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The glycoprotein gp350 of EBV – an *in vivo* antigen and a promising druggable target molecule

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München, den 27.08.2020

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1. Abstract

Epstein-Barr virus (EBV) infects more than 90% of the human population worldwide. The virus is directly responsible for acute infectious mononucleosis (IM), and is related to several malignancies like Hodgkin lymphoma (HL), Burkitt lymphoma (BL), nasopharyngeal carcinoma (NPC) and posttransplant lymphoproliferative diseases (PTLD). EBV is also associated with various autoimmune diseases like Multiple Sclerosis (MS).

The present work aimed at investigating the biology of EBV in long-term infected healthy carriers, thereby focusing on the expression of the lytic envelope glycoprotein gp350. The protein is well known for its crucial role for the viral infection of human B cells and is therefore considered an attractive target for therapeutic approaches, and also a vaccine candidate. The first part of this thesis was dedicated to investigating the presence of gp350 and its potential role *in vivo*. Since gp350-specificic antibodies can be detected life-long in EBV seropositive individuals, there might be a constant trigger of the immune system by gp350. This hypothesis was corroborated by my observation that certain latently EBV infected cell lines express significant amounts of gp350 on the cell surface and/or release it via extracellular vesicles (EVs). Moreover, I could show for the first time that the gp350 protein is present on a subset of B cells from healthy EBV-seropositive donors, and that the protein is also present in sera. Intriguingly, I could observe a direct correlation between antibody and protein titers. Moreover, I could show that gp350 carrying vesicles isolated from donors reactivate autologous gp350-specific $CD4^+$ T cells, implicating a potential immunological function, e.g., in maintaining viral latency and stable co-existence with the host. This is the first time that gp350 was detected in the blood of healthy donors. Additional studies are needed to elucidate its origin, exact status and biological function.

Therapeutic monoclonal antibodies are a success story in modern medicine. Of particular potential are fully human IgG antibodies which combine the unprecedented specificities of antibodies with a very low immunogenicity. However, fully human antibodies targeting gp350 are not existent. In the second part of this thesis, we therefore developed a new technique to generate fully human IgG antibodies in

humanized mice in collaboration with Prof. R. Stripecke (Med. Hochschule Hannover). These humanized mice were immunized with Epstein-Barr-virus like particles (EB-VLPs), and splenocytes were then immortalized with EBV *in vitro*. The supernatants of the EBV immortalized B cells were tested for human gp350-specific antibodies with neutralizing potential. The most promising antibody from the biological and neutralization assays was cloned. The immunoglobulin sequence was obtained, and classified the antibody as human IgG3, an Ig class strongly related with infectious diseases. The collaboration with the Stripecke lab resulted in the first fully human IgG antibodies generated in humanized mice.

In summary, the present study showed for the first time that gp350 is present in sera from EBV-positive healthy carriers without previous viral reactivation. Moreover, this protein is able to reactivate specific cellular immune responses. Finally, a fully human IgG3 gp350-specific antibody was produced, that may be developed into a therapeutic tool for the treatment of EBV-associated diseases.

1.1. Zusammenfassung

Das Epstein-Barr-Virus (EBV) infiziert mehr als 90% der Weltbevölkerung. Das Virus ist Auslöser der akuten Infektiösen Mononukleose (IM) und ist darüber hinaus mit mehreren malignen Erkrankungen wie Hodgkin-Lymphom (HL), Burkitt-Lymphom (BL), Nasopharynxkarzinom (NPC) und der Posttransplantations-lymphoproliferativen Erkrankung (PTLD) assoziiert. EBV steht außerdem im Verdacht verschiedene Autoimmunerkrankungen wie Multiple Sklerose (MS) zu begünstigen.

Die vorliegende Arbeit zielte darauf ab, die Biologie von EBV bei langzeitinfizierten, gesunden Trägern zu untersuchen. Im Fokus stand dabei das lytische Glykoprotein gp350, ein Hauptbestandteil der Virushülle, das eine entscheidende Rolle bei der Infektion menschlicher B-Zellen spielt und daher als attraktives Ziel für therapeutische Ansätze und auch als Impfstoffkandidat gilt. Im ersten Teil dieser Arbeit wurde das Vorhandensein von gp350 und seine potenzielle Rolle *in vivo* untersucht. Bei EBV-seropositiven Personen können lebenslang gp350-spezifische Antikörper nachgewiesen werden, was durch eine ständige Aktivierung des Immunsystems durch gp350 bedingt

sein könnte. Diese Hypothese wurde durch meine Beobachtung unterstützt, da einige latent EBV-infizierte Zelllinien signifikante Mengen von gp350 auf ihrer Oberfläche exprimieren und/oder über extrazelluläre Vesikel freisetzen. Außerdem konnte ich erstmals zeigen, dass das gp350 Protein auf peripheren B-Zellen von gesunden EBV-seropositiven Spendern vorhanden ist und dass das Protein selbst in Serumproben nachgewiesen werden kann. Interessanterweise konnte eine direkte Korrelation zwischen Antikörper- und Proteintitern beobachtet werden. Darüber hinaus wurde gezeigt, dass gp350 tragende Vesikel gp350-spezifische CD4+ T-Zellen reaktivieren können, was eine mögliche immunologische Funktion impliziert. So wäre es möglich, dass solche Vesikel zur Aufrechterhaltung der viralen Latenz und der stabilen Koexistenz mit dem Wirt beitragen. Dies ist das erste Mal, dass das gp350 Protein im Blut gesunder Spender nachgewiesen wurde. Weitere Studien sind nun erforderlich, um die Herkunft, den exakten Status und die biologische Funktion aufzuklären.

Therapeutische monoklonale Antikörper sind eine Erfolgsgeschichte in der modernen Medizin. Von besonderem Potenzial sind vollständig humane IgG-Antikörper, die die beispiellose Spezifität von Antikörpern mit einer sehr geringen Immunogenität kombinieren. Es gibt jedoch bislang keine vollständig humanen, gp350-spezifische monoklonale Antikörper. Im zweiten Teil dieser Arbeit haben wir deshalb in Zusammenarbeit mit Prof. R. Stripecke (Med. Hochschule Hannover) ein Protokoll entwickelt, um humane monoklonale Antikörper in humanisierten Mäusen herzustellen. Diese Mäuse wurden mit EBV-ähnlichen Partikeln (engl. *virus-like particles*, VLPs) immunisiert, Splenozyten wurden isoliert und *in vitro* mit EBV immortalisiert. Die Überstände dieser immortalisierten B-Zellen wurden auf humane gp350-spezifische Antikörper mit neutralisierendem Potenzial getestet und der vielversprechendste Antikörper aus den Erkennungs- und Neutralisationstests wurde kloniert. Die Immunglobulinsequenz wurde identifiziert und der Antikörper konnte als humanes IgG3 klassifiziert werden, eine Immunglobulinklasse, die stark mit Infektionskrankheiten assoziiert ist. Diese Zusammenarbeit führte zu den ersten vollständig humanen IgG-Antikörpern, die je in humanisierten Mäusen erzeugt wurden.

Zusammenfassend zeigte die vorliegende Studie erstmals das Vorhandensein von gp350 in Seren von EBV-positiven, gesunden Trägern ohne erkennbare vorherige virale Reaktivierung. Darüber hinaus ist dieses Protein in der Lage, spezifische zelluläre

Immunantworten zu reaktivieren. Schließlich wurde ein vollständig humaner gp350-spezifischer IgG3 Antikörper hergestellt, der eventuell zur Therapie EBV-assoziierter Erkrankungen eingesetzt werden kann.

2. Introduction

2.1. Epstein-Barr virus (EBV)

In 1964, M.A. Epstein, Y. Barr and B.G. Achong identified “small viral particles” in cultured Burkitt lymphoma cells that they analysed with electron microscopy. “Their structure and morphology link it with the herpesvirus family”, they described their discovery, but unlike the other herpesviruses known until that day, these particles were inert, meaning that they did not destroy the infected cells (Epstein et al. 1964).

Today, these particles are known as Epstein-Barr virus (EBV), also called human herpesvirus 4 (HHV-4). EBV is a gamma-herpesvirus with a double-stranded DNA genome of 172 kb in size. This genome exists in two different forms, it is either linear when it is contained in viral particles, or circular, known as episome, when it is contained and propagated in the nucleus of infected cells (Küppers 2003).

EBV persists life-long asymptomatically in healthy individuals thanks to its ability to remain latently in circulating memory B cells thereby hiding from the host's immune system. This strategy, which is characteristic for all herpesviruses, normally results in an asymptomatic co-existence (Thorley-Lawson 2005).

In vitro, EBV is able to effectively transform human B cells into lymphoblastoid proliferative cell lines (LCLs) and is therefore often used as a model system to investigate various aspects of cancer development, among others (Diehl et al. 1968) (Pope et al. 1968). The two known different types of EBV (type 1 and 2) mainly differ in the sequences of two latent genes, namely the *Epstein-Barr nuclear antigen (EBNA)-2* and *EBNA-3*. EBV type 1 is more prominent in the world than EBV type 2, and has a higher capacity to transform B cells into LCLs (Rickinson et al. 1987).

In fact, EBV was the first virus to be recognized as a human oncovirus. Today, it is known that EBV is associated with more than 200,000 new cases of cancer worldwide every year (Cohen et al. 2011). EBV is well extended in the human population all over the world, and it is particularly associated with Burkitt lymphoma (BL), nasopharyngeal carcinoma (NPC) and Hodgkin lymphoma (HL). The virus is also related with other malignancies like gastric cancer (GC) and some types of T and NK cell lymphomas. In

immunocompromised patients, like people with acquired immune deficiency syndrome (AIDS) or transplant patients, EBV can cause lymphomas and post-transplantation lymphoproliferative diseases (PTLD). Collectively, these facts make EBV a relevant pathogen.

2.1.1. EBV's life cycle: Lytic and Latent phase

Primary infection with EBV normally occurs during childhood and is asymptomatic, but when it is delayed into adolescence or adulthood it regularly causes infectious mononucleosis (IM, see chapter 2.1.2 for more details). During IM, a strong $CD8^+$ T cell activation and proliferation takes place (Hislop et al. 2002), which, together with the concomitant cytokines released, is responsible for symptoms like a strong swelling of the tonsils and lymph nodes, fever and fatigue. Nevertheless, IM is a normally self-limiting disease that does not cause severe long-lasting health damage (Rickinson et al. 2014). EBV has a very strong tropism for human B lymphocytes. It enters the body through the saliva, and penetrates the epithelium surrounding the adenoids and tonsils, where it infects underlying naïve B cells and starts the viral life cycle (Figure 2.1). The infection of B cells occurs through the binding of the major viral envelope glycoprotein gp350/220 to cellular CD21, also known as complement receptor 2 (CR2). Thereafter, a second viral envelope glycoprotein, gp42, binds to a human leukocyte antigen (HLA) class II molecule, and together with viral gHgL and gB, forms a complex that allows the fusion of the virus envelope with the plasma membrane (Chesnokova et al. 2015). EBV type 2 sporadically also infects T cells and is associated with T cell lymphomas (Coleman et al. 2018). Moreover, EBV can penetrate into normal epithelial cells, but it is still not clear if it is able to establish latency there, although it does so in epithelial malignancies like NPC (Hutt-Fletcher 2017).

Nevertheless, a few years ago, experiments in CD21 negative pre-B cells transduced with CD21, CD35, or both, probed that gp350/220 can also interact with cellular CD35, also known as complement receptor 1 (CR1) (Ogembo et al. 2013). In addition, in the interaction of EBV with epithelial cells, gp350 is less critical as can be seen from the fact that EBV can infect cells with low or completely absent CD21 expression. Here, the

envelope glycoproteins gHgL seems to be more important due to their ability to bind to αV integrins that can trigger virus entry (Chesnokova and Hutt-Fletcher 2011).

During primary infection of B cells, initially a pre-latent phase takes place, characterized by the expression of latent and a limited set of lytic genes (Figure 2.1). Thereafter, the establishment of latency starts with the so-called latency III program, which is typical for in vitro growth-transformed lymphoblastoid B cell lines (LCLs) and EBV associated lymphoproliferative diseases in immunocompromised hosts. Latency III is characterized by the expression of nine viral proteins, namely the EBV nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C and -LP), and the latent membrane proteins LMP1, LMP2A and LMP2B. Also expressed at high levels are two types of non-translated RNAs, the *EBERs* and *BARTs* (BamH1-A rightwards transcripts) (Cai et al. 2006) (Qiu et al. 2011). All these proteins and non-translated RNAs are driving the naïve B cells to enter the follicles where they differentiate into mature B cells. Once the B cells are in the follicles, EBV initiates a latency II program, as it is also present in malignancies like HL and NPC, with the expression of only 3 viral proteins, EBNA1, LMP1 and LMP2, that regulate the differentiation into memory B cells. Finally, the virus shuts down LMP-1 and -2 protein expression completely, driving mature B cells to class-switch recombination (CSR) and somatic hypermutation (SHM), to become resting memory B cells, where the virus persists life-long undetectable in healthy carriers, in its latency 0 program (Küppers 2003) (Thorley-Lawson et al. 2008). In latently infected, dividing memory B cells, EBNA1 is expressed, which is essential for EBV genome replication and proper segregation. This so called latency I program is characteristic for Burkitt lymphoma cells (Kang and Kieff 2015). An overview of the different latency programs and where they can be found is given in Figure 2.1.

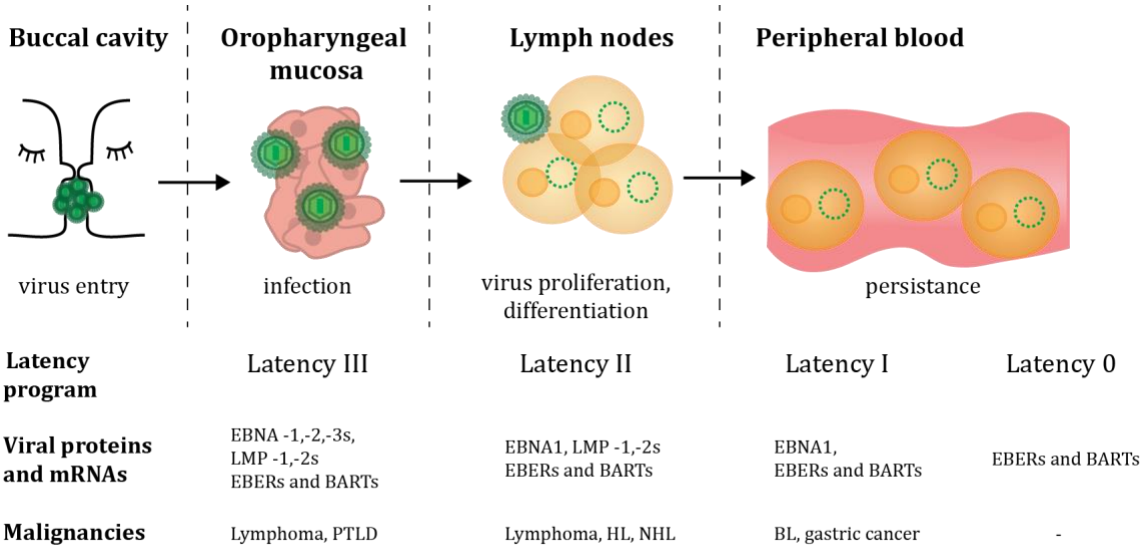


Figure 2.1. The EBV life cycle. (*Adapted from Bollard, Rooney and Heslop, 2012*) EBV enters the host through the saliva and penetrates the oropharyngeal mucosa, where it infects underlying naïve B cells. Immediately following infection, EBV starts a transient pre-latent phase, where viral latent and a subset of lytic genes are expressed. Later, the virus starts its latency programs from III to I, before it finally shuts down almost completely into latency 0 to persist in circulating memory B cells and to evade the immune system.

Cells latently infected with EBV can, in some cases, reactivate and start the viral lytic phase. For this transition, the expression of the two immediate early viral transcription factors *BZLF1* (also called *ZEBRA, Zta, EB1* or *Z*) and *BRLF1* (also known as *Rta* or *R*) is essential (Kalla and Hammerschmidt 2012). Afterwards, the early lytic genes like *BMRF1* or *BALF5* are expressed, followed by the late lytic genes, which mainly encode structural proteins of the viral capsid and envelope, like VCA-p18 and gp350/220. In healthy individuals, this reactivation takes place when infected circulating memory B cells re-enter lymphoid organs like the adenoids and tonsils, where they also start generating infectious viral particles that eventually will infect naïve B cells in the vicinity. It however remains unknown what exactly triggers this reactivation and if it has a pre-determined periodicity (Laichalk and Thorley-Lawson 2005) (Thorley-Lawson 2015).

2.1.2. EBV associated diseases and malignancies

EBV is well known for causing infectious mononucleosis (IM), a benign disease that regularly occurs when EBV infects adolescents or adults. Around 75 % of the IM patients have typical symptoms, like fever, fatigue, sore throat and acute hyperproliferation of $CD8^+$ T lymphocytes. Transmission of the virus normally occurs during kissing (that's why IM is also termed "kissing disease"), but in some cases can occur via solid organ or stem cell transplantation or even by blood transfusion. The incubation period until symptoms emerge is around 6 weeks. IM is normally a mild, self-limiting illness, although in some cases can disable the patient for a long period of time of up to several months (Balfour et al. 2013) (Dunmire et al. 2015).

EBV is also directly related with several malignancies, summing up to more than 200,000 new cases every year (Table 2.1). Annually, tens of thousands of cases of gastric cancer (GC) and nasopharyngeal carcinoma (NPC) are related with EBV (Young et al. 2016), and the virus is also associated with a high proportion of Hodgkin lymphomas (HL) and Burkitt lymphomas (BL), as well as with post-transplant lymphoproliferative diseases (PTLD) in solid organ and stem cell transplant patients. Moreover, EBV is also linked to a number of autoimmune diseases like systemic lupus erythematosus (SLE) and multiple sclerosis (MS), as well as with lymphoproliferative diseases in immunocompromised individuals, like AIDS patients. All these clinical data support the need for the development of a EBV vaccine (Cohen 2018).

Table 2.1. Estimated new cases of EBV-related malignancies all over the world yearly. (Cohen et al. 2011)

Cancer	Nr. of cases	Nr. of cases attributable to EBV
Burkitt lymphoma (BL)		
Developed countries	400	100
Less-developed countries	7800	6600
Gastric carcinoma (GC)	933,900	84,050
Hodgkin lymphoma (HL)	62,400	28,600
Nasopharyngeal carcinoma (NPC)	80,000	78,100
Total		197,450

2.1.3. EBV-specific immune responses upon infection

The immune system reacts to an EBV viral infection with humoral and a set of innate and adaptive cellular immune responses (Figure 2.2).

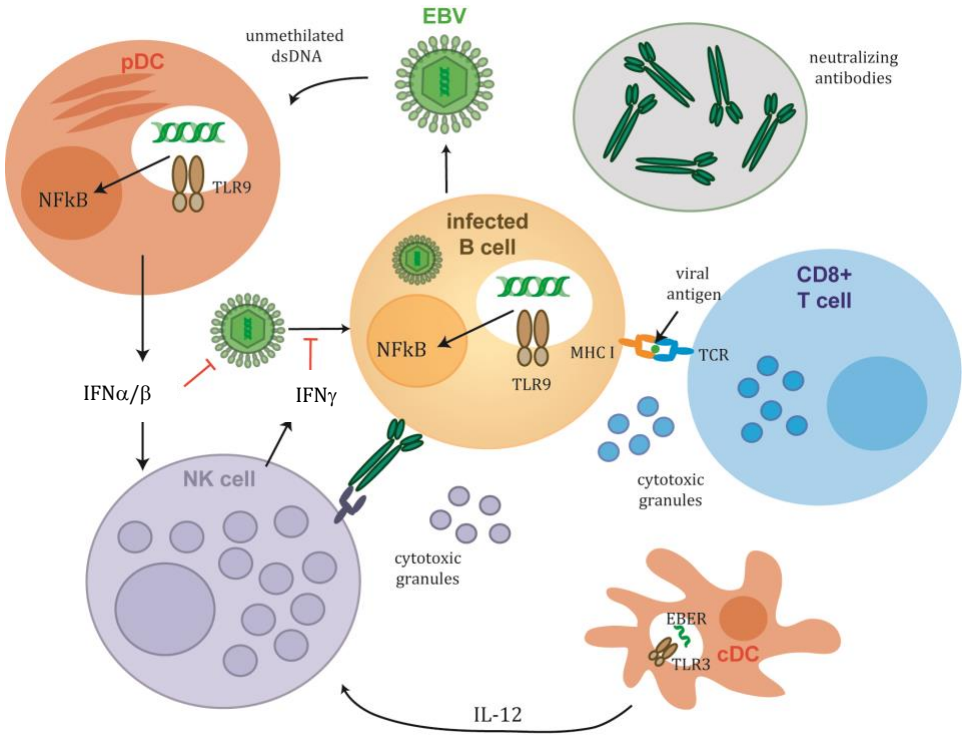


Figure 2.2. Schematic picture of innate and adaptive immune responses against EBV. (*Adapted from Chijioke et al., 2013 and van Zyl et al. 2019*). Infected B cells and plasmacytoid dendritic cells (pDCs) recognize viral unmethylated CpG dsDNA via TLR9. This leads to an activation of the NFkB signalling pathway, resulting in the secretion of IFNα/β that will block EBV infection. Viral dsRNAs, like EBERs, are recognized via TLR3 by conventional DCs (cDCs) and monocytes. In peripheral blood, $CD56^{dim}$ NK cells respond to infected cells by secreting cytotoxic granules. In tonsils and other lymph nodes, $CD56^{high}$ NK cells, upon IL-12 activation, secrete IFNγ that will block EBV infection and B cell transformation. CD8+ T cells recognize viral antigen on MHC class I molecules, mainly epitopes from lytic genes, and release cytotoxic granules and other cytokines to kill infected cells. Humoral responses consist of the production of neutralizing antibodies to avoid viral spread.

The first innate immune response is mediated by the Toll-like receptors (TLRs) in B cells, dendritic cells (DCs) and monocytes / macrophages. The TLR9, present in plasmacytoid

DCs (pDCs) and B cells, recognizes the unmethylated EBV genome as it is contained in virions, resulting in NFkB activation followed by the transcription of proinflammatory cytokines that will activate additional immune cells (Lünemann 2015). Upon EBV infection, the TLR9 in B cells is down-regulated by viral proteins like LMP1 during the latent phase, and by BGLF5 during the lytic phase (Chijioke et al. 2013). During latency, the non-translated EBERs expressed in infected B cells can be released in extracellular vesicles (EVs), and these can be detected by the TLR3 present in conventional DCs (cDCs) and monocytes, which start expressing IL-12 that, in turn, efficiently activates natural killer (NK) cells (Iwakiri et al. 2009). Thus, also NK cells play an important role in controlling EBV infections. In tonsils, mature $CD16^{-}/CD56^{bright}$ NK cells play a role in the control of EBV replication via IFNγ secretion, thereby blocking B cell transformation. And in the peripheral blood, $CD16^{+}/CD56^{dim}$ NK cells regulate viral dissemination via antibody-dependent cellular cytotoxicity (ADCC)-mediated secretion of cytotoxic granules and cytokines (Lopez-Montanes et al. 2017).

After this first wave of innate immune responses, the adaptive immune system begins to monitor the viral infection and to contain the virus. The cellular and humoral responses take place with the activation of T cells and the secretion of neutralizing antibodies. $CD8^{+}$ T cells, present in tonsils and other lymphoid organs, mainly account for the adaptive cellular responses following primary infection by recognizing viral antigen epitopes presented by MHC class I molecules on infected B cells. Most of these epitopes are derived from immediate early (IE) lytic genes, and less are from early (E) and late (L) lytic genes (Pudney et al. 2005). The $CD8^{+}$ T cells also recognize epitopes from latent genes, but at a significantly lower percentage, and these are mainly derived from the EBNA3 proteins and LMP2. The role of $CD4^{+}$ T cells during primary infection is still not well understood. They are probably more important in controlling established infections (Hislop 2015).

The characterization of humoral responses to EBV is used today in routine clinical diagnostics to determine the status of the infection (Figure 2.3). Normally, the first contact with the virus results in the early generation of IgM antibodies against the viral capsid antigen (VCA), but also IgG antibodies against the early antigen (EA) start to evolve. Thus, EA and VCA antibody titers are used as markers for an acute primary infection. Once the virus has established a prevalent latent infection, the VCA IgM

antibodies decrease rapidly and are replaced by VCA IgG antibodies that are maintained life-long. Due to their long persistence, IgG antibodies against EBNA1 are equally used as a robust readout to determine if a patient or transplant donor is EBV seropositive (Middeldorp 2015).

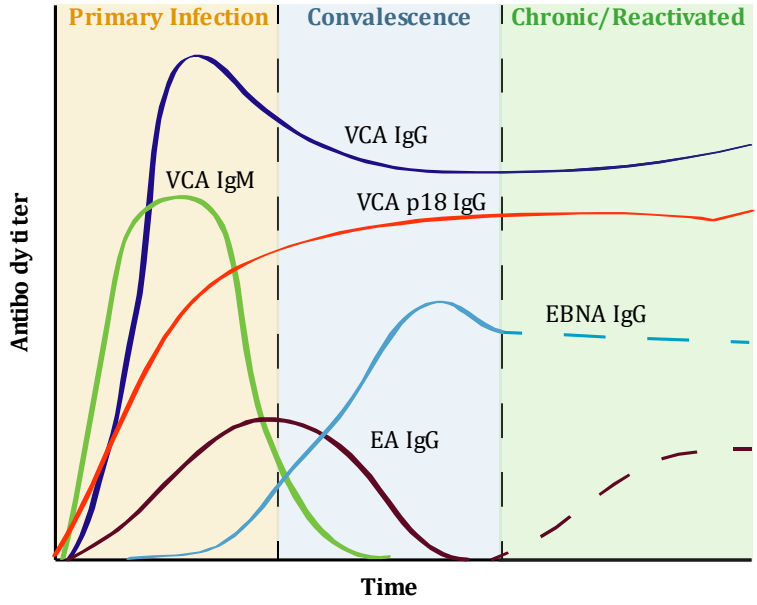


Figure 2.3. Kinetics of the humoral response upon primary EBV infection. (*Adapted from J.M. Middeldorp*, 2015). In the clinics, antibody titers against several EBV proteins like the VCA complex, VCA p18, EA and EBNA1 are used to determine the EBV status of the patient. The *dashed line* for EA indicates the presence of IgG antibodies, that correlate with a reactivation of the virus and possibly appearance of an EBV-associated malignancy.

2.1.4. Glycoprotein 350 (gp350)

The EBV genome encodes 13 different glycoproteins (gp), most of which are only expressed during the lytic cycle. Only one glycoprotein, BARF1, is also expressed during the latent cycle in epithelial malignancies like NPC or GC (Hutt-Fletcher 2015) (Hoebe et al. 2018). Most glycoproteins are part of the envelope and play a role either in virus entry or in the assembly of virions. gp350, encoded by the *BLLF1* open reading frame (ORF), is the most abundant viral glycoprotein, and plays a key role in the attachment of the virus to B cells by binding to cellular CD21 (also known as CR2) or CD35 (also known as CR1) (Ogembo et al. 2013). gp350 is a type I transmembrane protein with a

large N' terminal extracellular domain, a single transmembrane domain and a short C' terminal intracellular domain. The protein is highly glycosylated, especially at the CR2 binding region located in the N' terminal domain, with 14 glycan chains. gp220 is a splice variant of gp350 that lacks one internal exon (Hoebe et al. 2018). The function of gp220 is still unknown, but conserves the binding region for CR2 and the glycosylation structure. Studies in B95.8 cells showed that gp220 is expressed at a much lower level than gp350 (Whang et al. 1987) (Szakonyi et al. 2006).

gp350 is the key viral protein for the infection of B cells. Moreover, gp350-specific monoclonal antibodies (mAbs) that neutralize infection have been reported in EBV-seropositive individuals (Thorley-Lawson and Geilinger 1980). Studies in sera from patients with EBV-associated diseases like NPC, HD, IM and chronic symptomatic EBV infection (CEI), have also shown the presence of gp350/220 specific mAbs (Xu et al. 1998).

gp350 is present on the viral envelope and also on the surface of some EBV infected cells and therefore is a druggable target molecule. E.g. the 72A1 mouse gp350-specific mAb (Hoffman et al. 1980) has been used *in vitro* and in a phase I clinical trial to prevent EBV infection, but some participants developed human-anti mouse antibodies (HAMA) and experienced complications like peripheral cyanosis, hypotension, and vomiting, that led to an interruption of the treatment (Haque et al. 2006). In order to reduce these adverse effects, humanized versions of the 72A1 antibody have been developed. The humanized 72A1 (hu72A1) antibody neutralized EBV infection of Raji cells, but did not block the interaction of mouse 72A1 antibody with gp350, indicating structural changes during humanization of the antibody, showing the challenges of antibody humanization (Tanner et al. 2018).

2.1.5. Therapeutic strategies against EBV

Examples of preventive vaccines against herpesviruses are limited to Varicella-zoster virus (VZV) and Marek´s disease virus (MDV), where attenuated live viruses are used as a vaccine to induce protective immune responses comparable to regular infections. From VZV vaccination, we know that live-attenuated herpesviruses can persist after

vaccination in infected individuals (Hambleton et al. 2008). This, together with the fact that EBV is an oncovirus with a very complex life cycle and able to infect several types of cells using different sets of glycoproteins, contradicts the use of an attenuated live virus as an EBV vaccine (Van Zyl et al. 2019).

First studies on an EBV vaccine have been performed already in 1985 (Figure 2.4). Cotton-top tamarins, considered a reliable animal model for EBV at that time, that were intraperitoneally immunized with isolated cell membranes from infected B cells or with liposomes containing purified gp350, developed neutralizing antibodies and were protected from EBV-induced tumours (Epstein et al. 1985). The first human clinical trial was performed in China in 1995, when EBV positive adults and negative children were vaccinated with a modified vaccinia virus expressing gp350. The vaccine induced neutralizing antibodies but the cohort was very small, and nowadays this technique is no longer in use due to the high risk of side effects (Gu et al. 1995). In 2007, a double-blind placebo-controlled clinical trial with recombinant gp350 was performed. No differences were found in the EBV infection rate between placebo and vaccine groups, but the number of patients who developed clinical symptoms from IM was significantly lower in the vaccinated group (Sokal et al. 2007). Nevertheless, the development of the vaccine has been discontinued by GSK for unknown reasons. Another phase I trial using an EBNA3A specific peptide was performed in 2008, which showed $CD8^+$ T cell responses and a certain extent of IM protection. Due to the tetanus toxoids (TT) used as an adjuvant to stimulate T cell responses, all vaccinated individuals developed inflammation at the injection site. Although the peptide vaccination showed some successful results, the large number of peptide combinations that will be needed to cover the HLA repertoire of the entire population makes this option not suitable for a global EBV vaccination (Elliott et al. 2008). Yet another gp350 vaccine was tested in 2009 without significant effects in children with chronic renal insufficiency undergoing transplantation (Smith 2015). In 2011, an alternative strategy to create an EBV vaccine took form by the use of virus-like particles (VLPs, see 2.2.1 for more details). Mice were immunized with VLPs containing different EBV envelope and capsid proteins, they developed broad EBV-specific cellular and humoral responses, including neutralizing antibodies (Ruiss et al. 2011). VLPs from non-enveloped viruses are efficient vaccines

against viruses like hepatitis B and human papillomavirus (HPV) and may therefore be attractive candidates for an safe and potent EBV vaccine (Ogembo et al. 2015).

Taken together, EBV is associated with a relevant number of malignancies and an unknown number of IM every year. In addition, the virus is supposed to be causally involved in a number of other diseases like MS, SLE and Chronic Fatigue Syndrome (CFS), among others. A safe and efficient vaccine would be an important achievement for the prevention of these EBV-associated malignancies thus reducing the global burden of EBV.

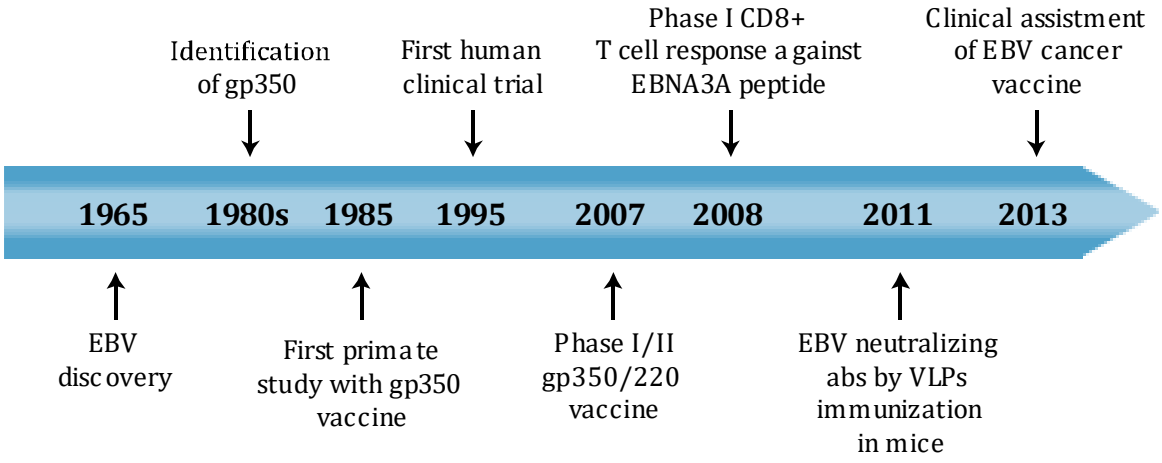


Figure 2.4. Timeline of important hints for the development of an EBV vaccine. *Adapted from Cohen 2015 and Smith et al. 2015*. The timeline reflects the important events take place during the discovery of EBV and clinical trials made with potential EBV vaccines.

2.2. Subviral particles: Virus-like particles (VLPs) and Extracellular vesicles (EVs)

Subviral particles are particles which are self-assembled structures released by virus infected cells and carry one or more viral proteins but no viral genome. They can have different sizes and structures depending on the virus from which they are derived, with a diameter between 25 – 100 nm, and up to 150 nm in enveloped viruses. Subviral particles carry proteins from the capsid and, if applicable, an envelope composed of

cellular membranes decorated with viral glycoproteins, in particular the most abundant ones, in their native conformation, mimicking the structure of the parental virus (Roldão et al. 2010). These particles differ in their composition, but all of them are non-infectious. Hepatitis B virus (HBV) infected cells release spherical or filamentous subviral particles with equal surface composition as virions, and their concentration in serum can be 1,000 times higher than that of infectious virions (Bruss 2007). Another example is human immunodeficiency virus (HIV) that uses the exosome morphogenesis machinery in infected cells to release extracellular vesicles (EVs) carrying different HIV proteins like Tat, Vpr, and also RNA (Patters and Kumar 2018). Also, within the *Herpesviridae* family there are some examples of subviral particles, like the L-particles from Herpes simplex 1 virus (HSV1), that are common for all α-herpesviruses. The L-particles contain envelope glycoproteins and tegument proteins, and it has been suggested that they are involved in immune evasion strategies of the virus (Heilingloh and Krawczyk 2017). Human cytomegalovirus (HCMV) produces "dense bodies" (DB) after *in vitro* infection of fibroblast cell cultures. These DB carry viral envelope glycoproteins and tegument proteins, above all pp65 and gB, and are able to induce humoral and cellular immune responses *in vitro* (Pepperl et al. 2000). In contrast, subviral particles of the human γ-herpesviruses, EBV and KSHV, have not been described so far.

2.2.1. EB-VLPs: characteristics, immune responses and applications

VLPs of EBV (EB-VLPs), as they were used herein, do not exist in nature but are rather engineered in dedicated HEK293-derived producer cell lines. These cell lines harbor a mutated EBV genome that lacks the viral packaging signals, the so called terminal repeats (TR) (Delecluse et al. 1999) (Hettich et al. 2006). Consequently, the VLPs released from these cell lines do not contain the viral genome as has been described for example by sensitive qPCR analysis (Ruiss et al. 2011). This means that, in contrast to the wildtype virus, VLPs are not infectious, and incapable to replicate. It has also been shown that EBV-VLPs transport not only functional viral proteins, but also transduce viral RNAs (tvRNAs) that are translated into functional proteins in freshly infected cells. (Jochum et al. 2012).

Due to their small size, EB-VLPs can easily migrate through the lymphoid system and are efficiently internalized by antigen-presenting cells (APCs), especially B cells and dendritic cells (DCs). After uptake, VLP-derived proteins are processed and presented in conjunction with major histocompatibility complex (MHC) class I and II molecules to finally trigger T-cell activation. It has been shown that VLPs can induce strong T cell independent B cell responses, due to the presence of repetitive viral epitopes on their surface, which lead to high titers of IgM antibodies against particular VLP protein epitopes (Frietze et al. 2016). In addition, the repetitive epitope structures of the VLPs create pathogen-associated molecular pattern motifs (PAMPs) that can activate innate immune responses by Toll-like receptors (TLRs) and others pattern-recognition receptors (PPRs) (Crisci et al. 2012).

The broad and potent cellular and humoral immune responses generated by VLPs, together with their ability to freely move through the different organs, and their lack of viral genomes make EB-VLPs a potent candidate to be used as a tool for vaccine development. However, as of today, only VLP-based vaccines against three non-enveloped viruses, hepatitis B (HBV), like Recombivax HB, Engerix-B, Elovac B, Genevac B and Shanvac B; hepatitis E (HEV), like Hecolin, and human papilloma virus (HPV), like Gardasil and Cervarix, are marketed, and several more are in clinical development (Donaldson et al. 2018). EB VLPs as potential vaccine candidate were investigated e.g. by *Ruiss et al. 2011*. The VLPs, secreted by a producer cell line, contained various EBV proteins including gp350 and LMP2a, reactivated EBV-specific $CD4^+$ and $CD8^+$ T cells *in vitro*, and induced broad cellular immune responses and neutralizing antibodies in immunized mice (Ruiss et al. 2011).

2.2.2. Extracellular vesicles (EVs)

Cells constitutively release different types of vesicles like exosomes and microvesicles, which differ in their composition, their morphogenesis and probably also their function. These vesicles are today collectively termed extracellular vesicles (EVs). EVs have been described for the first time already in 1967 as “dust” released from reticulocytes (Wolf 1967). Nowadays it is known that almost all cells secrete EVs and that they constitute an important route of intercellular communication.

What all EV subclasses have in common is that they are surrounded by a phospholipid bilayer and that they contain a "cargo" consisting of proteins, lipids, and different types of RNAs, which they transport from the cells of origin to target cells. EVs are small vesicles with a 100 nm average size and in sucrose gradients float at a density of 1.13 to 1.19 g/ml. EVs can transfer information from cells of origin to target cells, but they are also related with viral infections, immune responses, cancer and neurological diseases (Colombo et al. 2014) (Kalluri and LeBleu 2020).

The role of EVs in the regulation of immune responses has been intensively studied during the last decade. It was found that EVs released from APC carry MHC class I and MHC class II molecules and stimulate both $CD8^+$ and $CD4^+$ T cells (Robbins and Morelli 2014). Also, engineered EVs carrying CD40L, gp350 and pp65 HCMV protein led to a strong and specific reactivation of $CD4^+$ and $CD8^+$ T cells from healthy donors and even from patients with chronic lymphocytic leukemia (CLL) (Gärtner et al. 2019). Moreover, EVs containing LMP1, as released from EBV transformed B cells and present in serum and saliva of NPC patients, interfere with the NF-κB signalling pathway and thereby contribute to tumour immune evasion and thus progression (Houali et al. 2007) (Hurwitz et al. 2017).

There are three main techniques to isolate EVs from biological fluids, (i) differential centrifugation with several steps of ultracentrifugation, (ii) density gradient centrifugation using sucrose or iodixanol, and (iii) size exclusion chromatography (SEC). Each technique has its advantages and disadvantages, but the main problem for all of them is the loss of sample material during the isolation process (Pluchino and Smith 2019). Isolation of EVs from peripheral blood is particularly complex due to the plethora of draining proteins and other types of vesicles like liposomes. Moreover, it is known that during coagulation to produce serum, platelets release additional particles, so that for the analysis of EVs in the blood, plasma is the better choice. Several protocols have therefore been developed based on a combination of techniques like ultracentrifugation and SEC. It has been shown that the EVs numbers in plasma vary a lot from donor to donor, with, in average, higher numbers in patients with infectious, autoimmune or malignant diseases than in healthy individuals (Karimi et al. 2018) (Yáñez-Mó et al. 2015).

2.3. Therapeutic monoclonal antibodies

2.3.1. Brief story of antibodies

From the first documented smallpox inoculation in humans by Lady Mary Wortley in 1710s, to the serum transfer for the treatment of diphtheria in animals by Behring and Kitasato in 1890, a lot of important achievements have been made in the field of immunology, and especially in the field of antibodies (Figure 2.6). But the first real step in the history of antibodies started in 1900, when Paul Ehrlich proposed his antibody theory. He postulated the existence of receptors ("side-chains") that bind to specific antigens. This hypothesis became later known as the famous "magic bullet" theory (Strebhardt and Ullrich 2008). In 1959, Edelman and Porter published at the same time the structure of an immunoglobulin after papain treatment (Edelman 1959) (Porter 1959). Finally, in 1975 monoclonal antibodies (mAb) evolved, when Köhler and Milstein successfully generated the first stable hybridoma along a protocol that is still in use for the production of mAbs (Köhler and Milstein 1975). Since then, more than 500 different mAbs have been tested with therapeutic purposes in clinical trials, and around 80 mAbs have been approved by the United States Food and Drugs Administration (US FDA) (Lu et al. 2020).

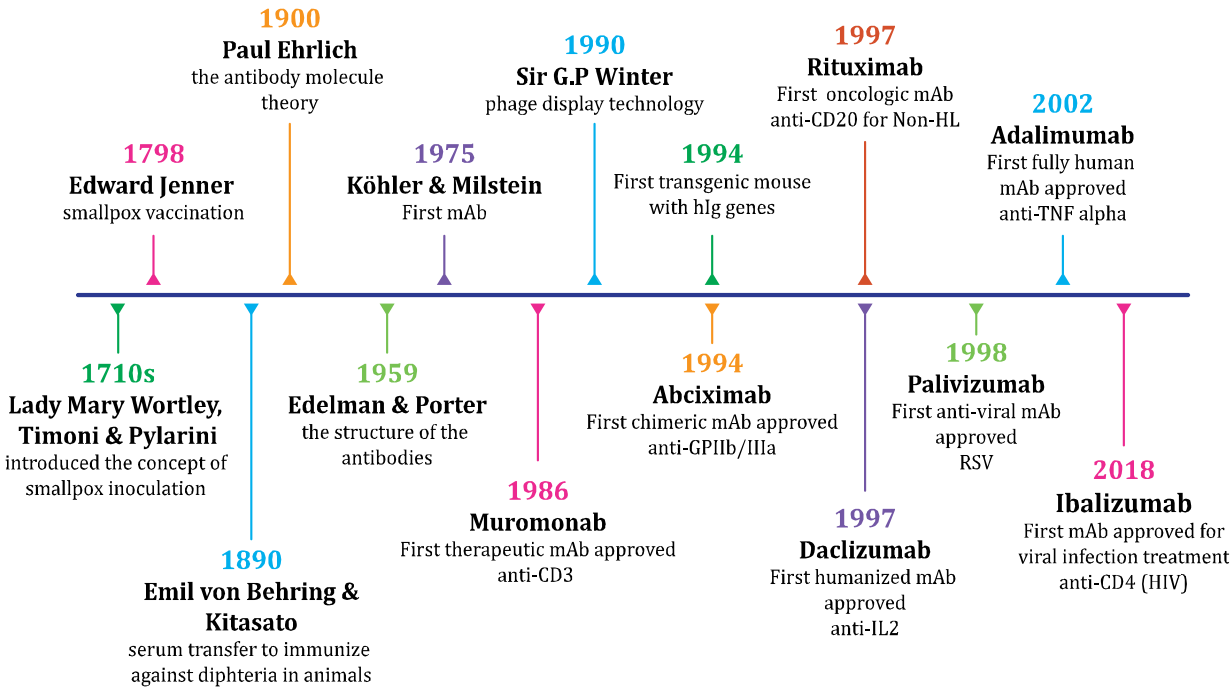


Figure 2.6 Timeline of the important goals achieved in the history of monoclonal antibodies (mAbs). The timeline shows important achievements in the antibody field. From the first concept of smallpox inoculation in 1710s, the Muromonab, the first murine mAb that was approved, to the first mAb approved for the treatment of a viral infection in 2018.

2.3.2. Structure and function of antibodies

Antibodies are the soluble form of the immunoglobulins, that are the B – cell receptors (BCR). They are produced by antibody secreting cells (ASC), that are mature, differentiated B cells, plasmablasts and plasma cells, whose main function it is to secrete large amounts of antibodies into the blood. In humans, antibodies consist of two immunoglobulin heavy (H) and two immunoglobulin light (L) chains creating a Y-shaped basic structure (Figure 2.7), with a variable (V) region at the N-terminal end of the molecule, consisting of two domains (VH and VL) where the antibody binds its specific antigen, and a constant region (C) forming the core with four domains (CL, CH1, CH2 and CH3), that are responsible for the effector functions of the antibodies. The structure of the heavy chain is responsible for their classification as IgM, IgD, IgG, IgA and IgE. The light chains are classified into kappa (κ) or lambda (λ), with a κ:λ ratio in humans of

about 2:1. Changes in this ratio can indicate an anomalous proliferation of a B cell clone. Treatment of an antibody with papain cuts it at the hinge region and releases two Fab fragments and one Fc fragment. The Fab fragment, or antigen binding fragment, is formed by the variable region and the first constant region of the heavy and the light chain, which maintains the full antigen binding capacity. The fragment crystallizable region, or Fc fragment, is formed with the constant domains of the heavy chain, and is responsible for the effector functions of antibodies like antibody dependent cellular cytotoxicity (ADCC), activation of the complement system, and opsonization. Moreover, the Fc fragment is responsible for the transport of the immunoglobulins across epithelial barriers. The hinge region that joins the Fab with the Fc part is partially responsible for the flexibility of the antibodies, facilitating the binding of the antibody even to epitopes that are located at long distances from each other (Murphy 2016).

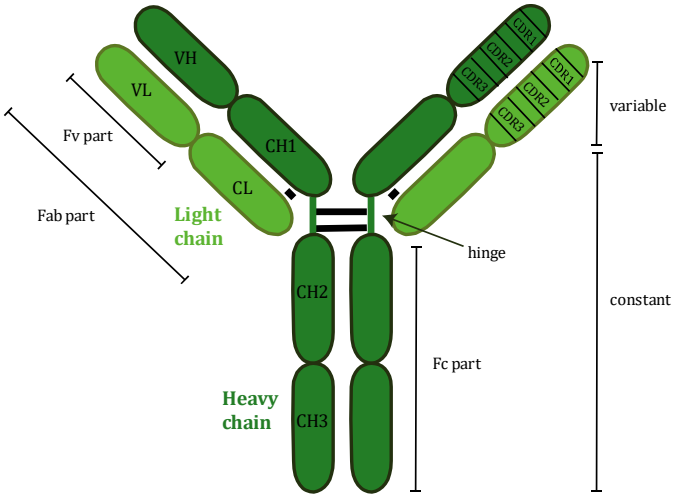


Figure 2.7. Structure of an immunoglobulin (IgG). Every immunoglobulin molecule has two light and two heavy chains that consist of a variable region (V) and one or more constant regions (C). The immunoglobulins can be divided into three parts, the variable fragment, Fv, the antigen binding fragment Fab and the fragment crystallizable region Fc. Embedded in the variable regions are the hypervariable domains, named CDR regions, which form the paratope, i.e. the part of the antibody that binds to the antigen.

The variable region is different for each antibody, but this high variability is mainly concentrated in three regions denominated hypervariable (HV) regions, which are

separated by less variable sections denominated framework regions (FR). The hypervariable regions create the binding site for the antigen, i.e. they are complementary to the antigen that they bind, so they are denominated complementary – determining regions (CDRs). The broad repertoire of specificities that antibodies have, their affinity to a specific antigen are due to variations in length and amino acid sequence of the six CDRs of each Fab fragment. (Murphy, K., Travers, P., Walport, M., & Janeway 2017).

2.3.3. The humanization processes

Since the generation of the first hybridomas in 1975, monoclonal antibodies (mAbs) have become important tools for the treatment of various diseases like inflammatory diseases and cancer. Therapeutic mAbs can be classified into murine, chimeric, humanized and fully human (Figure 2.8). The immunogenicity of the therapeutic mAbs can be ranked as *negligible*, *tolerable* and *marked*. When less than 2% of the treated patients develop an anti – antibody response (AAR) the mAb is classified as *negligible*, 2 – 15 % is classified as *tolerable*, and more than 15 % as *marked*, these mAbs are not usually used in the clinics (Hwang and Foote 2005).

The first therapeutic mAbs were of mouse origin and caused serious adverse events up to anaphylactic shocks. Patients treated with murine mAbs that induced human anti – mouse Abs (HAMA), and sometimes allergic reactions. In addition, they only have a limited capacity to induce antibody-dependent cellular cytotoxicity (ADCC) reactions due to the murine Fc region that inefficiently interacts with human Fc receptors (Lu et al. 2020).

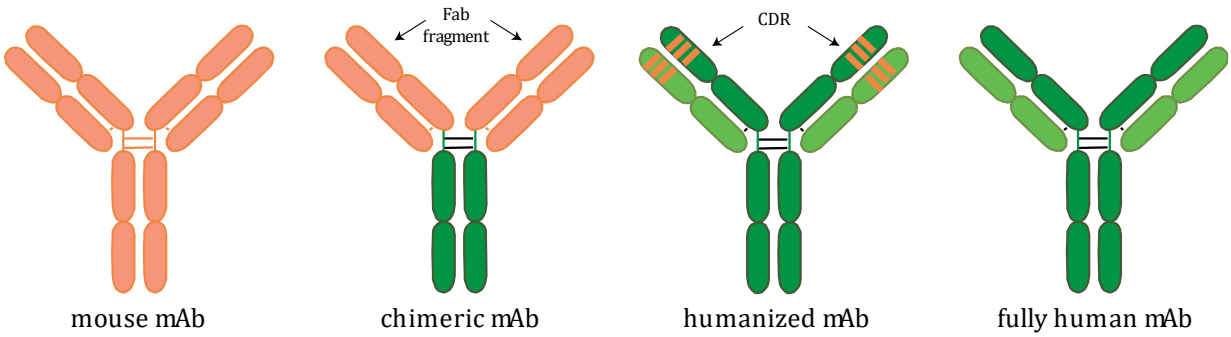


Figure 2.8. Schematic description of the different classes of therapeutic monoclonal antibodies (mAbs). (I) murine mAbs, the whole mAb is of mouse origin, (II) chimeric mAb, that contains murine Fab fragments while the Fc fragment is derived from a human immunoglobulin, (III) humanized mAbs, which have murine CDRs grafted into a human immunoglobulin, and (IV) fully human mAb.

In order to reduce the immunogenicity of murine mAbs, chimeric mAbs were developed. They still contain the murine Fab part but the Fc part is replaced by a human Fc fragment. However, many individuals treated with chimeric mAbs still produced human anti – chimeric antibodies (HACA) but these were less pronounced, i.e. only 40 % of them were described as *marked* (Hwang and Foote 2005).

The next major step towards deimmunization was the advent of the CDR-grafting technique, in which the CDRs of a (normally) mouse mAbs are grafted into a human IgG molecule, giving rise to so called humanized antibodies. Although most of a humanized mAb is of human origin, patients can still develop human anti – humanized antibodies (HAHA), mostly with a *negligible* AAR (Hwang and Foote 2005). Overall, humanized mAbs are well tolerated by most patients and are in daily clinical use for the treatment of an ever increasing number of diseases (Harding et al. 2010). Nevertheless, humanized antibodies carry murine CDRs that can provoke immune reactions, especially when applied repeatedly. For this reason, several approaches are aiming at the generation of fully human therapeutic antibodies.

2.3.4. Technologies to generate fully human monoclonal antibodies

Due to the therapeutic effects and economic success of therapeutic mAb throughout the last years, technologies have evolved for the generation of fully human antibodies which should, in principle, be the least immunogenic class of therapeutic mAbs. There are three main protocols to generate and engineer human mAbs, (i) phage display, (ii) transgenic mice and (iii) recombinant expression of the immunoglobulin light and heavy chains isolated from B cells of patients recovered from a particular disease (Marasco and Sui 2007).

The phage display technique, that was invented in 1985 (Smith 1985), is one of the display technologies to produce large amounts of a peptide repertoire in bacteriophages. Genes encoding VH and VL human fragments are amplified by PCR and cloned into bacteriophages to create a combinatorial phage library of human variable region fragments (scFv or Fab), the fragments are presented in the surface of phages and can be used for binding and affinity screenings. The selected fragment can then be isolated from the phage, cloned and used to develop a human IgG antibody (Carter 2006). One of the antibodies in the market developed by phage display is Adalimubab, better known as Humira®, a TNFα-specific mAb used in patients with rheumatoid arthritis and psoriatic arthritis (den Broeder et al. 2002). The second technique uses transgenic mice, in which the endogenous immunoglobulin loci are replaced by human immunoglobulin genes. Hence these mice produce human mAbs upon immunization (Lonberg et al. 1994) (Mendez et al. 1997).The first human mAb developed with transgenic mouse models that was approved was Panitumumab (Vectibix®), specific for the epidermal growth factor receptor (EGFR), which is used in patients with colorectal cancer (Tyagi 2005). The third protocol developed makes use of EBV's potential to immortalize human B cells (LCLs) from recovered patients into LCLs (Antonio Lanzavecchia 1985) (Traggiai et al. 2004). These LCLs are then single-cell cloned, their supernatants are tested for specific antibodies, and the specific immunoglobulins of the producer LCLs are amplified and cloned. Several improvements have been achieved during the last decade to implement the transformation efficiency. Another variant for the single B cell technology combines the use of single human B cell with state-of-the-art gene cloning technology. Here, primary B cells from infected individuals are directly single-cell sorted by flow cytometry and the heavy and light chain of single cells are amplified by RT-PCR (Tiller et al. 2008). Afterwards, the sequences are cloned into a suitable expression vector and

transfected into suitable cells like CHO or HEK293 to express the human mAbs (Wang 2011). The disadvantage of this technology is that normally a large number of different mAbs has to be recombinantly expressed before their specificity can be tested. Thus, this technology is expensive and very laborious. However, many mAbs have been developed with this technology against viral infections, e.g., Ebola virus (Rijal et al. 2019), HIV (Walker et al. 2009), or SARS coronavirus (Traggiai et al. 2004)

2.3.5. Human monoclonal antibody therapy of viral infections

The first reports of using antibodies against viral infections dates back to 1907, when sera from recovered measles patients was used as a prophylactic treatment. Later, sera were substituted by pooled human immunoglobulins as they are still in use against rubeola or hepatitis A, or hyperimmune IgG against human cytomegalovirus (HCMV), respiratory syncytial virus (RSV), hepatitis C (HCV), rabies, vaccinia, etc. The problem with these polyclonal preparations is that they only contain a small fraction of specific antibodies and that different batches can vary in their composition and thus efficacy (Marasco and Sui 2007).

With the development of new protocols for mAbs production, the treatment of viral infections has been successfully improved. Today, there are several mAbs in clinical phases 2 or 3 against HCMV, at least five mAbs against influenza virus in either phase 1 or 2a, and one mAb already approved by the FDA against HIV and others are pending, several mAbs against HRSV, that affects neonates and infants, and one mAb in review against Ebola. Antibodies against Zika virus, rabies or dengue have been intensively studied in research institutions all over the world but none has reached the clinical state until now (Salazar et al. 2017).

2.4. Aim of this thesis

The Epstein – Barr virus (EBV) is a human herpesvirus that normally infects children, mostly without clinical symptoms. In the developed world however, an increasing number of primary infections only occur during adolescence or adulthood, where they regularly cause a transient disease called infectious mononucleosis (IM). EBV was also the first human oncovirus described, which, as we know today, is directly related with approximately 200,000 cases of new cases of cancer worldwide every year. EBV infections can also be life-threatening in immunocompromised patients.

EBV, like all herpesviruses, persists life-long asymptomatically in healthy carriers. How the virus co-exists in equilibrium with its host despite broad antiviral humoral and cellular immune responses, including high EBV-specific antibody titers, and significant numbers of T cells, is not currently understood.

gp350 of EBV is well characterized as the late lytic viral envelope glycoprotein that interacts with human CD21 on B cells, thereby significantly triggering infection and internalization. Untypically, the protein is also expressed on latently EBV-infected cells and is released in extracellular vesicles.

The roles of gp350 as an antigen in EBV-seropositive individuals and as a druggable molecule for targeted therapeutic approaches are less well understood. Likewise, its expression on the surface of EBV-infected (tumour) cells makes gp350 an attractive target molecule for specific therapeutic regimens.

The aim of this study was therefore

• to analyse the presence of gp350-specific antibodies and protein in the blood of healthy EBV-seropositive donors.

• to exploit, in collaboration with Prof. R. Stripecke (MHH), a new humanized mouse model for the generation of the first fully human gp350-specific monoclonal antibody.

3. Materials

3.1. Bacteria

NAME	DESCRIPTION
E.coli DH5α	*F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK- mK+), λ* - (Hanahan 1983)

3.2. Eukaryotic cells

NAME	DESCRIPTION
B- Blast	Conditional immortalised B cells by CD40L stimulation from LL8 cells and 2ng/ml IL-4, provided by Andreas Moosmann group (Wiesner et al. 2008)
B95-8	Marmoset lymphoblastic cell line immortalized by EBV infection (Miller et al. 1972)
HEK 293	Human embryonic kidney cells transformed after transfection with DNA from human Adenovirus Type 5 (Graham et al. 1977)
HEK293 2089	Human embryonic kidney cell line (HEK293) stably transfected with the maxi-EBV plasmid (p2089) (Delecluse et al. 1998)
HEK293 6008	HEK293 stably transfected with the maxi-EBV plasmid (p6008) (Generated by M.B., Hammerschmidt lab)
HEK293 6507.8	HEK293 stably transfected with the maxi-EBV plasmid (p6507.8) (Generated by W. Hammerschmidt)
LCL	Lymphoblastoid cell line derived from B cell transformed with EBV (B95.8 strain) (Diehl et al. 1968) (Pope et al. 1968)
LL8	CD40-ligand-expressing LL8 mouse fibroblast (kindly provided by A. Moosmann group) (Rancan et al. 2015)
PBMCs	Peripheral blood mononuclear cells from healthy donors obtained by gradient centrifugation

PCI-1	Laryngeal squamous cell carcinoma (Heo et al. 1989)
PCI-1/gp350	PCI-1 cells stably transfected with a gp350 expressing vector (p2385) (Generated by K.G., Zeidler lab)
Primary B cells	B cells purified from human adenoid tissue and used for infection with EBV, different anonymous donors
Raji	Human Burkitt lymphoma cell line, EBV-positive (Pulvertaft 1964)

3.3. Mice

All mice were maintained at the animal facility of the Stripecke group, Hannover Medical School (MHH), according to the institutional guidelines.

NAME	DESCRIPTION
NSG-HLA2.1	NOD.Cg-Mcph1 *Tg(HLA-A2.1)1Enge* Prkdc *scid* Il2rg *tm1Wjl*/SzJ. This mutant strain expresses human HLA-A2.1 MHC class I molecule. NSG-HLA2.1 mice were transplanted with HSC (Salguero et al. 2014)

3.4. Plasmids

NAME	DESCRIPTION
p509	*BZLF1* expression plasmid under the control of the CMV promoter (Hammerschmidt and Sugden 1988)
p2385	*gp350* expression plasmid
p2670	*BALF4* expression plasmid under the control of the CMV promoter
p6926	MP71 retrovirus with BZLF1-ERT2 fused to T2A and tNGF-R (based on the retroviral vector MP71Gpre, a derivative of Moloney Murine Leukemia Virus, Mo-MLV)

3.5. Virus

NAME	DESCRIPTION
EBV 2089 (p2089)	Recombinant EBV; contains a BAC, based on B95.8 with a CMV-promoter controlled by hygromycin resistant and eGFP; Virus production is induced under transient transfection with p509 and p2670
EBV 6008 (p6008)	Prototypic EBV genome based on B95-8 genomic DNA plus M-ABA sequences complementing the deletion in B95-8. Construct is based on 5854 wild type maxi-EBV with puromycin and EGFP
EBV 6507.8 (p6507.8)	EBV Δmir all maxi-EBV with mutated *BFRF1A* locus, *LMP1* deletion, lox71 and lox66 inserted into left and right prokaryotic flanks, respectively, right oriLyt, CMV-puro cassette inserted downstream of lox66

3.6. Cell culture

3.6.1. Media and solutions

NAME	SOURCE OF SUPPLY
Alpha-thioglycerole (α-TG)	Sigma Aldrich
Amphotericin B	Gibco by Life Technologies
BamBanker	GC Lymphotec Inc
Bathocuproinedisulfonic acid (BCS)	Sigma-Aldrich
Cyclosporin A (CsA)	Novartis
Defibrinated sheep blood	Thermo Fisher Scientific
Foetal calf serum (FCS)	Bio & SELL
Geneticin (G418)	Milipore

Hygromycin B	Invitrogen
IL-4	R&D Systems
L-Glutamine 200mM	Gibco by Life Technologies
MEM Non-essential amino acids (NEAA) (100x)	Gibco by Life Technologies
ODN 2006 (CpGs)	MACS Miltenyi
OptiMEM®	Gibco by Life Technologies
Pancoll (Ficoll)	PAN Biotech
PBS	Sigma-Aldrich
Penicillin/streptomycin	Gibco by Life Technologies
Polyethylenimine (PEI)	Sigma-Aldrich
Puromycin	PanReac Applichem
RPMI 1640	Gibco by Life Technologies
Sodium Pyruvate 100mM	Gibco by Life Technologies
Sodium Selenite	Gibco by Life Technologies
Trypsin / 0,05% EDTA	Gibco by Life Technologies

3.6.2. Consumables

PRODUCT	SOURCE OF SUPPLY
Butterfly needle, Vacutainer safety-lock green	BD Bioscience
C-Chip (Disposable Hemocytometer)	NanoEnTek

Cell culture plates	Thermo Fisher Scientific
Cell scraper	TPP
Cell strainer (100 µm)	BD Falcon
CELLSTAR® for suspension cells	Greiner Bio-One GmbH
Cryo tubes 1,8 ml	Thermo Fisher Scientific
Disposable Pasteur pipette 3,2 ml	Carl Roth
Disposable scalpel	Aesculap; Swann-Morton
Filter units 0.45 µm pore size	Merk Millipore
Filter units 0.8 µm pore size	Schleicher & Schuell
Flat-bottom plates (6-/12-/24-/96-well)	BD Falcon
Serological pipette (5, 10 and 25 ml)	Greiner Bio-One
Syringes for blood extraction, INFUJECT 50/60 ml	Dispomed
Syringes for cell supernatant	Ecojet
T25, T75 and T175 cell culture flask	Thermo Fisher Scientific

3.6.3. Equipment

PRODUCT	SOURCE OF SUPPLY
Cell culture centrifuge (Rotanta 460R and 46 RSC)	Hettich
CO_2 Incubator	Heraeus, CO_2-Auto-Zero // Binder
Fluorescence microscope	Axiovert200M, Carl Zeiss

Irradiation device Gammacell 40 (Cs-137)	Atomic Energy of Canada Ltd
Laminar flow hood	BDK, Sonnenbühl-Genkingen
Light microscope	Axiovert25, Carl Zeiss

3.7. Antibodies

3.7.1. Antibodies for flow cytometry

Name (clone)	Clone Nr.	Origin	Source of supply	Dilution
anti-gp350 – Alexa647	6G4	Rat	R. Zeidler, HMGU	1:200
anti-human CD19 – FIT	LT19	Mouse	MACS Miltenyi Biotec	1:100
anti-human CD19 – BV605	HIB19	Mouse	BioLegend	1:100
anti-human CD27 - BV605	O323	Mouse	BioLegend	1:100
anti-human CD3 – PECy5	HIT-3a	Mouse	BioLegend	1:100
anti-human IgA-PE	IS11-8E10	Mouse	MACS Miltenyi Biotec	1:50
anti-human IgG - Alexa647	polyclonal	Donkey	Jackson ImmunoResearch	1:500
anti-human IgG - Alexa647	HP6017	Mouse	BioLegend	1:100
Anti-human IgG - PE	G18-145	Mouse	Bioscience	1:50
anti-human IgG - PE/Cy7	HP6017	Mouse	BioLegend	1:50

anti-human IgM -Pacific Blue	MHM-88	Mouse	BioLegend	1:50
anti-human kappa – APC	HP6062	Mouse	Invitrogen	1:200
anti-human lambda – PE	HP6054	Mouse	Invitrogen	1:200

3.7.2. Antibodies for dot blot, WES and ELISA

Name (clone)	Origin	Source of supply	Dilution	Application
anti-CD63 (24F9)	Rat IgG2b	R. Zeidler, HMGU	1:10	Dot blot
anti-gp350 (6G4) supernatant / purified	Rat IgG2a	R. Zeidler, HMGU	1:5 / 1 µg/ml	Dot blot / Neutralizing assay
anti-gp350 (72A1) purified	Mouse IgG1	Monoclonal Antibody Core Facility (MAB), HMGU	5 µg/ml	WES
anti-gp350 (7A1) purified	Rat IgG2a	R. Zeidler, HMGU	1µg/ml	ELISA
anti-gp350 (OT-6)	Mouse	J. M. Middeldorp, The Netherlands	1:2000	WES
anti-gp350-HRP (6G4)	Rat IgG2a	R. Zeidler, HMGU	1:1000	ELISA
anti-His-HRP	Rat	Monoclonal Antibody Core Facility (MAB), HMGU	1:200	ELISA
anti-human IgG-HRP (1 mg/ml)	Goat	Carl Roth	1:6,000	ELISA/Dot blot
anti-mouse IgG-	Horse	Cell Signaling	1:2000	Dot blot

HRP				
anti-rat IgG-HRP	Goat	Jackson ImmunoResearch	1:10,000	Dot blot

3.8. Oligonucleotides

PRIMER	SEQUENCE
3'CG-CH1_RV	GGAAGGTGTGCACGCCGCTGGTC
3'IGG (INTERNAL)_RV	GTTCGGGGAAGTAGTCCTTGAC
3′Cκ494_RV	GTGCTGTCCTTGCTGTCCTGCT
3′Cκ543_RV	GTTTCTCGTAGTCTGCTTTGCTCA
Igkappa-const-Schanz _RV (Schanz et al. 2014)	AGATGGTGCAGCCAC
OPR-IGHV-1_FW	ATGGACTGGACCTGGAGCATCC
OPR-IGHV-10_FW	ATGAAACACCTGTGGTTCTTCCTCCTCC
OPR-IGHV-11_FW	ACATCTGTGGTTCTTCCTTCTCCTGGTG
OPR-IGHV-12_FW	GCCTCTCCACTTAAACCCAGGCTC
OPR-IGHV-13_FW	ATGTCTGTCTCCTTCCTCATCTTCCTGC
OPR-IGHV-14_FW	ATGGAGTTGGGGCTGAGCTGG
OPR-IGHV-15_FW	ATGGGGTCAACCGCCATCCTC
OPR-IGHV-2_FW	ATGGACTGGACCTGGAGGATCCTC
OPR-IGHV-3_FW	ATGGACTGGACCTGGAGGGTCTTC
OPR-IGHV-4_FW	ATGGACTGGATTTGGAGGGTCCTCTTC

OPR-IGHV-5_FW	ATGGACACACTTTGCTACACACTCCTGC
OPR-IGHV-6_FW	ACTTTGCTCCACGCTCCTGC
OPR-IGHV-7_FW	GGCTGAGCTGGGTTTTCCTTGTTG
OPR-IGHV-8_FW	GGCTCCGCTGGGTTTTCCTTGTTG
OPR-IGHV-9_FW	CACCTGTGGTTCTTCCTCCTGCTG
oPR-IGKV-1_FW	ATGAGGCTCCTTGCTCAGCTTCTGG
oPR-IGKV-2_FW	ATGGAAGCCCCAGCTCAGCTTC
oPR-IGKV-4_FW	TGGTGTTGCAGACCCAGGTCTTCATTTC
oPR-IGKV-5_FW	GTCCCAGGTTCACCTCCTCAGCTTC
oPR-IGKV-6_FW	GCCATCACAACTCATTGGGTTTCTGCTG
oPR-IGKV-7_FW	TCCCTGCTCAGCTCCTGGG
oPR-IGKV-8_FW	CCTGGGACTCCTGCTGCTCTG
oPR-IGV-3_FW	CCCAGCTCAGCTTCTCTTCCTCCTG
RACE abridge anchor primer (AAP)	GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG

3.9. Enzymes and recombinant proteins

PRODUCT	SOURCE OF SUPPLY
EBV gp350 Protein (His-tag)	Sino Biological Inc.
GoTaq® Polymerase II	Promega
Platinum Taq	Thermo Fisher Scientific
Proteinase K	Carl Roth GmbH

qPCR reaction mix (SYBR Green Master I)	Roche
Reverse Transcriptase (SuperScript IV RT)	Invitrogen
RNase H (5 U/µl)	Invitrogen
RNase OUT (40 U/µl)	Invitrogen

3.10. Chemicals and reagents

PRODUCT	SOURCE OF SUPPLY
102 nm Standard beads (NTA)	Particle Metrix
2 – Propanol	Carl Roth
10x GoTaq® Flexi PCR buffer	Promega
Agarose	Gibco
Anti-Mouse Ig, κ/Negative Control Compensation Particles Set	BD Bioscience
Anti-Rat Ig, κ/Negative Control Compensation Particles Set	BD Bioscience
Cell lysis Buffer (10x)	Cell Signaling Technologies
Chloroform	Merck Millipore
Dimethyl sulfoxide (DMSO)	Carl Roth
DNA ladders (GeneRuler 1Kb and 100bp)	Thermo Fisher Scientific
DNA loading dye (6x)	Thermo Fisher Scientific
dNTPs (10mM)	Invitrogen
ECL detection reagent	self-made

Ethanol	Carl Roth
Ethidiumbromide	AppliChem
FACS Flow / Clean / Rinse	BD Bioscience
Ficoll-Hypaque	PAN Biotech
Human BD Fc Block™	BD Bioscience
Non-fat dried milk poder	Carl Roth
Nuclease free water	Thermo Fisher Scientific
OptEIA substrate solutions	BD Bioscience
PEI transfection reagent	Sigma-Aldrich
Phenylmethylsufonyl fluoride (PMSF)	Roche Diagnostics
Protease Inhibitor tablets (EDTA-free)	Roche Diagnostics
TRIzol® reagent	Invitrogen
Tween-20	Carl Roth
TMB (3,3', 5,5' tetramethylbenzidine)	BD Bioscience

3.11. Kits

PRODUCT	SOURCE OF SUPPLY
5´ RACE System for Rapid Amplification of cDNA Ends	Invitrogen
Amersham ECL select western blotting detection Reagent	GE Healthcare
anti-mouse Detection Kit	ProteinSimple

Bradford Protein Assay	BioRad
EBV Early Antigen IgG ELISA	Immunolab GmbH
EBV VCA IgG ELISA	Immunolab GmbH
EndoFree Plasmid Maxi-Kit	Qiagen
Human IFNγ ELISA ALP Kit	Mabtech
IgG (Total) Human Uncoated ELISA Kit with Plates	Invitrogen
Jess & Wes Separation Module	ProteinSimple
Monarch® Total RNA Miniprep Kit	BioLabs Inc.
MycoAlert mycoplasma detection kit	Lonza
PCR clean-up Gel extraction	Macherey-Nagel
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific
RDT EBV IgG Assay	Bio-Rad
RNeasy 96 Kit	Qiagen

3.12. Buffers and solutions

Buffer	Composition
Dot blot blocking buffer	5 % Milk in 1x TBST
Dot blot wash buffer	0.05 % Tween-20 in TBS (TBST)
ECL solution 1	0.1 M Tris/HCL (pH 8.8.), 200 mM p-Courmaric acid, 1.25 mM luminol
ECL solution 2	3 % H_2O_2

ELISA blocking buffer 1	5 % FCS in DPBS
ELISA blocking buffer 2	5 % Milk in DPBS
ELISA dilution buffer	2 % FCS in DPBS
ELISA STOP solution	2 N H_2SO_4
ELISA wash buffer	0.05 % Tween-20 in DPBS (PBST)
FACS buffer	2 % FCS, 2 mM EDTA, 1x DPBS
MACS buffer	1 % BSA, 1x DPBS
RIPA lysis buffer	50 mM TrisHCl, 0.1 % SDS, 1 % (v/v) NP40, 0.5 % DOC, 150 mM NaCl, pH 8.0
Size Exclusion Chromatography (SEC) cleaning buffer	0.5 M NaOH
Sorting buffer	1 % FCS, 1x DPBS
Thiols buffer	20 nM BCS, 0.433 % α-TG in 10 ml PBS

3.13. General consumables

PRODUCT	SOURCE OF SUPPLY
5 ml polystyrene round bottom tube (FACS tubes) with or without cell-strainer	Falcon
ELISA plates (REF 439454)	Thermo Fisher Scientific
PCR tubes	Brand
Pipet tips	Gilson; Rainin; Eppendorf
Polystyrene tubes (15 ml, 50 ml)	Falcon

qPCR 96-well plates with cover follies (LC480)	Roche
Reaction tubes (1.5 ml, 2.0 ml), DNA low binding (1.5 ml), Protein low binding (1.5 ml)	Eppendorf
Tubes for ultracentrifugation	25 x 89 mm, Kisker Biotech 11 x 60 mm, Beckman Coulter 9.5 x 38 mm, Beckman Coulter

3.14. General equipment

PRODUCT	SOURCE OF SUPPLY
Agarose gel chamber	PeqLab
Analytical balance	Kern and Sohn GmbH
Centrifuges	5415R, Eppendorf; Heraeus PICO 21, Thermo Scientific
ELISA HydroSpeed™ washer	Tecan
FACS machine / cytometer (FACS Canto™, LSR Fortessa™ and Aria™)	BD Biosciences
Fluorescence plate reader (CLARIOstar®)	BMG Labtech
Gel documentation imaging (Quantum ST5)	Vilber
Imaging System (Fusion Fx®)	Vilber
Light Cycler 480 II	Roche
Multichannel pipets (2,5-20, 20-200)	Brand
NTA instrument (ZetaView®)	Particle Metrix
Pipetboy 2, Pipetgirl	Integra

Pipets (P2, P20, P200, P1000)	Gilson
Rotating mixer 5 rolls	neoLab
Rotors for ultracentrifugation (SW60Ti, SW28, SW32, TL-100)	Beckman Coulter
Simple Western system (WES®)	ProteinSimple
Spectrophotometer NanoDrop 1000	PeqLab
Sprout™ mini-centrifuge	Biozym®
Thermo cycler T gradient 96	Biometra
Thermostat plus	Eppendorf
Ultracentrifuges Optima L-60 & L-70	Beckman Coulter
Vortex mixer (Vortex 2 Genie)	Scientific Industries
Water bath	Memmert

3.15. Software

Name	Application	SOURCE OF SUPPLY
Adobe Illustrator CS5	Graphic design	Adobe Systems Inc.
BD FACSDiva™ Software	FACs analysis and FACs sorting	BD Bioscience
Compass for SW	WES analysis	ProsteinSimple
FlowJo 10.6.1	FACs analysis	Tree star
GraphPad Prism 6	Basic statistics, graph processing	GraphPad Software Inc.

LightCycler 480 Software SP3	qPCR analysis	Roche
MacVector 17.0.8	Analysis of nucleotides and protein sequences	MacVector Inc.
Mendeley Desktop 1.19.4	Literature citation	Mendeley Inc.
Microsoft Office® 2017	Text, tables and presenting processing	Microsoft
MARS Data analysis software	Fluorescence data analysis	BMG Labtech

4. Methods

4.1. Cell culture

All the cell culture from this thesis was done under sterile conditions in a biosafety level 2 (BSL-2) laminar flow hood. All the cells were cultured in incubators at 37 °C with 95 % humidity and 5 % CO_2. Most of the cells were cultivated in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10 % foetal calf serum (FCS). LCLs were cultivated during the first weeks with a special enriched RPMI 1640 medium containing 20 % FCS, 2 % Glutamine, 1 % NEAA, 1 mM sodium pyruvate, 50 µM α-TG, 1 % Penicillin/Streptomycin and 0.5 % Amphotericin B. HEK293-6507.8, 6008 and 2089 cell lines were cultivated with RPMI 1640 supplemented with 8 % FCS, 1 % Penicillin/Streptomycin, 1 mM sodium pyruvate, 100 nM sodium selenite, 0.43 % α-TG (thiol buffer) and the corresponding selection antibiotic, 100 µg/ml Hygromycin for 2089 and 500 ng/ml puromycin for 6008 and 6507.8. PCI-1/gp350 cells were cultivated under the selection of 1 mg/ml geneticin (G418) in the medium.

All the cells were regularly tested for the presence of mycoplasma contamination with the MycoAlert mycoplasma test, following the instructions of the manufacturer. Positive cells were discarded.

4.1.1. Cultivation of suspension cells

Raji cells were cultivated and maintained at a concentration of approximately 5 x 10^5 cells/ml. LCLs were cultivated and maintained at a minimum concentration of 1 x 10^6 cells/ml. The cells were resuspended and passaged at a dilution of 1:8 for Raji cells and between 1:2 and 1:3 for LCLs.

4.1.2. Cultivation of B Blast cells and feeder cells

B Blast cells were cultivated always onto LL8 cells and IL-4 (2 ng/ml), at a concentration of around 1 x 10^6 cells/ml. LL8 cells were harvested like other adherent cells, resuspended in 25 ml of fresh medium and irradiated at $\geq$180 Gy for 6 h in a ^{137}Cs

device. Afterwards, the cells were centrifuged at 1500 rpm for 10 min at room temperature (RT). The cell pellet was resuspended in fresh medium (25 ml) and cells were plated at 1 x 10^6 cells per plate in 12-well plate.

4.1.3. Cultivation of adherent cells

All adherent cells were washed once with 1x PBS and incubated with Trypsin at 37 °C for detachment. Afterwards, the cells were resuspended in fresh medium to block trypsinaction, and diluted between 1:8 and 1:12 into a new sterile cell culture flask. LL8 cells were passaged once a week, 1:25 diluted, until passage 30 and subsequently, the cells were discarded.

4.1.4. T cell assay

To determine the presence of gp350 specific vesicles in the plasma of EBV-seropositive donors, samples containing vesicles fractions and protein fractions from ultracentrifugated plasma were incubated, overnight at 37 °C in a 96-well plate, with 5 x 10^5 mini LCLs, that were used as antigen presented cells. The next day, 5 x 10^5 gp350-specific $CD4^+$ T cells were incubated with the mini LCLs. After 16-20 hours, the supernatant was collected and used to perform an IFNγ ELISA, or stored at -20 °C.

4.1.5. Storage of cells

For long-term storage, 1 x 10^7 cells were centrifuged at 1500 rpm for 7 min at 4 °C, and the pellet was resuspended in 1 ml of commercial freezing medium (Bambanker) and transferred into 1.8 ml cryotubes. The cell stocks were first slowly frozen for at least two days at -80 °C in the freezing container (Qualilab) containing isopropanol that enables a freezing rate of 1 °C/min before transfer to storage facilities in liquid nitrogen.

4.1.6. Cell counting

Cell counting was performed with a haemocytometer (commercial Neubauer chamber). Cell cultures were well resuspended and 10 µl were mixed with 10 µl of trypan blue solution and introduced into a commercial cell counting chamber. The cells were counted under the microscope. In the four big squares, living/dead cells were counted and the mean was calculated according to the following equation 4.1.

$$\frac{Living\ cells}{ml} = \left(\frac{sum\ of\ 4\ squares}{4}\right) x\ {}^{1}/_{2}\, x \frac{10^4}{ml}$$

Equation 4.1. Formula used to calculate the number of cells per ml.

4.1.7. Induction of virus production from producer cell lines

This protocol was used to induce virus production from any of the producer cell lines, HEK293-2089, HEK293-6008 or HEK293-6508.

One day prior to transfection, cells were splitted 1:6 and seeded on a 15 cm^2 dish. For an optimal transfection, the cells were incubated until 70 – 80 % confluency was reached. The cells were transfected using PEI with a plasmid mix containing 6 µg of p0509 (*BZLF1*) and 6 µg of p2670 (*BALF4*). The plasmid mix was incubated with 2 ml of OptiMEM® for 2 min at RT. At the same time, the PEI (6 µl per DNA µg) was incubated also with 2 ml of OptiMEM®. Afterwards, the plasmid mix was incorporated to the PEI mix and the mixed solution was incubated for 30 min at room temperature. In the meantime, the cells were washed once with PBS to eliminate the rests of FCS and antibiotics, and 10 ml of OptiMEM® were used to cultivate the cells. Then, the plasmid-PEI mixed solution was added dropwise to the cells. After 6 h of incubation at 37 °C, the medium was removed and replaced with fresh RPMI medium in absence of selection antibiotics. Three days after transfection, the cell supernatant was collected in 50 ml Falcon tubes and centrifuged once at 1200 rpm for 10 min at 4 °C. After centrifugation, the supernatant was filtrated with a 0.8 µm diameter pore filter and stored in a falcon tube at 4 °C with the lid covered with parafilm to avoid evaporation.

4.1.8. Virus titration

To determine the virus titer Raji cells were used and this calculation is known as Green Raji Units (GRU). 5 x 10^4 Raji cells were seeded onto a 48-well plate in 100 µl of fresh RPMI medium. Different amounts of virus supernatant (20 µl, 50 µl, 100 µl, 150 µl and 200 µl) were added to the cells, and more medium was added up to a final volume of 700 µl. The cells were incubated for three days and analysis for GFP expression was done by flow cytometry. The GRU were calculated according to the following equation 4.2.

$$GRU = Nr.cells\ x\ \left(\frac{\%\ GFP\ positive\ cells}{100}\right) x \left(\frac{1000\ \mu l}{x\ \mu l\ of\ virus\ supernatant}\right) x10^5$$

Equation 4.2. Formula used to titrate EBV and calculate the GRU.

GRU per ml indicates the concentration of virus in a given sample. This number was used to obtain a certain multiplicity of infection (MOI).

4.1.9. Virus neutralization assay

To analyze the neutralizing activity of the antibodies produced by the LCLs a neutralization assay was performed using Raji or primary B cells. The EBV 2089 and EBV 6008 virus at a MOI 0.2 were incubated with different amounts (20, 50, 100, 150 and 200 µl) of LCL supernatant for 3 h rolling at 37 °C. As a control, the neutralizing gp350-specific antibody clone 6G4 (1 µg/ml) was used. Afterwards, the virus with the supernatant was incubated with 5 x 10^4 cells in a 48-well plate. Three to five days post-infection the cells were analyzed by FACS.

4.1.10. Isolation of human peripheral blood mononuclear cells (PBMCs)

Blood was drawn from healthy adult donors. To avoid coagulation, 100 µl of 0.5 M EDTA per 10 ml of blood were added in the syringe. PBMCs were isolated from the blood by density gradient centrifugation. 30 ml of blood was mixed with 10 ml of PBS and carefully a layer of 7 – 10 ml of Ficoll was added. The tube was centrifuged at 2000 rpm for 40 min at RT with slow acceleration and deceleration in order to allow separation by

layers (Figure 4.1). Afterwards, around 25 ml of plasma was collected into a new 50 ml Falcon tube and centrifuged at 4600 rpm for 20 min at RT and finally stored at -20 °C. The PBMCs layer was carefully collected into a new 50 ml Falcon tube and diluted to 50 ml with PBS to wash the cells from the Ficoll, cells were spun down by centrifugation at 1800 rpm, 10 min at RT. The PBS was discarded and the cell pellet was washed again with PBS, and again centrifuged at 1500 rpm, 10 min at RT. This step was repeated twice.

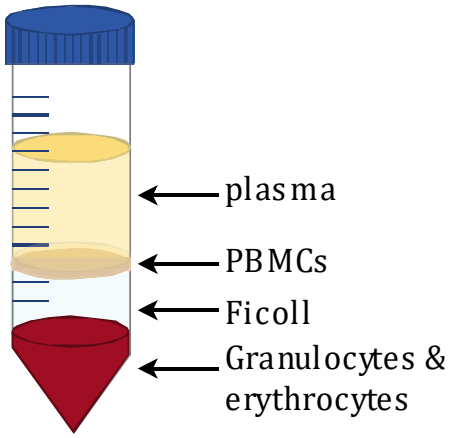


Figure 4.1. Scheme of the different layers of the blood after density gradient centrifugation. The human blood was separated by Ficoll gradient into different layers. From top to bottom, we observed the plasma layer, the PBMCs layer, the Ficoll layer and finally a pellet with the granulocytes and the erythrocytes.

The cells were resuspended in BamBanker and divided into aliquots of 1 – 2 x 10^7 cells per cryotube, and finally stored at -80 °C (Methods 4.1.4).

4.1.11. Isolation of B cells from adenoid tissue

The primary B cells were isolated from adenoids obtained from young children undergoing routine adenoidectomy at the Klinikum Großhadern, Munich. The ethic committee of the LMU has granted the usage of the tissue samples. First of all, the adenoids were cut in small pieces with the help of two scalpels. These small pieces of tissue were passed through a 100 µm cell strainer and collected in a 50 ml falcon tube. All was washed with PBS up to a final volume of around 20 ml. 500 µl of sheep blood was added to the falcon tube and incubated for 15 min at room temperature to deplete T

cells by rosetting. Finally, the B cells were isolated by Ficoll gradient, as described previously (Methods 4.1.8).

4.1.12. Establishment of LCLs

In order to develop fully human antibodies against gp350 and other EBV proteins, splenocytes from immunized hNSG mice (Methods 4.6) were transformed into lymphoblastoid cell lines (LCLs) to make possible its analysis and maintenance.

In a 96-well plate with flat-bottom, 1 x 10^6 irradiated LL8 cells (Methods 4.1.2) were cultivated. After three days, the mouse splenocytes were thawed, resuspended in fresh medium and incubated with EBV (2089) (MOI 0.1) rolling at 37 °C for 3 h. In the meantime, the medium of the feeder plates was removed and substituted by fresh medium with IL-4 (2 ng/ml), CsA (1 µg/ml) and 25 x 10^3 irradiated LENL5 cells/well. The LENL5 were irradiated at $\geq$180 Gy for 1.5 h in a ^{137}Cs device. Finally, the infected splenocytes were centrifuged at 1200 rpm for 10 min at RT to remove the unbounded virus, and resuspended in fresh medium. The splenocytes were plated in the feeder plates at different cell density (5 x 10^4, 2.5 x 10^4 and 1.25 x 10^4 cells/well) (Figure 4.2).

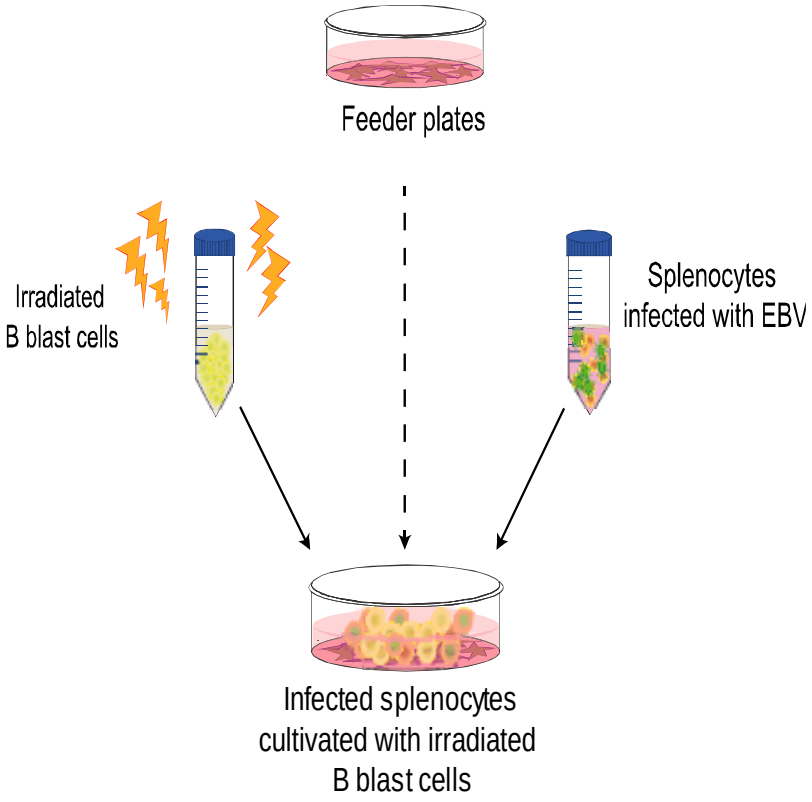


Figure 4.2. Scheme of the immortalization protocol flowed to establish the LCLs used in this thesis. The splenocytes were incubated with the EBV, and together with irradiated B blast cells were cultivated on top of feeder cells.

Once a week half of the medium was replaced with fresh medium, and during four weeks the treatment with CsA was performed to block the T cells. After approximately ten to twelve weeks, the first LCLs were passaged. The cells were always kept in the same feeder plate during the whole experiment.

4.2. Purification and characterization of extracellular vesicles (EVs)

4.2.1. Isolation of EVs

The extracellular vesicles (EVs) were isolated from either blood or cell culture supernatants. Isolation of EVs from cell supernatant was done exclusively by serial centrifugation, while the EVs from blood were isolated by size exclusion

chromatography followed by a final ultracentrifugation step or iodixanol density gradient.

4.2.1.1. Isolation by serial centrifugation

The PCI-1 and PCI-1/gp350 cells were grown under normal cell culture conditions until a cell density of approximately 70 % was reached. Afterwards, the cells were washed and incubated for three days in RPMI without antibiotics or FCS. The cell supernatant was collected and the cell debris was depleted by serial centrifugation (10 min at 1200 rpm, 20 min at 4600 rpm at 4 °C) followed by filtration (pore size 0.45 µm). The EVs were pelleted by centrifugation at 100,000 x g, 4 °C for 2 h in a swinging-bucket rotor (SW32 or SW28). Then, the supernatant was removed to 1 ml, and 100 µl of PBS containing proteinase inhibitor were added. The EVs were resuspended by agitation for 1 h on ice.

To further pellet the EVs, all the supernatant from the ultracentrifugation tubes were collected and centrifugated again at 100,000 x g, 4 °C for 1.5 h with the TL-100 rotor. After centrifugation the supernatant was removed and the EVs were resuspended in 20 µl of PBS containing proteinase inhibitor. The purified EVs were stored at 4 °C for several months.

4.2.1.2. Isolation by size exclusion chromatography (SEC)

To isolate the EVs from blood samples qEV original columns (Izon) with a pore size of 70 nm were used. First of all, the column was washed with approximately 20 ml of PBS, then 1 ml of pure plasma was added and afterwards, PBS was added until collect 30 fractions of about 500 µl. The EVs were located in fractions 6 – 12, and free proteins and other small particles and protein aggregates in fractions 13 – 26. The columns were washed with 20 ml of 5 M NaOH and with other 20 ml of PBS, and finally stored at 4 °C. Each column can was used up to five times.

The collected fractions were separated into two groups, the vesicle samples (6 – 12 fraction) and the high protein content samples (13 – 26 fraction). Each group was

combined and ultracentrifugated with the TL-100 rotor (Methods 4.2.1.1) to get a final volume of approx. 20 µl.

4.2.1.3. Isolation by iodixanol density gradient

The EVs from blood samples were also isolated by floatation into a bottom-up iodixanol density gradient. 400 µl plasma were mixed with 500 µl of 60 % Optiprep. This mixture was added carefully to a 4 ml ultracentrifugation tube, then 2.5 ml of 30 % Optiprep (diluted in PBS) was added carefully and finally, 600 µl of PBS to reach a final volume of 4 ml. The tubes were balanced and centrifuged overnight (o/n) at 160,000 x g, 4 °C. On the next day, 8 fractions of 500 µl were collected from top to bottom and stored at 4 °C for further analysis.

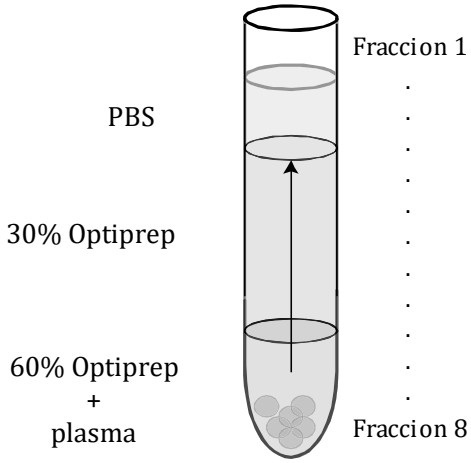


Figure 4.3. Scheme of the Optiprep density gradient protocol. The plasma was mixed with 60% Optiprep and added to the bottom of the ultracentrifugated tube, then carefully a 30% concentrated Optiprep solution mas carefully added without breaking the gradient, and finally the PBS was added. After the density gradient centrifugation, the particles from the plasma will be localized at its corresponding density.

4.2.2. Quantification of EVs

To quantify and characterize the EVs a Nanoparticle-Tracking Analysis (NTA) system was used with the ZetaView® PMX110 instrument (Particle Metrix). The instrument was calibrated with 102 nm polystyrene standard beads. The EVs were diluted in PBS for the measurement. The settings used for the camera were the following: shutter 50, sensitivity 70 and temperature 23 °C.

4.3. Immunological methods

4.3.1. Plasma isolation

The human plasma was isolated from fresh blood samples from different healthy donors. All the plasma was collected after PBMCs isolation by density gradient centrifugation (Methods 4.1.8). After collection, the sample was centrifuged once more at 4500 rpm for 20 min at 4 °C to eliminate cell debris. The supernatant, was finally transfected to a new collection tube and stored at -20 °C until use.

4.3.2. EBV serology

The IgG ELISA against Early Antigen (EA) and Virus Capsid Antigen (VCA) was used to determine the EBV infectious status of the different blood donors used in this thesis. The ELISA was performed following the instructions of the manufacturer. The absorption was measured at 450 nm with the Fluorescence plate reader CLARIOstar®. A third test was also used to determine the EBV status, the RDT EBV IgG Assay, an immunofiltration test against EBNA1 and VCA p18 recombinant antigens. The test was done following the manufacturer protocol.

4.3.3. Gp350 ELISA

To measure the gp350 antibody titers in either cell supernatant or plasma, ELISA plates were coated with 1 µg/ml of commercial recombinant gp350-His protein diluted in PBS. After overnight incubation, the plates were washed four times with 300 µl of washing buffer (Material 3.12) and incubated for 2 h at 37 °C with blocking buffer (300 µl of 5 % FCS in PBS). To avoid evaporation, the plates were always shelled for every incubation

step. After blocking, the plates were washed again four times, and 50 µl of the samples and the controls (diluted in 2 % FCS in PBS) were added in duplicates and incubated for 2 h at 37 °C. Finally, after another washing step, the plates were incubated for 1 h at 37 °C with anti-human-HRP antibody diluted 1 to 6,000 in 2 % FCS in PBS. To develop the signal, the plates were incubated for 30 min with 100 µl of OptEIA substrate solutions diluted 1:1. Reaction was stopped with 100 µl of 2N H_2SO_4. The horseradish peroxidase (HRP) activity was measured at 450 nm with the fluorescence plate reader ClarioStar®.

4.3.4. 7A1 – gp350 ELISA

To measure the gp350 concentration in plasma, ELISA plates were coated with 5 µg/ml of specific gp350 ab (7A1 clone) diluted in PBS. After overnight incubation, the plates were washed four times with 300 µl of washing buffer (Material 3.12) and incubated for 2 h at 37 °C with blocking buffer (300 µl of 5 % Milk in PBS). To avoid evaporation, the plates were always shelled for every incubation step. After blocking, the plates were washed again four times, and 50 µl of the samples (plasma 1:1 diluted) and the controls (diluted in 5 % Milk in PBS) were added in duplicates and incubated for 2 h at 37 °C. Finally, after another washing step, the plates were incubated for 1 h at 37 °C with another gp350 specific antibody HRP coated (clone 6G4) diluted 1 to 1000 in 5 % Milk in PBS. To develop the signal, the plates were incubated for 15-30 min with 100 µl of OptEIA substrate solutions diluted 1:1. Reaction was stopped with 100 µl of 2N H_2SO_4. The horseradish peroxidase (HRP) activity was measured at 450 nm with the fluorescence plate reader ClarioStar®.

4.3.5. IFNγ ELISA

To measure the IFNγ concentration, ELISA plates were coated with 2 µg/ml of specific IFNγ ab (mAb 1-D1K1 clone) diluted in PBS. After overnight incubation, the plates were washed four times with 300 µl of washing buffer (Material 3.12) and incubated for 1 h at RT with blocking buffer (200 µl of 8 % FCS in RPMI). To avoid evaporation, the plates were always shelled for every incubation step. After blocking, the plates were washed

again four times, and 50 µl of the samples and the controls (diluted in regular RPMI medium) were added in triplicates and incubated for 2 h at RT. Then, after another washing step, the plates were incubated for 2 h at RT with a biotinylated specific antibody (mAB 7-B6-1-biotin clone) diluted 1 to 1000 in PBS. Finally, after another washing step, the plates were incubated with a Streptavidin-ALP antibody for 1 h at RT. To develop the signal, the plates were incubated with 100 µl of pNPP substrate and measure several times until the standard curve was linear. The ALP activity was measured at 405 and 620 nm with the fluorescence plate reader ClarioStar®.

4.4. Flow cytometry

The detection and analysis of specific surface markers and proteins was done by flow cytometry. 1 – 5 x 10^5 cells were collected and washed in FACS buffer. The cells were pelleted and resuspended in 50 – 100 µl of first antibody diluted in FACS buffer (Material 3.12) and incubated for 30 – 60 min at 4 °C in the dark. Afterwards, the cells were washed once with FACS buffer and resuspended in 100 µl of second antibody and incubated for 30 min at 4 °C and protected from the light. Finally, the cells were washed twice with FACS buffer and resuspended in 200 – 400 µl of FACS buffer and analyzed with a flow cytometry device.

In case a directly coupled antibody was used, the antibodies were diluted in FACS buffer according to the manufacturer recommendations, and the cells were incubated with the antibody for 30 min at 4 °C in the dark. After staining, the cells were washed twice and resuspended in FACS buffer for further analysis.

For the analysis of the virus titration, the cells were directly analyzed for GFP expression with the flow cytometry device without any previous washing step.

4.4.1. Compensation controls

For multi-staining panels, the compensation controls were done with anti-mouse compensation beads (Material 3.10). One drop of negative beads and one drop of positive beads were mixed with 1 µl of the corresponding antibody, each antibody from

the panel separately, and incubated for 5 min, 4 °C, in the dark. A negative control, without antibody, was always included. After measuring, the compensation matrix was done with the Diva software and applied to all the samples from the experiment before been measured.

4.4.2. FACS sorting of LCLs

LCLs were sorted using FACS Aria IIIu device according to the instructions of the manufacturer to get to isolate the different cell populations contained in LCLs. 3 x 10^8 cells were collected, washed with FACS buffer and treated with Fc Block (2.5 µg/ml diluted in FACS buffer) for 10 min at RT. Afterwards, the cells were centrifuged down and stained with direct-coupled antibodies against CD19-FITC, CD27-BV605, IgM-PB and IgG-PE (Material 3.7.1). After 60 min of incubation in the dark, the cells were washed twice with FACS buffer and filtrated through a 35 µm cell strainer to obtain a single cell suspension.

The sorting was performed with a 100 µm nozzle in a "4-way purity" mode. The gating criteria included: (1) living cells, (2) single cells, (3) CD19-positive cells, (4) CD27-positive cells, (5) IgM-negative vs IgG-positive. The cells were sorted to obtained an IgM-negative, IgG-positive population.

4.4.3. Flow cytometry measurement

After the antibody staining and subsequent washing, the cells were transferred into FACs tubes and measured with the FACS cytometer Canto™ or LSR Fortessa™, depending from the fluorescence marker used. The recorded FACS data was exported to a USB stick and analyzed later with the help of the FlowJo software.

4.5. Molecular biology

4.5.1. Quantitative *real-time* PCR (qRT-PCR)

The presence and frequency of RNA transcripts was by quantitative *real-time* PCR in 96-well plate using the LightCycler 480 from Roche. The SYBR Green (Roche) was used as reagent to be intercalated between dsDNA. Total reaction volume was reduced to 10 µl, and each sample was performed as triplicate. 2 µl of dilutes cDNA (1:5 diluted in H_2O) was mixed with 10 pmol of primer mix (forward and reverse) and 5 µl 2x SYBR Green reaction mix. The efficiency of each primer was calculated in a previous experiment. The relative expression level of each RNA was calculated using the LC480 software. For the normalization of the data, GAPDH reference transcript was used.

4.5.2. Cloning of the heavy and light chain of the human antibody

For the cloning of the heavy and the light chain of the human immunoglobulin produced by LCLs, the cells were first sorted to obtained a clear $CD19^+$ $CD27^+$ IgM^- IgG^+ population, RNA was isolated from the sorted cells, and cDNA was produced from the isolated RNA. Two different approaches were done for the amplification of the heavy and light chain. PCRs were performed with specific gamma heavy chain and kappa light chain primers. The sequencing was done by the company Eurofins in Robert-Koch-Straße 3A/Haus 2, 82152 Planegg. The analysis of the sequences was done using the website *http://www.abysis.org*, specialized in the analysis and prediction of antibody sequences and structures.

4.5.2.1. RNA isolation

For the RNA isolation the *RNeasy 96 Kit* was used. This kit is designed to isolate RNA from 10 cells to 1 x 10^6 cells. 4100 sorted LCLs cells were used. The cells were centrifuged at 0.3 x g for 7 min, the supernatant was removed, and 100 µl of RTL lysis buffer were added to the tube. After mixing, 100 µl of 70 % etOH were added to the tube, the sample was pipetted vigorously to mix it. The lysate was transferred to a RNeasy 96-well plate that was plated on top of a square-well block. The well plate was centrifugated at 4600 rpm, for 4 min, at RT. The flow-through was discard, and 800 µl of RW1 washing buffer were added to the 96-well plate. The plate was centrifugated at 4600 rpm, for 4 min, at RT. After two more washing steps with the RPE washing buffer

the plate was centrifugated at 4600 rpm, for 10 min, at RT. Finally, the RNA was eluted with 45 µl of RNase-free H_2O, the sample was centrifugated at 4600 rpm, for 4 min, at RT. The RNA was stored at -20 °C.

4.5.2.2. Reverse transcription of mRNA

The RNA obtained from the sorted cells was low that it was not possible to measure. For that reason, the maximal volume was used (11 µl). The reverse transcription was done using Random Hexamers and oligo-dNTPs mixture, so the reverse transcription (RT) from mRNAs with and without poly-A tail could be performed. The RT was done using the SuperScript IV reverse transcriptase, following the manufacturer protocol. Additional incubation step with RNase H was done to eliminate rests of possible genomic DNA. The cDNA was stored at -20 °C.

4.5.2.3. Human IgG cloning nested PCR

The cloning of the immunoglobulin heavy chain from LCLs was done by nested PCR protocol. The forward primers used in this protocol were designed in the lab from Prof. Klein in Köln (Kreer et al. 2020), the reverse primers were previously described (Tiller et al. 2008).

PCR protocol:

2.5 µl		0.1 µl
0.75 µl		16.15 µl
1.5 µl		
0.5 µl		
0.5 µl		
0.5 µl		
2.5 µl	(10x) PCR Buffer (no magnesium chloride)	

50 mM MgCl2
KB Extender
dNTPs mix (10 mM each)
Forward primer mix
Reverse primer
Template
Platinum Taq DNA polymerase
H_2O

PCR program:

1st PCR			**2nd PCR**		
94 °C	2 min		94 °C	2 min	
94 °C	30 sec		94 °C	30 sec	
57 °C	30 sec	50 cycles	57 °C	30 sec	50 cycles
72 °C	55 sec		72 °C	45 sec	
72 °C	5 min		72 °C	5 min	
4 °C	hold		4 °C	hold	

4.5.2.4. Human Ig Kappa cloning RACE PCR

The cloning of the immunoglobulin light chain from LCLs was done by RACE PCR protocol. The forward primers used in this protocol were from the kit, the reverse primers were previously described (Schanz et al. 2014).

PCR protocol

26 µl	H_2O
10 µl	5x GoTaq® Flexi PCR Buffer
3 µl	25 mM $MgCl_2$
1 µl	10 mM dNTP mix
5 µl	dC-tailed cDNA
2 µl	PCR primer (10 µM)
2 µl	Anchor primer (10 µM)
1 µl	GoTaq® pol II

PCR program:

95 °C	90 sec	
95 °C	30 sec	35 cycles
55 °C	30 sec	
72 °C	60 sec	
72 °C	6 min	
5 °C	Hold	

4.5.3. PCR product sequencing

The PCR product (20 µl) was loaded in a agarose gel with 1 % EtBr. The gel was run at 60 V for 70 min. The corresponding bands were cut and cleaned following the indications of the kit manufacturer (*PCR clean-up gel extraction, MN)*. The cleaned product was sent for sequencing at Eurofins following the company indications.

4.6. Protein analysis

To analyze the presence of specific proteins in EVs or human plasma, simple western blot was performed with cell lysates and purified EVs. To study the presence of specific antibodies, dot blot was one of the chosen methods.

4.6.1. Preparation of cell lysates

The cells were collected and washed 1x with PBS. After centrifugation, cells were resuspended in one-time RIPA buffer (Material 3.12) mixed with one-time protease inhibitor (50 – 400 µl, depending on the cell number). After 30 min of incubation on ice, the lysed cells were centrifuged at full speed for 30 min. The supernatants were transferred into a new reaction tube and stored at -20 °C. PBMCs were lysed with 20 – 50 µl of a commercial cell lysis buffer (Material 3.10) diluted 1:10 in H_2O and mixed with 1mM PMSF (proteinase inhibitor).

The concentration of the protein was determined by a quantification assay based on the principle of Bradford's protein assay. The dye reagent (Material 3.11), diluted 1:5 in H_2O, was mixed with 1 µl of protein lysate. As blank, pure diluted reagent was used. For the EVs or PBMCs lysates, where the protein concentration was presumably very low, the commercial BCA kit (Material 3.11) was used to measure the protein concentration.

4.6.2. Simple western blot (WES)

The Simple western blot (WES) is a new automated western blot method, very useful when your sample volume is limited. The whole process takes place in the WES instrument (Material 3.14) with a duration of around 4 h. The WES method does not use membranes, instead uses a capillary system to detect the protein signal.

To performed simple western blot, 5 µl of EVs sample or cell lysate were used (Material 3.11). The WES was done following the manufacturer protocol. As primary antibody, 72A1 (5 µg/ml) and OT-6 (1:2000) antibodies (Material 3.7.2) were used, both diluted in the antibody diluent buffer from the kit. As secondary antibody, the ready-to-use anti-mouse antibody from the kit was used.

	1	2	3	4	5	6	7	8	9	10	11	12	13
A	Biotinylated Ladder 5 µl	Sample 5 µl											
B	Antibody Diluent 10 µl												
C	Antibody Diluent 10	Primary antibody 10 µlk											
D	Streptavidin-HRP 10 µl	Secondary antibody 10 µl											
E	Luminol/Peroxide Mix 15 µl												
F	empty												

Figure 4.5. Scheme of pipetting protocol for WES. First column always corresponds with the biotinylated ladder, running control from the WES. All the components are loaded into a 13 or 24-well chamber following manufacturer indications, avoiding bubbles that can interfere with the results at the end.

WES protocol:

Separation time (min)	25.0
Separation voltage (volts)	375
Antibody diluent time (min)	5.0
Primary antibody time (min)	90.0
Secondary antibody time (min)	30.0
Detection	

The WES data were analyzed with the Compass for SW program (ProteinSimple).

4.6.3. Dot blot

Dot blot is an immunodetection technique to detect proteins in the native conformation. 1 – 2 µl of sample (cells lysate, SEC samples, density gradient fraction) were pipetted directly onto a nitrocellulose membrane. The dots were dried for 10 min and blocked with 5 % Milk in TBST for 60 min at RT. The membrane was incubated overnight (o/n) at 4 °C with the first antibody diluted in blocking buffer. On the next day, the membrane was washed three times for 10 min with washing buffer (Material 3.12), then the membrane was incubated for 2 h at RT with the HRP-conjugated secondary antibody. Afterwards, the membrane was four times washed for 15 min. The chemiluminescence signal was developed with our home-made ECL or with the commercial ECL (Material 3.11), depending on the expected intensity of the signal. The visualization of the signal was done with the Fusion FX imaging instrument (Material 3.15).

4.7. NSG-HLA2.1 mouse immunization

The animal experiments were performed by Simon Danisch from the Stripecke group, Hannover medical School (MHH). In order to reconstitute the human immune system completely, 17 NSG-HLA2.1 mice were transplanted with 2 x 10^5 CD34-positive human stem cells (HSC) following the protocol from the Stripecke lab (Salguero et al. 2014). The mice were separated in two groups, untreated group, with 8 mice, and VLPs group, with 9 mice. VLPs group were treated with EBV 6507.8 (5 x 10^6 particles) four times. The first

VLPs were injected intravenous (iv) 10 weeks after HSC transplantation. Afterwards, VLPs were injected three times more (11, 14, 16 week after HSCT), 18 weeks after HSC transplantation animals were sacrificed, back bone, lymph nodes and spleen were prepared, cells were extracted, partially stored at -80°C. Fresh splenocytes were sent to our lab to start the work for this thesis. Over the course of the experiment, blood samples were collected to monitor EBV infection (Methods 4.3.2) and serum antibody levels were measured by ELISA (Methods 4.3.3).

5. Results

Part I: Study of the presence of gp350 in the blood of EBV-seropositive donors

5.1. *In vitro* and *in vivo* expression of gp350

gp350, encoded by the *BLLF1* open reading frame, is the major envelope glycoprotein of EBV. It is responsible for the attachment of the virus to human B cells during infection by interacting with the human CD21 complement receptor (Chesnokova et al. 2015) or with the human CD35 complement receptor (Ogembo et al. 2013). gp350/220 is a protein expressed during the late lytic cycle of the virus. Nevertheless, the presence of gp350 protein has been detected on the surface of LCLs by flow cytometry and on extracellular vesicles (EVs) released by these cells by immunoblotting (Vallhov et al. 2011). We were therefore wondering whether gp350 is generally present on EBV-infected cells and whether the protein is also expressed *in vivo*, assuming a potential biological role e.g. in EVs. In a first experiment, the expression of the gp350 protein on the surface of different EBV positive cell lines was analyzed by flow cytometry. The B95.8 cell line is a cell line derived from an infection of peripheral blood B lymphocytes from a cotton-top tamarin with EBV (Miller et al. 1972). B95.8 is known to release infectious EBV viral particles and to express various lytic proteins, like BZLF1and gp350. Therefore, the B95.8 cell line was used as a positive control. All cell lines analyzed were stained with the gp350-specific antibody 7A1, which has been developed in our lab, and with a secondary antibody carrying the Alexa647 fluorochrome. As expected, this analysis revealed that a fraction of B95.8 cells expresses gp350 on the surface (Figure 5.1).The presence of gp350 on the surface of LCLs cell lines and BL cell lines differed from cell line to cell line, where two out of three, and one out of three LCLs and BL cell lines, respectively, were clearly positive for surface gp350. The unique LCLs cell line stablished from a primary PTLD analyzed (PTLD 880) was clearly negative for surface gp350 expression (Figure 5.1). This experiment demonstrated that some latently EBV-infected cell line constantly express gp350, among these KEM I, a Burkitt lymphoma with a latency type I viral program.

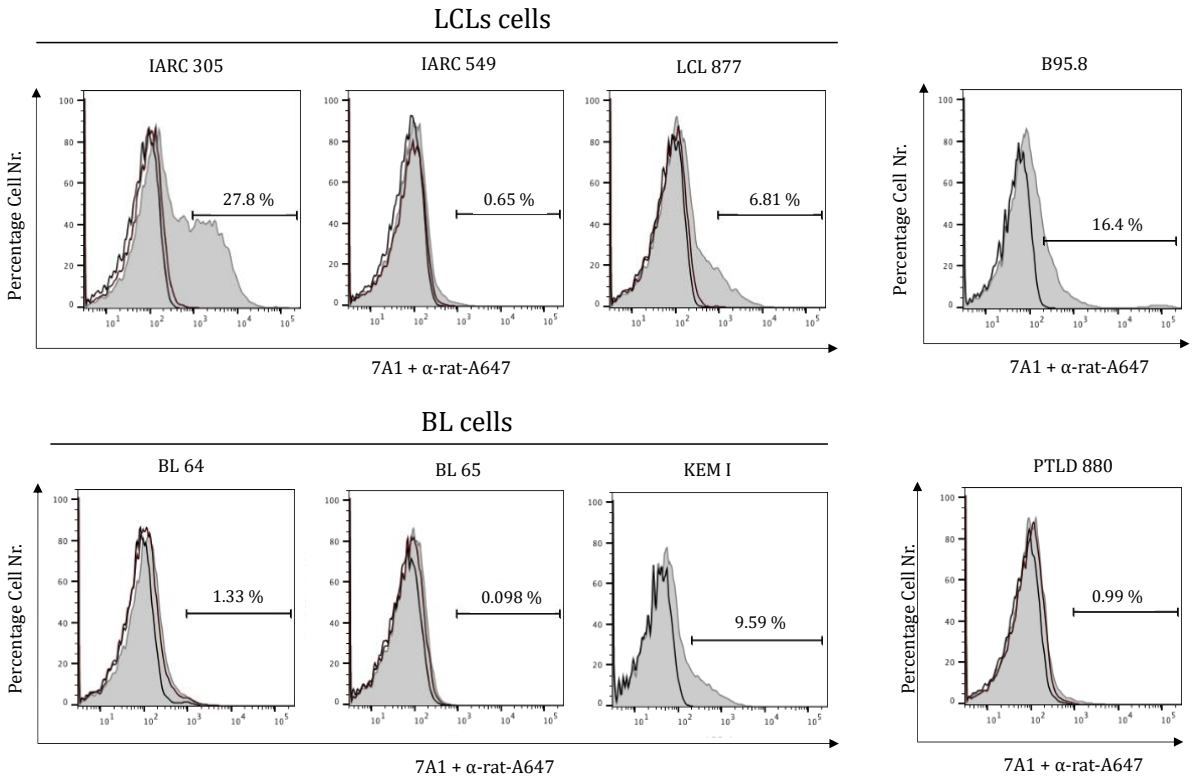


Figure 5.1. gp350 protein is expressed on the surface of several latently infected EBV positive cell lines. 1 x 10^6 cells were incubated at 4 °C for 1 h with the gp350- specific antibody 7A1 (1 µg/ml) (grey histogram). After washing, cells were incubated for 30 min with anti-rat-Alexa647 antibody (diluted 1:500 in FACS buffer). Prior to analysis, the cells were washed twice with FACS buffer. Black line: isotype control. The FACS data were analyzed with the Flow Jo software.

To investigate gp350 expression *in vivo*, we first isolated by serial steps of ultracentrifugation (2,000 g, 10,000 g, 100,000 g as described in detail in Materials and Methods) EVs from a series of buffy coats from healthy donors. As shown in Figure 5.2, five out of twelve samples gave a clear positive result with the murine gp350-specific antibody 72A1 that is widely used in the field for many years (Hoffman et al. 1980). VLPs from a HEK293 derived producer cell line (TR–) served as a positive control and EVs isolated from cord blood (A) as a negative control. The pan-EV marker CD63 was used to proof the successful isolation of EVs.

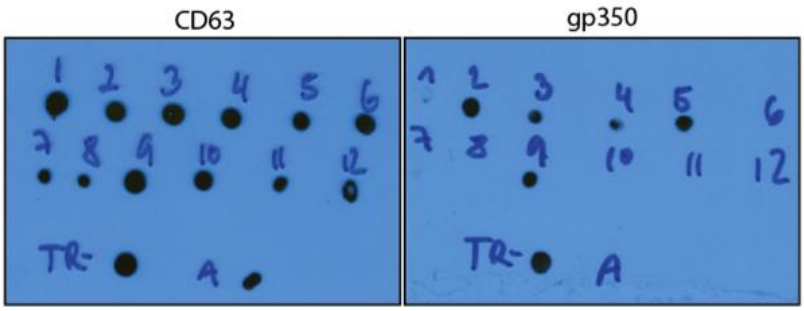


Figure 5.2. **gp350 on extracellular vesicles (EVs) obtained in sera of healthy donors.** EVs from buffy coats of different healthy donors were isolated by ultracentrifugation. The samples were stained for CD63 (pan-EV marker) and gp350 with the gp350-specific antibody 72A1. As a positive control EVs obtained from the TR- cell line, a HEK293-based cell lines that, upon induction of the lytic cycle, releases EB-VLPs (Delecluse et al. 1999) (a gp350-producer cell line) were used, and as a negative control EVs obtained from cord blood (named A in the blot).

On the basis of these data, we decided to investigate the expression of gp350 *in vivo* in more detail.

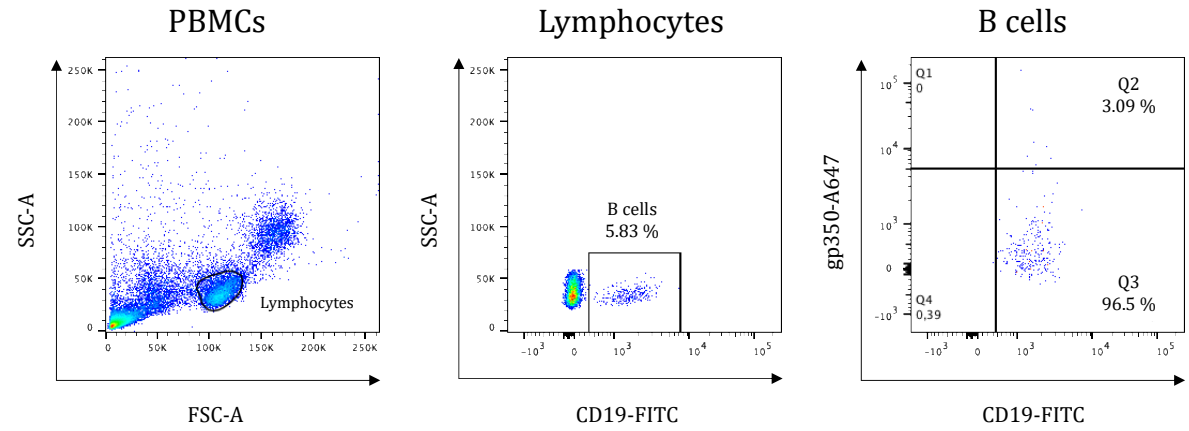
5.2. Study on the presence of the late lytic gp350 protein in the blood of healthy EBV seropositive donors

The detection of EBV-specific antibodies in the peripheral blood is a daily routine both for IM diagnostic and recurrent EBV infection. While IgM antibodies against EBV viral capsid antigen (VCA) are characteristic of an acute IM, later in time IgG antibodies against VCA will appear and persist life-long. Likewise, IgG antibodies against EBV nuclear antigen 1 (EBNA1) also persist life-long in the individual (Henle et al. 1987). Studies done in students of an American university, following the antibody titers of several immediate early (IE), early (E), late lytic (L) and latent EBV proteins, showed the presence of EBV-specific neutralizing antibodies at least 6 months after the IM episode. For some individuals, the presence of gp350 antibodies was detected up to 3 years after the IM episode (Bu et al. 2016).

After been shown that EBV-positive cell lines express gp350 on their surface, that gp350-specific antibodies are present in the blood of EBV-seropositive healthy donors

(Sashihara et al. 2009) (Panikkar et al. 2015) (Bu et al. 2016), and knowing that gp350-specific extracellular vesicles (EVs) are detectable in the blood of healthy donors (Figure 5.2), we wanted to investigate whether gp350 protein can also be found on the surface of peripheral blood mononuclear cells (PBMCs) of EBV-seropositive healthy individuals. For this, PBMCs, obtained by density gradient centrifugation, were stained with directly-labeled antibodies against CD19 and gp350. First of all, to analyze the percentage of gp350 positive cells from the totality of B cells population, PBMCs were gated by SSC-A vs FSC-A for lymphocytes population and afterwards, lymphocytes were gated by SSC-A vs CD19 (pan-B cell marker) for B cells population. Finally, B cells population was analyzed for the presence of gp350 on their surface by gating CD19 vs gp350 (Figure 5.3 A). B cells expressing gp350 could be detected in every EBV-positive healthy donor tested. On average 2.36 % of B cells stained positive for gp350. On the analyzed EBV-negative donor, no B cells were detected positive for the expression of gp350 (Figure 5.3 B).

A



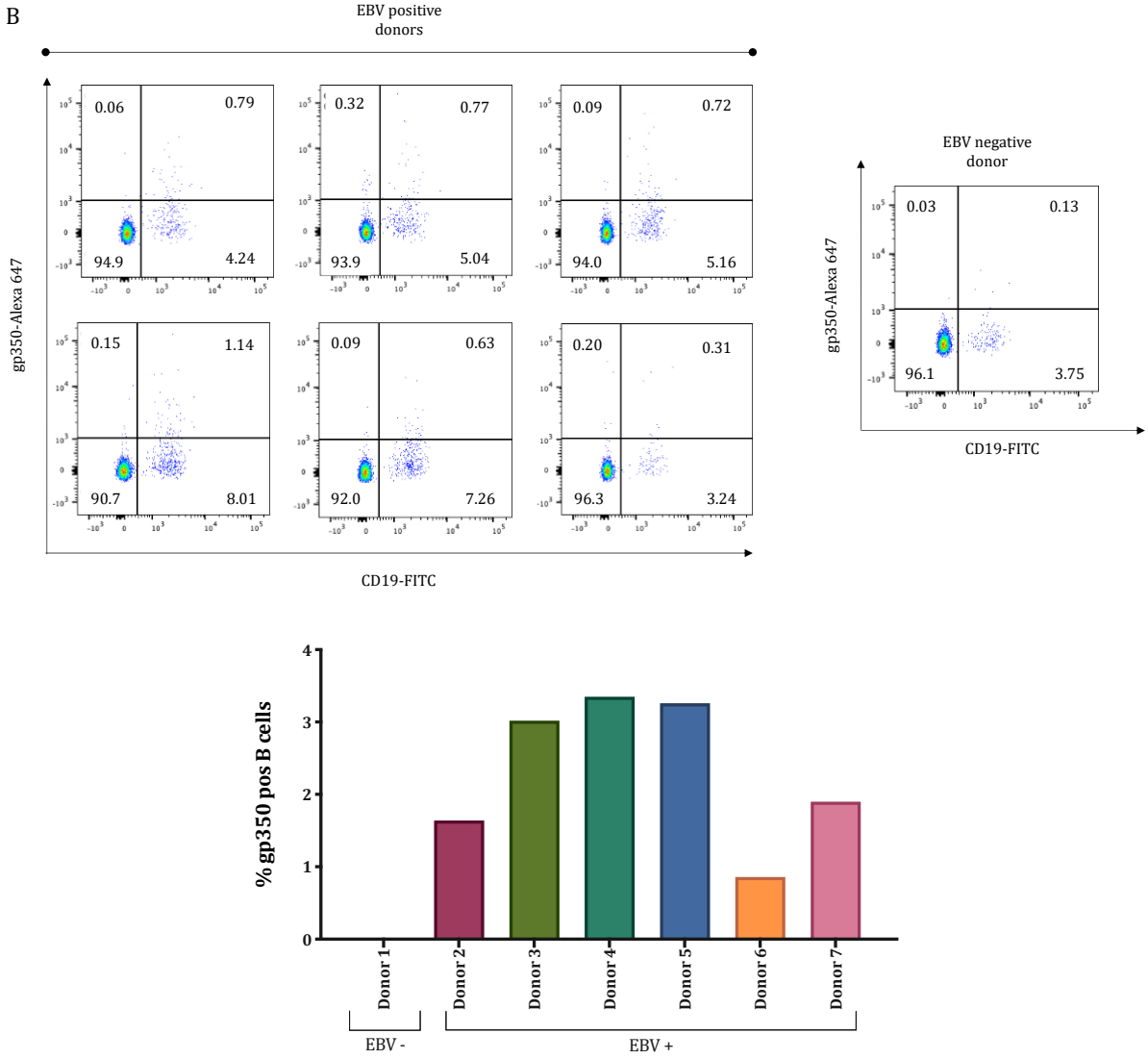


Figure 5.3. In EBV-seropositive donors, from total PBMCs, a portion of B cells express gp350 on its surface. A. Gating strategy: PBMCs were gated for lymphocytes population, then lymphocytes were gated for B cell population and finally, B cells were analyzed for the presence of gp350 on their surface. **B.** B cells from six EVB-positive and one EBV-negative healthy donors were analyzed for the presence of gp350 on B cell surface. The cells were stained with directly-labelled antibodies against CD19 and gp350. The lower graph summarizes the flow cytometry data.

After having analyzed PBMCs for the presence of surface gp350, we next analyzed the presence of gp350-specific IgG antibodies in sera of healthy EBV-seropositive donors. For this, the plasma of 19 healthy individuals, 17 EBV-seropositive and 2 EBV-seronegative, was analyzed by ELISA. The plasma was presented against a commercially

available gp350 protein with a C-terminal His-tag, and the signals were developed with an anti-human IgG-HRP antibody. gp350-specific IgG antibodies could be detected in every EBV-seropositive donor tested, albeit at variable titers. For 8 out of 17 seropositive donors, these titers were highly significant (3 x the mean of the negative control). No gp350 antibodies were detected in both EBV-seronegative donors (Figure 5.4).

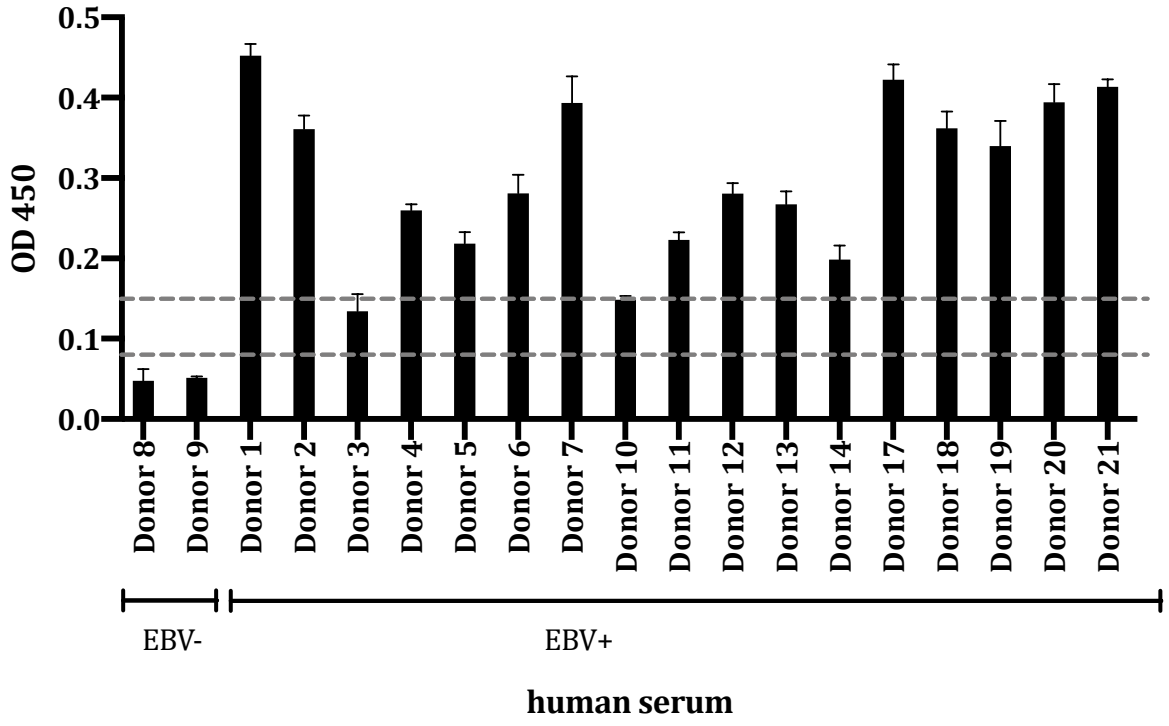


Figure 5.4. The plasma of all EBV-seropositive donors contains significant titers of gp350-specific antibodies. Human plasma obtained after centrifugation of blood of several EBV-seropositive and -seronegative donors, was used to perform a gp350 ELISA. The ELISA plates were coated with gp350-His protein (Sino Biological, 1 µg/ml diluted in PBS). As a positive control, the anti-His-HRP antibody, diluted 1:200 was used. The human plasma was diluted 1:250 in 2 % FCS in PBS. The donors 8 and 9 are EBV-seronegative donors. The second antibody was a donkey anti-human IgG-HRP, diluted 1:6,000 in 2 % FCS in PBS. The "cut-off" threshold corresponds with the mean of the negative controls + 2x SD, and the second threshold correlates with 3x the mean of the negative control.

5.3. Particles containing the EBV gp350 protein are present in the blood of healthy seropositive donors

During the last decade, the role of the extracellular vesicles (EVs) in the regulation of immune responses has been intensively studied. EVs are particles that are constantly released by most, if not every, eukaryotic cell. EVs contain proteins, DNA and various species of RNAs of the mother cells, which they transfer to recipient cells. They have been associated with a broad range of biological processes like cancer progression, immune responses or viral infections (Kalluri and LeBleu 2020).

As mentioned before, also LCLs release EVs, and these EVs have been shown to contain gp350. Of interest, it has been demonstrated that gp350+ EVs interfere with the infection of B cells with EBV (Vallhov et al. 2011), indicative for a potential biological or immunological role also *in vivo*. This was corroborated by the detection of gp350 in the supernatants of buffy coat preparations. Also, the presence of gp350-specific antibodies in the plasma of EBV-seropositive donors raised the question whether gp350 protein is also present in the serum. For this, the human plasma samples were incubated in a 96-well plate, coated with a gp350-specific antibody (clone 7A1), and the signal was developed with the HRP-labeled gp350-specific antibody 6G4 which binds to a different epitope (Figure 5.5 A). gp350 protein was detected in 4 out of 11 EBV-seropositive donors. For the EBV-seronegative donor the signal was undetectable (Figure 5.5 B).

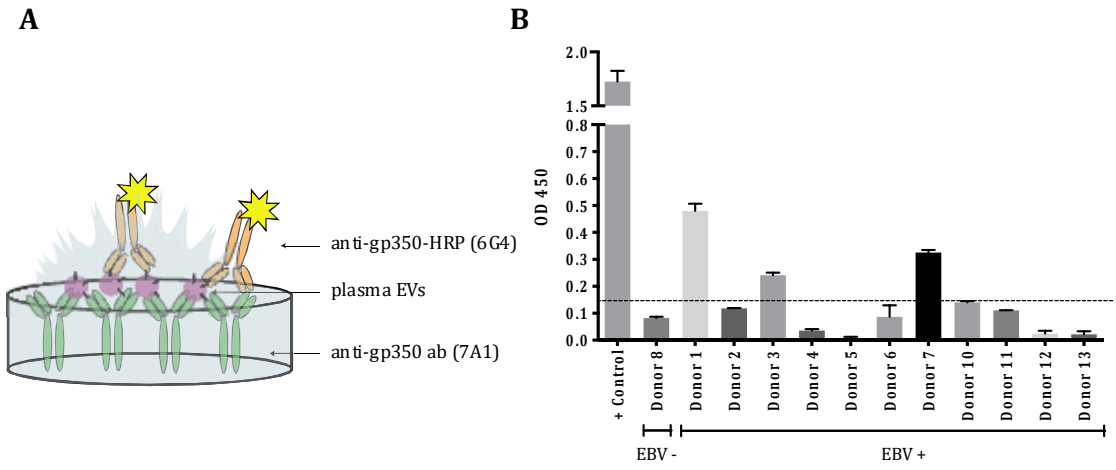


Figure 5.5. gp350 protein can be detected in the plasma of EBV positive donors. A. Scheme of the EVs ELISA assay to measure gp350 protein in plasma. The ELISA plates were coated with purified

gp350-specific antibody 7A1 (5 µg/ml diluted in PBS). After overnight incubation, the plates were washed and blocked with 5 % milk in PBS at 37 ° C for 2 h. Afterwards, plasma samples of EBV-seropositive and -seronegative donors (diluted 1:1 in PBS) were incubated at 37 °C for 2 h. As a positive control, EVs obtained from the supernatant of PCI-1/gp350 cell line were used. Finally, the gp350-specific HRP antibody 6G4 (diluted 1:1000) was used for detection. After a final washing step, the samples were incubated with a commercial TMB substrate for 15 min, and the reaction was stopped with 2 N H_2SO_4. The chemiluminescence signal was measured with a fluorescence plate reader. **B.** gp350-specific ELISA with plasma of EBV-seropositive and -seronegative donors. The threshold was set by doubling the chemiluminescence signal of the negative control.

5.3.1. Characterization of the particles present in the human plasma

The ELISA assay described above revealed the presence of detectable amounts of gp350 protein in some EBV-seropositive donors. But this assay did not explain whether the protein is present on EVs, some other type of vesicles or as free protein. We therefore aimed at characterizing the nature of gp350 in more detail. First, the plasma samples were fractionated by either size exclusion chromatography (SEC) or by iodixanol (Optiprep®) density gradient centrifugation. These are the two most common and standardized techniques used nowadays for the enrichment and isolation of EVs, based on their size in a range of 40 to 160 nm, and a density of 1.1 g/ml in iodixanol (Iwai et al. 2017).

For this purpose, 0.5 ml each of the plasma of an EBV-seropositive donor and of an EBV-seronegative donor were loaded onto a qEV SEC column (Izon, pore size of 70 nm) which was then washed intensively with PBS. From the flow-through, 40 fractions of approximately 500 µl each were collected, analyzed for protein concentration with a commercial BSA kit and for vesicle numbers with a Nanoparticle Tracking Analysis machine (NTA) called Zetaview®. The vesicle numbers obtained of the EBV-positive and the -negative donor are depicted in blue and red, respectively, while the protein concentration in the different fractions of the EBV-positive and -negative donor are given in green and violet, respectively. The highest vesicle numbers were present in fractions 8 to 11, as expected from the provider, while lipoproteins, protein aggregates and free protein were found in later fractions 15 to 27 (Böing et al. 2014). The vesicle

numbers as well as the protein concentration were almost similar for both blood donors (Figure 5.6 A).

For the iodixanol (Optiprep®) density gradient separation, the plasma was mixed 1:1 with 60 % of iodixanol previously added to the tube, then layers of 30 % iodixanol diluted in PBS and pure PBS were added on top. The tube was ultracentrifuged overnight (o/n) at 100,000 x g, then the sample was separated into 8 fractions of 500 µl each. The fractions were analyzed for vesicle numbers and protein concentration as described above. The vesicles were mainly found in fractions 1 to 3 and free protein, protein aggregates and lipoproteins in fractions 6 to 8. Surprisingly, for one of the EBV+ donor, vesicles were also found at fraction 8 at high numbers, where normally only free protein and small particles are found (Figure 5.6 B).

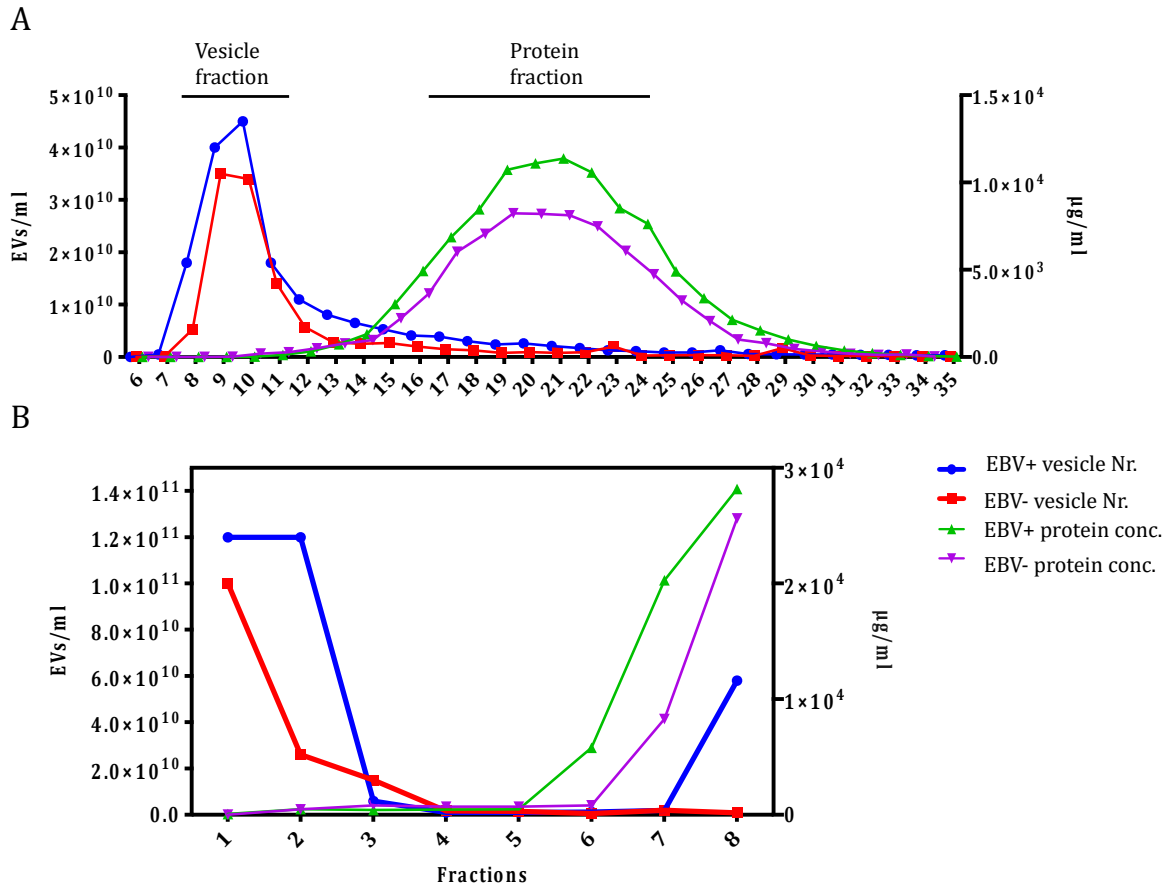


Figure 5.6. The plasma of EBV-seropositive and -seronegative donors contain EVs that can be separated by either size exclusion chromatography (SEC) and Optiprep density gradient centrifugation. A. 0.5 ml of human plasma was loaded onto a SEC column with a pore size of 70 nm.

35 fractions of approximately 500 µl were collected. The fractions were analyzed with the ZetaView to measure the number of EVs in each fraction. The total protein concentration was calculated with a BCA test. The graph shows in blue and red the concentration of EVs in each fraction, and in green and violet the protein concentration of each fraction. **B.** 0.5 ml of plasma was separated in 8 different fractions by Optiprep density gradient centrifugation. The different fractions were analyzed for EVs numbers and protein concentration. The graph shows in blue and red the number of EVs, and in green and violet the protein concentration of each fraction.

The fractions obtained by Optiprep® gradient were analyzed by ELISA for the presence of gp350 protein. With the same protocol as for the plasma, the plates were coated with the gp350-specific antibody 7A1, the samples were incubated with the antibody, and the signal was finally developed with the gp350-specific antibody 6G4 labeled with HRP. The gp350 signal was mostly detected in the fractions of highest densities, this was also observed in previous analysis of gp350+ EVs of LCLs (Vallhov et al. 2011). Moreover, at the EBV-negative donor, gp350 signals where found in the fractions 7 and 8, possibly due to background problems coming from the high antibody and protein concentration in the human plasma (Figure 5.7). In summary, this assay did not give a clear answer to the question whether gp350 can be detected from small volumes of sera and whether gp350+ EVs are present in the blood of seropositive donors.

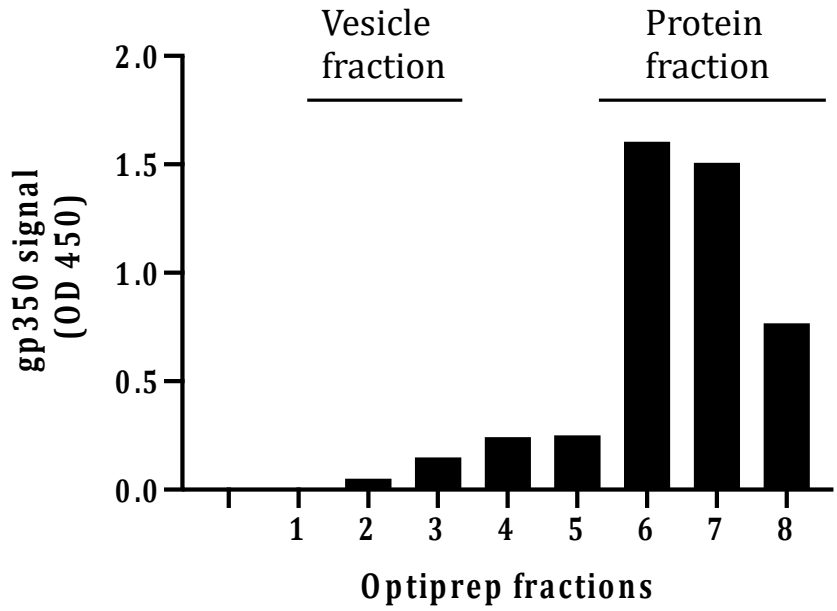


Figure 5.7. The Optiprep gradient fractions of EBV-seropositive plasma with a high protein concentration contains high amounts of gp350. The fractions collected after Optiprep® density gradient centrifugation were used for a gp350 ELISA. The plates were coated with the gp350-specific antibody 7A1 (5 µg/ml diluted in PBS). After overnight incubation and three washing steps the plates were blocked with 5 % milk in PBS, then the plates were incubated with the different fractions overnight. Finally, the plate was developed with the gp350-specific antibody 6G4 coupled with HRP. To develop the gp350 signal a TMB substrate was used. The EBV- data were subtracted from the EBV + data.

To further characterize the presence of g350 in vesicles from blood of EBV-seropositive donors, immunoblotting assays using the sensitive Wes™ system were performed. The advantages of this system are its high reproducibility with an intra-assay confident value of less than 15 %, and the small amount of sample and antibodies that are needed. Due to the limited numbers of vesicles we were able to isolate from serum and plasma for obvious reasons, we decided to use the Wes™ system to analyse the SEC samples described above. The samples were processed as recommended by the manufacturer, and the signal was developed using the murine gp350-specific antibody 72A1, which was known to work with the Wes system. The gp350 signal was detected in the SEC fractions of two EBV-seropositive donors, and also in the diluted plasma of the same donors, whereas both SEC fractions and plasma of two EBV-negative donors stained completely negative (Figure 5.8 A). With the Compass software from Simple Protein, the signal of the bands could be analysed, and the area of these bands was calculated and plotted in Excel graphs. The gp350 signal was detected in the SEC high protein fractions of the EBV-positive donors but not in the EV fractions. The gp350 signal was also detected in the plasma samples of the EBV+ donors (Figure 5.8 B).

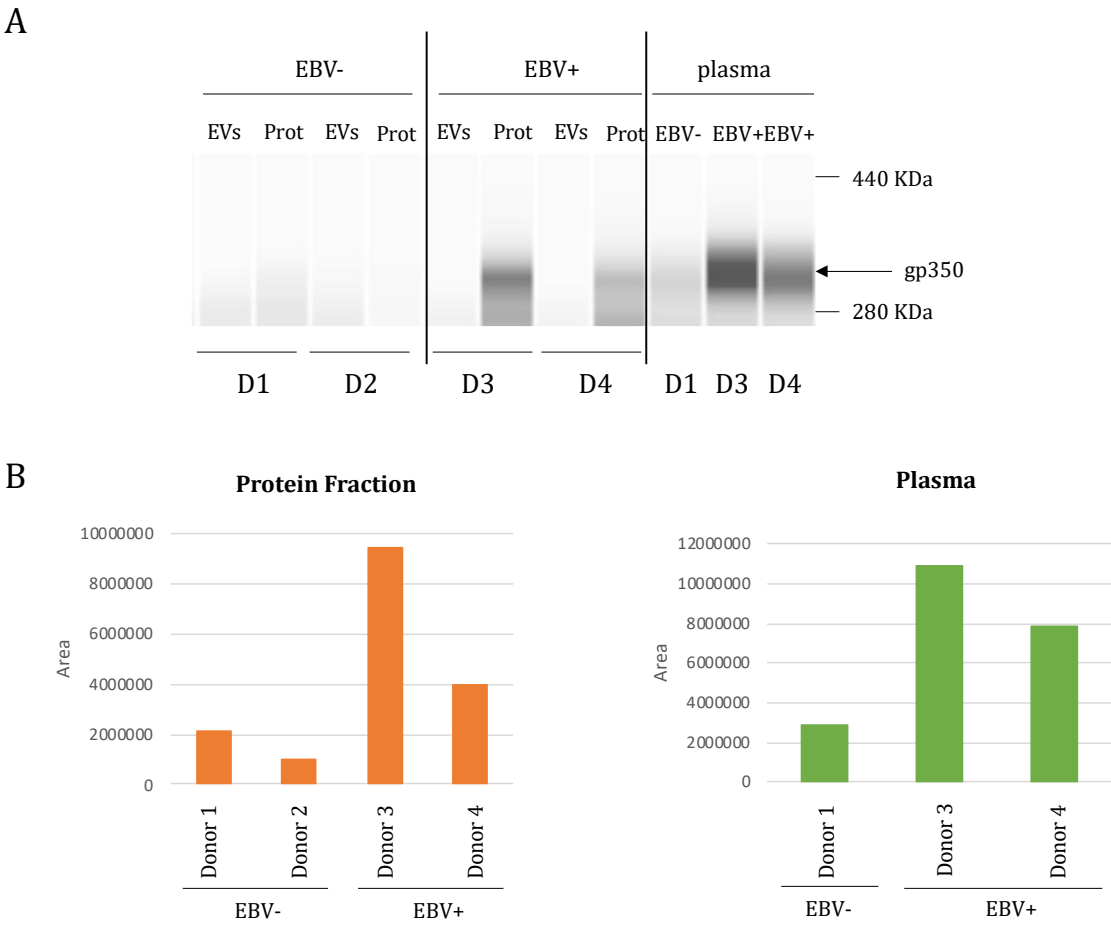


Figure 5.8. The presence of gp350 in the SEC protein fractions of EBV-seropositive donors was confirmed by simple western (WES). A. 5 µl of each sample were loaded into the WES cartridge, together with the primary antibody (5µgr/ml of the gp350-specific 72A1 antibody diluted in the antibody diluent buffer from the WES kit). The WES data were analysed using the Compass for SW software. **B.** The area of the corresponding gp350 band was compared between EBV-seropositive and -seronegative donors. The data were plotted in a graph with Excel.

5.4. Activation of the adaptative immune system by EBV-specific particles from the blood of seropositive donors

Antigen presenting cells (APCs) can internalize exogenous particulate antigens by endocytosis and present them to $CD4^+$ T cells in association with human leukocyte antigen (HLA) class II molecules. Because the presence of gp350 on EVs could not be unambiguously demonstrated by ELISA and immunoblotting, we decided to apply a sensitive T-cell assay, based on APCs loaded with EVs isolated from plasmas, and a

gp350-specific CD4+ T-cell clone. Therefore, 5 x 10^4 mini-LCLs (Kempkes et al. 1995) were used as APCs and incubated overnight with EVs (100 EVs/cell) or protein (30 pg) fractions of EBV-seropositive and -seronegative donors. One day later, 5 x 10^4 gp350-specific autologous CD4+ T cells were confronted with the mini-LCLs (ratio 1:1) o/n and one day later an ELISA was performed to quantify IFNγ in the supernatants as a marker for T-cell activation (Moosmann et al. 2002) (Figure 5.9). IFNγ was detectable within 1 out of 5 EV fractions and within 4 out of 5 protein fractions of EBV-seropositive donors. In contrast, no signals were detected in the plasma of EBV-seronegative donors.

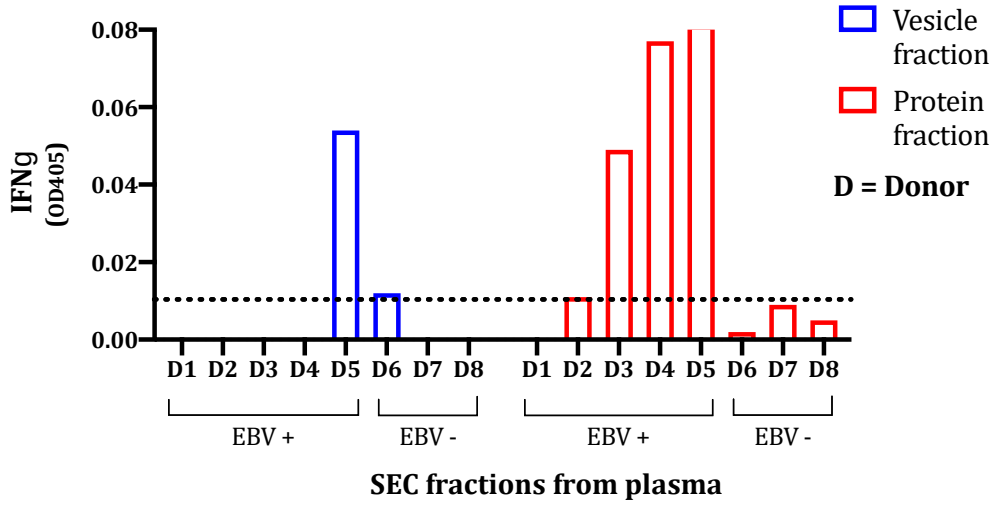


Figure 5.9. Vesicles and protein SEC fractions from plasma of EBV-seropositive donors reactivate gp350-specific CD4+ T cells. 5 x 10^4 mini LCLs, used as APCs, were incubated with either EVs (100 vesicles/cell) or protein fraction (30 pg) samples at 37 °C overnight. The next day, 5 x 10^4 autologous gp350 specific CD4+ T cells were added to the cell culture. The co-culture was incubated overnight at 37 °C. Finally, the supernatant was collected and used to perform an IFNγ ELISA.

Part II: Development of a gp350-specific human monoclonal antibody

5.5. Experimental design for the development of human monoclonal antibodies

Fully human monoclonal antibodies (hmAbs) have emerged as potent therapeutic tools in modern medicine. Since 1980, when the first hmAb was produced by fusion of human splenocytes with human myelomas, several new approaches have been developed in order to produce stable hmAb such as the use of transgenic mice, single-cell sequencing and B cell transformation by Epstein-Barr virus (EBV) (Wang 2011). Also humanized mice have already been successfully used for the generation of hmAbs, but these antibodies were of the IgM subclass because successful class switch had obviously not occurred in the animals (Becker et al. 2010) (Akkina 2013).

In a completely new approach, we used NSG-HLA-A2.1 immunodeficient mice reconstituted with a human immune system by transplantation of human $CD34^+$ hematopoietic stem cells (HSCT) isolated from cord blood (Salguero et al. 2014). In order to challenge the immune system to produce EBV specific antibodies, virus-like particles (VLPs) derived from a recombinant maxi-EBV containing the wild type genome version were used (Delecluse et al. 1999). Mice were infected with VLPs intravenously 4 times with 5×10^6 particles each at week 10, 11, 14 and 18 after HSCT. VLP-immunized and control mice were sacrificed 18 weeks after HSCT and their spleens and blood samples were collected (Figure 5.10 A). All work with the humanized mice was performed in the lab of Prof. R. Stripecke (Hannover).

Sera were prepared from the blood samples and were analyzed for the presence of EBV specific antibodies by an enzyme-linked immunosorbent assay (ELISA). The gp350 glycoprotein is known to be highly immunogenic (Ogembo et al. 2015) and was therefore selected for this evaluation to proof that an immune reaction had taken place in the immunized mice. The sera from 8 control mice and 8 treated mice were presented against a commercial gp350 protein consisting of the external and the cytoplasmic domain. The ELISA revealed that the sera from immunized animals #168 and, in particular, #261 contained the highest titers of human gp350-specific IgG antibodies (Figure 5.10 B). Therefore, splenocytes of animal #261 were used for the subsequent

experiments aiming at the production of fully human monoclonal antibodies of the IgG subtype.

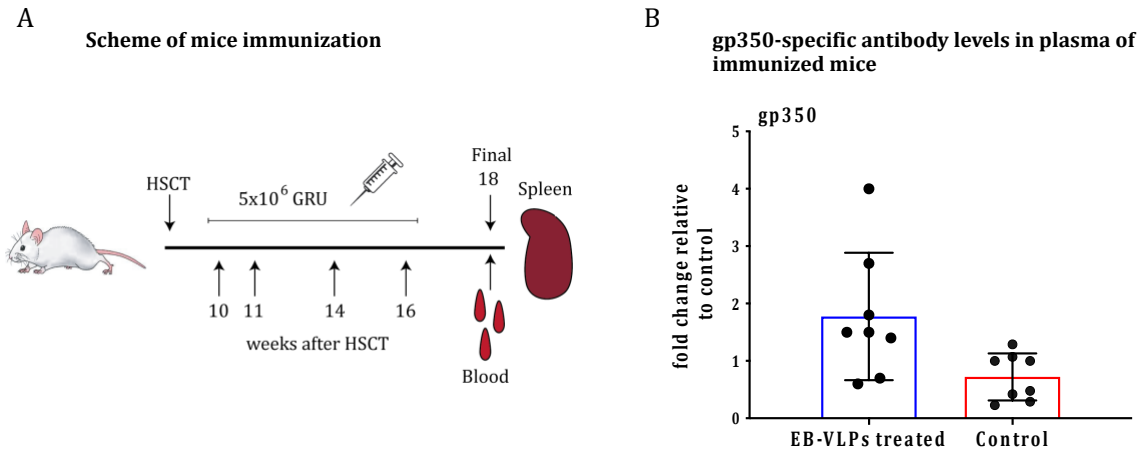


Figure 5.10. Immunodeficient humanized mice immunized with EBV VLPs produce human EBV-specific antibodies. A. NSG-HLA-A2.1 mice were irradiated and subsequently transplanted with $CD34^+$ human stem cells isolated from cord blood. 10 weeks after transplantation, the animals were immunized four times with EB-VLPs (5 x 10^6 particles) at weeks 10, 11, 14 and 16 following HSCT. 18 weeks after transplantation, sera and spleens were collected from the sacrificed animals for further analysis. **B.** The sera from immunized mice were used for an ELISA to detect gp350-specific human IgG antibodies. VLP-immunized animals are shown in blue and the control animals in red. The ELISA plates were coated with His-tagged gp350 protein and incubated with undiluted sera from the mice. An anti-human IgG-HRP antibody (1:6,000 diluted in 2% FCS in PBS) was used as a capture antibody and the chemiluminescence signal was developed with commercial TMB solution, and after 30 min the reaction was stopped with 2 N H_2SO_4. The signal was measured at 450 nm.

5.6. Generation of lymphoblastoid cell lines (LCLs) from splenocytes of VLPs immunized NSG-HLA-A2.1 mice

The strategy to develop human monoclonal antibodies (mAbs) followed throughout this thesis, was to immortalize the splenocytes with EBV, in order to secure their long-term cultivation. A special immortalization protocol was created due to the observed particular fragility of the splenocytes. For infecting the splenocytes, a wild type EBV (2089) strain at a multiplicity of infection (MOI) of 0.1 was used (Steinbrück et al. 2015). The infected cells were then co-cultivated with irradiated B blasts (5 x 10^4 cells/well)

and LL8 feeder (1 x 10^6 cells/plate) cells that expressed CD40L on the surface, and provide to the B cells stimulatory signals via the CD40 receptor (Wiesner et al. 2008) in 96-well cell cluster plates. The expansion and cytolytic activity of EBV-specific T cells was blocked by adding Cyclosporin A (CsA) to the medium for the first four weeks of culture (Figure 5.11). 8-12 weeks after infection, the outgrowth of transformed lymphoblastoid cell line (LCL) became visible in some wells. These were isolated and further cultivated.

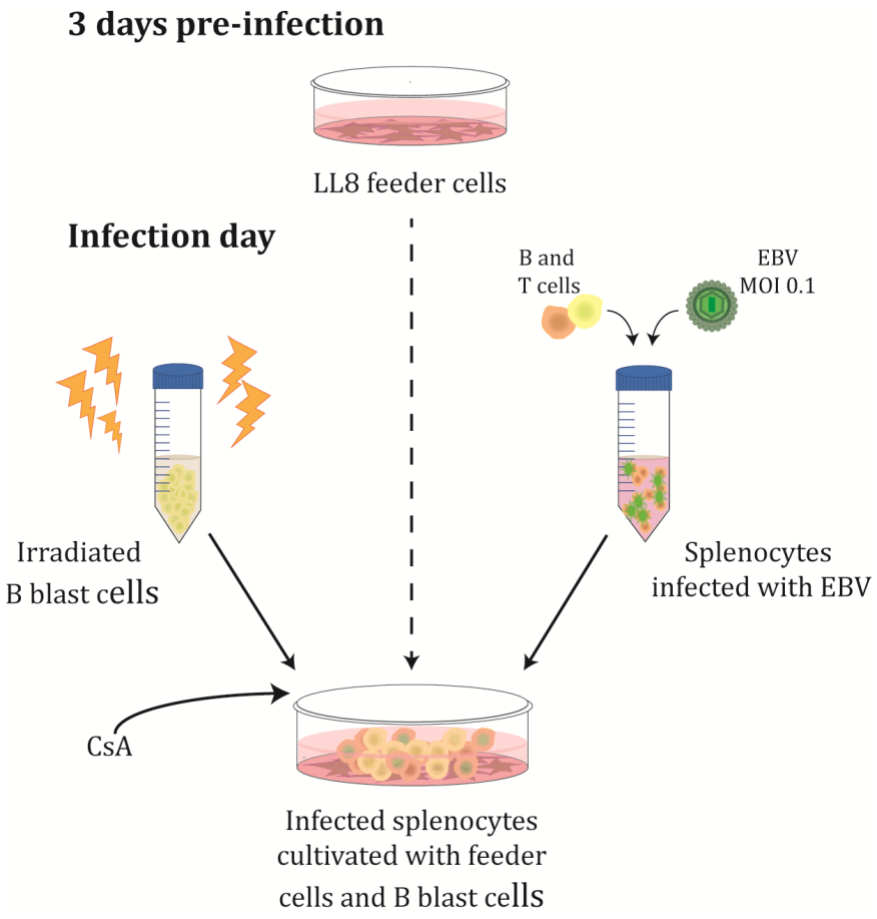


Figure 5.11. LCLs generation from splenocytes of VLPs immunized mice. Splenocytes from mouse #261, that had the highest titer of gp350-specific human IgG antibodies, were infected with EBV to create LCLs. 3 days before infection, CD40L+ LL8 feeder cells were irradiated and plated in 96-well flat bottom plates (1 x 10^6 cells per plate). Splenocytes were thawed and infected in fresh medium with EBV 2089 (MOI 0.1) for 2 h. In the meantime, B blasts were irradiated. 2.5 x 10^4 EBV-infected splenocytes were then co-cultivated with 5 x 10^4 irradiated B blasts on top of feeder cells for 8-10 weeks. Half of the supernatant was replaced by fresh medium once per week. During the first 4 weeks, 1 µg/ml of CsA was added to the culture to suppress expansion and cytolytic activity of EBV-specific T cells.

In total, growth of EBV-infected B cells was observed in 46 out of 160 wells, indicating a low efficacy of growth transformation of the splenocytes as compared to B cells isolated directly from human donors, which, under similar conditions, usually results in the growth of LCLs in each well (not shown). The LCLs were supposed to be oligoclonal, as 2.5×10^4 cells were plated per well which was found the minimal cell number for consistent outgrowth of LCLs. All the LCLs were tested for human gp350-specific IgG antibodies by ELISA, FACS and/or dot blot. The results are summarized in Table 5.1, indicating in green the positive lines that were further analysed, and in red the lines that gave conflicting results in the different tests.

Table 5.1. All LCLs were tested for the production of gp350-specific antibodies. The table summarizes the number of "cell lines" that were obtained from the immortalization of splenocytes from VLP-immunized humanized mice, and the tests that were done to test them for the production of gp350-specific human IgG antibodies. Marked green are those supernatants that were reliably positive in all assays, while those that gave inconsistent results are marked red. FP means false positive, that is, supernatants that were positive on PCI-1 cells, an EBV negative human cell line derived from a hypopharyngeal carcinoma, and on PCI-1/gp350 cells, i.e. PCI-1 cells stably transfected with a gp350 expression plasmid, indicating that they bound to a cellular protein present on both lines. The presence of antibodies directed against human proteins can be explained by the fact that the envelope of VLPs is cell-derived and thus mainly consists of human proteins.

Line name	ELISA	Dot Blot	FACS
1B4	+	+	-
1B5	-	FP	FP
1C3	-	FP	-
1D5	-	-	
1D8	+	+	FP
1D9	+++	-	++
1E2	+++	FP	FP
1E4	+	FP	-
1E7	+	+	+
1F6	-	FP	+
1F7	+	+	+
1F8	-	+	+
1F9	-	+	-
1G8	-	-	-
2B4	++	FP	-
2B9	-	FP	FP
2C7	+++	FP	-
2C8	-	-	-
2D2	+++		FP
2D9	-	-	FP
2E10	-	+	
2E3	+++	+	FP
3C8	+	FP	FP
3D3	-	-	-
3D6	-	FP	FP
3D7	-	+	
3E10	+	+	+
3F4	-	+	+
3F6	-	-	-
3F9	-	FP	FP
3G5	+++	+	+++
4B3	+++	+	FP
4B8	-	-	-
4C10	+++	-	++
4D4	-	-	-
4D5	+++	FP	FP
4E10	+	+	
4E7	+	+	++
4F3	-	+	
4F5	+	+	+
5C3	+		
5C9	++	FP	FP
5D3	-	FP	-
5D3	-	FP	
7B8	-	-	-

5.7. Analysis of LCLs for gp350-specific antibodies

The selected LCLs were analyzed by flow cytometry for different surface protein markers, in order to define their stage of differentiation. The cells were stained with the pan-B cell marker, CD19, and with CD27, a member of the tumor necrosis factor (TNF) receptor family, that plays an important role in long-term immune responses and that is expressed on memory B cells (Jacquot 2000). As expected, all the cells were positive for CD19, while the presence of CD27 on the surface of LCLs was different between lines, meaning that not all LCLs were derived from memory B cells but probably from more immature B cells (Figure 5.12 B).

The type of surface immunoglobulin (Ig) was also analyzed by flow cytometry. There are two types of light chains, kappa (κ) and lambda (λ). In humans, the ratio kappa : lambda is approximately 2:1, and LCLs analyzed predominantly expressed the kappa light chains, as expected (Murphy, K., Travers, P., Walport, M., & Janeway 2017) (Figure 5.12 A). Based on their heavy chains, immunoglobulins are classified as either IgA, IgD, IgE, IgG or IgM. The cells were analyzed for the presence of IgM, the Ig expressed in early stages of the B cell development, IgG, present in mature B cells, and accounting for long-term immune responses, and IgA, also present in mature B cells, but predominantly present in mucosal tissues and secretions, like saliva or breast milk, but also to some extent in serum (Schroeder and Cavacini 2010). FACS analysis of LCLs revealed the presence of IgM on the surface of all LCLs tested, while some cells expressed IgG. Astonishingly, a subpopulation of cells expressed on their surface both IgG and IgM simultaneously (Figure 5.13 C). None of the four LCLs analyzed stained positive for IgA (Figure 5.13 D)

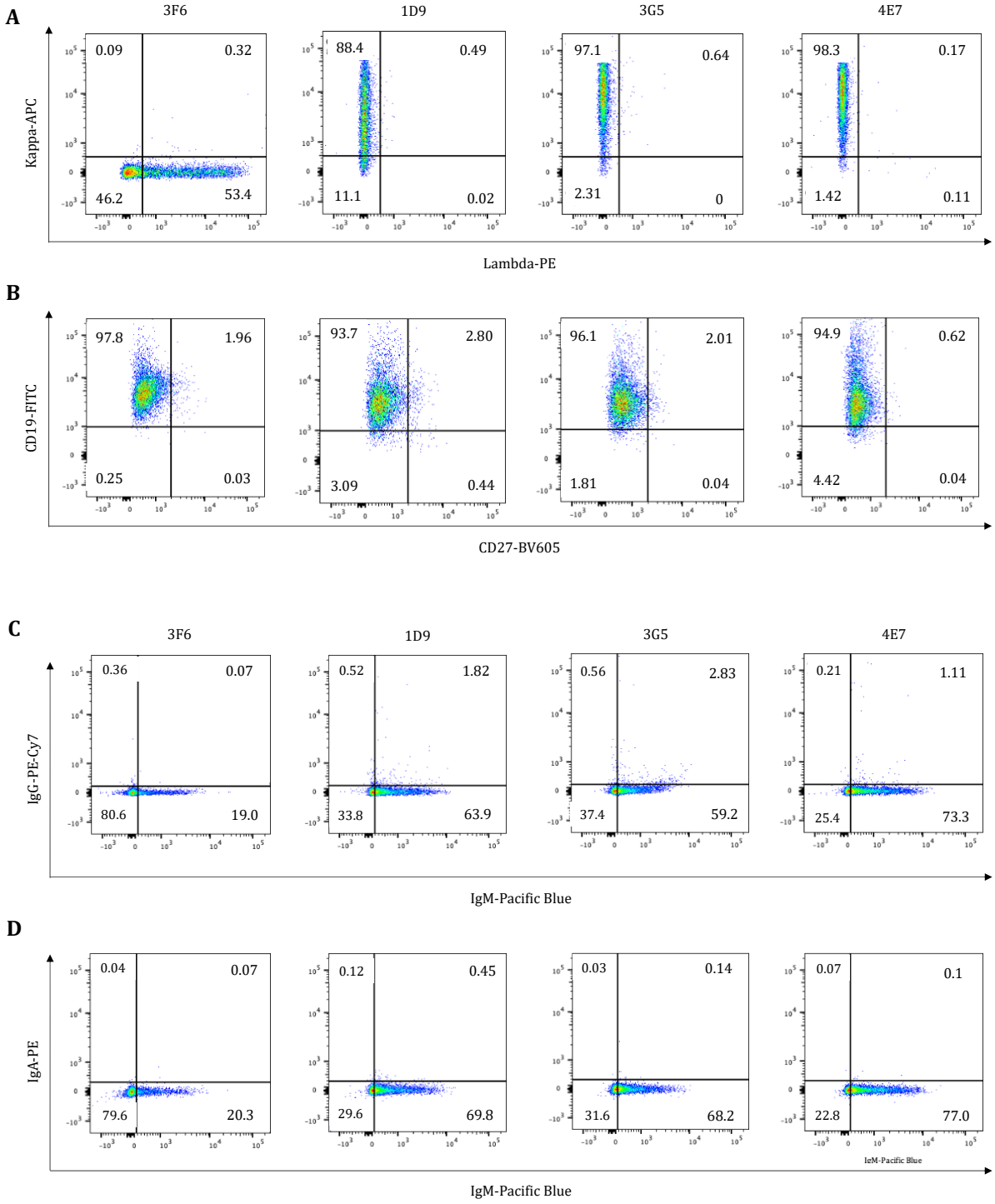


Figure 5.12. LCLs derived from splenocytes express IgG and/or IgM. LCLs were stained for CD19-FITC (1:100), a pan-B cells marker; CD27-BV605 (1:100), memory B cell marker; human IgG-PE-Cy7 (1:50); human IgM-Pacific blue (1:50); human IgA-PE (1:50); light chain kappa-APC (1:200) and lambda-PE (1:200). All antibodies were diluted in FACS buffer and compensated using BD compensation beads. The cells were incubated for 1 h with the antibodies. Prior to analysis, the cells were washed twice with FACS buffer. The majority of cells expressed surface IgM, while a subpopulation expressed IgG and another subpopulation stained positive for both IgM and IgG.

5.8. Analysis and characterization of the antibodies expressed by gp350 positive LCLs

LCLs were kept in culture at a density of approximately 1 x 10^6 cells per ml in an enriched RPMI medium, and the antibodies in the supernatants were analyzed for their specificity against gp350. The supernatants were filtrated (0.2 µm) to avoid the presence of cell debris that could distort the results.

5.8.1. LCLs 4E7, 1D9 and 3G5 produce IgG gp350 specific antibodies

For the analysis and characterization of the antibodies released from LCLs into the supernatant, PCI-1 human head and neck cancer cells stably transfected with a gp350 expression plasmid (already available in our lab) were used. To validate the expression of gp350 in these cells, dot blots were done with PCI-1/gp350 and parental PCI-1 cell lysates (Figure 5.13 B). The gp350 signal was detected with the gp350-specific antibody, 7A1. As expected, the lysate from the transfected cells stained positive for gp350, while the parental cell line was negative.

Flow cytometry analyses revealed that the LCLs 4E7, 1D9 and 3G5 released gp350-specific human IgG antibodies since they specifically bound to PCI-1/gp350 cells but not to parental PCI-1 cells. In contrast, clone 3F6 did not release gp350-specific antibodies and was therefore further on used as negative control (Figure 5.13 A). To validate these results, the chimeric 7A1 gp350-specific antibody (ch7A1, which has a human IgG1 Fc part) was used as a positive control. This experiment corroborated the results described in Table 5.1.

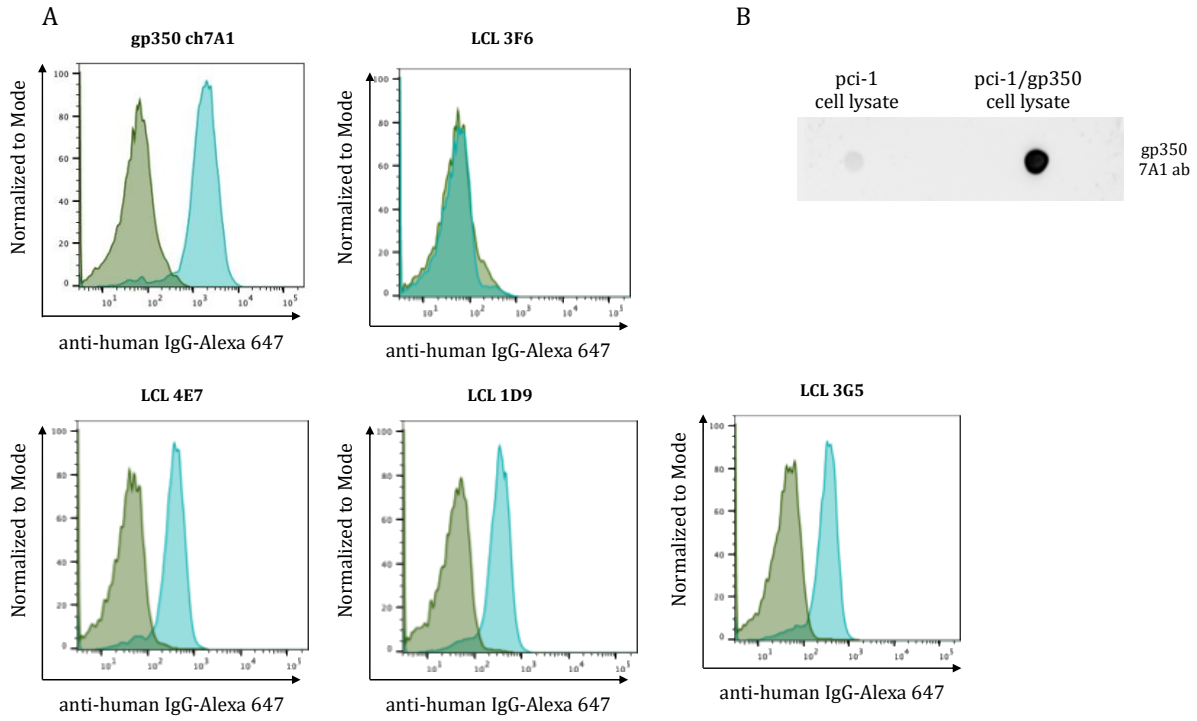


Figure 5.13. LCLs express gp350-specific human IgG antibodies. A. PCI-1 cells and PCI-1/gp350 cells were incubated with 100 µl of different undiluted LCL supernatants. In green, the staining for PCI-1 is shown, and in turquoise, the staining for PCI-1/gp350. As a positive control, both cell lines were incubated with 1 µg/ml of ch7A1 ab (chimeric gp350-specific antibody 7A1). After incubation with the first antibody, the cells were incubated with anti-human IgG-Alexa647 antibody (1:500 diluted in FACS buffer) and analyzed by flow cytometry. **B.** The cell lysate from PCI-1 and PCI-1/gp350 cells were analyzed by dot blot for the presence of gp350 protein. 1 µl of cell lysate was dropped on a nitrocellulose membrane and incubated with the gp350-specific antibody 7A1 (diluted 1:5 in 5% milk in TBST). As a second antibody, anti-rat IgG-HRP antibody (diluted 1:10,000 in TBST) was used. The chemiluminescence signal was developed with ECL.

The secretion of gp350-specific human IgG antibodies from the different LCLs was also confirmed by ELISA. A specific protocol was designed for the analysis of LCL supernatants (Figure 5.14 A). The cell supernatants were presented against a commercial gp350 protein and the signal was developed with a polyclonal anti-human IgG antibody. As expected, gp350-specific human IgG antibodies were detected in the supernatants of LCLs 3G5, 1D9, 4E7, but not of clone 3F6. Two additional LCLs, 4C10

and 4F5, gave a minimal signal against gp350 specific antibodies and were not analyzed in more detail (Figure 5.15.B).

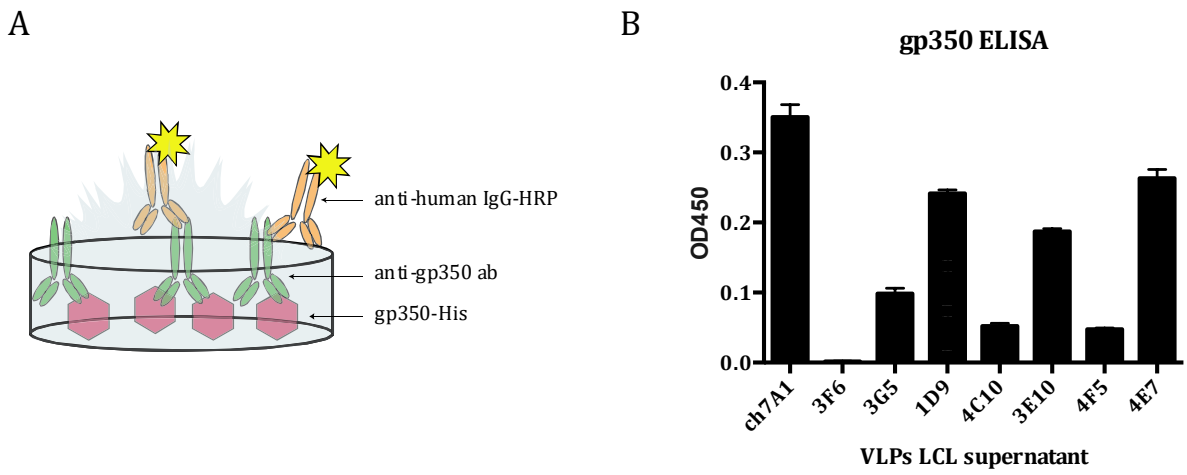


Figure 5.14. LCLs express human IgG antibodies specific to gp350. A. Scheme of gp350 ELISA. The ELISA plates were coated with gp350, incubated with supernatants of various LCLs and developed with a human-IgG-specific antibody coupled with HRP. **B.** The ELISA for gp350 was done with supernatants of various LCLs, the chimeric 7A1 antibody was used as a positive control, and clone 3F6, an LCL that stained negative in FACS, was included as a negative control. The data shown are blank corrected.

To further validate the specificity of the antibodies of interest, dot blots were performed using lysates from PCI-1-gp350 cells and parental PCI-1 cells. This assay confirmed the gp350 specificity of some LCLs (3G5, 3E10, 5E7, 4E10 and 4F5), while other LCLs (4C10 and 1D9) gave no result at all, which may be due to the low concentration of antibodies in the supernatants of these LCLs, or that they recognize non-linear epitopes in natural conformation that are not present in lysates which contained 0.1% SDS (Figure 5.15).

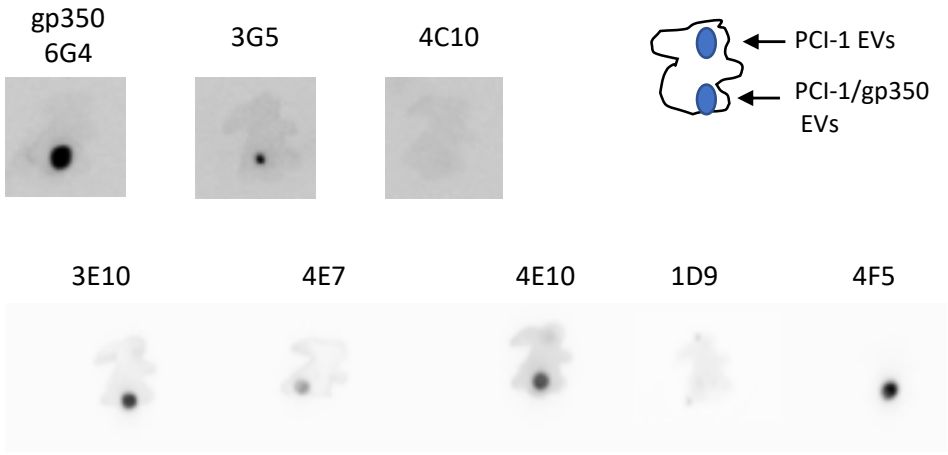


Figure 5.15. LCLs release gp350-specific antibodies that can be detected by dot blots. 1µl each of lysates from PCI-1/gp350 or PCI-1 cells were spotted onto a nitrocellulose membrane. The membranes were blocked for 1 h with 5 % milk in TBST and incubated with LCL supernatants at 4 °C overnight. A gp350-specific antibody supernatant (clone 6G4), diluted 1:5 in 5 % milk in TBST, was used as a positive control. Afterwards, the membranes were washed three times for 10 min with TBST and incubated for 2 h with the secondary antibody, an anti-human-IgG antibody coupled with HRP for the LCL samples and an anti-rat-HRP for the 7A1 antibody. After a final washing for 15 min, the signals were developed with ECL.

5.8.2. LCLs secrete very low concentrations of IgG in comparison with other B cell lines

Primary human B cells cannot be cultivated in long-term as they quickly die. Therefore, they have to be either transformed into "conditionally immortal" cells by CD40L stimulation and IL-4, giving raise to so-called B blasts (Wiesner et al. 2008), or by EBV infection, giving raise to LCLs (Diehl et al. 1968) (Pope et al. 1968) or mini-LCLs (Kempkes et al. 1995). LCLs created from splenocytes of VLP-immunized mice secreted 56 – 145 ng/ml of human IgG within three days of culture, approximately 10 times less than normal LCLs or B blasts (Table 5.2).

Table 5.2. LCLs from splenocytes of humanized mice express very low concentrations of human IgG antibodies. The IgG concentration of each LCL clone was calculated using a commercial kit from Invitrogen, following the manufacturer's protocol. 1 x 10^6 cells/ml were incubated for 3 days in 15 ml of fresh medium. The cells were kept at high densities for maximal antibody concentrations.

LCL	IgG concentration (ng/ml)
3G5	84.4
1D9	144.6
4C10	56.5
3E10	101.6
4F5	142.8
4E7	133.9

5.8.3. LCLs secrete gp350-specific antibodies with neutralizing activity

Once the gp350 specificity of the antibodies produced by the LCLs 3G5, 1D9 and 4E7 was confirmed, their capacity to interfere with the infection of human B cells by EBV was tested. Since the gp350 protein plays an important role in the attachment of EBV to the B cells, the first functional assay with these antibodies was a neutralization assay using "Raji" cells, an EBV positive cell line derived from a Burkitt lymphoma (Pulvertaft 1964).

The supernatant of the LCLs (50 µl) was incubated for 2 h with EBV 2089. Afterwards, Raji cells were infected with the mixture of virus and antibody at an MOI of 0.2 for three days. Because the 2089 EBV virus encodes for GFP, infected cells can be easily identified and quantified by e.g. flow cytometry. To control the overall efficacy of the EBV infection, Raji cells were incubated with virus only. The strongly neutralizing gp350-specific antibody (clone 6G4; 1µg/ml) was used as a positive control. The three tested supernatants (3G5, 1D9 and 3E7) had clear neutralizing activities on the infection of Raji cells. To control that this effect was specific for the antibodies contained in the LCLs supernatants, and not a side effect from the supernatants, the neutralization assay was also performed using the supernatant 3F6, which did not contain gp350-specific antibodies according to the experiments described above (Figure 5.16 A).

The percentage of infected Raji cells (Figure 5.16 B) and the percentage of inhibition of infection (Figure 5.16 C) were calculated based on flow cytometry data. As expected, the neutralizing gp350 antibody (6G4) blocked the infection of the Raji cells almost completely. The antibodies produced by 3G5, 1D9 and 4E7 LCLs blocked the infection of

Raji cells by approximately 60 - 80 %, whereas clone 3F6 and the supernatant of a normal LCL (1034) reduced the infection by up to 30%. The reduction of infection by an LCL supernatant that does not contain an gp350-specific antibody is probably to the presence of antibodies against a viral protein other than gp350, and/or by gp350-carrying EVs which have been shown to be released by LCLs (Vallhov et al. 2011).

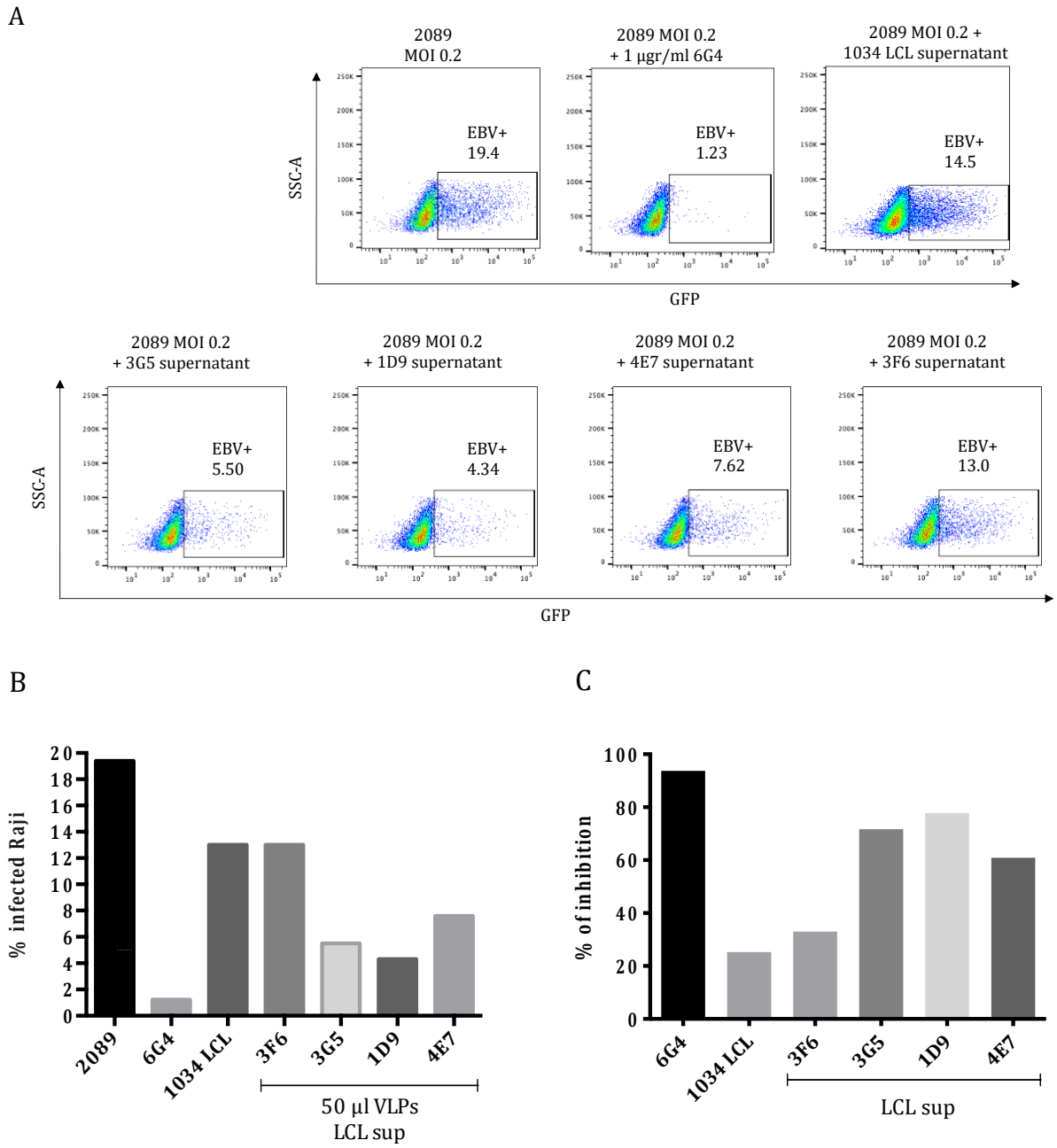


Figure 5.16. LCLs produce gp350-specific antibodies that neutralize the EBV infection in Raji cells. A. EBV 2089 was pre-incubated with the different LCLs supernatants for 2 h. Afterwards, Raji

cells were infected with the EBV- LCL supernatants mixture at a MOI of 0.2 at 37 °C for 3 days. Then, the Raji cells were analyzed for GFP signal by flow cytometry. As a positive control, the neutralizing gp350 antibody (6G4) was used at a concentration of 1 µg/ml. LCL 1034 is a normal LCL and included as a negative control. **B.** The FACS data and the neutralizing effects of the supernatants were calculated with Graphpad Prism. Representative experiment out of 3. The percentage of inhibition was calculated using the following formula:

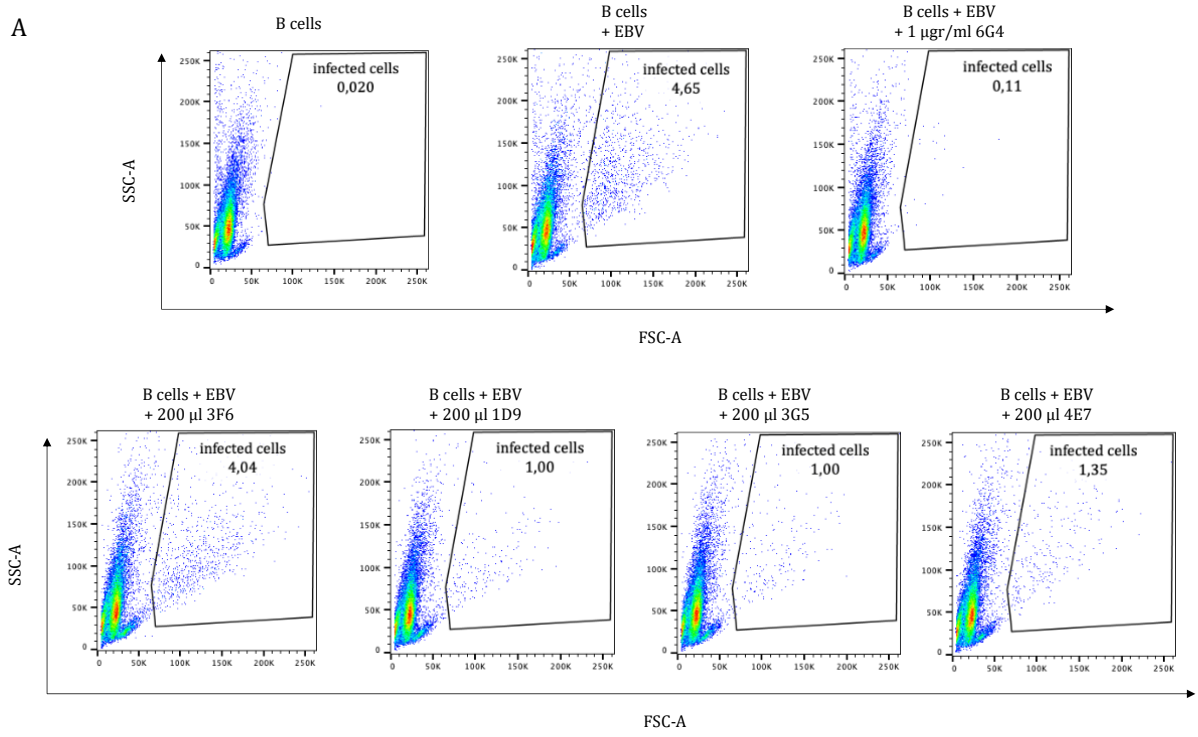
$$100 - \left(\frac{infected\ cells\ with\ ab}{infected\ cells\ with\ 2089}\ x\ 100\right)$$

The neutralizing effect of the antibodies was also tested with primary B cells. EBV infects human B cells and transforms them into LCLs. Very early during the transformation from B cell to LCL, the cells go through several metabolic and phenotypic changes, which result in an increase in size and granularity (Mrozek-Gorska et al. 2019). Therefore, B cells were obtained from adenoid tissues and infected with EBV 2089 as described before. After 3 days of incubation, the B cells were analyzed by flow cytometry. The measurement of the forward scatter (FSC) vs the sideward scatter (SSC) allowed us to differentiate infected and uninfected cells.

As described above, EBV 2089 was pre-incubated with the LCL supernatants for 2 h. Then, these supernatants were used to infect primary B cells at a MOI of 0.1. To control the infection, primary B cells were infected only with the virus, and as a positive control, the neutralizing gp350-specific antibody was used (clone 6G4; 1 µg/ml). Infected cells were analyzed by flow cytometry four days later. The three tested LCL supernatants (3G5, 1D9 and 4E7) interfered with the infection in a dose-related manner and blocked B cell transformation. To control that this effect was caused by the antibodies contained in the LCL supernatants rather than a side effect of the supernatants, primary B cells were also infected with a virus – antibody mixture using the 3F6 supernatant that didn't contain gp350-specific antibodies (Figure 5.17 A).

The percentage of infected primary B cells was calculated as described above and found to be considerably reduced when the EBV 2089 had been pre-incubated with the supernatants that contained gp350-specific antibodies (LCLs 3G5, 1D9 and 4E5) in comparison with the supernatant that did not contain gp350-specific antibodies (clone 3F6), and this effect was dose-dependent (Figure 5.17 B). Still, supernatant 3F6 had a

slightly neutralizing effect, but this effect is not dose-dependent. This “neutralizing” effect of the LCLs supernatants on the EBV infection of primary B cells is intrinsic to every LCL due to the presence of extracellular vesicles containing gp350 in the supernatants of the LCLs (Vallhov et al. 2011).



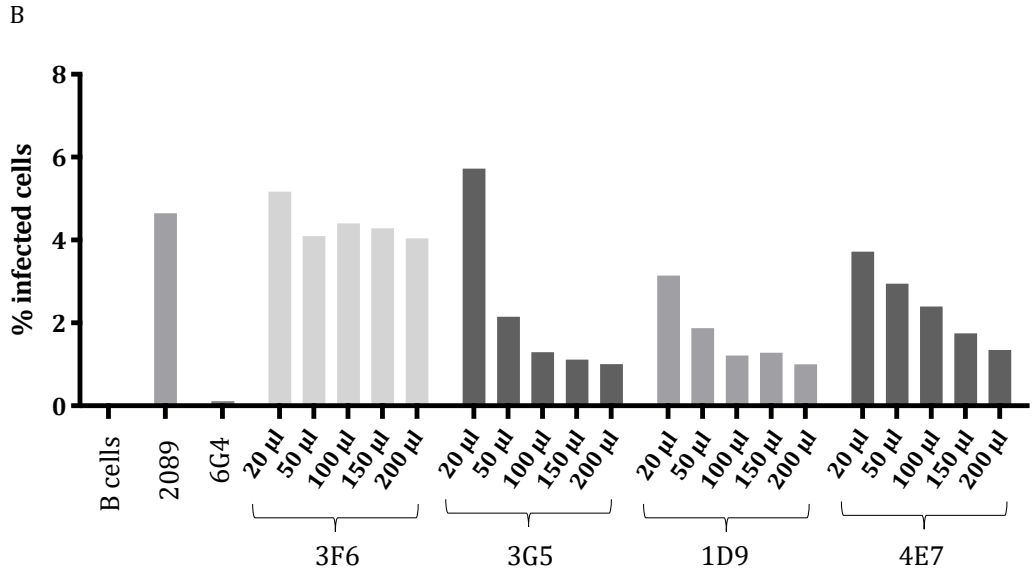


Figure 5.17. LCLs derived from splenocytes produce gp350-specific antibodies that block primary B cells infection. A. Primary B cells were incubated with EBV 2089, which had been pre-incubated for 2 h with supernatants of the LCLs indicated. Four days later, the B cells were analyzed by flow cytometry. As a positive control, the neutralizing gp350-specific antibody (clone 6G4) was used at a concentration of 1 µg/ml. **B.** The percentage of infected B cells was analyzed with the Graphpad Prims based on the FACS data. Different amounts of supernatant were used (20, 50, 100, 150 and 200 µl, showed from left to right in columns in the figure)

5.9. Sequencing strategy of the immunoglobulin heavy and light chain from LCL.

The vast diverse repertoire of antibodies that a human being possesses is generated by somatic recombination of the heavy and light immunoglobulin chains. Due to this extremely high variability, the sequencing of the human immunoglobulins is still challenging. For this, two different approaches were used to amplify the immunoglobulin heavy and light chain. Firstly, $CD19^+$ $CD27^+$ IgM^- IgG $^+$ cells were positively sorted. Due to the small number of cells obtained, the RNeasy 96 kit from Qiagen was used that is specially designed for the isolation of total RNA from 10 to 10^5 cells. The reverse transcription of the obtained RNA was done using random hexamers to ensure a complete cDNA library. The immunoglobulin heavy chain template was amplified using a nested PCR, a forward primer mix specific for the leader sequence (Kreer et al. 2020), and two different reverse primers specific for the constant region (Tiller et al. 2008) were used to performed the amplification (Figure 5.18 A). The amplified fragment was analyzed by electrophoresis through a 2 % agarose gel, and the size of the band was 510 bp for the immunoglobulin heavy chain as expected.

For the amplification of the immunoglobulin light chain a 5’ RACE PCR method was used, in which firstly a dCTP tale is added at the 3' end of cDNA template and secondly, the immunoglobulin was amplified by PCR using primers corresponding the dCTP tail and the non-variable constant region (Schanz et al. 2014) (Figure 5.18 B). The amplified fragment was analyzed by electrophoresis through a 2 % agarose gel, and the size of the band was 525 bp, as expected.

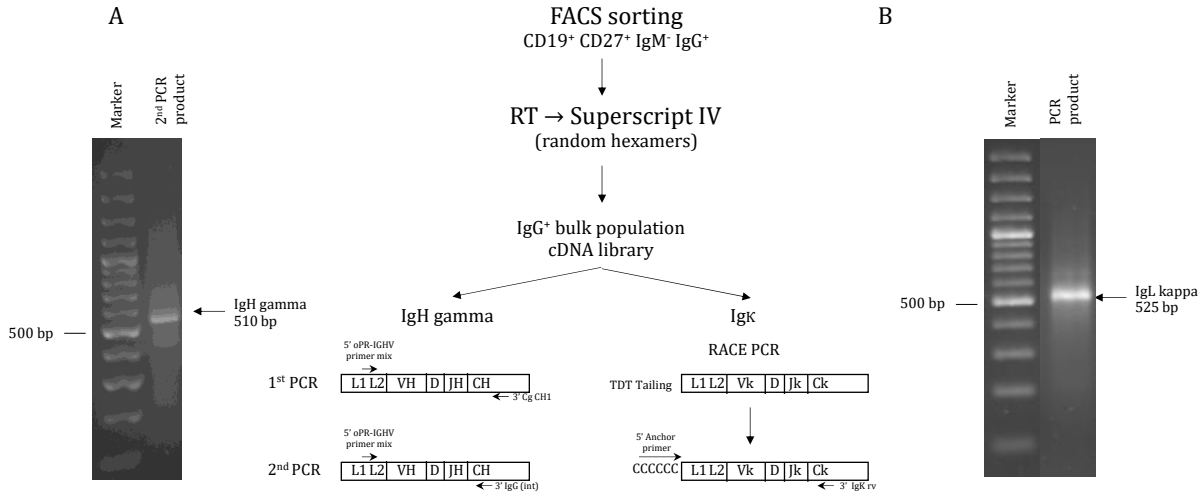


Figure 5.18. The immunoglobulin heavy and light chains of LCLs 1D9 can be amplified by nested PCR. The CD19+ CD27+ IgM- IgG+ cells were sorted, lysed and the RNA was extracted and reverse transcribed into cDNA using random hexamers. **A**. This cDNA library was used to performed a nested PCR. The first PCR was done using a 5' forward (FW) primer mix of the leader sequences for the immunoglobulin heavy chain, and a reverse (RV) primer specific for the constant region. For the second PCR the same FW primer mix was used and a RV primer specific for an internal sequence of the constant region. The PCR product was analyzed by electrophoresis using a 2 % agarose gel. The correct band size of at 510 bp was extracted from the gel, purified and sent for sequencing. **B.** The cDNA library was purified and a dCTP tale was added at the 3´ ends. Finally, a regular PCR was performed using as forward primer an anchor primer (AAP) and as reverse primer, a design primer for the constant region of the kappa light chain. **B.** The PCR product was analyzed by electrophoresis using a 2 % agarose gel. The correct band size at 525 bp was extracted from the gel, purified and sent for sequencing.

The PCR products were purified and sent for sequencing. The sequence was analyzed with the Mac Vector program and annotated through the website http://www.abysis.org. This website identified the sequence as a human IgG3 heavy chain and a kappa light chain, with complementary determining regions (CDRs) separated by the so-called framework (LFR) (Figure 5.19).

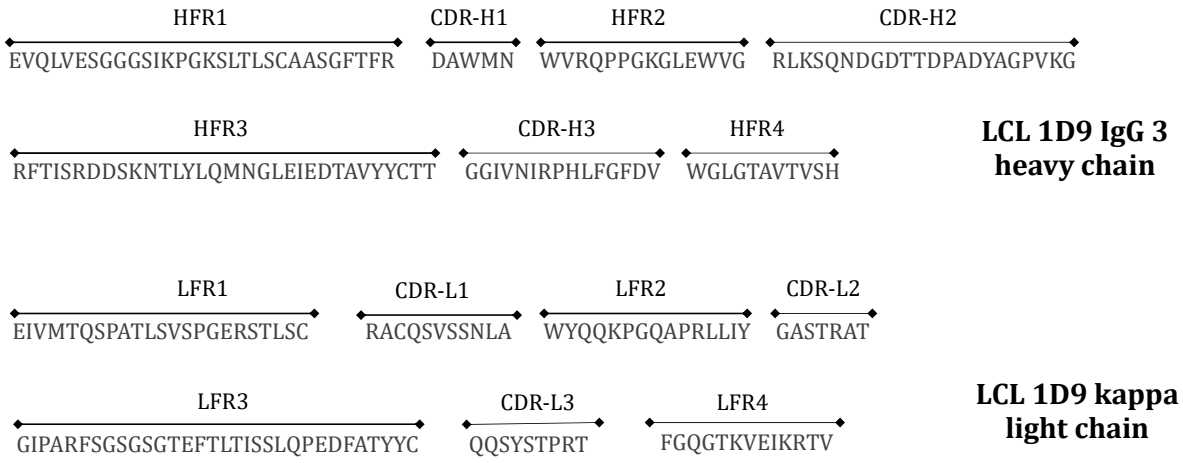


Figure 5.19. The LCL 1D9 encodes a human IgG3 heavy chain and a kappa light chain amino acid sequence. The sequenced PCR products were analyzed with the Mac Vector program, and annotated using the annotated tool from the specialized website http://www.abysis.org.

The sequencing of the immunoglobulin from the LCL 1D9 clone confirmed that this clone secreted a human IgG antibody. The immunoglobulin light chain sequence classified it as kappa, as it was shown by flow cytometry, and the heavy chain sequence classified the antibody as IgG3, typical for viral infection immune responses.

6. Discussion

EBV, like other herpesviruses, has a lytic and a latent phase and is able to persist life-long asymptomatically in resting memory B cells from healthy individuals. But how the virus successfully and stably establishes a life-long latency within a functional immune system is far from clear (Young et al. 2016). The aim of this work was to study in more detail the viral late lytic protein gp350, which interestingly is also expressed in latently infected cells, and thus independently from completion of the lytic productive cycle, as well as gp350-specific immune responses in healthy EBV-seropositive donors.

6.1. Viral lytic gene and protein expression in EBV-derived cancer cell lines

After EBV infection, the virus starts the latent phase (latency III, II or I programme) within infected cells. The latency programme that EBV expresses depends on the B cell maturation level from which the malignancy derived (Rowe et al. 1992), e.g., in LCLs and PTLD derived cells, EBV stablishes the latency III program, the growth program in which all the latent proteins and RNAs are expressed. In BL cells, EBV stablishes the latency I program, a persistence phase where only a few proteins and RNAs are expressed to avoid the immune system (Küppers 2003) (Kang and Kieff 2015).

The expression level of EBV genes in different cell lines derived from EBV-related tumours, such as BL, PTLD and NPC, have been studied mainly for latent genes and also for early lytic genes (Kelly et al. 2013) (Navari et al. 2014). However, not so many efforts have been put in the study of the late lytic proteins, which mainly comprise genes that encode structural proteins of the virion. One of the most important genes of the late lytic phase is *BLLF1*, that encodes for the gp350 glycoprotein. gp350 is the major envelope glycoprotein of EBV and is responsible for the virus attachment to the B cells by interacting with the human CD21 complement receptor (Chesnokova et al. 2015). As a late lytic gene, gp350 is supposed to be only expressed in the context of a full-blown productive cycle that is initiated by *BZLF1* expression and that ends in the production and release of progeny virus (Hammerschmidt 2015). However, gp350 can also be

detected on latently infected cells, i.e. cells in which mainly latent viral genes are expressed and that do not release detectable infectious viral particles. Vallhov et al. described recently surface gp350 on LCLs, in which EBV initiates type III latency program. Here, I could confirm this observation and extend it to Burkitt lymphoma cells in which EBV initiated a type I program characterized by the expression of a single latent gene, EBNA1 (Figure 5.1). However, it remains unknown why some LCLs and BL express gp350, while others don't. It is tempting to speculate that either the developmental state of the cell or the EBV subtype accounts for this phenomenon.

6.2. The late lytic protein gp350 is present in the blood of healthy EBV-seropositive donors

gp350 expression on EBV-infected peripheral B cells has already been described previously, but only in vitro after stimulation with CD40 ligand (Al Tabaa et al. 2009). Here, I describe for the first time that surface gp350 can also be detected on circulating B cells *ex vivo*. This is shown in Figure 5.3, where between 1 to 4 % of peripheral B cells of healthy EBV-seropositive donors expressed surface gp350. It is known that memory B cells undergo plasma cell differentiation upon either host signal activation or T cell stimuli, together with spontaneous division. Some groups have been suggesting that this mechanism to renew the memory B cells pool in the human body could be used by EBV to maintain its long-term presence in healthy carriers (Laichalk and Thorley-Lawson 2005). It has also been proposed that these plasma cells with late lytic protein expression are destroyed by EBV-specific cytotoxic T cells in order to control the balance between EBV and its host (Bernasconi et al. 2002) (Thorley-Lawson 2015). Nevertheless, the exact role of either a full or abortive lytic cycle activation is still unknown.

Serological studies in healthy donors have shown that EBV-neutralizing antibodies after IM infection persist for long time in the patient sera, maybe even life-long (Hewetson et al. 1973). Recent studies on the kinetics of EBV antibodies in students after primary infection corroborates this result. Moreover, it was shown that specific antibodies for several late lytic viral proteins like gp350 or gp42 persist more than 1,000 days after primary infection (Bu et al. 2016). In these studies, individuals were followed up to a

maximum of three years after primary infection, whereas here we can demonstrate that gp350-specific IgG antibodies are present in the blood of heathy EBV-seropositive donors even up to 20 to 30 years after primary infection (Figure 5.4). These results strongly suggest that life-long EBV humoral responses are not only against latent proteins like EBNA1 but also against gp350. This goes in line with the above-mentioned hypothesis in which EBV would use the renewal memory B cell mechanisms for a silent life-long maintenance in healthy carriers. Interestingly, although the number of donors investigated was low, those who had developed IM in adolescence or early adulthood also had the highest gp350 antibody titers. The reason for this is currently not understood (unpublished observation).

In this study I demonstrated the expression of gp350 in LCLs and other EBV-positive cell lines and showed that even *in vivo*, a small fraction of circulating B cells of healthy EBV-seropositive donors stained positive for surface gp350. Vallhov et al. ascribed gp350-positive EVs released from LCLs a biological function in that they interfered with EBV infection in vitro (Vallhov et al. 2011). Whether this is also the role of gp350-positive EVs *in vivo*, remains to been elucidated. The presence of gp350-specific antibodies in EBV-seropositive donors with no history of a known EBV reactivation prompted us to study the presence of gp350 protein *in vivo*, and its role in the balance between EBV and its host immune system.

6.2.1. Isolation and characterization of gp350 particles

In this study I isolated EVs from the plasma of EBV-seropositive donors using density gradient and SEC, the most frequent methods used for blood samples (Karimi et al. 2018). As shown in figure 5.6, the EVs could be successfully separated from other matter like protein aggregates and free proteins. The applied techniques led to two types of fractions, one with high vesicle numbers and low protein concentration and another with high protein concentrations and low vesicle numbers.

gp350 protein was detectable in sera of EBV-seropositive donors (Figure 5.5), but the source and the exact nature of this protein remains elusive. Clear signals in ELISA, immunoblotting and T-cell assays were obtained within the high protein fractions, and

only in one case also from the EV fraction after Optiprep gradient. Additional experiments like immune electron microscopy, which will be performed in the future in our lab, are necessary to get a deeper insight into the origin and nature of gp350 EVs *in vivo*. Overall, this is the first time that surface gp350 was detected on circulating B cells from EBV-positive donors *ex vivo*. Its possible role could be a contribution to the stable equilibrium between EBV and the host's immune system.

In this respect it is worth mentioning that APC loaded with the high protein fractions of four out of four EBV+ donors and, the EV fraction of one out of four donors efficiently reactivated an autologous gp350-specific CD4+ T-cell clone (Figure 5.19). This particular donor had the highest titers of gp350-specific antibodies and also amounts of gp350 protein of all donors tested, assuming that gp350-carrying EVs are probably present in sera of EBV-positive donors at variable numbers, but at too low numbers to be detected in the assays performed.

All together, these results demonstrate for the first time that gp350 protein is present in sera of healthy EBV-infected individuals without previous known viral reactivation, and that the protein can reactivate specific T-cells. However, the nature, and the role of gp350 remain to be elucidated. Stable life-long latency within a functional immune system is characteristic for all herpesviruses but still far from understood. It is therefore tempting to speculate that gp350 (as soluble or particulate antigen) contributes to the maintenance of latency by constantly triggering the immune system at a low level, thereby backing the dynamic immunological equilibrium between the virus and the host.

6.3. Development of fully human monoclonal antibodies

Monoclonal antibodies (mAbs) have become very useful tools for the treatment of several types of malignancies, viral or bacterial infections, and autoimmune diseases (Marasco and Sui 2007). Until December 2019, 79 therapeutic monoclonal antibodies have been marketed (Lu et al. 2020). One major reason for their clinical success is the possibility to de-immunize murine monoclonal antibodies through chimerization or humanization, or to even generate fully human antibodies (hmAbs), the latter being the preferred form as they probably produce the least adverse immune reactions in patients.

There are several protocols to engineer hmAbs, like phage display, transgenic mice, immortalized B cells and sequencing of the immunoglobulin chains from single B cells, all with advantages and shortcomings (Traggiai et al. 2004) (Tiller et al. 2008) (Akkina 2013).

In the present study, we used a new mouse model with reconstituted human immune system, capable of producing human antibodies of the IgG subclass. These humanized mice had a fully reconstituted human immune system with B and T cell engraftment and differentiation (Volk et al. 2017) (Theobald et al. 2018). EBV virus-like particles (VLPs) have been successfully used in the past to trigger specific immune responses, including the production of neutralizing antibodies, in immunocompetent mice, (Ruiss et al. 2011), were used here as an immunogen to generate gp350 hmAbs. The potential of this new model has been explored in a collaboration with the team of Prof. R. Stripecke in Hannover, for two model antigens, namely gB of HCMV and gp350 of EBV. While the gB-immunized mice were mainly analyzed in Hannover, I was working on the EBV-VLP-immunized mice because all tools and techniques necessary for such analysis were established in our lab.

Previous attempts to generate fully human monoclonal antibodies in humanized mice showed that these animals were unable to generate human IgG antibodies due to incomplete T- and B cell maturation, and a lack of germinal centers (Becker et al. 2010). An initial serology analysis of the sera of the mice used in this study revealed that they in fact produced IgG gp350-specific hmAbs, indicating successful Ig class switching (Figure 5.10). Nevertheless, the human IgG concentration in these mice were extremely low. These results strongly indicated the principle usability of the model on the one hand, but also a presumed poor efficacy on the other hand. Consequently, we expected only a very small number of B cells producing gp350-specific IgG human antibodies.

In vitro long-term cultivation, and thus analysis, of primary human B cells is not possible unless they are either stimulated with CD40L and IL-4 (Wiesner et al. 2008) or transformed into proliferating lymphoblastoid cell lines (LCLs) by EBV infection. Having shown that sera from VLP-immunized mice contained gp350-specific human IgG antibodies, we decided to immortalize and analyze the splenocytes from the animal with the highest antibody titer, using a protocol specially designed for this study (Figure

5.11) as we observed an extreme sensitivity of the human B cells recovered from the Hu mice. The transformation of the cells with EBV gave rise to several potentially oligoclonal LCLs that were tested for IgG gp350-specific antibodies using different bioassays like serology, dot blot and flow cytometry. The presence of gp350-specific IgG antibodies was probed in several LCLs, and these LCLs were subsequently analyzed in more detail.

6.3.1. Evaluation of the human B cell development in VLP immunized Hu mice after EBV immortalization

The engraftment and maturation of B and T cells in Hu mice has always been a limitation of the model system. B cells from Hu mice after hematopoietic stem cells transplantation (HSCT) normally do not differentiate further than to a transitional B cells stage. Whereas the maturation state of the human T cells in the Hu mouse model used here has been previously studied , demonstrating that immunized mice had mature T cell and secondary lymphoid organs development (Volk et al. 2017), the status of B cells has been less investigated. Flow cytometry analysis of immortalized splenocytes showed that all stained positive for the B cell lineage marker CD19, of which many also stained positive for CD27, which is a marker for memory B cells and plasma cells, demonstrating that complete B cell development has occurred. Similar results have been obtained from comparable mouse models recently (Yu et al. 2017).

The analysis of surface immunoglobulins in the LCLs generated in this project revealed the presence of IgM^+ IgG^- and IgM^+ IgA^- as the majority of cells, and that therefore B cell maturation has stopped at a transitional state. However, a small number of $CD27^+$ IgM^- IgG^+ cells were also present, and these results correlated with serological data, demonstrating the presence of hmAbs in VLP-immunized mice. Intriguingly, a fraction of cells stained positive for both IgM and IgG, and this phenomenon has also been observed in the gB-immunized mice which have been analyzed in the Stripecke lab (personal communication) (Figure 5.12). A possible explanation for the presence of these IgM^+ IgG^+ double positive LCLs is that these cells present both immunoglobulins on their surface due to incomplete allelic exclusion, which will result in B cells expressing both IgH alleles. This phenomenon has previously been described in a patient suffering from

Chronic Lymphocytic Leukemia (CLL) (Katayama and Sakai 2000). It is known that IL-7 is relevant for the allelic exclusion in large pre-B cells, and despite the B and T cell engraftment and proliferation was optimal in our hu mice model, it is still unknown if the lack of certain human cytokines could affected the maturation process at some level.

6.3.2. The LCLs 3G5, 1D9 and 4E7 produce neutralizing human IgG gp350 specific antibodies

The aim of this study was the generation of a gp350-specific fully human IgG monoclonal antibody using a new humanized mouse model. From immortalized human splenocytes from VLP-immunized animals, I could generate several LCLs that secreted detectable amounts of such human IgG antibodies into the supernatants. This could be probed by serology, flow cytometry and immunoblotting (Figure 5.13, 5.14 and 5.15). However, the concentrations of human IgG molecules secreted by these LCLs was very low in comparison with "normal" transformed human B cells isolated directly from human donors, and also much lower than those secreted by CD40/IL-4 stimulated B blasts (unpublish data from Dr. A. Moosmann) (Table 5.2). This phenomenon could be due to insufficient maturation in the hu mice due to a lack of relevant human cytokines mandatory for B cell allelic exclusion and immunoglobulin class-switch. Nevertheless, this is just an hypothesis to explain the particular fragility of the immortalized cells and the low amounts of antibodies secreted.

However, the supernatants of those LCLs that secreted gp350-specific IgG antibodies were subsequently used in neutralization assays. Although the concentration of antibodies in the supernatants was low, several supernatants clearly showed a neutralizing activity, and this neutralizing effect correlated with the volume of supernatants used in the assay, as shown in figure 5.16 and 5.17.

The sequencing of the immunoglobulin heavy chain from the LCL that secreted the antibody with the highest neutralizing capacity classified it as a human IgG3 molecule, the subclass that is particularly related with humoral immune responses against bacterial and viral infections. It is also known that IgG3 has a short half-live in comparison with other IgG immunoglobulins, as they are normally the "first responders" to infections. Also, IgG3 antibodies have more potent effector functions than other IgG molecules (Damelang et al. 2019). The analysis of the IgH sequence also revealed that

the LCL was of a monoclonal origin, indicating that the growth transformation efficacy of Hu mice-derived human B cells was particularly low, probably also due to the reasons mentioned above.

Taken together, fully human IgM antibodies have already been successfully isolated from EBV-immortalized B cells isolated from humanized mice (Becker et al. 2010). In collaboration with the Stripecke lab we are the first to successfully describe this technique for human IgG antibodies. Future analysis will show the quality and biopotency of the identified antibodies.

6.4. Concluding remarks and outlook

The present study represents the first description and characterization of a gp350-specific immune reaction in EBV-seropositive patients that went through infection more than twenty years ago. I demonstrated the presence of gp350-specific antibodies in sera of all EBV-positive donors tested, and also the presence of gp350 protein in sera of several of these donors. Interestingly, high antibody titers correlate nicely with the presence of gp350 protein in sera. Moreover, all the donors that had detectable amounts of gp350 protein in sera reported infectious mononucleosis (IM) during adolescence or as adults. To verify this possible link between IM and high gp350-specific antibody titers and the presence of gp350 protein in sera, a bigger cohort should be analyzed. Unfortunately, we could not specify the source of the gp350 protein in sera, but it is possible that the protein is carried by small vesicles rather than as free protein or as protein aggregates. To confirm this assumption electron microscopy analysis could be performed to see if the gp350 protein is detectable in EVs. The role of the gp350 protein in EBV-positive donors is still unclear, but since it can reactivate specific $CD4^+$ T cells, it is tempting to speculate that it must play a role on EBV long-term regulation of viral latency and co-existence in the host.

EBV still presents a challenge for human health. So far, there is no vaccine available and also no fully human monoclonal antibody for the treatment of EBV related malignancies and diseases. In this thesis, I developed a fully human IgG gp350-specific antibody that specifically binds to gp350 and that neutralizes EBV infection of Raji cells and PBMCs *in vitro*. Also, I identified the Ig sequences of this fully human antibody. The next steps would involve expressing this antibody in a suitable cell line like CHO and characterizing it in more detail, e.g. by comparing its biopotency to other non-human gp350-specific antibodies. This is now ongoing work in our lab. These results will show whether the antibody can be developed into a therapeutic candidate.

7. Literature

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8. Abbreviations

%	percent
°C	degree Celsius
A	adenine
ab	antibody
ADCC	ab-dependent cellular cytotoxicity
AIDS	Acquired Immune Deficiency Syndrome
APC	antigen-presenting cell
BL	Burkitt Lymphoma
bp	base pair
C	cytosine
CD	cluster of differentiation
cDC	conventional dendritic cell
cDNA	complementary DNA
CpG	cytosine-phosphatidyl-guanosine
CR	complement receptor
DC	dendritic cell
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
EA	early antigen
EBNA	EBV-encoded nuclear antigen
EBV	Epstein-Barr virus
ECL	enhance chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
et al.	et alia
etc.	et cetera
EtOH	ethanol
EV	extracellular vesicle
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
G	guanine

gp	glycoprotein
GRU	Green Raji units
h	hour
H_2O	dihydrogen monoxide; water
HBV	hepatitis B virus
HEV	hepatitis E virus
HHV4	Human Herpesvirus 4
HL	Hodgkin Lymphoma
HLA	human leukocyte antigen
HPV	human papilloma virus
HRP	horseradish peroxidase
HSC	human stem cells
i.v.	intravenous
IgG	immunoglobulin G
IM	infectious mononucleosis
IFNγ	Interferon gamma
kb	kilo base
kDa	kilo Dalton
LCL	lymphoblastoid cell line
LMP	latent membrane protein
M	molar
MDV	Marek´s disease virus
MHC	major histocompatibility complex
min	minute
ml	millilitre
mM	millimolar
MOI	multiplicity of infection
NFkB	nuclear factor "kappa-light-chain-enhancer" of activated B cells
NK	natural killer
nm	nanometre
NPC	nasopharyngeal carcinoma
NTA	nanoparticle tracking analysis
OD	optical density

PBMCS	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PEI	polyethyleneimine
PPR	pattern-recognition receptor
PTLD	Post-transplant lymphoproliferative diseases
RNA	ribonucleic acid
rpm	rounds per minute
RT	room temperature
RT	reverse transcription
sec	second
SEC	size-exclusion chromatography
T	thymine
TCR	T-cell receptor
TLR	toll-like receptor
V	Volt
VCA	virus capsid antigen
VLP	virus-like particle
VZV	Varicella-zoster virus
WES	simple western blot
wt.	wild type
µgr	microgram
µl	Microliter

9. Presentations

Parts of this work were presented in international meetings:

June 5th – 9th, 2016	**Summer School on Infection Research**
Buchenau, Germany	Poster: "Novel neutralizing antibodies with clinical potential against viral infections in the immunocompromised host"
November 24th – 26th, 2016	**Annual Meeting of the German Center for Infection Research**
Köln, Germany	Poster: "Novel neutralizing antibodies with clinical potential against viral infections in the immunocompromised host"

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