and that a considerable amount of antibodies in human polyclonal sera are reactive with. These hidden epitopes are referred to as cryptic epitopes or cryptotopes and have already been described in several other pathogens and abundance of antibodies reactive with these type of epitopes have been associated both with a positive and negative prognosis for the course of an infection.
We hypothesise therefore that cryptic epitopes also play an important part in the immune reaction of humans towards CMV infection and that detailed investigation of CMV's cryptic epitopes could have a tremendous impact on CMV therapy and prophylaxis.

# ■ Zusammenfassung Deutsch

Humanes Cytomegalovirus (CMV) ist ein weit verbreiterter Angehöriger der Herpesviridae Familie. Während dieser Virus in gesunden Menschen nur äußerst selten Beschwerden hervorruft, kann eine CMV Infektion für immunsupprimierte Patienten lebensbedrohlich sein. Auch für die Entwicklung von ungeborene Kinder stellt eine Infektion ein Risiko dar.
Trotz jahrelanger Arbeit von Wissenschaftlern aus aller Welt, ist es bis zum heutigen Tag nicht gelungen einen schützenden Impfstoff gegen dieses Virus zu entwickeln und auch die Möglichkeiten CMV Infektionen medikamentös zu behandeln sind bis zum heutigen Tage noch stark eingeschränkt.

In dieser Arbeit zeigen wir, dass Behandlung von CMV Lysat mit denaturierenden Chemikalien zu einer Freilegung von zusätzlichen Epitopen führt, die auf dem nativen Protein versteckt sind. Ein erheblicher Anteil an Antikörpern in humanen CMV seropositiven Immunseren reagiert mit diesen Epitopen. Diese versteckten Epitope, oder auch Cryptotope, wurden bereits in anderen Pathogenen beschrieben und Auftauchen von Antikörpern gegen diesen Epitop Typ wurde sowohl mit einer guten als auch mit einer schlechten Prognose für den Krankheitsverlauf assoziiert.
Wir vermuten dass eine Immunreaktion des Körpers auf diese Cryptotope eine wichtige Rolle in der Immunabwehr gegen CMV spielt und dass eine weitere Untersuchung dieses Themas einen großen Einfluss auf die Zukunft der CMV Therapie und Prophylaxe haben könnte.

Wir vermuten daher, dass cryptische Epitope auch in einer CMV Infektion eine wichtige Rolle spielen und dass eine detailliertere Untersuchung einen großen Effekt auf die Zukunft der CMV Therapie und Prophylaxe haben könnte.

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# ■ Chapter 1: Introduction

## 1.1 Human Cytomegalovirus (CMV)

Human Cytomegalovirus (CMV) is one of the most abundant viruses found in the population all over the world. It is a member of the Herpesvirus family, which are widely distributed among vertebrates and were found to infect various cell types.
Three different subfamilies , the Alpha-, Beta- and Gammaherpesviridae, have been identified based on their sequence similarity and affinity for host cell types (Penkert, 2011) so far – besides muromegalovirus and roseolovirus, CMV belongs to the group of Betaherpesvirirdiae.
The major characteristic of this virus family is their ability to stay latent in cells for their hosts entire life – However, reactivation can occur (Modrow et al., 2010).

## 1.2. CMV Structure

Like most herpesviruses also CMV is composed of an icosahedral capsid containing the double-stranded DNA, a tegument layer and an outer lipid envelope.

So far three different types of CMV virus particles could be isolated from human fibroblast growth medium. First “classical” infectious virions , which are made up of a core, a capsid, a tegument layer and a lipid envelope. Additionaly two other types of non-infectious particles do exist: the dense bodies and the noninfectious enveloped particles referred to as NIEPs.
Dense bodies are non-replicative particles lacking DNA and a capsid, which can be found in the blood of most CMV infected individuals. Merely composed of the tegument protein pp65 as well as some glycoproteins they have been observed to enter target cells easily and being able to elicit both cellular and humoral immune response (Pepperl et al., 2000).
The structure of NIEPs is very similar to those of infectious virions except for their lack of DNA. Furthermore a 35kDa protein can be found in NIEPs which are neither present in infectious virions nor dense bodies. Like dense bodies, they are also a byproduct of virus production by host cells, which can be observed upon infection of cells by all different CMV strains investigated so far (Irmiere et al., 1983; Roby et al., 1986; Varnum et al., 2004)

### 1.2.1. Genome

The CMV genome is composed of linear double stranded DNA and with a size of 229.354 bp and 252 ORFs is the largest known genome of a viral humanpathogen. The genome is terminal redundant – there are 2 regions referred to as the unique long (UL) and the unique short (US) sequence. These sequences are flanked by inverted repeats, TLR/TRS and ILR/IRS.

It has been found that there is a high variability in the amino acid sequences between clinical strains – though it could be observed that in patients sequences were stable over a certain amount of time (Cunningham et al, 2009; Dolan et al., 2004; Murphy et al, 2003).
Additional to the DNA also viral and some cell-specific mRNAs can be found in the virion. What the function of these mRNAs is is not clear, but there is evidence that they are incorporated into the virion randomly according to their frequency in the host cell (Modrow et al., 2010; Terhune et al., 2004).

### 1.2.2 Capsid

The CMV capsid is about 105nm in diameter and is composed of 162 so-called capsomeres, proteins essential for the icosahedral structure of the capsid.
Four major capsid proteins are building the outer shell of the capsid are known: MCP, the largest of these proteins, SCP, the smallest and UL85 as well as UL46 which are both minor capsid proteins. MCP forms hexons and pentons which are connected by the two minor capsid proteins. On the surface of the capsid the small capsid protein SCP can be found. All four of these proteins can be detected in the mature virion.
Whereas the large and the minor of these capsid proteins share a highly similar structure, SCP is much more diverse and is of particular interest for scientists as this 8kDa protein was shown to be essential for entering the host cell (Borst et al., 2001; Yu et al., 2005).
Additional to those four proteins, three other capsid proteins could be detected, which can be found in the capsid internally: protein A and $C_{pra}$ as well as the protein AP.
Interestingly the precursor of protein AP, pAP, was found to be essential for the transport of MCP into the nucleus of the host cell. After the assembly of the procapsid pAP is then cleaved into AP, which prevents further interaction of the protein with MCP.
Inside the capside the DNA is coiled onto a fibrillar protein matrix, whose ends are attached to the underside of the capsid (Brignole et al., 2007; Modrow et al., 2010; Wood et al., 1997)

### 1.2.3 Tegument

The tegument can be found between the lipid envelope and the capsid of CMV. At least 25 proteins can be found here, meaning it provides more than a half of proteins which can be found in an infectious CMV virion.
Tegument protein pp65 and UL32 have always been particularly interesting for scientific research: pp65 is the most abundant protein in the CMV virion and is furthermore a highly immunogenic protein. Pp150 (UL32) is essential for replication of the virus and elicits both a strong humoral and cellular immune response.
Most of the proteins found in the tegument are highly phosphorylated, but if and what functions these phosphorylations have has not been investigated in detail yet. However, it is known that tegument proteins play an important role in the life cycle of CMV, as they are essential during cell entry and for the regulation of proper viral gene expression. The tegument has been thought to be unstructured for a long time, but studies indicated an icosahedral composition due to protein-protein interactions within this viral layer (Chevilotte et al., 2008; Tomtishen 2012).

#### 1.2.3.1. pp65

The most abundant protein found in the tegument is UL83 encoded phosphoprotein pp65 – also referred to as Lower Matrix Protein - which is also the main component of extracellular virions and dense bodies. Inter-

estingly, despite its great abundance, pp65 depleted virus was found to still be capable of infecting host cells in vitro and replicate within these (Chevilotte et al., 2008; Schmolke et al., 1995).
Directly after infection of host cells pp65 is transported to the nucleus, a process dependent on host cyclin-dependent kinases (CDKs) activity. Also pp65 newly expressed during early-late phase of infection can be found in the nucleus, but 48 hours postinfection no pp65 is left over in the nucleus but can only be found in the cytoplasm (Sanchez et al., 2007).

Both humoral and cellular immunity target pp65, though not all sera are able to detect pp65 by Western Blotting indicating a relatively weak humoral response towards this protein. In contrast to that cytotoxic T-cells were shown to respond strongly towards pp65 – up to 90 percent of cytotoxic T cells recognising CMV infected cells are specific for this protein (Wills et al., 1996).

#### 1.2.3.2 pp150

The second most abundant CMV tegument protein is pp150, encoded by the gene UL32. This protein is present in extracellular virions as well as NIEPs, but not so much in dense bodies.
In contrast to pp65, pp150 is essential for virus replication in infected cells as studies using UL32-null mutants have shown (AuCoin et al., 2006). After translation of pp150 is finished, the protein is highly phosphorylated. Furthermore pp150 is posttranslationally modified by the addition of O-linked N-acetlyglucosamine (O-Glcn-Nacs) at two Serine residues. The distinct effect of these modifications have not been described yet, However they do not seem to play a too essential role as substitution of the two concerned serine residues with alanine residues did not have an noticeable effect on the virus life cycle (AuCoin et al., 2006; Greis et al., 1994).

pp150 has been shown to be highly immunogenic by eliciting both humoral immunity as well as a CD4+/CD8+ immune response, which is usually easily detectable long after acute infection has been cleared by the host (Landini et al. 2000).

### 1.2.4 Envelope

The outermost layer of the CMV virion is the liquid envelope in which at least different viral proteins are embedded. This envelope is about 10nm thick and essential for the virus for being able to infect the next cell. After infection cell CMV elevates fatty-acid synthesis of its host cell – the newly synthesised membranes accumulate at the AC. Interestingly the glycerophospholipid composition of the viral envelope is quite different from this of the cell membrane but resembles more the composition of synaptic vesicles, which leads to the assumption that CMV buds into exosome-like vesicles. Although no direct proof for this hypothesis has been shown yet, it is strengthened by the fact that depletion of SNARE complex parts, which are essential for exocytosis of synaptic vesicles, CMV yield was found to be decreased. Furthermore SNARE complexes are known to be $Ca^{2+}$ dependent – $Ca^{2+}$ levels have been found to be elevated in CMV infected human fibroblasts, which is further supporting this hypothesis (Liu et al., 2011; Munger et al., 2008).

Nine viral encoded glycoproteins can be found embedded in the envelope of CMV virions. One of the most prominent is the UL55 encoded glycoprotein B (gB), whose homologues can be found in all different herpesviruses.

### 1.2.5 Glycoprotein B

Glycoprotein B is the most abundant glycoprotein found in CMV.
Directly after synthesis it has a size of 105kDa, but then is highly glycosylated in the Golgi or ER at the N-terminus of the protein, leaving it with a size of 130 to 160kDa. The active protein has to be cleaved by the cellular protease furin, which cleaves it into a amino-terminal 116kDa fragment and a carboxy-terminal 55kDa fragment. These two monomers oligomerize and fold while travelling through the ER, a process shown to take quite long, but which is essential for full functionality of the protein and for transport out of the ER to the cell surface (Boyle et al., 1998; Britt et al., 1992).
gB is thought to be critical for generating a stable bond to the target cell as well as for the fusion of the cellular with the viral membrane which allows viral tegument proteins and DNA to enter the cell. Essential for these functions is the association of gB with the heparan sulfate proteoglycan heparin. Interestingly gB also incorporates a highly conserved disintegrin-like domain, which seems to be essential for fusion events by the use of cellular integrins as receptors (Feire et al., 2004; Isaacson et al., 2009).

Glycoprotein B elicits a strong immune response as nearly 100% of CMV infected individuals develop antibodies against this viral protein, most of which were found to be neutralizing (Schoppel et al., 1997).
So far five different antigenic domains have been described.

Antigenic domain 1 (AD-1) is a continuous, that is linear, epitope stretching from gB residue 552 to 635. Containing about 80 amino acids it is a surprisingly complex domain. It was shown that the complete stretch is needed for antibody binding and that simple point mutations can heavily influence antibody binding properties to this domain. AD-1 has been in the focus of research's interest for a long time as nearly all CMV infected individuals elicit antibodies against this domain. Not all of these antibodies bind to the same part of the AD-1 domain, but all of them seem to be dependent on cysteine residues 573 and 610.
(Schoppel et al., 1997; Speckner et al., 1999; Wagner et al., 1992).
Along with AD-1, antigenic domain 2 (AD-2) was the first antigenic domain discovered on gB.
Located at the N-terminus of gB, AD-2 is composed of two distinct sites. Site I is a linear epitope, composed of 8 amino acids (aa) and stretches from residue 69 to residue 78 on gB – the sequence of this site is the same in all different CMV strains and was found to elicit neutralizing epitopes.
In site II sequence differences can be found between strains and no neutralizing antibodies against this site have been detected so far. However, only about 50% of CMV infected individuals seem to carry antibodies against this epitope (McLean et al., 2005; Pötzsch et al., 2011)

Whereas AD-1 and AD-2 are located at the gB ectodomain, AD-3 can be found at the endodomain. It is a linear epitope and due to its location inside the virion does not induce neutralizing epitopes, which might be the reason AD-3 has never been of strong interest in research and not too much is known about this epitope (Kniess et al., 1991; Silvestri et al., 1991).

Antigenic domain 4 and 5 (AD-4, AD-5) have only been discovered quite recently.
Whereas AD-5 is located between aa residues 133-143, AD-4 is a discontinuous domain stretching from aa 121-132 and 344-438 and hence lie in highly conserved regions of gB. Both domains were found to elicit highly neutralizing antibodies - 90% of antibodies were tested positive for the AD-4 domain, 50% positive for the AD-5 domain. The exact mechanisms of how neutralization is conferred is not known yet though it was shown that these antibodies do not prevent attachment of the virion to the target cell but function in a postabsorption step (Pötzsch et al., 2011).

## 1.3. Human Cytomegalovirus Life Cycle

Like in most herpesviruses infection of a cell starts upon fusion of the cell membrane and the viral lipid envelope, which leads to translocation of the nucleocapsid in the inner of the cell. In CMV this fusion is done by binding of its glycoproteins to several receptors on the target cell.

After entry of the cell the nucleocapsid enters the nucleus, where the viral DNA is released.
Viral genes are expressed in a cascade like manner, starting off with the immediate early (IE) genes.
The major IE gene products, IE72 and IE86 are generated from a primary transcript by differential splicing and polyadenylation. Both proteins are essential for the expression of viral early genes.

For productive virus replication, CMV needs to manipulate its host cell by elevating some cellular and alter its cell cycle regulatory machinery. IE86 was shown to cause progression of the host cell to S-phase and then stop ongoing of the cell cycle. Interestingly it was found that CMV also has mechanisms to accelerate the cell cycle – it is hypothesised that the virus blocks cellular DNA replication in favour of rash replication of viral DNA that way.

Only after expression of early genes, which is essential for starting the replication of viral DNA, delayed early and late gene expression is allowed, which encode for structural components of the virus and often are involved in the assembly of the virus.
The most important proteins involved in virion assembly are pp150, pp28 and UL97 (reviewed in Tomtishen, 2012, Murphy et al., 2000).
pp150 can be detected both in the cytoplasm as well as in the nucleus of the host cell during infection. Translocation of pp150 to the nucleus starts at the beginning of migration, where it binds to capsid structures via its amino one third (Baxter et al., 2001) – it remains attached to the nucleocapsid during the whole maturation process. In the cytoplasm pp150 was found to accumulate in a juxtanuclear structure, referred to as the assembly compartment (AC). Various other CMV encoded proteins, among them glycoprotein B and H as well as tegument protein pp65 co-localise there. Hence it has been suggested that these structures provide a site for virus assembly in the cytoplasm (Sanchez et al., 2000).
The localization to the AC is thought to be dependent on the interaction of pp150 with the cellular protein Bicaudal-1 (BicD1) – the exact mechanism of this interaction is still unknown, but yet of crucial importance for

viral replication as depletion of fully functional BicD1 was shown to decrease assembly of infectious virus (AuCoin et al., 2006; Indran et al., 2010; Sanchez et al., 2000).
After tegument proteins and the nucleocapsid have arranged at a specific locus, pp28 is essential for the particles to be enveloped to form viral particles – the exact mechanism of this function is not known yet though.
UL97 is known to have a kinase activity, which allows it to phosphorylate other viral proteins. Although it has been suggested that this tegument protein plays a role in several events during the CMV life cycle, i.e.DNA synthesis, its major function seems to be the phosphorylation of proteins at the assembly compartment, a process which is thought to make the packaging of proteins to the virion easier (reviewed in Tomtishen 2012; reviewed in Kaljeta, 2008).

After primary infection CMV has the ability to stay latent in its host and that way establish life-long persistence. In healthy individuals reactivation of CMV infection only seldom occurs.
In latent CMV infection no viral particles are produced and the viral DNA stays as extrachromosomal episome in the nuclear plasma. The mechanisms mediating latency are not entirely understood yet. However it has been shown that inhibition of the IE genes is essential for establishment of a latent state. The cellular protein Daxx was shown to interact with a histone acetylase (HDAC), which induces a repressive chromatin state at the major IE gene locus. This state can be reversed if the viral tegument protein pp71 binds to Daxx. In latent infected cells pp71 and Daxx are found in different compartments, pp71 is not able to travel to the nucleus and therefore not capable to inhibit Daxx (Saffert et al., 2007).
How exactly reactivation of CMV occurs is not known yet in detail either – it was found though that reactivation of virus infecting bone marrow progenitor cells happens after macrophages and dendritic cells start to differentiate (Sinclair et al., 2006).

## 1.4 Clinical Relevance

### 1.4.1 Epidemiology and Transmission

Human cytomegalovirus is widely distributed in populations all across the world. In the developed world overall CMV seropositivity is 60% - seroprevalence was found to strongly depend on ethnicity and income levels though (Munro et al., 2005).

Transmission of the virus from one host to the other can occur on various routes.
Infectious virions can be found in saliva, as well as in urine or blood – especially CMV positive children either cogenitally infected or younger than 3 years were shown to shed great amounts of CMV in their body fluids (Stowell et al., 2012). Additionally, infectious viral particles were found in semen and in cervicovaginal secretions indicating that also transmission during sexual contact is common (Johnson J. et al., 2012).

Transmission can also happen from mother to child either post-natal by breast-feeding or pre-natal by congenital infection. 1-8% of CMV seronegative pregnant women develop a primary CMV infection, in 40% of the cases the virus is transmitted to the unborn child (Rahvad, 2007). Additionally reactivation in seropositive mothers due to hormonal changes can occur during the pregnancy referred to as non-primary infections.

Though it is thought that in these cases the embryo is partially protected by the mother's immune system and immune IgG travelling through the placenta, the infection can still have grave effects on the foetus (Ludwig et al., 2009)
Furthermore the virus can be transmitted by organ transplants as well as by blood transfusions.

### 1.4.2 Clinical Symptoms

After a 4 to 8 week incubation period after CMV primary infection, some patients experience malaise with fever episodes over 38°C which can last more than 2 weeks. Also headache, pharyngitis and lymphadenopathy have been described, but these first symptoms can hardly be distinguished from an Epstein-Barr infection (Johnson et al., 2012; Just-Nübling et al., 2003).
However, in most healthy adults CMV primary infection is completely asymptomatic.

On the contrary, CMV infections can be critical and even lethal for congenital infected children as well as for immunocomprised patients and transplant recipients.
CMV primary infection in pregnant women often stays undetected and so does intrauterine contagion of the unborn children. Estimated 400 newborns in the United States die per year due to the consequences unrecognised congenital CMV infection (Cannon et al., 2005). Furthermore congenital CMV infection is a leading cause for mental retardation, neurological deficits (Leung et al., 2003) and is thought to be the reason for one-third of cases of sensineural hearing loss in children. Treatment of these children is especially difficult as 90% of infected infants show no signs of infection after birth, but will suffer from progressing hearing loss over several years (Arvin et al., 2004; Barkai et al., 2013).

As well as for unborn children also for immunocompromised patients CMV infection can be life-threatening. Patients suffering from AIDS often undergo CMV primary infection or reactivation due to the loss of cell mediated immunity – in 50% of individuals viral particles with an $CD4^+$ count lower than 100 cells per µl viral shedding can be detected in their urine - in other words CMV infection usually happens in a late stage of the disease and seems to independently predict the upcoming death of a patient (MacGregor et al., 1995; Vidal et al., 2003). Cases of acute CMV infections as well as patients undergoing transient episodes of viremia have been reported so far – the clinical importance of this viremia episodes is not entirely known yet, though it has been speculated that these could contribute to the dispersion of viral particles through the organs and thus leading to end-organ disease (Jacobson et al., 2006a).
CMV infection in AIDS patients can have various clinical manifestations: 80 to 90% of individuals with a $CD4^+$ count lower than 50 cells per µl suffer from Chorioretinitis, an inflammation of the choroid which leads among others to a blurred vision and sensitivity to light (Drew et al., 2006).
Furthermore interstitial pneumonia - characterised a.o. by dyspnea and non-productive cough – due to CMV infection has been found to be highly frequent and to often contribute to the death of affected patients. Therefore it is particular problematic that the diagnosis of CMV induced pneumonia often is difficult as CMV induced pneumonia often does not cause distinctive abnormalities. Additionally the virus co-exists with other pneumonia causing pathogens, e.g. Pneumocystis jirovecii, in the lung of these patients, making it difficult to examine which of them is actually causing the disease.

Apart from these two diseases also gastrointestinal and central nervous system disease due to CMV infection has been reported (Drew et al., 2006; Rodrigues-Barradas et al., 1996; Wallace et al., 1967).

Another high risk group concerning grave CMV infection are transplantant recipients as these patients usually are treated with immunosuppressive drugs to prevent allograft rejection. Especially seronegative recipients receiving either a solid organ or a bone merror transplant from a CMV seropositive donor (D+/R- transplants) are endangered. Surprisingly some studies have shown that not the D+/R- group but D+/R+ group have the worst prognosis for 3 year graft and patient survival (Brennan, 2001; Schnitzler et al., 1997)
A total of 75% of patients are either newly infected by CMV or experience reactivation after the intervention. Although in modern medicine highly effective antiviral drugs are available, medication can not be given forever due to the development of resistant viral strains as well as due to often occurring severe side effects these drugs elicit. Therefore antiviral drugs often just delay the onset of CMV infection - 35% of R+/D- transplant recipients experience a symptomatic CMV infection after antiviral medication is stopped (Liu et al., 2013; Freeman, 2009;).
Besides pulmonary, gastrointestinal and other typical diseases caused by CMV in immunocompromised patients, it was also found that late CMV infection in transplant recipients can cause graft loss - main reasons for this loss is cardiovascular death and chronic rejection.
Chronic rejection is characterised by a distinct form of arteriosclerosis, myointimal thickening. Studies have shown that CMV seropositive individuals have a greater risk to suffer from restenosis after a coronarangiography than seronegatives. Whether increased CMV infected smooth muscle cell proliferation (SMC) due to high p53 levels, which correlate with CMV IE84 expression patterns, or increased SMC migration triggered by CMV chemokine receptor US28 are responsible for this phenomenon is not clear yet. However, it was shown that infection of SMCs by CMV leads to an increased LDL uptake, a factor which has already been identified in the development of arthersclerosis. Nevertheless it has to be further investigated how and if these factors also play a role in rapid graft loss after late CMV infection (Brennan, 2001; Speir et al., 1994; Streblow et al., 1999; Zhou et al., 1996).

### 1.4.3 CMV Prophylaxis

Due to the high prevalence and huge complications CMV infection can cause in immunocompromised patients and congenitally infected children, the development of a vaccine against this disease has been in focus of scientists and physicians since the 1970s. Although many of the vaccines developed so far have succeeded in phase I clinical trials, they were not able to give satisfactory results in phase II clinical trials (reviewed in Rieder et al., 2013).

Most of these developed vaccines are either composed of living attenuated viruses or subunits which target a specific pathogenic protein (Schleiss et al., 2008).
One of the most common strains worked with in the development of a live attenuated virus vaccine is the clinical CMV strain Towne. A clinical trial with transplant patients showed that though the vaccine could not prevent CMV disease, vaccinated patients showed severe CMV disease more seldom than non-vaccinated

individuals. Another trial investigating seronegative mothers with children attending a day-care showed that vaccination could not protect these mothers from being infected by their children. However, it could also be shown that vaccination could save already seropositive mothers from infection with a new CMV strain (Adler et al., 1995; Plotkin et al., 1994). All these data indicate that a Towne vaccination only provides little protection against CMV infection. An essential reason for the failure of whole virus vaccines is that laboratory strains genetically differ a lot from clinical strains (reviewed in Rieder et al., 2013).

Several different strategies have been developed to improve immunogenicity and hence also protection from CMV infection after Towne vaccination, like for example recombining CMV strain Towne with the CMV strain Toledo or co-administration of the vaccine with human IL-12. However, these vaccines are still in clinical trial and it is hard to predict whether or not these strategies will be more successful than their antecessors (Heinemann et al., 2006; Jacobson et al., 2006b; Sung et al., 2010).

Additional to these live, attenuated virus vaccines also subunit vaccines have been developed.
A common CMV protein used for those subunit vaccines is CMV glycoprotein B. All CMV seropositive individuals carry antibodies against this viral protein, and a great amount of the neutralizing antibodies present in the host are specific for epitopes found on gB (Navarro et al., 1993).
Besides gB also other proteins were tested as subunit vaccines e.g. the lower matrix protein pp65 and the Immediate Early Protein 84 (IE84). Both of these proteins were shown to elicit specific recognition of cytotoxic T-lymphocytes recognising CMV (Wills et al., 1996; Sung et al., 2010).

A DNA vaccine currently in phase II clinical trial is the DNA vaccine TransVax, encoding for both gB and pp65. The combination of these two proteins was chosen as pp65 is known to elicit a strong cellular adaptive immune response, whereas gB is a popular target for B- as well as for T-cell.
A trial in CMV seropositive bone marrow transplantation patients, receiving bone marrow from CMV seropositive donors, found that vaccination with TransVax could not only reduce acute CMV infection episodes but also extended the time span between two viremia episodes (reviewed in Rieder et al. 2013).

Further investigation has to be done to see whether or not any of these vaccines will be a strong protection against CMV infection for both immunocompetent, immunocompromised and congenital infection.

## 1.5. CMV and the immune system

Human cells react to CMV infection with full activation of both the innate and the adaptive immune system. Although the human immune system uses many cellular and humoral techniques to prevent CMV infection, usually these efforts are fruitless as the virus has developed many mechanisms to suppress both the innate and adaptive immune system and so maintain latency in its host.

### 1.5.1. Innate Immune System

Upon invasion of pathogens into the human body the innate immune system is the first line of defence reacting to possibly dangerous invaders. Besides mechanical barriers, like the skin and saliva in the nasopharynx, leukocytes are involved in this process.

#### 1.5.1.1. Phagocytes

The main function of phagocytes is to remove dead or dying cells from the body. However, some also act as antigen presenting cells (APC). Macrophages, mast cells, neutrophils and dendritic cells all have the ability to phagocytose pathogens.

They express the so-called Pattern Recognition Receptors (PRRs) which recognise so called PAMPs (pathogen associated molecular pattern) on pathogenic species.
Two types of PRRs have been described so far: signaling and endocytic PRRs. Whereas binding of PAMPs to endocytic PRRs only leads to activation of phagocytes, activation of signaling PRRs starts intracellular signal cascades.
A prominent PRR are the Toll-like-Receptors – in humans ten different TLRs have been described, which all are specific for another PAMP, e.g. LPS (TLR-4). Binding of the ligand to the respective TLR starts a signalling cascade which leads to production of proinflammatory cytokines and NfκB.
Also CMV is recognised by these type of receptors - Toll like receptor 2 (TLR-2) was shown to be activated by CMV glycoproteins B and H (gB, gH), leading to the up-regulation of NF-kB and SP-1. Interestingly it has been found that liver transplant patients bearing a homozygous single nucleotide polymorphism (SNP) in TLR2 Arg53Gln were more susceptible to CMV disease. Recently it was shown that parts of a gB vaccine were also recognised by a specific SNP bearing TLR-7 leading to increased B-cell proliferation and antibody secretion, a finding which could contribute to the development of a fully protective CMV vaccine (Arav-Boger et al., 2012; Kijpittayarit et al., 2007). TLR-2 has also been shown to be essential for the secretion of inflammatory cytokines during CMV infection and that the main triggers for this activation are CMV gB and gH (Boehme et al., 2006).
However, not only PRRs but also other receptors can be expressed on the membrane of phagocytes.

Macrophages and dendritic cells (DC) develop from monocytes and are professional APCs. Both cell types have the ability to ingest pathogens by phagocytosis, digest them and present these antigens on MHC to T-cells.
Macrophages have a superior life span compared to other immune cells. They wander to sites of infection or tissue damage by chemotaxis and there enclose dying or already dead cells with arm like structures, referred to as pseudopodia, and ingest pathogens via a vesicle. Inside the cell the vesicle fuses with a lysosom and pathogen is killed.
Dendritic cells (DC) can mainly be found on areas of the body which are frequently exposed to the environment. Like macrophages, also DCs ingest and digest pathogens and stimulate cells of the adaptive immune system by presentation of antigens on MHC class II.

Mast cells are commonly associated with allergic disease, However they have also shown to play an important role in the innate immune defense to pathogens as they are usually concentrated in places constantly exposed to environmental factors (e.g. the skin.). It was shown that activation of pathogens can occur by pathogen binding to IgE bound to the FcεRI. This pathway was shown to be especially important in host defense against malaria and other parasites. Various other ways how mast cells can be activated by pathogens have been described as well. However most of them are only scarcely characterised.
Mast cells can fight pathogens either directly by phagocytosis and release of reactive oxygen species or indirectly by releasing pro-inflammatory mediators like histamine, chemo- and cytokines which activate other immune cells (reviewed in Urb et al., 2012). Furthermore mast cells have the ability to act as antigen presenting cells and activating CD-8+ T cells, which was shown to provide protection in pulmonary CMV infection (Ebert et al., 2012).

With an abundance of 50-60% of total circulating leukocytes, neutrophils are the most common phagocytes in the blood stream. Attracted to sites of infection by chemotactic agents released both by phagocytes and the invaders themselves, neutrophils have been found to kill pathogens in three different ways.
First by phagocytosis: via their Fc receptor neutrophils bind opsonised pathogens, ingest it and then kill it by the release of ROS and/or hydrolytic enzymes.
A second way of killing is the release of granules: neutrohils have been found to be rich in cytoplasmit granules containing microbial agents.
Thirdly neutrophils were shown to release granule proteins and chromatin which build up the so-called neutrophil extracellular traps (NETs), which bind and kill pathogens (Brinkmann et al. 2004; Campbell et al. 2006; Martinko et al., 2006; Smith 1994).

#### 1.5.1.2. Granulocytes

Eosinophils, basophils, neutrophils and mast cells all contain granules containing toxic chemicals, which are released upon activation through pathogens.

Eosinophils are traditionally associated with the defense against helminthic parasites as well as with allergic diseases like asthma. They recognise and are activated by binding of their FcεRI to pathogens coated with IgE antibodies – once they are detached to the pathogen lytic enzymes, e.g. the major basic protein which is a strong helminthotoxin, but can also cause damage of epithelial cells (Fischer et al., 2013).

Like mast cells and eosinophils also basophil granulocytes express the FcεR on their surface with which they recognise IgE. Additionally they have been shown to function as Antigen Presenting Cells (APC) – basophils express MHC class II and by uptake and processing of antigen – mostly IgE coated pathogens – they can induce Th2 differentiation (Nakanishi, 2010). It was shown that basophils also play a minor role in viral infections, However if and how this cell type plays a role in clearance of CMV infection has not been reported yet.

#### 1.5.1.3. Natural Killer Cells

Natural killer (NK) cells are an essential part of the innate immune system for the recognition and control of viral infection. Healthy cells present self markers on MHC I – by interacting with this receptor NK cells can discriminate between virus infected, cancer and healthy cells. Furthermore NK cells can be activated in an antibody dependent way via the FcYRIII and by cytokines released by virus infected cells (e.g. IL-12, IL-2). Activation of NK-cells leads to the release of granules containing perforins and granzymes near target cells. Perforin form holes in the membrane of these infected cells, through which the granzymes can travel and induce apoptosis.
CMV infection was shown to be important for NK cell development – several studies propose that CMV infection does accelerate NK cell differentiation and significantly shapes NK cell repertoire in humans (Benziat et al., 2013; Della Chiesa et al., 2013).

### 1.5.2. Adaptive Immune System

Besides the innate immune system also the adaptive immune system tries to protect individuals from invading pathogens. In contrary to cells of the innate immune system, cells of the adaptive immune system do not recognise patterns shared by many pathogens, but a multitude of different structures referred to as antigens. T- and B- lymphocytes are the major cell types contributing to adaptive immune system. They differentiate in the bone marrow from pluripotent stem cells to lymphoid cells – when wandering to the thymus these cell type differentiates into T-cells, but when remaining in the bone marrow they become B-cells (Campbell et al. 2006; Martinko et al., 2006).

#### 1.5.2.1. The T-cell Response

Lymphoid cells migrate to the thymus where they differentiate to the different subsets of mature, naïve T-cells, a progress which is referred to as thymatopoesis.
However, cells do not only undergo differentiation but also a strict selection process, allowing only T-cells capable of interacting with MHC molecules but not recognising self antigen to leave the thymus to enter the rest of the body – about 98% of thymocytes are sorted out during this process.
Another important event during thymatopoesis is the so-called beta-selection, a process during which V(D)J recombination creates a multitude of receptors all specific for another antigen. In T-cells, the receptor affected by this process is the T-cell receptor (TCR), which is expressed on the surface of T-cells.
These receptors interact with the major histocompatibility complex (MHC), of which two subgroups have been described. MHC class I is expressed on the surface of all nucleated cells, hence all cells despite erythrocytes. MHC class II however is only expressed on the surface of professional APCs.

After differentiation and selection mature, naïve T-cells leave the thymus to migrate through the body. Dependent on which molecular marker (cluster of differentiation, CD) these cells express they can be divided into T-cell sub-populations which fulfil various tasks:

T-helper cells, or CD4+ T cells, recognise antigen-MHC class II complexes presented on the surface of APCs. After recognition T-cells proliferate and differentiate into three different T-helper cell subgroups: effector T-cells, memory T-cells and regulatory T-cells.
Whereas memory T-cells reside in the blood to act as effector T-cells during another exposure of the same antigen, regulatory T-cells suppress the immune system to prevent an overreaction of the immune system and autoimmune reactions.
Effector T-cells further differentiate in either Th1 or Th2 effector cells. Th1 cells are mainly involved in the cellular immunity, Th2 cells are essential for the activation of the humoral immunity.

T-cells expressing CD8 on their surface are referred to as cytotoxic T-cells or CD8+ cells. Activation of these requires two signals: first interaction of the TCR with antigen bound to MHC class I and second by an APC or alternatively by the release of IL-2 by Th1 cells. After activation cytotoxic T-cells release cytotoxins, e.g. perforin which forms pores in the target cell's membrane – influx of water and ions then leads to lysis of this cell. Also, CD8+ cells can express Fas ligand on their surface after activation – binding to the Fas Receptor expressed leads to apoptosis of the target cell (Campbell et al. 2006; Martinko et al., 2006).

Compared to other viruses, CMV elicits an extraordinary large and long-lived number of CMV specific T-cells in healthy individuals. Though these large numbers of T-cells do neither have the ability to prevent transmission nor to clear infection, they are still essential to prevent disease in healthy humans (reviewed in LaRosa et al., 2012).

#### 1.5.2.2. The B-cell Response

Like T-cells, B-cells differentiate from pluripotent stem cells to lymphoid cells too. However, whereas T-cell progenitors travel to the thymus, B-cells develop in the bone marrow. Each differentiation step is characterised by a change (VDJ recombination) in the B-cell receptor (BCR), which is basically a membrane bound antibody.
After VDJ recombination has finished, B cells are excessively tested for recognition of self antigens and only then allowed to wander as mature naïve B-cells to the secondary lymph nodes. When antigen binds to the BCR, the Ig-antigen complex are internalised, digested and the generated peptides are then presented to Th2 cells on MHC class II. This leads to release of cytokines, mainly IL-4 and IL-5 by the Th2 cells which leads to clonal expansion antigen specific B-cells. These B-cells, which are all specific for the same antigen then, further differentiate into plasma cells or memory B-cells. Whereas the plasma cells have only a short life time, memory B-cells stay in the body for a long time and rapidly convert to plasma B-cells after second antigen exposure (LeBien 2000; Martinko et al., 2006).

Plasma cells are essential for the production and secretion of antibodies, also called immunoglobulins. All of these immunoglobulins are composed of two identical heavy and two identical light chains which are linked together by disulfide bonds. Both light and heavy chains contain a constant and a variable region, with which antigens are bound. The five different types of immunoglobulins (isotypes) differ in the constant region of the heavy chain.

Directly after exposure to antigen, B-cells express and secrete class M immunoglobulin (IgM). However, in the course of an infection also antibodies of other classes are expressed, a mechanism which is referred to as Ig class switch. As the immunoglobulins have different effector functions and are secreted in different parts of the body, this switch is essential to establish full protection against a pathogen (Wang et al., 2004). Immunoglobulin G (IgG) is the major antibody type circulating in the blood stream – it is the only immunoglobulin able to cross the placental barrier and hence is essential for protection of an unborn child. IgAs are circulating antibodies as well, but they can not only be found in the blood but also in secretions (e.g. saliva) of the body. Furthermore IgA is found in high amounts in mucosa, e.g. the gut, where they are essential for preventing colonisation of pathogens. IgE is mainly associated with autoimmunity, but has been found to be essential for immune response to parasitic worms. Like IgM, also IgD has been found to act as BCR – however, unlike IgM which is already present on B-cells in the bone marrow, IgD is only expressed on B-cells which have already left the bone marrow (Geisberger et al., 2006; Martinko et al., 2006).

IgM secreted during first exposure to an antigen usually do not have a high affinity for their respective antigen. It has been observed though that repeated exposure to the same antigen leads to the creation of antibodies with greater affinities. The underlying process is referred to as somatic hypermutation – in this process the variable domain of the antibody is rapidly changed by point mutations ($10^{-3}$ changes/base pair/cell cycle). APCs then present antigen to the B-cells – only the B-cells having a high affintiy for this antigen are allowed to proliferate, less affine B-cells undergo apoptosis (reviewed in Saribasak et al. 2012; reviewed in Schroeder et al., 2010).

Antibodies do not recognise whole antigens, but single sections on a protein, which are referred to as epitopes or antigenic determinants. These epitopes are usually 5 to 10 amino acids long – hence a single protein can contain several distinct epitopes.
Epitopes composed of discontinuous amino acids, which come together by the protein's tertiary structure are referred to as conformational epitopes – the amino acids building up these type of epitope can be located far away from each other on the primary structure of the protein. Most of B-cell epitopes are thought to be conformational ones. Continuous epitopes are made up by a stretch of succeeding amino acids and are also referred to as linear epitopes. Also a third epitope type has been described, the so-called cryptotopes. This type of epitope is hidden in the native form of the protein and is only exposed after denaturation of the antigen (Ansari et al., 2010; Campbell et al., 2006; Kumar et al., 2013).

Though antibodies do not directly destroy pathogens, they support the immune system in various ways.
First by activation of the complement system: proteins of the complement system contain a domain which is able to bind to antibodies (mostly IgG and IgM) which are attached to a pathogen. Activation of the complement can have two consequences: first by formation of the so-called MAC which forms pores in the membrane of target cell's leading to direct lysis of the cell. Second complement proteins attached to pathogens activate phagocytes – this process is referred to as opsonisation.
Antibodies can also agglutinate extracellular bacteria or virus – these precipitates can be recognised and ingested by phagocytes more easily than single pathogens.

Furthermore binding of antibodies to an antigen can result in the loss of its protein function, a process which is called neutralisation (Campbell et al., 2006).

### 1.5.3. CMV Evasion from the human IS

Approximately 108 million years of coevolution with humans have given cytomegalovirus a lot of time to develop methods to counteract the human immune system and to prevent total clearance of virus from its host. Many proteins CMV expresses have the ability to modulate the immune response and are associated either with immune evasion or increased inflammation.

As natural killer cells are the first line of defence against viruses, it is not surprising that CMV has developed several methods to hinder NK cell activation and function.
The main target to stop NK cell activation is MHC class I – as already mentioned NK cells can distinguish between healthy and virus infected cells by this receptor. CMV was found to express homologs to MHC class I, the most prominent of them being UL18. This protein interacts with the NK cell inhibitory receptor LIR-1 and hence hinders activation of the NK cells. Also two other CMV protein products, UL16 and UL142 have been shown to be MHC class I homologs, which interfere with NK cell activation and clearance of virus infected host cells (Prod'homme et al., 2007; reviewed in Schleiss 2011).
Not only NK cell response is modulated by CMV infection: there is evidence that CMV causes downregulation of chemotactic receptors and reorganises the cytoskeleton of macrophages, leading to a decreased mobility of these cells (Frascaroli et al., 2009).
Also Interferons, mainly Interferon alpha and beta, are significantly engaged in virus control - they can be generated by almost all cell types in the presence of viral and bacterial nucleic acids. Binding of Interferon alpha and beta to their receptors activates the JAK-STAT pathway, which leads to the expression of genes involved in the suppression of viral proteins and activation of the immune system.
Two genes, IE 72 and IE86, have been shown to interfere with this pathway. IE72 binds to STAT proteins, forming a complex with these proteins and hence inhibiting the JAK STAT Pathway. IE86 was observed to interfere with the expression of interferone beta – the exact mechanism of this process is not known yet (Paulus et al., 2006; Taylor et al., 2005).
Furthermore some CMV protein products have been shown to interfere and modulate chemokine and cytokine immune response. CMV UL111a for example is a homolog of human interleukin 10 (IL-10), which has anti-inflammatory effects (reviewed in Schleiss, 2010).

Besides methods to evade the innate immune system, CMV has also developed strategies to escape the adaptive immune system. Most of these strategies target presentation of antigens via MHC class I and are fulfilled by CMV proteins encoded in the US region of the virus.
US3, US2, and US11 all target MHC class I: US2 and 11 gene products translocate MHC class I from the ER to the cytoplasm, where it is degraded by proteosomes. US3 encodes for a gene product which settles in the ER and there binds to MHC class I which cannot be relocated any more (reviewed in Schleiss , 2010).

Healthy human individuals infected with CMV usually develop a broad CMV specific antibody spectrum. Many of these antibodies also show good neutralisation capacities, however, lack the ability to completely clear virus from the host.

An example for this phenomenon is the monoclonal antibody MSL-109, specific for CMV glycoprotein H (gH), which was isolated from a healthy CMV seropositive human donor. Although this antibody was shown to prevent infection in vitro, it was not able to protect from infection in vivo. Further investigations showed that cells infected with the virus took up MSL-109 which was then incorporated in the virion. This process did not only protect the virus from the antibody, but also facilitated its entry to other cells (Manley et al., 2011).

Glycoprotein B's antigenic domain 1 (AD-1) is another example of how CMV escapes neutralising antibodies. It was shown that this domain elicits the production of a multitude of antibodies – many, but not all of them have the ability to neutralize the virus. However, binding of the complete set of antibodies in humans was shown not to neutralize the virus completely as both neutralizing and non-neutralizing antibodies bind to this domain. It has been hypothesised that the competitive binding of these antibody species is a mechanism of the virus to evade their host's immune system (Schoppel et al., 1997; Speckner et al., 1999; Wagner et al., 1992).

# ■ Chapter 2: Motivation

CMV infection is quite abundant in the world's entire population and is associated with life-threatening disease in immunocompromised patients. Furthermore congenital CMV infection is the leading infectious cause for sensoneural deficits in new borns.
The development of a protective CMV vaccine has been ranked as top priority by the US Instiute of medicine, yet effective treatment and prophylaxis is still not available.

In an attempt to develop an in-house CMV specific ELISA we observed that treatment of virus lysate with denaturing agents leads to an increase in signal compared to those antigens treated with a buffer not containing protein disrupting chemicals. Three different types of epitopes have been described on pathogens: conformational, linear and cryptic epitopes. Those cryptic epitopes are hidden in the protein's native form and are only exposed when the protein changes its conformational structure.

Cryptic epitopes, also referred to as cryptotopes, have been described in several other human pathogens as well and immune response specific to these cryptotopes has been associated both with a positive and negative diagnosis.
We therefore wanted to isolate the antibody portions reacting to the different types of epitopes on CMV to be able to characterise them further and to see whether they differ in neutralizing capabilities.

CMV glycoprotein B (gB) is one of the most abundant proteins in the virion and was found to elicit a strong antibody response. All CMV seropositive sera include antibodies specific to CMV gB and a great portion of these antibodies are neutralizing, which is the reason why gB is the most commonly used CMV protein in vaccine development. However, most of these vaccines were found to offer limited protection against CMV primary infections.
To see whether cryptic epitopes are also abundant on CMV gB and whether they have an impact of overall humoral immunity against the virus, epitopes located on gB, referred to as antigenic domains, were cloned in the mammalian cell vectors and expressed in HEK293 cells. We hope to purifiy these recombinant proteins via their His-Tag and study antibodies reactive with these antigenic domains more accurately.

With the help of these data we hope to improve the understanding of how CMV manages to escape from the (humoral) immune system and to contribute to the development of better CMV prophylaxis and therapy regimes.

# ■ Chapter 3: Results

## 3.1. Development of an CMV specific ELISA

To develop an in-house indirect ELISA (Enzyme linked Immunosorbent assay) specific for the detection of CMV specific IgG antibodies, the optimal lysis conditions for CMV virions were investigated. For this purpose CMV lysate was diluted with Bicarbonate buffer pH 10, as it is commonly used in ELISA protocols, at ratios ranging from 1:1 to 1:10 in two fold step. Furthermore incubation time and temperature were varied to see which conditions resulted in the most effective lysis of CMV. 1:2 dilution with the buffer as well as incubation for 12 hours at 4°C were found to optimally prepare lysate for the successive assay.

To evaluate the optimum concentration of antigen and human serum to obtain the best signal to noise ratio, a checkerboard titration was performed according to standard protocols.
For this purpose CMV lysate was diluted in Bicarbonate buffer in two fold steps, concentrations ranging from 10µg/ml to 0.313µg/ml . A representative CMV-positive human serum was selected according to results obtained with use of a commercially available assay (Medac). This serum was diluted at a ratio from 1:200 to 1:12.800 in two fold steps.

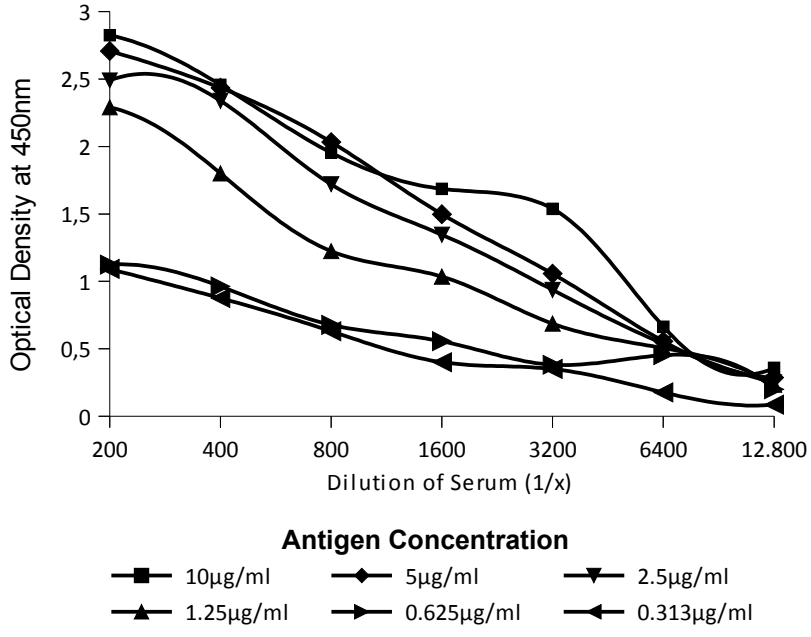


**Figure 1: Checkerboard Titration of CMV whole virus lysate**
CMV lysate was diluted in Bicarbonate buffer. A representative human serum (n=1) was chosen for the assay and applied in singlets.

As expected the signal was dependent on both antigen concentration and serum dilution (Figure 1). However, we did not obtain a logarithmic curve as aspired – nevertheless as an optimum to measure signals in our further experiments we determined an antigen concentration of 2.5µg/ml. Optimal serum dilution was found to be 1:2500, however serum dilution was often varied in our assay.

In a subsequent experiment Tris buffer pH 10 was evaluated besides Bicarbonate buffer and found to obtain even better results than the Bicarbonate buffer.

## 3.2 Treatment of CMV lysate with buffers containing denaturing chemicals causes an increase in signal in indirect ELISA

To optimize our assay also other buffers were evaluated to see whether they cause an increase in signal – Tris buffer both at pH6 and pH 10, and a buffer based on the chemical natriumdihydrogenphosphate (furthermore referred as NP buffer), all of which are often used in standard ELISA protocols, as well as a 8M Urea buffer to test under denaturing conditions were included in this experiment.
CMV seropositive sera (n=3) were diluted in two fold steps, ranging from 1:200 to 1:256.000. Antigen concentration was kept stable.

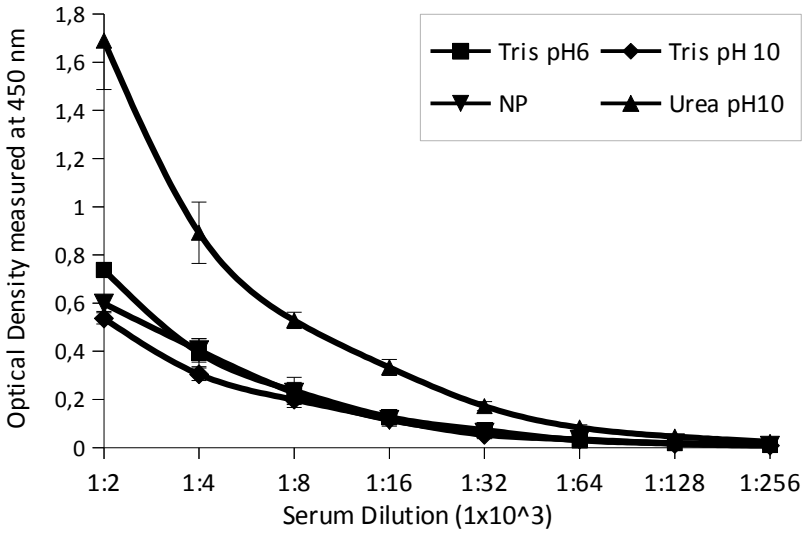


**Figure 2: Influence of treatment of CMV lysate with different buffers**
Tris pH6 - Tris Buffer pH 6; Tris pH10 – Tris buffer pH 10 ; NP – NaH2PO4 Buffer pH 10 ; Urea pH10 – 8M Urea Buffer;
Graph shows one representative serum. Signals obtained with CMV seronegative serum controls were substracted. All serum samples were measured in duplicates. Error Bars indicate the Standard Error.

Compared to the signals measured with the used native buffers, there was a significant increase in signal upon treatment of antigen with Urea Buffer (Figure 2). Mean signals measured with the three immune sera were 287% higher with Urea than with Tris pH 10 treated Antigen. The effect was not dependent on the serum dilution (Standard Error 8.9).
Considering the effect Urea had on the signal, one could suspect that treatment of CMV lysate with a slightly acetic pH would also lead to an increased signal. However, variation of pH of Tris did not have a significant effect on OD450 measured in the indirect ELISA.
In a subsequent experiment we also investigated whether a pH shift makes a difference when Urea buffer was used - however, no significant difference was observed (data not shown).

As Urea is known to denature proteins, we hypothesised that the elevated signal measured in indirect ELISA and hence the greater occurrence of antibodies reacting to Urea treated CMV antigen, might be due to a denaturation of viral proteins leading to an exposure of additional epitopes which might be hidden in the native form. However to rule out that the observed effect was only due to other interactions limited to Urea buffer, also other denaturing buffers were used for antigen preparation.
For this purpose we compared the effect of Tris buffer pH 10 on the signal with the effect of Urea buffer, as well as of a Tris based buffer containing 0.1% SDS and a 6M Guanidin Buffer. These three chemicals are chaotropic agents, which disrupt non-covalent protein bonds and cause denaturation of proteins.
18 CMV seropositive sera, 5 of these gathered from female donors, were chosen for this experiment. Age was ranging from 25 to 72 years (median: 50.5 years).

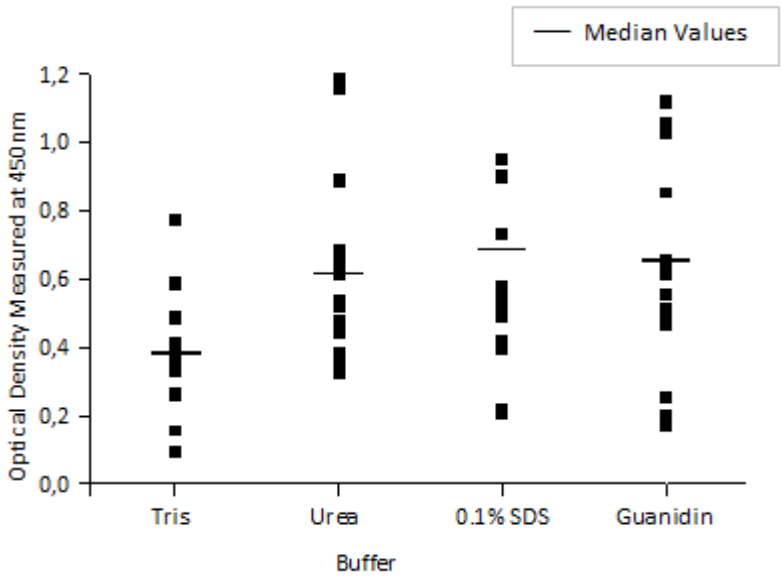


**Figure 3: Effect of CMV lysate treatment with Buffers containing denaturing chemicals on the reactivity of human immune sera (n=18);**
Tris buffer: 50mM Tris, 150mM NaCl, 5% Glycerol; Urea Buffer: 8M Urea, 100mM-NaH2CO3, 10mM Tris ; 0.1% SDS: 50mM Tris, 150mM NaCl, 5% Glycerol; Guanidin Buffer: 6M Guanidin-HCl;
All samples were tested in duplicates.

Treatment of virus lysate with buffers containing chaotropic chemicals leads to a significant increase in signal (Figure 3 ), indicating that denaturation of proteins exposes B-cell epitopes, which are hidden in the native structure of the protein. These hidden epitopes have already been described in other pathogens, e.g. HIV, and are referred to as cryptic epitopes or cryptotopes.

## 3.4. Urea-treatment of CMV lysate does not cause a complete denaturation of antigens

Though Urea is known to be a potent denaturans for proteins, it has also been described that upon removal of this chemical, refolding of proteins can happen. As after coating of the ELISA plate, residual Urea is removed by washing and does not get replaced during the rest of the ELISA protocol, we wanted to test whether Urea treated antigens stay denatured during the entire assay or if a partial refolding does happen.
To answer this question a competitive ELISA was performed.

For competition, a representative CMV seropositive human serum sample (n=1) was incubated with CMV lysate - antigen was diluted in parallel either with Tris or Urea buffer in two-fold steps, protein concentrations ranging from 20µg/ml to 2560µg/ml.
As positive controls serum was incubated both with buffer not containing any antigen and with S. aureus lysate (c= 2560µg/ml).
Competition was tested on 96-well ELISA plates coated with either Tris or Urea treated antigen.

As expected, there was greater competition for antibodies and hence a lower signal measurable when higher protein amount for competition was used (Figure 4).
We found that signals on the Tris detected plate were very similar no matter whether competition took place with Tris or Urea treated CMV lysate. In contrary the kinetic of the signals was different when antibodies were tested on a 96-well plate coated with Urea treated antigen. While competition with rising concentrations of Urea treated antigen constantly decreased the signal in the ELISA, competition with Tris treated antigen could not further decrease signal even when high amounts of antigen were used for competition.

These data indicate that competition with Urea treated antigen removes antibodies binding to conformational and linear epitopes present on the native antigen, as well as to cryptic epitopes – hence we assume that during the assay refolding of Urea denatured proteins does happen. Still this renaturation seems to be only partial, as otherwise cryptic epitopes would be again hidden in the native form of the protein and increased OD450 upon Urea treatment of antigens could not be explained.
Competition with Tris treated antigen withdraws only linear and conformational epitopes on the native protein – antibodies against cryptic epitopes, however, still are and remain available for antigen, explaining why also elevated antigen concentrations used for competition does not cause a decrease in signal.

We hypothesise that treatment of CMV lysate with denaturating chemicals exposes cryptotopes, while the majority of epitopes which can be found on native proteins still are present. Referring to the aviditiy index,

which has already been described for CMV, we developed a method to determine the ratio of antibodies specific for epitopes present on either native or denatured proteins.

The so-called cryptotope index (CI) was calculated as following: 1- ($OD_{Tris}$/$OD_{Urea}$). An index number >0 indicates the presence of antibodies binding to cryptic epitopes.

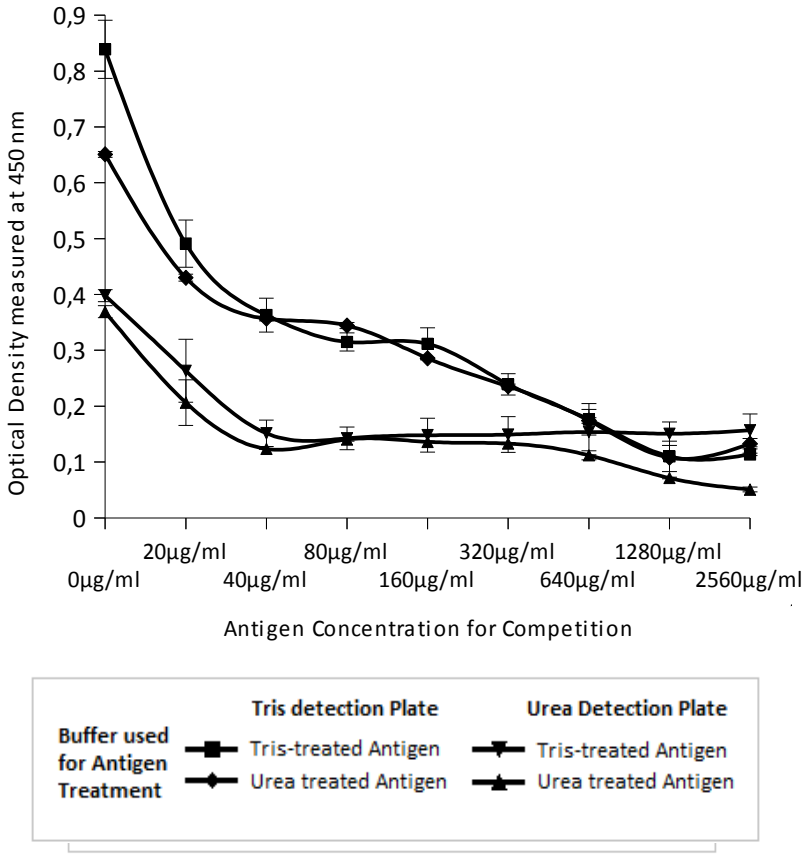


**Figure 4: Competitive ELISA**

Serum sample (n=1) was diluted 1:2000; all samples were tested in triplicates.

Error bar indicates the standard error.

In all our subsequent experiments and validations Tris pH 10 and Urea pH 10 were used as native and denaturing buffer.

## 3.5. Antibodies reactive to cryptic epitopes make up a great amount of total CMV specific antibodies in human polyclonal serum

To test if our finding was restricted to the human sera previously tested or if the increased responsibility towards CMV lysate previously treated with denaturating chemicals was common in a larger cohort of healthy individuals, 150 human sera were collected from healthy blood donors (Red Cross Vienna) and tested in the indirect ELISA.
These sera were serially diluted in 3-fold steps ranging from 1:500 to 1:3000.
As negative control three seronegative sera were tested with all samples and the cutoff for seropositives was calculated to be 0,099.
117 of these sera, that is 78%, were found to contain IgG antibodies reactive to CMV epitopes – these data correlate with already published data, declaring the overall seroprevalence in the European population to be 50-85% (Lopos et al., 2011; Reddehase, 2013; Varga et al, 2011).

Compatible with our previous experiments we found that at all antibody dilutions signal for Urea treated antigen was higher than for Tris treated (Figure 5a) – the cryptotope Index was also significantly similar for all three dilutions (Figure 5b).
In 94.9% of tested CMV seropositive sera signal was increased when antigen was treated with Urea and not Tris. It was conspicuous that the remaining 5.1% immune sera which reacted stronger to Tris treated than Urea treated CMV lysate all had a relatively low signal (mean OD450 0,115, Standard Error 0,004), which were only slightly above the calculated cut-off value.
These data indicate that there is a larger amount of IgG antibodies reacting to Urea treated antigen than to the native antigen and hence that a significant part of antibodies produced to fight CMV infection is specific for cryptotopes.

As it has been shown that CMV infection plays an important part in immunosenescence (Derhovanessian et al., 2009), we investigated whether there was a connection between age and increased recognition of Urea treated antigen by polyclonal serum samples.
In the statistical analysis of the data, using Spearman-rho correlation coefficient, we could not find any correlation between age of blood donors and CMV seropositivity ($\rho$=0.048, P= 0,556).
Spearman-rho correlation coefficient analysis also could not reveal any correlation between sex and seropositivity of patients.

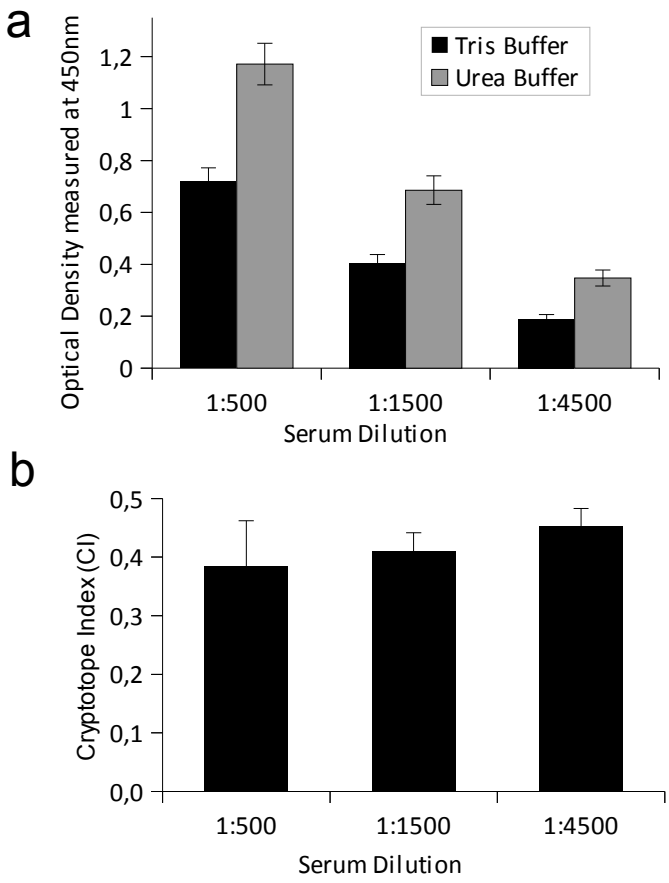


**Figure 5: Signal of tested CMV seropositive Sera against CMV Antigen (n=117)**

a) Mean signal of CMV seropositive sera reacting with either Tris or Urea treated CMV lysate: Sera were prepared at three dilutions. Error Bars indicate the Standard Error.

b) Cryptotope Index: Error Bars indicate the Standard Error.

## 3.6. Urea has the same effect on the antibody recognition of some other, but not all, pathogens

To test whether these findings also hold true for other human pathogens, two bacterial and two viral species were chosen, which are commonly infecting the human body: *Escherichia coli* strain JM109, *Staphylococcus aureus*, Influenza virus strain H1N1 as well as respiratory syncitial virus (RSV). These pathogens were chosen due to their high endemicity in the population. Furthermore antibodies against these pathogens can be found ubiquitously in the general population, due to cross-reactivity of antibodies.

Antigens were treated in parallel with Tris- or Urea buffer and reactivity of the polyclonal sera was tested in an indirect ELISA.
As primary antibody the samples collected from the Red Cross Vienna blood donors (see Chapter 3.5.) were used for this experiment. As expected all of the sera used contained antibodies reactive to all of these pathogens. Calculation of a cut-off value was not possible, as all available control sera previously tested in ELISA, showed a clearly positive signal in the assay.

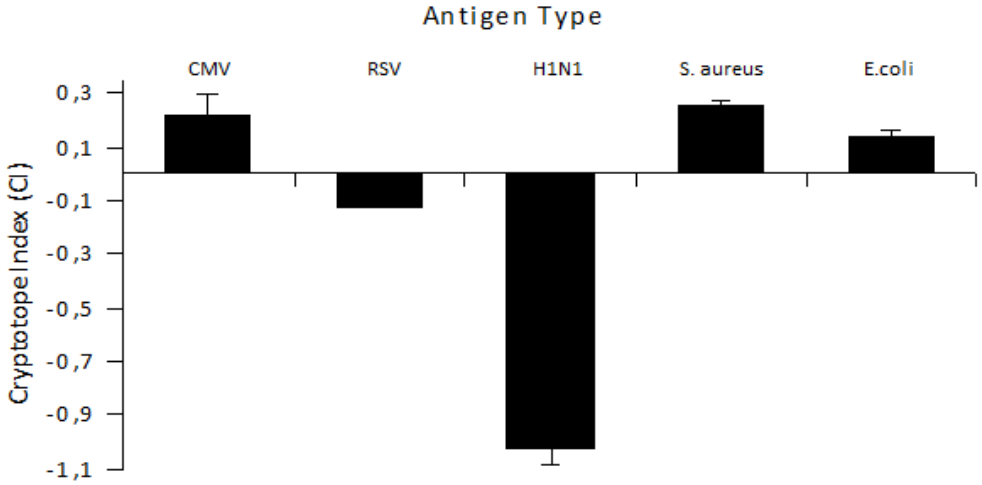


**Figure 6: Comparison of cryptotope indices with respect to antigen preparations from different viral and bacterial pathogens.**
Bars indicate the mean CI, error bars represent the Standard Error (SE) of all samples.
Number of sera tested: CMV n= 150; RSV n=139; H1N1 n=140; S.aureus n= 150; E.coli n=100

As can be seen in Figure 6, antibodies showed an elevated response to Urea treated bacterial antigens, but reacted stronger to native Influenza H1N1 and RSV antigen.

| | Antigen Type | | | | |
|---|---|---|---|---|---|
| | CMV | RSV | H1N1 | S.aureus | E. coli |
| Mean CI | 0,218 | -0,120 | -1,027 | 0,256 | 0,140 |
| SE | 0,076 | 0,008 | 0,057 | 0,013 | 0,023 |

**Table 1: Mean Cryptotope Index**
SE = Standard Error

Variability of cryptotope index CI was low (Table 1), indicating that CI is pathogen and not human specific. As with CMV antigen, we also tested if there was any connection between age and antibody reactivity, however no statistical analysis could not reveal any correlation between age and signal with the other pathogens neither. Values for OD450- Urea treated antigen and for OD450-Tris treated antigen of each seropositive sample was plotted in a scatter blot.

We found that the CI was independent of OD reading with limited interindividual variation for CMV, RSV and E.coli (Figure 7). This indicates that sera with an high overall antibody amount reactive to CMV antigen also contain many antibodies specific for cryptic epitopes and that there are no humans developing antibodies only to cryptic or epitopes found on the native protein.

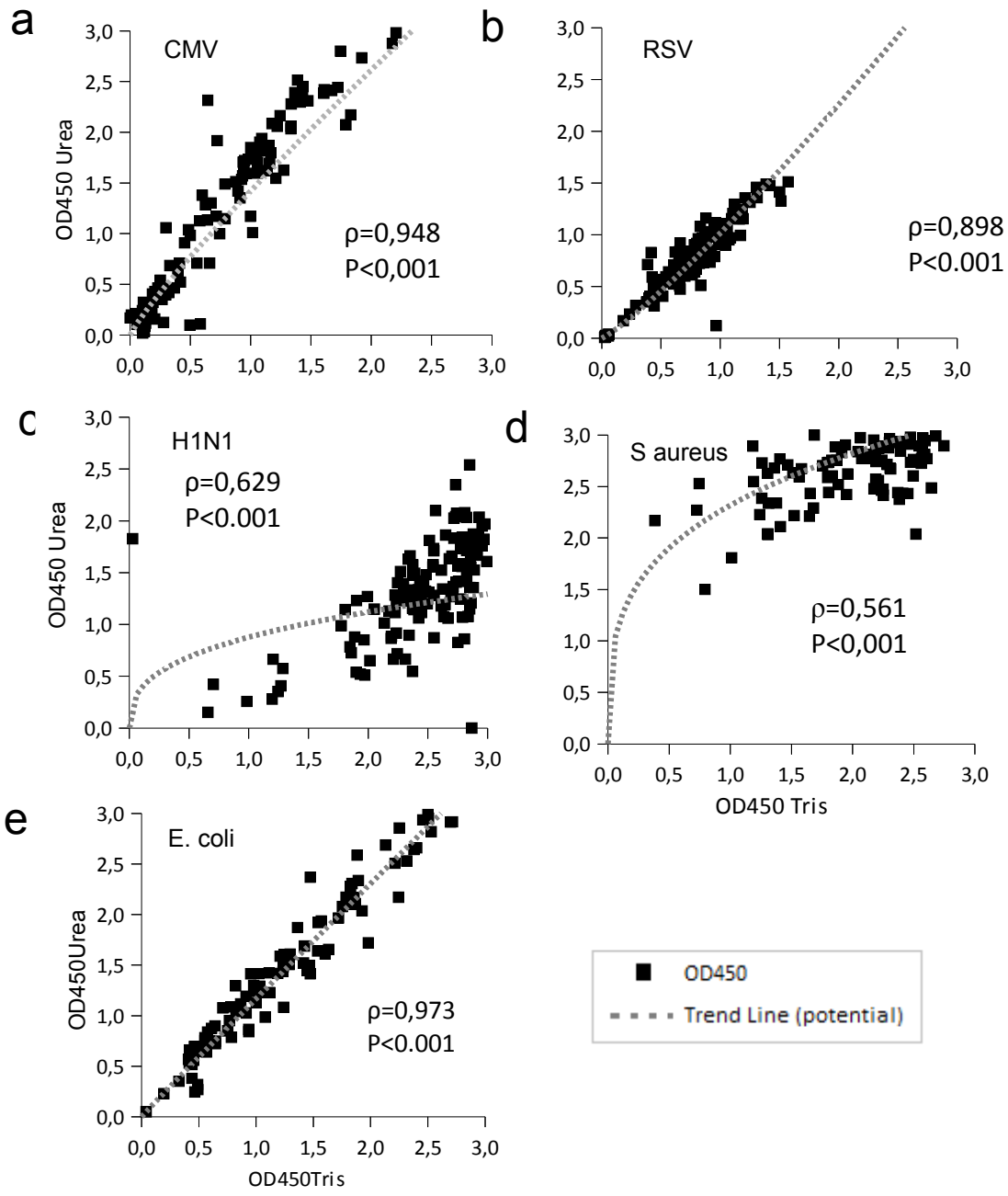


**Figure 7: Correlation of OD450 Tris and OD450 Urea**

a) CMV antigen; b) RSV, antigen concentration c= 5µg/ml; c) H1N1, c=2µg/ml; d) S. aureus, c=5µg/ml; e) E. coli, c=2.5µg/ml

Statistical analysis was done using Spearman-rho correlation coefficient.

## 3.7. Treatment of CMV recombinant proteins with Urea-buffer does not always cause an elevated OD450

As in our last experiments total virus lysate was used, we wanted to investigate whether all individual CMV proteins contain cryptic epitopes. For this purpose three CMV proteins were chosen, which are both known to be very abundant in the virion and to be prominent targets of the human immune response: Glycoprotein B (gB) as well as the tegument proteins pp65 and pp150.
Vector pcDNA 3.0 containing the genes encoding for these three proteins as well as an empty vector, which was used as a control, were transiently transfected in HEK293 cells.
To see whether the transient transfection was successful or not, a Western Blot was performed.

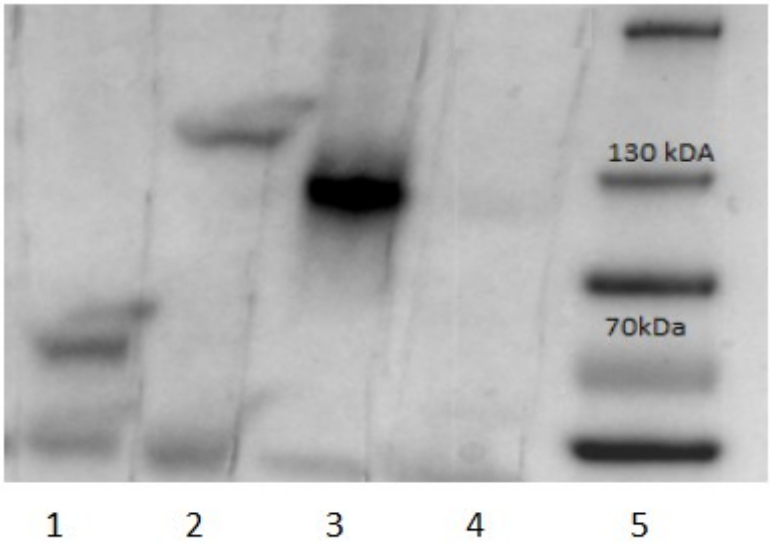


**Figure 8: Western Blot with transiently transfected HEK293 cells;**
All Samples were added in a concentration of 25µg/cm. Protein- PAGE: 4-15% Protein Gel (Mini-PROTEAN TGX, BioRad, Cat.No. 456-1083);
Primary antibody: representative CMV seropositive immune serum, dilution 1:5000
Secondary antibody: mouse anti-human IgG HRP labelled, dilution 1:100.0000
Lane 1 – pp65; Lane 2 – UL32; Lane 3- gB; Lane 4 – Mock transfected negative Control;
Lane 5 - PageRuler Plus Prestained Protein Ladder (ThermoScientific, MA 02454 USA);

As can be seen in Figure 8, transient transfection of the plasmids to HEK293 cells was successful: gB (130k-Da) as well as UL32 (150kDa) are clearly distinguishable. As human serum was used as primary antibody in this western blot, the band indicating the abundance of pp65 (65kDa) is rather weak as expected, yet it is still visible.

To perform an indirect ELISA, the mammalian cells were lysed with either Tris Buffer pH 10 or Urea Buffer pH10. 18 serum samples from the Red Cross Blood Sample stock, which have been found to be CMV sero-positive, were randomly chosen and tested in this assay.

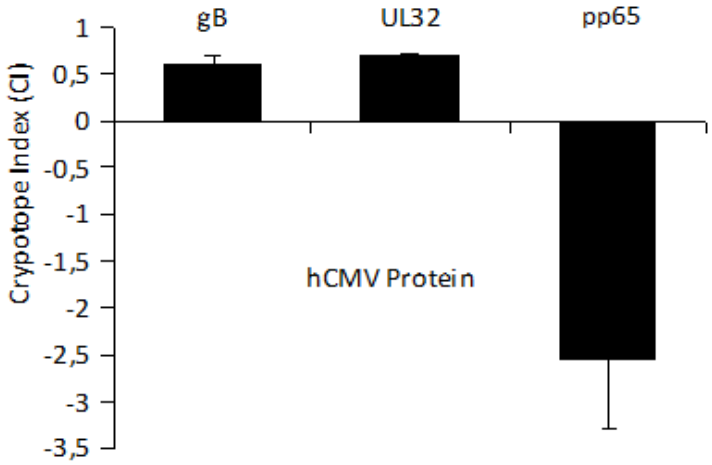


**Figure 9: Indirect ELISA with CMV recombinant Proteins**

gB – Glycoprotein B; UL32 – pp150; pp65; Protein concentration applied on plate: 25µg/ml representative Sera tested n=18; Serum Dilution 1:300; all samples were measured in triplicates.

Bars indicate the mean CI, error bars represent the Standard Error (SE) of all samples.

Interesting we found that the CI was > 0 for UL32 and gB but < 0 for pp65, indicating that on pp65 mainly conformational epitopes are located (Figure 9).

These data indicate that on gB as well as on UL32 cryptic B-cell epitopes are available for antibody binding.

## 3.8. Pre-Adsorption of human polyclonal Sera

For being able to describe the portions of antibodies binding to either native or Urea treated antigen a bit better, we planned to preadsorb human polyclonal serum with Tris or Urea treated CMV lysate followed by elution to gather the antibodies which did bind to the respective antigen.
As binding of antigens to our hitherto used 96-well plate was found to be not strong enough to endure treatment with an elution buffer, Amine-binding, Maleic Anhydride Activated Plates (Thermo Scientific, Cat. No. 15100) were chosen for our following experiments.

A simple experiment was performed to investigate which pH and which incubation time was the most suitable to elute antibodies without damaging their binding capacities. For this purpose one serum, which we knew to have a high antibody titer and good reactivity to CMV antigen, was pre-adsorbed with either Tris or Urea treated virus lysate. Eluted antibodies were detected on Tris or Urea coated plates using indirect ELISA. As can be seen in Figure 10 lower pH was more effective in eluting antibodies. Furthermore these data indicate that short incubation with elution buffer is preferable – this finding is not surprising as long incubation with a low pH can damage proteins hence antibodies as well.

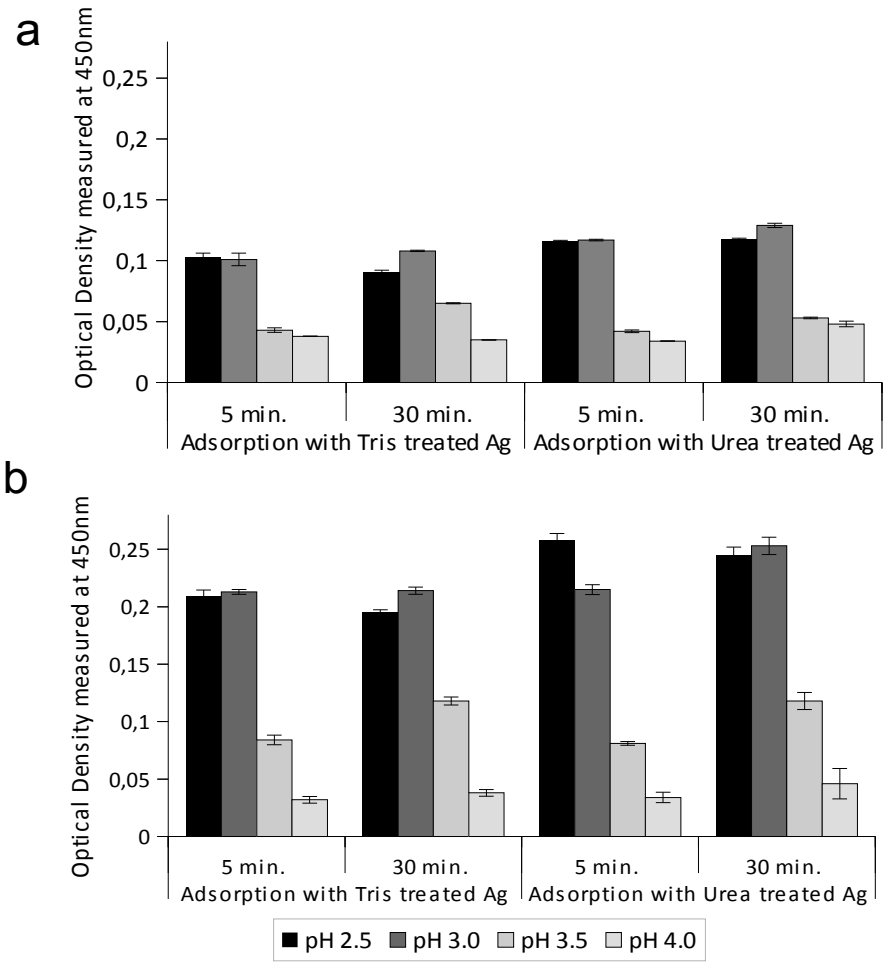


**Figure 10: Antibody yields obtained after preadsorption of human sera to CMV whole virus lysate pretreated with different buffers.**

a) Detection on plate coated with Tris treated Ag; b) Detection on plate coated with Urea treated Ag; Serum was applied in Triplicates; error bars indicate the Standard Error SE

### 3.9. Creation of Recombinant Proteins expressing gB and gB Antigenic Domain

CMV Glycoprotein B (gB) is a popular target for the human immune system and five antigenic domains (AD), which elicit a vast antibody response, have been described so far. As our previous experiments indicate that also multiple cryptic B-cell epitopes are located on this protein further research seemed interesting.
To investigate the reaction of antibodies to gB further plasmids encoding for the antigenic domains 1 (AD-1), 3 (AD-3) and 5 (AD-5) were produced.

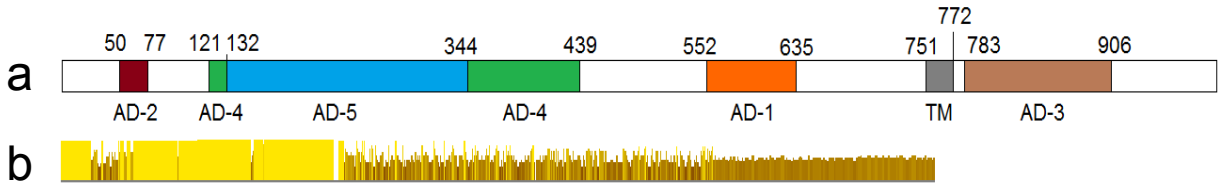


**Figure 11: Location of Antigenic Domains on CMV Glycoprotein B (AD169)**
a) Location of Epitopes (Antigenic Domains) on CMV gB
b) Alignment of gB Ectodomain sequences from clinical CMV isolates (n=3)

AD-1 and AD-5 are both located on the ectodomain part of gB, respectively ranging from amino acid (aa) 552-635 (AD-1) and aa 132-344 (AD-5). AD-3 is located downstream the transmembrane domain at the endodomain of gB, ranging from aa 783-906 (Figure 11).
For expression of these proteins the Kozak sequence (GAATCC) and a Stop codon (CTA) had to be inserted and for cloning restriction sites (BamHI, HindIII) were introducted at the 3' and 5' of the proteins. Additionally a 6x His- Tag was added for purification of expressed proteins.
This was accomplished by performing a mutagensis PCR (Figure 12) – this PCR was specifically designed to anneal long primers which include sequences not complementary to the target sequence highly specific to the DNA template.

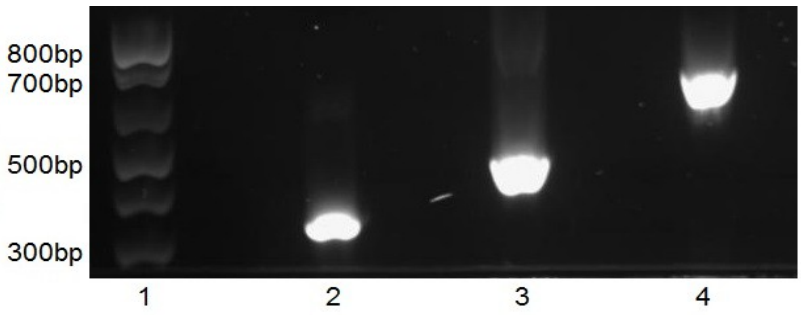


**Figure 12: Mutagensis PCR products of gB AD-1, 3 and 5**
*Lane 1*: Marker – MassRuler Low Range DNA Ladder, Ready to use (Thermo Scientific, MA 02454 USA); *Lane 2*: Antigenic Domain 1 – 303bp, *Lane 3:* Antigenic Domain 3 – 426bp
*Lane 4*: Antigenic Domain 5 – 684bp

PCR products were cloned into the vector pcDNA3.1. This vector has both a T7 and a CMV promotor and hence can be amplifiied in both bacterial and mammalian cells. Furthermore it contains two antibiotic resistance cassettes, Ampicillin and Neomycin.

Plasmids were first transformed in E. coli for amplification and then sequenced to make sure that no mutations or frame shifts did occur during the cloning procedure.

For expression of recombinant proteins the constructed plasmids were transiently transfected into HEK293 cells.
In our first attempt the recombinant proteins were tagged with a 6x His Tag at their N-terminus - curiously proteins were not expressed in HEK293 cells, although success of transfection could be confirmed by a specific PCR. Only after the construction of new plasmids, bearing the His Tag at the protein's C-terminus, protein could be detected in transiently transfected HEK293 cells (Figure 13).

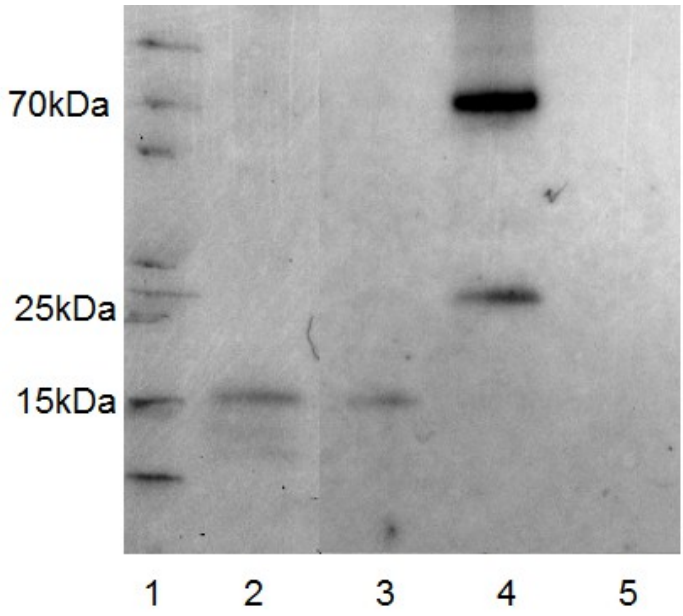


**Figure 13: Western Blot of transiently transfected HEK293 cells**

Samples were added in a concentration of 25µg/cm. Protein- PAGE: 4-15% Protein Gel (Mini-PROTEAN TGX, BioRad, Cat.No. 456-1083)
Lane 1 – PageRuler Plus Prestained Protein Ladder (ThermoScientific, MA 02454 USA);
Lane 2 – gB Antigenic Domain 1, approx. size 11kDa; Lane 3- gB Antigenic Domain 3, approx . size 13kDa; Lane 4 – gB Antigenic Domain 5, approx. size 23kDa; Lane 5 – Mock transfected negative Control.

# ■ Chapter 4: Materials and Methods

## 4.1. Transfection of mammalian cells

Transfection was performed following the Lipofectamine 2000 protocol provided by Invitrogen.

For optimal transfection HEK293 cell were grown in DMEM Glutamax high pyruvate +10% FCS (DMEM) (Invitrogen, CA 92008 – USA) until confluence. Cells were detached one day prior to transfection with 0.05% Trypsin-EDTA (Invitrogen, CA 92008 – USA). Cell number was counted in a Neubauer counting chamber (Laboroptik, BN15 8TN - UK) and the appropriate number of cells were plated in cell culture dishes.
Prior to transfection medium was changed to Opti-MEM Medium I Reduced Serum Medium (Opti-MEM) (Invitrogen, CA 92008 – USA).
Plasmid DNA and Lipofectamine 2000 (Invitrogen, CA 92008 – USA) were incubated in Opti-MEM for 5 minutes separately and next mixed together for complexes to build. After 30 minutes incubation at room temperature, complexes were added to HEK293 cells for 6 hours. Amount of chemicals used see Table 2.

| Component | 24-well | 6-well |
|---|---|---|
| Adherent cells | 1-4x10^4 | 0.25-1x10^6 |
| OptiMEM Medium for Cell Incubation | 250µl | 700µl |
| Lipofectamine 2000 | 5µl | 15µl |
| total amount of plasmid DNA | 5µg | 14µg |
| OptiMEM Medium for Lipofectamine/Plasmid Incubation | 50µl | 150µl |

**Table 2: Amounts of chemicals/cells used for transfection of mammalian cells**

For *transient transfections* cells were incubated for three days and detached from the cell culture plate by addition of 0.05% Trypsin-EDTA.
To produce *stably transfected clones* cells were transferred to 75cm3 culture flasks after 3 days and selective antibiotics - G418 or Hygromycin - were added to the medium for at least 2 weeks. Medium was changed regularly to provide all nutrients the cells needed.

### 4.2. Preparation of viral, bacterial and mammalian antigen

#### 4.2.1. Viral antigens

For long time propagation of CMV virions (strain AD169), human foreskin fibroblasts were cultured in Eagle's minimal essential medium containing 10% FCS (Invitrogen, CA 92008 – USA) and grown in 175cm2 flasks till they were confluent and then incubated for 3 additional days at 37°C.
Prior to infection cells were detached from the cell culture flask via 0.05% Trypsin + EDTA, resuspended in 20ml of DMEM + 10%FCS and then incubated for 1 to 1 ½ hours at 37°C. Virus at MOI 0.05 was added to the cells and incubated for 1 to 2 hours at 37°C. After that medium was removed and replaced by fresh DMEM + 10% FCS. Total CPE was usually reached 8 to 10 days after infection and cells were then harvested.

Influenza virus strain H1N1 as well as respiratory syncitical virus (RSV) were kindly provided by the Institute of Virology, Vienna.

To collect and purify virus samples, host cells had to be removed by centrifugation (10 min., 3000xg, room-temperature) before viral particles were collected by ultracentrifugation (90 min., 33.000xg, 4°C). Supernatant was discarded and pellets were resuspended in 1x TBS + 0.05% Tween-20 (TBS-T). To inactivate the virus samples were stored for 12 hours at 4°C and next sonicated (4 Bursts, each 10 sec., 40 Ampere). This was followed by another incubation at 4°C for 12 hours. Samples were stored at – 20°C.

#### 4.2.2. Bacterial antigens

*Escherichia coli* strain JM109 was cultured in LB medium for 12 hours at 37°C.
*Staphylococcus aureus* was cultured in Caso-Bouillon (Merck, Darmstadt - Germany) for 12 hours at 37°C and inactivated at 100°C for 15 minutes.

Bacteria were collected by centrifugation (20 min., 4500xg, room-temperature). Pellets were resuspended in 1x TBS-T and sonicated (4 Bursts, each 10 seconds, 40 Ampere). Samples were stored at -20°C.

#### 4.2.3. Mammalian antigens

1. Cells were detached from the cell-culture plate with 0.05% Trypsin + EDTA (Invitrogen, CA 92008 – USA) washed with 1x PBS (Invitrogen, CA 92008 – USA) and collected by centrifugation (5 minutes, 500xg, room-temperature). To lyse cells samples were resuspended in mammalian lysis buffer (50mM Tris, 150mM NaCl, pH 10) and sonicated (2 Bursts, each 10 seconds, 20 Ampere). Samples were stored at -20°C.

#### 4.2.4. Protein Concentration

Protein concentration of all samples were measured with the Quick Start Bradford Protein Assay Kit (Bio-Rad, CA 94547 – USA) according to the manufacturer's instructions. As proteins standards BSA samples at concentrations ranging from 2mg/ml to 0.125mg/ml were used (Bio-Rad, CA 94547 - USA).

## 4.3. Indirect Enzyme-linked Immunosorbent Assay (ELISA)

### 4.3.1. Human immune Sera

Human immune Sera were collected with approval of the ethics committee in the course of a prospective cohort study (EK26/2012).
Samples were collected from 150 healthy donors. Age was ranging from 24 to 74 years, median was 55 years. 132, that is 88%, of blood donors were male.

### 4.3.2. Pre-Testing of human immune sera

Selected sera were tested for CMV specific IgGs with an CMV-IgG-ELISA PKS Kit (Medac, 22880 Wedel – Germany) according to the manufacturer's instructions.

### 4.3.4. CMV IgG specific in-house ELISA

Viral and bacterial antigens (see Chapter 4.2.1. and 4.2.2) were diluted at a ratio of 1:3 in the respective buffers and stored over night at 4°C. The following buffers were used in our experiments:

- Tris buffer: 50mM Trizma base, 150mM NaCl, 5% Glycerol
- Urea Buffer: 8M Urea, 10mM NaH2PO4, 10mM Tris
- SDS Buffer: 50mM Trizma base, 150mM NaCl, 5% Glycerol, 0.1% SDS
- Bicarbonate Buffer: 0.1M Na2CO3, 0.2M NaHCO3
- NP-Buffer: 50mM NaH2PO4, 300mM NaCl

25µl of diluted antigen (Table 3) was added to an ELISA 96-well plate (Corning, NY 14831 USA). To ensure optimal binding of the antigen to the plate it was incubated over night at 4°C.
Plates were washed four times with PBS+ 0.05% Tween-20 (PBS-T) before Starting Block Blocking Buffer (Thermo Scientific, MA 02454 USA) was added to each well. Human polyclonal serum was diluted (Table 3) in Blocking Buffer and incubated over night at 4°C. To remove sera plates were washed four times with PBS-T. Secondary antibody goat anti-human IgG HRP-labelled (Abcam, CB4 0FL – UK) was diluted 1:10.000 in Blocking Buffer and 25µl was added to each well for 2 hours at room-temperature.
Wells were washed with PBS-T. Directly prior to use TMB Microwell Peroxidase Substrate (KPL, MD 20878 – USA) was mixed in a ratio of 1:1 and 50µl of the solution was added to each well. Incubation took place for 20 minutes at room- temperature. The reaction was stopped by addition of 50µl 1M o-phosphoric acid.
Optical density was measured at a wavelength of 450nm (reference filter: 620nm).

| Antigen | Protein concentration | Serum Dilution |
|---|---|---|
| hCMV | 2.5µg/ml | 1:500 |
| H1N1 | 2µg/ml | 1:200 |
| RSV | 5µg/ml | 1:200 |
| E. *coli* | 2.5µg/ml | 1:1000 |
| S. *aureus* | 5µg/ml | 1:400 |

**Table 3: Antigen concentrations used for ELISA assays**

## 4.4. Competitive ELISA

For competition viral and bacterial antigen was diluted in either Tris or Urea buffer at a ratio of 1:3 and incubated over night at 4°C. Serum was diluted at a ratio of 1:2000 and incubated with the antigen over night at 4°C on a shaker.
Unbound antibodies were detected on a 96- well ELISA plate (coating procedure see Chapter 4.2).
The protocol was continued as already described in Chapter 4.2.

## 4.5. Western Blot

For separating proteins under denaturing conditions samples were mixed with a 5x buffer (125mM Tris-HCl pH 6.8, 50% Glycerol, 4% SDS, 0.2 w/v Orange G, 10% beta-mercaptoethanol) to a final concentration of 1x and incubated for 5 minutes at 99°C prior to loading.
Sample preparations were applied on a 4-15% Mini-PROTEAN TGX precast gel (Bio-Rad CA 94547 - USA). Gels were run at 150 Volts in either a native (25mM Trizma Base, 196mM Glycine) or a denaturing (250mM Trizma Base, 192mM Glycine, 35mM Sodium Dodecyl Sulfate) Running Buffer.
Proteins were blotted via a Wet Transfer protocol: for this purpose a PVDF membrane (Bio-Rad,CA 94547 - USA) was activated with methanol and the membrane as well as blotting paper and the protein gel (Bio-Rad, CA 94547 - USA) were incubated with 1x Transfer Buffer (48mM Trizma Base, 39mM Glycine, 0.01% SDS, 20% methanol). The transfer cell [Mini Trans Blot Electrophoretic Transfer Cell] (Bio-Rad, CA 94547 - USA) was assembled according to the instructor's manual.
Blotting took place over night at 4°C at 30 Volts.
To see whether protein transfer was successful or not, protein bands were visualised with Ponceau S stain (Sigma Aldrich, MO 63103 USA) and de-stained with deionized water.
The membrane was blocked with Starting Block Blocking buffer ((Thermo Scientific, MA 02454 USA) for 1 hour at room-temperature. Primary antibody was added to the membranes and added over night at 4°C. For this purpose human sera were diluted at a ratio of 1:10.000, monoclonal antibodies were diluted at different ratios. To remove unbound antibodies the membrane was washed four times with phosphate buffered saline containing 0.05% Tween-20 (PBS-T; Sigma Aldrich, MO 63103 USA) and secondary antibody was added for 1 hour at room temperature – either anti-human or anti-mouse HRP labeled antibodies were used at a dilution of 1:10,000. The membrane was then washed again four times with PBS-T. To develop the blot Pierce ECL Western Blotting (Thermo Scientific, MA 02454 USA) substrate was mixed in a ratio of 1:1 prior to use and then applied to the membrane.

## 4.6. Pre-adsorption of antibodies

CMV lysate was diluted in Bicarbonate buffer (0.1M Na2CO3, 0.2M NaHCO3 pH 10) to a concentration of 50µg/ml and 200µl of this solution was applied to Amine-binding, Maleic Anhydride Activated Plates (Thermo Scientific, MA 02454 USA). To ensure optimal binding of the protein to the plate, incubation took place over night at 4°C.

After removal of the supernatant 200µl of either Tris (50mM Trizma base, 150mM NaCl, 5% Glycerol or Urea Buffer ( 8M Urea, 10mM NaH2PO4, 10mM Tris) was added to each well and incubated on a plate shaker for at least 5 hours at room-temperature. Then the plates were washed with PBS-T four times and incubated 30 minutes at room-temperature with Starting Block Blocking Buffer (Thermo Scientific, MA 02454 USA). Polyclonal human sera was diluted in a ratio of 1:50 in Blocking Buffer and 200µl of these samples were added to each well. Incubation took place over night at 4°C.

After washing the wells 10 times with each 200µl PBS-T antibodies were eluted by addition of Elution Buffer (0.2M Glycine-HCl, pH 2.5) 5 minutes at room-temperature on a plate shaker. The liquid was then removed with a Gilson Pipette and immediately neutralized by addition of Neutralization Buffer (0.5M Tris-HCl, pH 9.9). Using a ratio of 1:3 Elution buffer: Neutralization buffer, pH of the solution should be 7.5.

## 4.7. Creation of N-terminal 6x His tagged recombinant proteins

### 4.7.1. Mutagenesis PCR

Kozak sequence (GCCACC), a stop codon (CTA) and a c-terminal 6x His-Tag as well as restriction sites (BamHI: AAGCTT; HindIII: GGATCC) were added to the sequences of gB AD-1, AD-3 and AD-5. For this purpose Mutagensis Primers were used (Table 4).

To enable binding of the long primers to the template two Cycle Steps were introduced (Table 6). After Cycle Step I and before Cycle Step II was performed short Primers were added to the samples (10µM, final concentration 0,5µM). Chemistry see Table 5.

| | | Long Primer (5'-3') |
|---|---|---|
| AD-1 | Left | ATGAGAGGATCCGCCACCATGACCATCAACCAAACCAGCGTCAAGGTGCTGCGTGATATG |
| | Right | TTGCGACTCGAGCTAGTGATGGTGATGGTGATGGCGTTTGAAGAGGTAGTCCACGTACTCGTAGGCCGAGTTCCC |
| AD-3 | Left | ATGAGAGGATCCGCCACCATGCAGAACCTCTTTCCCTATCTGGTGTCCGCCGACGG |
| | Right | TTGCGACTCGAGCTAGTGATGGTGATGGTGATGGACGTTCTCTTCTTCGTCGGAGTCTTTCAAGTGTCTGTAGCCG |
| AD-5 | Left | ATGAGAGGATCCGCCACCATGATCGTGGCGCACACCTTTAAGGTACGGGTCTACCAAAAGGTTTTG |
| | Right | TTGCGACTCGAGCTAGTGATGGTGATGGTGATGGACATTCTTCTCGTCCTGTATATCCCAAGAGATCACCGAGTCGGC |

| | | Short Primer (5'-3') |
|---|---|---|
| Universal | Left | ATGAGAGGATCCGCCACC |
| | Right | TTGCGACTCGAGCTAGTGATG |

**Table 4: Primersequences used for Mutagenesis PCR of gB AD-1, AD-3 and AD-5**

| | Final Concentration | µl/tube |
|---|---|---|
| dd H20 | x | 5,6 |
| 5x Buffer HF | 1x | 4 |
| MgCl2 (50mM) | 3mM | 0,6 |
| dNTPs (10mM) | 200µM | 2 |
| long Primer Left (10µM) | 0.25µM | 0,5 |
| long Primer Right (10µM) | 0.25µM | 0,5 |
| DMSO (100%) | 3% | 0,6 |
| iProof Polymerase (2U/µl) | 0.02U/µl | 0,2 |
| | template (AD169 DNA) | 4 |
| | total volume in tube | 17 |

**Table 5: Chemistry for Mutagenesis PCR**

| Cycle Step I | | Cycle Step II | | |
|---|---|---|---|---|
| Temperature | Time | Temperature | Time | |
| 98°C | 3' | 98°C | 30" | |
| 72°C | 4' | 62°C | 10" | |
| 98°C | 3' | 72°C | Variable * | |
| 72°C | 4' | 98°C | 30" | |
| 4°C | hold | 61°C | 10" | |
| | | 72°C | Variable * | |
| | | 98°C | 30" | |
| | | 60°C | 10" | |
| | | 72°C | Variable * | |
| | | 98°C | 30" | |
| | | 59°C | 10" | |
| | | 72°C | Variable * | 30 cycles |
| | | 72°C | 3' | |
| | | 4°C | hold | |

**Table 6: Cycler Settings for Mutagenesis PCR**

***** Depending on the length of the sequence Elongation time was adapted: AD-1/3 30 sec., AD-5 60seconds

### 4.7.2. Introduction of the PCR product into pcDNA3.1.

As backbone for our inserts the vector pcDNA3.1 (Invitrogen, CA 92008 – USA) was used.

PCR products were applied on a 0.8% Agarose gel, respective bands were cut out and purified with the qiaQuick Gel Extraction Kit (QIAGEN, MD 20874 – USA) according to the manufacturer's protocol.

Both PCR products and vector were treated with restriction enzymes BamHI and HindIII (New England Biolabs, MA 01938-2723 UK) for 1 hour at 37°C and purified by application on a 0.8% agarose gel and purification with the qiaQuick Gel Extraction Kit again.

DNA pieces were ligated with T4 DNA ligase (Promega, WI 53711 – USA) either over night at 4°C or for 2 hours at room-temperature.

### 4.7.3. Transformation of competent E. coli

Competent E. coli strain DH5alpha (Invitrogen, CA 92008 – USA) were slowly thawed on ice. After addition of plasmid DNA, samples were kept on ice for additional 30 minutes. To allow entry of DNA to the bacteria, the cells were heat-shocked at 42°C for 45 seconds. Afterwards they were incubated on ice for 5 minutes. To allow the bacteria to recover from stress 500µl S.O.C medium (Invitrogen, CA 92008 – USA) was added to each sample and incubated at 37°C for 1 hour. Next 100-300µl of this mix were streaked on LB Agar plates containing 100µg/ml Ampicillin, which was used as selection marker.
Plates were incubated over night at 37°C.

### 4.7.4. Detection of positive transformed clones

To screen bacteria colonies for uptake of the plasmid a Colony PCR was performed. Chemistry see Table 7. Cycler settings see Table 6 (Cycler Setting II).

| CHEMISTRY | Final Concentration | µl/sample |
|---|---|---|
| H20 | x | 10,6 |
| 5x Buffer HF | 1x | 4 |
| MgCl2 (50mM Stock) | 3mM | 0,6 |
| dNTPs (2mM Stock) | 200µM | 2 |
| Primer 1 (10µM Stock) | 0.25µM | 1 |
| Primer 2 (10µM Stock) | 0.25µM | 1 |
| DMSO (100%) | 3% | 0,6 |
| iProof DNA Pol (2U/µl) | 0.02U/µl | 0,2 |
| | Template | x |
| | total content | 20µl |

**Table 7: Chemistry for Colony PCR protocols**

PCR products were analysed on 0.8% Agarose gels.

### 4.7.5. Isolation of Plasmids from Bacterial Cell Cultures

Bacterial cell cultures giving a positive signal in the Colony PCR were inoculated in 100ml LB medium containing 100µg/ml Ampicillin and incubated on a shaker over night at 37°C.
Plasmids were isolated from cell cultures by the QIAGEN Maxi Prep Kit (QIAGEN, MD 20874 – USA).

Sequencing of plasmids was performed by LGC Genomics (TW11OLY – UK).

# ■ Chapter 5: Discussion

In our studies we demonstrate that treatment of CMV lysate with denaturing chemicals exposes previously hidden epitopes, with which a large portion of antibodies in human polyclonal serum is reactive with.
These hidden epitopes have already been described in literature and are also referred to as cryptic epitopes or cryptotopes (Kumar et al., 2013)

## 5.1. Cryptic Epitopes

Two origins of those cryptotopes are known so far: on the one hand these epitopes can be simply hidden in the native form of a protein and becoming only accessible for antibodies upon denaturation of the protein. On the other hand it has been observed that cryptic epitopes can develop when an alternate reading frame (ARFs) of a coding sequence is used (Schirmbeck et al., 2005). Why and how these ARFs are being expressed is not quite clear yet – it has been suggested though that it might be due to translation starting at an internal start codon or translation of alternatively spliced mRNA.
However, in our study only epitopes which are hidden due to the native protein structure were investigated.

Both cryptic B- and T-cell epitopes have been described. As T-cells recognize peptide fragments which are presented by interaction between the MHC-complex of Antigen presenting cells and the T-cell receptor, it is quite clear how T-lymphocytes detect cryptic antigens. The situation is a bit more complicated in B-cells, which are thought to detect antigens in their native form via their B-cell receptor or a membrane bound antibody. Hence it is not clear how B-cells actually detect cryptic epitopes and there are not many data available on this topic.
One explanation is that plasma cells internalize and process antigens and present these antigenic peptides via their MHC class II receptor for being activated by CD4+ cells – at that time cryptic epitopes could be exposed to the B-cell. Another possible explanation was provided by Lok et al. 2009, who suggested that hidden epitopes on Dengue virus might be shortly available for antibodies due to the dynamic motion of viral proteins.

Cryptic epitopes have been found in a multitude of pathogens. In human immunodeficiency virus, mostly ARF encoded cryptic epitopes have been found, the most prominent located on the glycoprotein gp120. Also in other viruses like Influenza A cryptic epitopes have been described, as well as in bacterial species like Bacillus anthracis and in parasitic protozoa like Plasmodium falciparum (Ekiert et al., 2009; Oscherwitz et al., 2009; Rathore et al., 2005).

It was assumed that cryptic epitopes are always linear epitopes – however, Laureiro et al showed 2007 that cryptic epitopes on ovine growth hormone are not linear but conformational epitopes which are not present on the native protein. As our data suggest that treatment of lysate with Urea does not cause a complete linearisation of antigens, this could also be the case for some or all cryptic epitopes on CMV.

Cryptic epitopes have also been with autoimmune reactions – as already mentioned T- and B-cells are subdued to extensive negative selection to isolate and destroy those lymphocytes that are reactive with self-antigens. However, it was demonstrated that disintegration of self proteins can lead to the exposure of previously hidden epitopes, that T- and B-cells can react with. It has been demonstrated that viral or bacterial infections followed by inflammation and increased protease activity in the cell can be one factor causing the exposure of these cryptic self epitopes (reviewed in Ercolini et al., 2009).

### 5.2. Cryptic Epitopes on CMV

For CMV cryptic epitopes have not been described so far, but our data strongly suggest that at least on Glycoprotein B as well as on UL32 cryptic B-cell epitopes are available for antibody binding.
Our data demonstrate though that treatment of gB and UL32 with Urea leads to a significant increase in antibody binding. Structural analysis of CMV Glycoprotein B has revealed that this protein is complexly folded – hence it is not surprising that denaturation of this protein can lead to the exposure of previously hidden antigenic determinants. However, our data are curious for pp150 as structural analysis has revealed that this protein is intrinsically unstructured, hence lacks a tertiary structure. If UL32 really was only available in its linear form, treatment of Urea should not make any difference in OD450. As already mentioned host derived BicD1 interacts with UL32 in the host cell, but is absent from the mature virion. We hypothesise that interaction between UL32 and BicD1 causes folding of UL32 which covers epitopes, that are then exposed when treated with a denaturating agent.

In addition to UL32 and gB we also included CMV pp65 in our protein. In contrary to gB and UL32, treatment of pp65 with Urea did not lead to an increase in signal, indicating that either no cryptic epitopes are available on pp65 or that they only play an inferior role in the immune response to this protein.
Generally pp65 was shown to not elicit a strong humoral immune response, but is a prominent target for cytotoxic T-cells. Hence it is absolutely possible that not many cryptic B- but T-cell epitopes are available on this protein.

It was eye catching that the difference in Tris/Urea signal was much more distinct in pp65 than in gB and UL32. A possible interpretation of these data is that treatment with Urea destroys the majority of native epitopes. However, this does not match our data obtained in the competitive ELISA, which suggest that also competition with Urea treated antigen removes the majority of antibodies reactive with native CMV lysate.

Another essential difference between gB, UL32 and pp65 is the appearance of glycosylations on these proteins – gB as well as pp150 is extensively glycosylated, whereas no glycosylations have been described on pp65 so far. This is of particular interest as it was found for self-antigens that change of glycosylation patterns can lead to the exposure of previously hidden cryptic epitopes (Purcell et al. 2008). Highly interesting it was also found that underglycosylated recombinant CMV glycoprotein N proteins were much more prone to antibody neutralization (Kropff et al., 2012).

### 5.3. Cryptic Epitopes on bacterial and viral antigens

We also investigated the effect of Urea on antigens other than CMV. We found that treatment of the bacterial, but not of the viral, strains with Urea also caused an increased signal in indirect ELISA.
The question arises why the effect of Urea to CMV was more similar to the bacterial strains than to the viral strains tested.

However, as already mentioned, cryptic epitopes have been described both in viral as well as in bacterial strains and hence our findings could also simply be by chance.

### 5.4. Impact of the Discovery of Cryptic Epitopes on CMV

The existence of antibodies and T-cells specific for cryptotopes has been associated with both a good and bad prognosis for infected individuals.

On the one hand it has been suggested that cryptic epitopes are another mechanism of pathogens and also tumor cells to evade clearance by the immune system. Indeed it is not surprising that pathogens may try to protect essential functional parts of proteins and enzymes from the immune system to keep them working properly and several studies have been published associating the abundance of lmyphocytes reactive with cryptic epitopes to a better diagnosis:
Simian immunodeficiency virus (SIV) seropositive individuals, which were able to effectively control viral replication in vivo, were found to bear high levels of CD+ T-cells recognizing a cryptic epitope on an envARF (Maness et al., 2007).
Invasion of the liver by Plasmodium falciparum can be easily blocked by antibodies reactive with the amino-terminal part of a circumsporozoite protein. However in its native form the epitope is immunologically unresponsive as it is hidden (Rathore et al., 2005).

On the other hand it was found that antibodies reactive with cryptic epitopes are not desirable as these antibodies to not have neutralising abilities (Laureiro et. al., 2007).
Interestingly it has been proposed that lactate dehyxdrogenase elevating virus (LDE) infected mice which were vaccinated with human growth hormone developed more antibodies against cryptic than against native epitopes compared to pathogen free mice. Furthermore this shift towards antibodies reactive with cryptic epitopes was associated with an increased production of auto-antibodies (Gomez et al., 2002) and hence harm the host additionally.

CMV infection still is life-threatening for immunocompromised individuals as well as problematic in early stages of pregnancy, but neither a fully protective vaccine nor effective treatment plans are available yet.
Our finding suggests that there are not only antibodies binding to linear or conformational epitopes, but also to a third kind of epitope referred to as cryptotopes.
In CMV it was already found that the binding of multiple various antibodies, some of them neutralizing, some not, to a single protein can lead to an overall reduction in neutralizing antibody activity. Hence we hypothesize that our finding can help improving already existing treatment regimes as well as vaccination strategies.

### 5.4.1. Cryptic Epitopes and IVIG Therapy

Pregnant women with primary CMV infection often receive IgG preparations (HIG) to minimize risk of passing the infection on to their child. In the beginning of HIG therapy mostly intravenous immunglobuline (IVIG) was used, which contains polyvalent IgG pooled from various plasma donors. Currently mainly CMV HIGs (CMVIG) are used, which are IgGs collected only from donors with high CMV specific IgG titers. Both of these preparations were found to be quite safe and side effects only seldom occur (reviewed in Walker et al, 2013).

However, though they are safe, none of the HIG preparations tested so far were able to fully protect unborns from vertical transmission in mothers undergoing seroconversion during pregnancy. One study published in 2005 (Nigro et al. 2005) claims that upon administration of CMV HIG transmission could be reduced from 40 to 16 percent, However, these data are not statistically significant (reviewed in Walker et al., 2013).

Organ transplant recipients usually receive antiviral drugs, mainly ganciclovir, intravenously and currently often CMVIGs are administered as well for virus prophylaxis – a study performed with hear transplant recipients showed that co-administration of CMVIG and ganciclovir was not only more efficient in preventing CMV disease than administration of ganciclovir alone but was also associated with lower graft rejection rates and and a better overall surival of patients (Valantine et al., 2001). Other studies report though that co-administration of CMVIGs did indeed prevent CMV infection, but was not able to prevent CMV disease and graft rejection (Ranganathan et al. 2010), or the other way round (Snydman et al., 1993).

Anyhow, fact is that therapy with CMVIgs to prevent CMV disease and infection is not as effective as was hoped and there is still room for improving this kind of treatment.

Failing of IVIg therapy in vivo has also been observed in other viruses, e.g. Hepatitis C. Here it was found that neutralizing antibodies isolated from HCV- positive sera had good neutralizing ability in vitro, but were mostly ineffective in vivo. Interestingly Zhang et al found in 2009 that on HCV E2 protein two epitopes were present, one of which induced antibodies with high neutralizing activity (epitope I), but antibody binding of the other epitope (epitope II) lead to complete failure of virus neutralization. However, removal of antibodies binding to epitope II by affinity depletion was able to restore the neutralizing activity of the serum – it has been proposed that the creation of IVIgs containing only antibodies for epitope I but not II could dramatically improve IVIg therapy in HCV infected patients (Zhang et al., 2009).

A similar mechanism could also operate on CMV proteins and its cryptic epitopes – for instance on Glycoprotein B. Both AD-1 and AD-5 elicit potent neutralizing antibodies, However full neutralization by these antibodies only seldom occurs. As already described in chapter 1.5.5 competitive binding of both non-neutralizing and neutralizing antibodies at least in AD-1 has been shown to lead to an overall reduced neutralizing capacity of sera. Therefore we hypothesise that removal of antibodies against one of the domains could drastically improve overall neutralization capacity of sera.

### 5.4.2. Cryptic Epitopes and CMV Diagnosis

Especially for CMV seronegative pregnant women as well as for transplant recipients an exact and confident method to diagnose acute CMV infection is essential. Due to scientific progress in the last few years several methods are now available to perform this task. Real time PCR (RT-PCR) as well as immunohistochemistry

(IHC) and pp65 Antigenemia tests have been found to be highly specific – these tests are costly though as they are not only expensive but also require extensive labour.
Furthermore these tests only test the appearance of viral DNA or proteins and can not give any information about the recency of the infection – serological tests are necessary for this purpose, which are mostly based on ELISA methods.
As IgM antibodies are usually associated with a recent infection, many tests are based on the capture of this antibody isotype. However, studies found these serological assays to be error-prone – results obtained in different commercially available CMV ELISA kits hardly correlated with each other. Additionally the presence of IgM in the blood does not always truly indicate primary infection as IgM can be detected in the blood stream long time after primary infection occurred.
Other assays trying to distinguish primary CMV infection from reactivation are based on IgG avidity. As already mentioned in Chapter 1.4.2.2., antibodies gain avidity for their respective antigen when they are exposed to the same pathogen more then once. In IgG avidity assays the binding strength of antibodies is measured by treating antibodies with denaturing agents. However, all of these tests lack sensitivity and specificity (reviewed in Ross et al., 2013).

In cancer, cryptic epitopes exposed have been found to elicit a strong humoral immune response and it has been hypothesised that antibodies reactive with these cryptotopes could have a huge diagnostic potential (Fischer et al., 2010). Also our data reveal that multiple antibodies specific for cryptic CMV B-cell epitopes are available in CMV seropositive individuals. The abundance of antibodies specific for cryptic epitopes could be a new marker in the diagnosis of CMV infection. However, further investigation of their diagnostic capabilities is necessary.

### 5.4.3. Cryptic Epitopes and CMV Vaccines

Although it has been ranked the highest priority by the US Institute of Medicine, almost forty years of CMV research have not been able yet to develop a fully protective vaccine against this virus. Although many vaccines have been developed, most were not able to pass phase II clinical trials.
Failing of these vaccines may have several reasons, including the use of laboratory strains which genetically differ a lot from clinical strains due to extensive propagation on fibroblasts.

Also the development of subunit vaccines has not been overly successful. One of the most promising subunit vaccines developed so far is gB/MF59. This vaccine is composed of purified CMV Glycoprotein B to which the squalene MF59 was added. Overall vaccine efficiency was shown to be 50% in healthy, postpartum, CMV seronegative women. A trial in kidney and liver transplantation patients showed that administration of gB/MF59 was associated with higher gB specific antibody titres correlating with shorter episodes of viremia, compared to the placebo control group. Vaccination was not able to reduce the risk of CMV infection in seronegative recipients receiving an organ from a CMV seropositive donor, though (reviewed in Rieder et al., 2013).

Our data indicate that cryptic B-cell epitopes are present on the gB ectodomain and that a major part of antibodies reactive with this protein are specific for these cryptotopes. Glycoprotein B subunit vaccines de-

veloped so far have used full length gB – hence it is most likely that antibodies specific to cryptic gB B-cell epitopes are also elicited after vaccination.
It was shown that vaccination with a single peptide of *Bacillus anthracis'* Protective antigen (PA), which is hidden in the whole protein, elicited high titres of PA specific antibodies with strong neutralisation capabilities in rabbits, whereas immunisation with whole PA was mainly uneffective (Oscherwitz et al., 2009). However, as already described before it has also been reported that antibodies against cryptic epitopes are non neutralising and exclusion of these epitopes from subunit vaccines should hence be attempted.
Therefore, in the case of gB further investigations are needed to clarify whether the abundance of antibodies reactive with cryptic epitopes is beneficial or not and hence whether cryptic epitopes should be restricted or should be increasingly integrated in subunit vaccines.

### 5.5. Outlook

Several experiments are planned to further investigate the role antibodies reactive with CMV cryptic epitopes have in protection against the virus.

To further characterise antibodies reactive with CMV glycoprotein B, gB antigenic domain 1, 3 and 5 have already been successfully inserted in a vector. To be able to purify these recombinant proteins, a 6x His Tag was inserted at the N-terminus of the protein – curiously these proteins were not expressed by HEK293 cells neither when transient nor when stable transfection was performed, although the success of the transfection was confirmed by PCR with sequence specific primers. As the Kozak Sequence as well as the start and the stop codon in these plasmids were identical with plasmids already successfully used for protein expression in our lab and only differed in the presence of the His-Tag, the 6x His Tag was relocated to the C-terminus of the recombinant protein. Indeed these new c-terminal tagged proteins could be expressed in transiently transfected HEK293 cells.
Although larger tags have already been described to interfere with the cell's expression machinery, the problems with the N-terminal His Tag were unexpected as with 6 amino acids this tag is comparably small. Bioinformatic analysis has not revealed any interactions between the 6x His-Tag and the rest of the plasmid, neither on DNA nor on mRNA level. Still we hypothesise that the failure of protein expression was due to secondary structures, not allowing the protein expression machinery to bind and express our recombinant proteins.
We plan to stably transfect the plasmids to HEK293 cells and then to His-Tag purify the recombinant proteins, which shall be used in ELISA assays.

A protocol to isolate antibodies reactive with Urea and Tris treated CMV antigen has already been developed and successfully used. The so-gathered antibodies are going to be tested and compared to each other in microneutralisation assay. These experiments are essential to determine the neutralisation capabilities of the antibodies. Additional to whole CMV lysate, we also plan to pre-adsorb antibodies with UL32, pp65, gB as well as gB AD-1, AD-3 and AD-5 recombinant proteins.
Especially for gB these data could be of great interest: it was found that this protein elicits a multitude of antibodies, many of which have neutralising capabilities, but that competitive binding of antibodies with different

neutralising abilities leads to incomplete neutralisation of the virus (Schoppel et al., 1997; Speckner et al., 1999; Wagner et al., 1992). As already mentioned it has been hypothesised that binding of antibodies to cryptic epitopes is due to the dynamic motion of viral proteins, which leads to short exposure of previously hidden epitopes. Furthermore it has been suggested that antibodies binding to epitopes which are always accessible on the protein need a smaller space, whereas antibodies binding to cryptotopes have to bind to a bigger part (Pierson et al. 2008).

We hope these experiments help us to understand the role antibodies against cryptic epitopes have during natural CMV infection and that the data gathered contribute to the development of efficient CMV vaccines and treatment strategies to save people all around the world.

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# Curriculum Vitae

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**Education:**
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2000-2007: Secondary school with focus on languages, GRG 23, 1230 Vienna
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**Lab-Skills**:
Development and execution of immunological assays (ELISA, Western Blot, IF); Development and execution of PCR; Design and creation of plasmids; Transfection of mammalian cells and transformation of bacterial cells; Mammalian cell culture, Isolation of RNA and DNA, Purification of mammalian and bacterial recombinant Proteins

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