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The CD4+ T cell response against structural antigens of EBV: the role of the lytic cycle antigens BNRF1 and gp350

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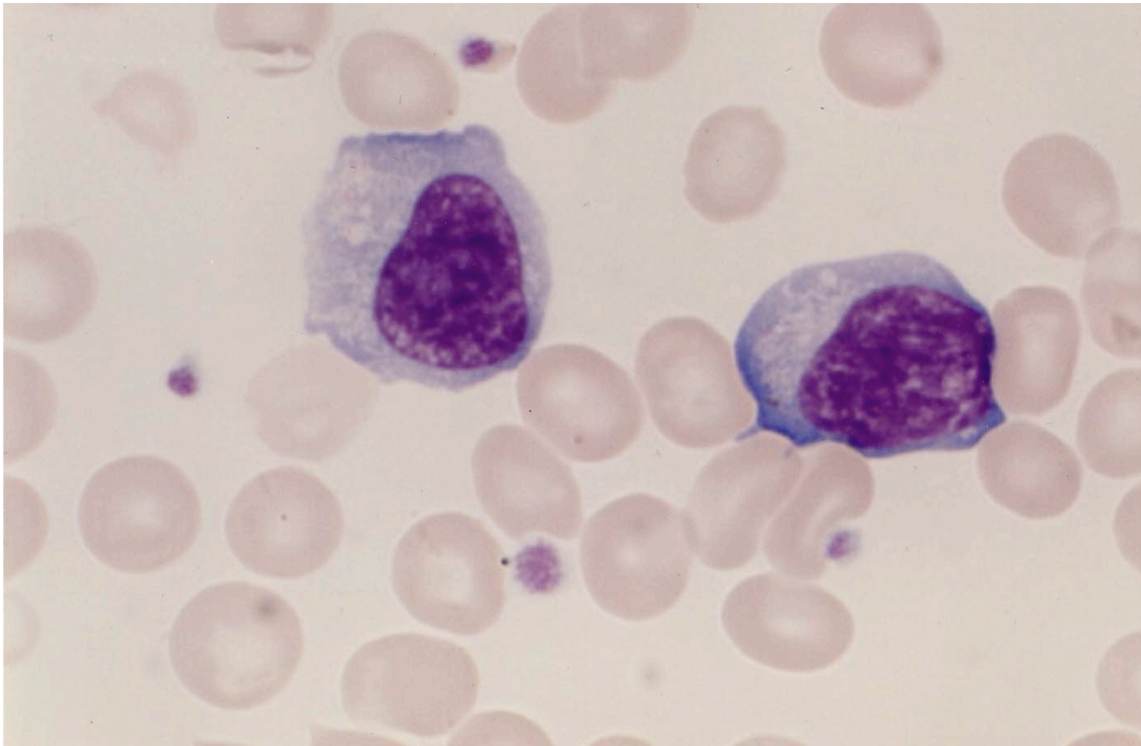

Pfeiffer cells are reactive T lymphocytes.
Blood smear, courtesy of Prof. Dr. med. Uta Behrends

ABSTRACT

The Epstein-Barr virus (EBV) is an ubiquitous human γ-herpesvirus that establishes lifelong persistent infections in the human host by latently infecting B cells. Primary infection usually occurs during early childhood by parent-to-child oral transmission in a mostly asymptomatic fashion. Delayed primary infection during adolescence or adulthood may cause the syndrome of infectious mononucleosis (IM). Although many infected individuals experience no serious virus-associated pathology, EBV is linked to a range of tumors of epithelial and lymphoid origin, autoimmune diseases, and other virus-associated disorders.

EBV infection is controlled by T cells as demonstrated by the incidence of EBV-associated post-transplant lymphoproliferative disease (PTLD) in immunosuppressed solid organ and hematopoietic stem cell transplant recipients, and by the successful treatment of such lesions by the infusion of EBV-specific T cell lines generated by repeated stimulation of T cells with EBV-infected B cells *in vitro*. Due to the often rapid progression of these tumors and the lengthy preparation of such T cell lines, adoptive T cell therapy still has a limited role in the management of EBV-associated diseases. A better understanding of the virus-specific T cell response may allow to expedite the preparation of EBV-specific T cell lines and, ultimately, to implement this treatment modality as a conventional therapeutic option for EBV-associated malignancies.

The aim of this work was to characterize the poorly-defined CD4+ T cell response against structural antigens of EBV in more detail using two different experimental approaches: (i) analyzing the frequency of virion-specific CD4+ T cells in patients with IM and healthy virus carriers *ex vivo*, and (ii) assessing CD4+ T cell responses against gp350 and BNRF1, two virion proteins of EBV, *in vitro*.

To quantify the virion-specific T cell response, CD4+ T cells were isolated from peripheral blood of EBV-positive donors and probed with autologous PBMC that had been pulsed with virus concentrate in interferon-γ ELISPOT assays. In healthy virus carriers, the frequency of EB virion-specific CD4+ T cells was found to be in the range of 0.1-0.01 ‰, and thus approximately 100-fold lower than the correspond-

ing CD8+ T cell response. Surprisingly, virion-specific CD4+ T cell responses were barely detectable during acute infection and reached levels of healthy virus carriers during convalescence, suggesting that these T cells develop late after infection, or that their delayed development results in symptomatic infection.

In the second part of the work, CD4+ T cells from ten healthy virus carriers were repeatedly stimulated *in vitro* with autologous PBMC pulsed with recombinant gp350 and BNRF1 proteins. While BNRF1-stimulated T cell lines failed to show specificity against the stimulator protein even after repeated rounds of stimulation, T cell lines specific for gp350 were obtained in half of the cases. Using a bacterial expression cloning approach, the antigenic peptides recognized by these T cells were identified and the restriction elements defined by probing the T cells with antigen loaded, allogeneic lymphoblastic cell lines (LCL).

These findings demonstrate that CD4+ T cell responses against gp350 are frequent in healthy virus carriers and target different epitopes presented on different human leukocyte antigen (HLA) molecules. Thus, EB virion-specific CD4+ T cell responses can be characterized with this approach despite their low magnitude which may ultimately lead to the development of faster and more efficient immunization strategies.

ZUSAMMENFASSUNG

Das Epstein-Barr-Virus (EBV) ist ein weltweit verbreitetes humanes γ-Herpesvirus, das in seinem Wirt eine lebenslang persistierende Infektion in Form einer latenten Infektion der B-Zellen hervorruft. Die Primärinfektion erfolgt meist in der frühen Kindheit über orale Transmission von den Eltern auf das Kind und verläuft gewöhnlich asymptomatisch. Eine spätere Primärinfektion in der Adoleszenz oder im Erwachsenenalter kann das Syndrom der Infektiösen Mononukleose hervorrufen (IM). Obwohl die meisten infizierten Menschen keine schwerwiegende Virus-assoziierte Pathologie entwickeln, ist EBV beteiligt an der Entstehung einiger Tumoren epithelialen und lymphoiden Ursprungs, von autoimmunen und anderen Virus-assoziierten Erkrankungen.

Die Infektion mit EBV wird durch T-Zellen kontrolliert. Ist diese durch die Immunsuppression im Rahmen einer Organ- oder Knochenmarktransplantation gestört, können EBV-assoziierte lymphoproliferative Erkrankungen (Post-transplant lymphoproliferative diseases, PTLD) auftreten. Diese können erfolgreich behandelt werden durch die Verabreichung von EBV-spezifischen T-Zellen, die *in vitro* durch repetitive Stimulation von T-Zellen mit EBV-infizierten B-Zellen hergestellt werden. Da diese Tumoren jedoch schnell voranschreiten und die Herstellung spezifischer T-Zellen zeitaufwändig ist, nimmt die adoptive T-Zell-Therapie in der Behandlung von EBV-assoziierten Erkrankungen bisher eine nachrangige Stellung ein. Ein besseres Verständnis der virus-spezifischen T-Zell-Antwort könnte diesen Herstellungsprozess beschleunigen und die adoptive T-Zell-Therapie zu einer konventionellen Behandlungsoption bei EBV-induzierten Neoplasien werden lassen.

Das Ziel der vorliegenden Arbeit war es, die bisher kaum definierte CD4(+)-T-Zell-Antwort auf Strukturantigene von EBV näher zu chrakterisieren. Dabei wurden zwei experimentelle Ansätze angewandt: (i) die Häufigkeitsanalyse von CD4(+)-T-Zellen in Patienten mit IM und gesunden EBV-Trägern *ex vivo*, (ii) das Hervorrufen einer CD4(+)-T-Tell-Antwort *in vitro* auf die beiden EB-viralen Proteine gp350 und BNRF1.

Um die virus-spezifische T-Zell-Antwort zu quantifizieren, wurden CD4(+)-T-Zellen aus dem peripheren Blut EBV-positiver Donoren isoliert und in Interferon-γ ELISPOT-Assays mit autologen PBMC getestet, die mit Viruskonzentrat inkubiert worden waren. In gesunden Virusträgern wurde eine Frequenz von EBV-spezifischen CD4(+)-T-Zellen von 0,1 bis 0,01 ‰ ermittelt, die damit etwa hundertfach unter der korrespondierenden CD8(+)-T-Zell-Antwort liegt. Überraschenderweise waren virus-spezifische CD4(+)-T-Zellen in der akuten Infektion kaum nachweisbar, erreichten aber das Niveau der gesunden Virusträger in der Rekonvaleszenz. Das legt nahe, dass diese T-Zellen spät nach Infektion entstehen, oder dass ihre verzögerte Produktion eine symptomatische Infektion zur Folge hat.

Im zweiten Teil der Arbeit wurden CD4(+)-T-Zellen von zehn gesunden Virusträgern wiederholten *In-vitro*-Stimulationen mit PBMC unterzogen, die mit rekombinantem gp350- oder BNRF1-Protein beladen worden waren. Während die T-Zell-Linien, die mit BNRF1 stimuliert wurden, auch nach wiederholten Stimulationen keine Spezifizität gegen das Stimulans entwickelten, zeigte die Hälfte der Linien eine Spezifizität gegen gp350. Mittels der Expression und Klonierung der DNA in Bakterien, wurden die Peptide, die als Antigene fungieren und von den T-Zellen erkannt werden, ermittelt, das Restriktionselement wurde durch die Testung der T-Zellen mit allogenen, Antigen-beladenen lymphoblastären Zelllinien (lymphoblastic cell lines, LCL) eruiert.

Diese Ergebnisse zeigen, dass eine CD4(+)-Antwort auf gp350 häufig in gesunden Virusträgern vorkommt, und dass verschiedene Epitope, präsentiert auf verschiedenen humanen leukozytären Antigenen (human leukocyte antigen, HLA), erkannt werden. Demnach kann die EBV-spezifische CD4(+)-zelluläre Immunantwort auch bei geringer Zellzahl charakterisiert werden. Das könnte zur Entwicklung von schnelleren und effizienteren Immunisierungsstrategien führen.

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When starting in the lab at the Helmholtz-Zentrum in Munich, I was introduced to experimental procedures, methodologies and theoretical background by Dr. med. Dinesh Adhikary, who patiently and always with a smile on his face helped me to get into the topics of EBV-virology, -immunology and –tumor biology and into daily lab routine. He was the best advisor I could have imagined for my first steps in the lab and he was a friend as well as a role model to me.

In the second phase of my practical and theoretical work - Dinesh had already gone to Cambridge and then later back home to Kathmandu – PD Dr. rer. nat. Josef Mautner, the deputy leader of the lab group, became the spiritus rector of my project. Indefatigably, he discussed all my results with me, provided constructive criticism, helped me considering the wider field of immunology and the recent results in the research on Epstein-Barr virus and tumor immunology. Furthermore, I was and still am very grateful for his sense of humour that motivated me a lot to accomplish my study.

In particular, I would like to thank my mother for all her support during all our years together, for explaining me the world and the life, educating me to be curious towards everything and to keep the horizon always as broad as possible, for listening to me, helping me resolving my problems, passing the times of trouble with me, encouraging me in all situations to find and follow my way.

Last not least, I received the motivation to finally conclude my work from Pablo Cuéllar Moro and I thank him so much for his empathy, patience and love.

Anna Maria Rupp Berlin, December 2011.

To my mother

What the Tortoise Said to Achilles

Lewis Carroll

Achilles had overtaken the Tortoise, and had seated himself comfortably on its back.

"So you've got to the end of our race-course?" said the Tortoise. "Even though it does consist of an infinite series of distances? I thought some wiseacre or other had proved that the thing couldn't be done?"

"It can be done," said Achilles. "It has been done! Solvitur ambulando. You see the distances were constantly diminishing; and so --"

"But if they had been constantly increasing?" the Tortoise interrupted "How then?"

"Then I shouldn't be here," Achilles modestly replied; "and you would have got several times round the world, by this time!"

"What the Tortoise Said to Achilles," Mind 4, No. 14 (April 1895): p. 278-280

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Abbreviations

Symbol	*Description*
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
B95.8	EBV-producer marmoset B cell line
BL	Burkitt's lymphoma
CAT	Chloramphenicol acetyltransferase
CD	Cluster of differentiation
CMV	Cytomegalovirus
CNS	Central nervous system
CO2	Carbondioxide
CSA	Cyclosporine A
CTL	Cytotoxic T lymphocytes
DLI	Donor lymphocyte infusion
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EA	Early antigen
EBV	Epstein-Barr virus
EBER	Epstein-Barr virus encoded small RNA
EBNA	Epstein-Barr virus nuclear antigen
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzyme-linked immunosorbent assay
Elispot	Enzyme-linked immunospot assay
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FDA	(US) Food and Drug Administration
FIM	Fatal infectious mononucleosis
GvHD	Graft-versus-host disease
Gp350	Glycoprotein 350
HEK293T	Human embryonic kidney cell line 293 (293T cells), transformed with adenovirus 5 and expressing SV40 large T antigen
HIV	Human immunodeficiency virus
HL	Hodgkin's lymphoma
HLA	Human leucocyte antigen
HSCT	Human stem cell transplantation
IBD	Inflammatory bowel disease
IFNγ	Interferon-gamma
IL	Interleukin
IM	Infectious mononucleosis
IPTG	Isopropyl-1-thio-b-D-galactoside
LCL	Lymphoblastic cell lines
LMP	Latent membrane protein
MA	Membrane antigen

MACS	Magnetic cell separation
MHC class I and II	Major histocompatibility complex class I and II
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
NHL	Non-Hodgkin lymphoma
NK cell	Natural Killer cell
NPC	Nasopharyngeal carcinoma
OD	Optical density
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PTLD	Post-transplantation lymphoproliferative diseases
RI	Reduced immunosuppression
RNA	Ribonucleic acid
SFU	Spot forming unit
SLE	Systemic lupus erythematodes
SOT	Solid organ transplanation
TIL	Tumor infiltrating lymphocytes
Th	T helper cells
TNFα	Tumor necrosis factor α

Treg	Regulatory T cells
VAHS	Virus-associated hemophagocytic syndrome
VCA	Viral capsid antigen
Wi38	Human fibroblast cell line

Any deviations from these conventions are described in the text wherever applicable.

Units of measurement

Symbol	*Description*
bp	base pair
°C	degree Celsius
X g	Times the relative centrifuge force (gravity)
g	gram
Gy	Gray
h	hour(s)
kb	kilobasepairs
l	liter
µg	microgram
µl	microliter
ml	millilitre
mM	millimolar
ng	nanogram
rpm	rotations per minute
U	Units

1 INTRODUCTION TO EPSTEIN-BARR VIRUS, ITS BIOLOGY AND ITS EFFECTS ON THE HUMAN HOST

Medicine is an applied science, its basis is ongoing biomedical research and the results thereof constantly contribute to the understanding of health, disease and the development of new therapies. It can nevertheless be helpful to have a look back into the history of medicine before talking about its latest efforts. Discovering the initial studies of a field one is working on is also a method to define and redefine the possibilities and aims of research itself and the specific experiments one is developing and evaluating. That may justify the following brief essay.

1.1 Historical facts about EBV

The Epstein-Barr virus is known since Anthony Epstein (*1921, London) and Yvonne Barr (*1932, London) identified, by examining electronic micrographs, a herpesvirus in cultured lymphoma cells. In London, Epstein, a pathologist and virologist, had been attending the lectures of Denis Burkitt on his observations made in Uganda. Burkitt (1911-1993) was serving as a British colonial surgeon in the Royal Army Medical Corps in Africa in the 1950s where he could observe many children with multifocally swollen jaws. By examining them, he discovered that this disease was caused by a particular type of extranodular lymphoma – a lymphoma endemic to the sub-Saharan region. Because malaria is holoendemic in this area, Burkitt supposed that an infectious agent was involved in the pathogenesis of this disease. Epstein obtained cells from biopsies of these tumours that are now called Burkitt's lymphoma after its discoverer and

describer. With the help of Epstein’s pathological studies, EBV was recognized in 1964 as the first oncogenic virus (Young, Rickinson, 2004).

Since the 19th century, infectious mononucleosis (IM) has been recognized as a clinical syndrome consisting of fever, pharyngitis, and lymphadenopathy. Since 1889, the term “Pfeiffer’sches Drüsenfieber" (“Pfeiffer's glandular fever”), named after Emil Pfeiffer, is used by German physicians. It was also described by the Russian pediatrician Nil Feodorovich Filatov (Moscow, 1887), by the Austrian internist Wilhelm Türk (Vienna, 1904) and by the physician Thomas Speck Sprunt (Baltimore, 1920) and is therefore also called Filatov’s disease, Türk’s lymphomatosis or Sprunt’s disease.

In 1920, Sprunt was the one to describe the syndrome first as Infectious Mononucleosis in the Bulletin of the Johns Hopkins Hospital - in an article entitled "Mononuclear leukocytosis in reaction to acute infection (infectious mononucleosis)".

The association between infectious mononucleosis and EBV was discovered in the late 1960s: Werner and Gertrud Henle, virologists at the children’s hospital in Philadelphia, who became interested in this novel virus, provided a simple and ingenious solution to trace the virus in the human population. They compared the sera of healthy individuals and Burkitt’s lymphoma patients by immunofluorescence, using virus produced by cultured lymphoma cells as viral antigen (Bornkamm, 2008), and thereby identified EBV as the causative agent of infectious mononucleosis in 1968.

The genomic structure of the prototype strain of EBV was published in 1984 (Baer et al., 1984) and was then the largest DNA contig ever sequenced and the first large virus genome to be fully sequenced in overlapping fragments (Farrell, 2005).

1.2 Epidemiology

EBV infection is very common around the world. In developing countries, primary infection usually occurs within the first three years of life. In industrialised countries, it is relatively delayed, so that about 50 % of the pre-adolescents are still uninfected. Altogether, the infection rate in the world population is considered to be around 90 %.

There are two strains of EBV, EBV-1 and EBV-2, with the EBV-1 strain mostly found in infected Caucasian and Southeast Asian populations. 80 to 90 % of EBV-positive individuals in these regions are infected with EBV-1. In the equatorial African and New Guinean population, both strains are nearly equally prevalent (Hjalgrim et al., 2007).

EBV-2 is more frequently found in individuals with higher numbers of sex partners and in homosexual as compared to heterosexual men, suggesting that EBV-2 is sexually transmitted (van Baarle et al., 2000). In contrast to earlier findings by van Baarle (1999), infection with EBV-2 and co-infection rates with both EBV-strains appear to be equal in both HIV-positive and -negative patients (Correa et al., 2007).

1.3 Clinical manifestations

1.3.1 Acute infection

In most cases, the primary contact in early childhood leads to a silent, chronic infection, but about 25 % of primary infections in adolescence or early adulthood manifest as IM. The symptoms of IM usually develop after an incubation period of 10 to 50 days, and can consist of mild fever for up to three weeks, lymphoadenopathy, pharyngitis, hepatosplenomegaly, exanthema and general malaise for up to several weeks. This clinical presentation results from the vigorous immune activation involving proinflammatory cytokines (Rickinson and Kieff, 2006).

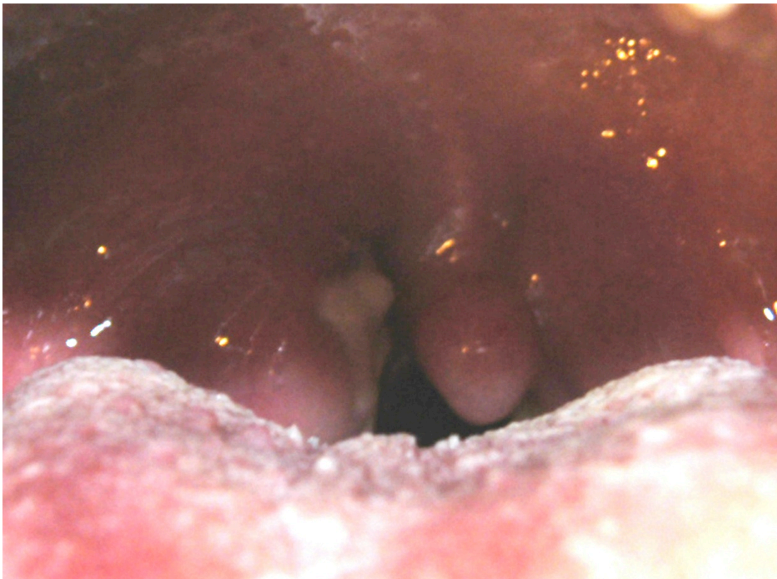


Fig. 1: Clinical symptoms of IM

Typical clinical picture of IM-associated tonsillitis in a child (courtesy of Prof. Dr. med. Uta Behrends).

As the virus is shed in the saliva, the infection usually occurs through the oral route – therefore its colloquial name “kissing disease”. As the virus replicates and persists in B cells, the infection can also be transmitted through blood transfusions or organ transplants.

There is no curative therapy, so the treatment is symptomatic. In cases of severe cervical tonsillar hypertrophy corticosteroids can be indicated to alleviate the swelling and prevent severe obstruction that otherwise may lead to dyspnea.

1.3.2 Complications and chronic infection

Complications of IM are quite rare, but nevertheless can be severe. They range from affections of the central nervous system to severe cardiological, nephrological, pneumological and dermatological complications as for instance myocarditis, hemolytic uremic syndrome, pneumonia, or pityriasis lichenoides, but also to neurological troubles. Severe immunological and haematological consequences can be hepatitis, spleen rupture and also EBV-induced lymphoma, mostly derived from B lymphocytes, especially in patients with preexisting immune defect.

A high susceptibility to EBV-induced malignancies – as to other diseases of infectious origin – is observed in patients with inherited or acquired immunodeficiencies, including patients with X-linked lymphoproliferative syndrome, combined variable immunodeficiency, Wiskott-Aldrich syndrome as well as HIV infection (Carbone et al., 2008).

In addition, iatrogenic immunosuppression administered after solid organ or bone marrow transplantion may lead to the manifestation of EBV-positive malignancies, and tumor incidence usually correlates with the degree of immuno-

suppression and thus with the type of the transplanted organ (Gupta et al., 2010).

In the immunocompetent host, EBV-associated malignancies are mainly Hodgkin's lymphoma (HL) and different kinds of non-Hodgkin lymphoma (NHL). Nasopharyngeal carcinoma (NPC) is an EBV-induced cancer that is often encountered in Southeast Asia, and more rarely in Western countries.

HL is a lymphoproliferative disease, arising from B cell transformation, and has two age peaks: the first between 15 to 35 years, the second in the over 55 years old population. About 40 % of all cases of HL are EBV-associated tumors (Huls et al., 2003). It usually arises in the immunocompetent host.

NHL can appear in the immunocompromised as well as in the immunocompetent patient, and include Burkitt's lymphoma (BL), diffuse large B cell lymphoma, extranodal NK-T cell lymphoma, aggressive NK cell lymphoma and angioimmunoblastic lymphoma (Pietersma et al., 2008). In the post-transplant situation of iatrogenic immunosuppression, PTLD are most frequent, in patients with acquired immunodeficiency syndrome (AIDS), primary central nervous system (CNS) lymphoma and primary effusion lymphoma are more typical (*ibidem*).

NPC is frequent mainly in Southeast Asia, where the incidence reaches in some areas up to 50/100 000 (Taylor et al., 2004), in North America and Western Europe its incidence is about 100-fold lower (Pagano, 1999).

EBV has also been shown to play a role in the development of several autoimmune diseases. The risk for multiple sclerosis (MS) is increased in case of late EBV-infection. Over 99 % of MS patients show EBV-specific antibodies (90 % in the normal population) and antibody responses against the EBV nuclear antigen 1 (EBNA1) are abnormally high. These findings suggested that certain viral antigens might resemble brain proteins and that this molecular mimicry enables EBV-specific cells to target the body's own brain tissue (Christensen, 2006).

Likewise, similarities between the systemic lupus erythematosus (SLE) - associated autoantigens Ro and Sm, and the EBV antigen EBNA1 have been described (Harley et al., 2006). Since SLE is often preceded by an EBV-infection and the mRNA expression of several antigens has been shown to be significantly elevated in SLE patients (Poole et al., 2009), EBV-infection is probably one epidemiological co-factor for a later SLE disease.

With regard to the pathogenesis of inflammatory bowel disease, the involvement of EBV is less clear. Although EBV DNA has been found in bowel biopsies (Knösel et al., 2009), evidence for a causal role of EBV in disease development is still lacking.

Table 1: EBV-induced diseases, the frequency of EBV-association and their incidence

EBV-induced diseases	Frequency of EBV-association	Incidence of disease
Infectious mononucleosis	100 %	6-8/1 000 from age of 10-19, <10 or >30 years less than 1/1000 (Ebell, 2004)
X-linked lymphoproliferative syndrome (Duncan's disease): - EBV-associated hemophagocytic lympho–histiocytosis (HLH)	100 % (there is also HLH associated with other viruses)	Prevalence 1/1 000 000 in males
Burkitt's lymphoma (BL)	95 % in Central Africa 40-50 % in HIV-patients 10-20 % in sporadic cases (Bornkamm et al., 2009)	Uganda: 2.4/100 000 (Ogwang et al., 2008) US: 0.3/100 000 (Morton et al., 2005)
Nasopharyngeal carcinoma	Nearly 100 %	50/100 000 in Southern China (Straathof et al., 2005)

Post transplant lymphoprolif-erative disease (PTLD)	100 % in early onset PTLD; late onset PTLD can be EBV neg. (Shroff and Rees, 2004)	7.3 % in pediatric and 2 % in adult SOT recipients (Straathof et al., 2003)
Hodgkin's lymphoma	40 % (Huls et al., 2003)	2.4/100 000 in white Americans (The Leukemia & Lymphoma society, 2009)
Non-Hodgkin lymphoma (NHL) other than BL and PTLD: - EBV-associated T/NK cell lymphoma (extranodal / nasal type / angioimmunoblastic T cell lymphoma) - HIV-associated lymphoprolif-erative disorders	 100 % (Suzuki et al., 2008) 60-100 % (Carbone et al., 2009)	 12 % of NHL, incidence higher in Eastern Asia and Southamerica (O'Leary et al., 2009) up to 30 % of HIV patients de-velop cancer, risk for NHL 10-150-fold increased (Angeletti et al., 2008)
Autoimmune diseases - Multiple sclerosis - Chronic inflammatory bowel disease - Systemic lupus erythematodes	 >90 %; in children 83 % remote infection (Christensen, 2006) 10 % EBV-DNA in biopsies (Knösel et al., 2009) in young patients: >99 % (Christensen, 2006)	 3.6/100 000 in females, 2.0/100 000 in male (Alonso et al., 2008) 6.3/100 000 for Crohn's disease, 12.0/100 000 for colitis ulcerosa (Herrinton et al., 2008) 7.89/100 000 in females, 1.53/100 000 in males in the UK (Somers et al., 2007)

1.4 Taxonomy and virus structure

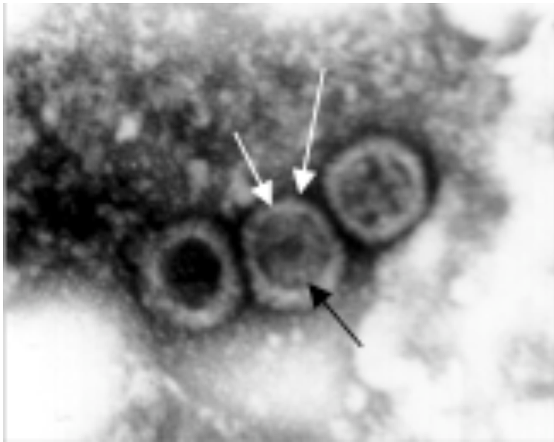


Fig. 2: Electronic micrograph of three extracellular EBV particles.

From the outer to the inner structure: virus envelope, tegument, nucleocapsid (courtesy of Regina Feederle, DKFZ).

EBV is a human gamma-herpesvirus and is also known as human herpesvirus 4 (HHV4) or, as it infects preferentially B lymphocytes, lymphocryptovirus (LCV) (Kieff and Rickinson, 2006). The viral genome consists of double-stranded DNA of a size of about 184 kb. At both ends of the DNA genome are variable numbers of so called terminal repeats, reiterated 0.5 kb direct repeats. The quantity of these repeats depends on the EBV strain and usually remains constant in latently infected cells. Inside the genome there are two largely unique sequences containing the main part of the virus' coding region, separated by a variable number of internal repeats of about 3 kb. The unique sequences also include perfect and imperfect tandem repeats of variable lengths, mostly within the nearly hundred open reading frames (ORF) identified in the EBV genome (Kieff and Rickinson, 2006).

An Epstein-Barr virion is a spherical particle of about 110 nm in diameter. Its surface is formed by an envelope derived from the host cell nuclear or plasma

membrane, studded with viral glycoproteins (Yamaguchi et al., 1967). From the envelope inwards is a protein tegument followed by an icosahedral nucleocapsid. The 162 capsomeres of the nucleocapsid enclose the viral genome that is wrapped around a toroid-shaped protein core.

The EB viral proteins are divided into latent and lytic phase proteins, the lytic phase proteins further into immediate early, early, and late phase proteins. The latent proteins comprise six EBV nuclear antigens (EBNA1, 2, 3A, 3B, 3C, LP) and three latent membrane proteins (LMP1, 2A, 2B).

The EBV genome was sequenced from a BamHI fragment library (BamHI is a restriction endonuclease type II, derived from Bacillus amyloliquefaciens). Consequently, EBV genetic elements like genes and promoters are referenced to the corresponding BamHI fragment. Thus, the EBV major envelope glycoprotein gp350 is also referred to as BLLF1, for BamHI L fragment, leftward ORF number 1. Such nomenclatures are still widely used. Alternative names are used for some well characterized gene products. For example, the EBV proteins expressed during viral latency are called EBNAs or latent membrane proteins (LMP).

1.5 Molecular biology of viral infection

1.5.1 EBV infection *in vivo*

EBV-positive healthy carriers intermittently shed virus from oral epithelial cells into the saliva and transmit the virus by the oral route. Most primary infections are caused by mother-to-child oro-oral transmission. The transmission by

mother milk does not seem to be a relevant way of infection as demonstrated in several studies (i.e. Kusuhara et al., 1997)

In vivo, EBV infects two main target cells: B lymphocytes and stratified epithelium. Studies on tonsillar tissue of IM patients failed to detect virus replication in epithelial cells but found plasmacytoid B cells situated near the crypt epithelium that expressed early lytic cycle antigens (Anagnostopoulos et al., 1995). Fully permissive replication is thought to occur elsewhere in the oropharynx as EBV DNA and lytic cycle transcripts have been found in throat washings of IM patients (Sixbey et al., 1984), although this mechanism was disputed later (Karajannis et al., 1997). Recent studies strongly suggest that lingual epithelium is a likely permissive site (Frangou et al., 2005).

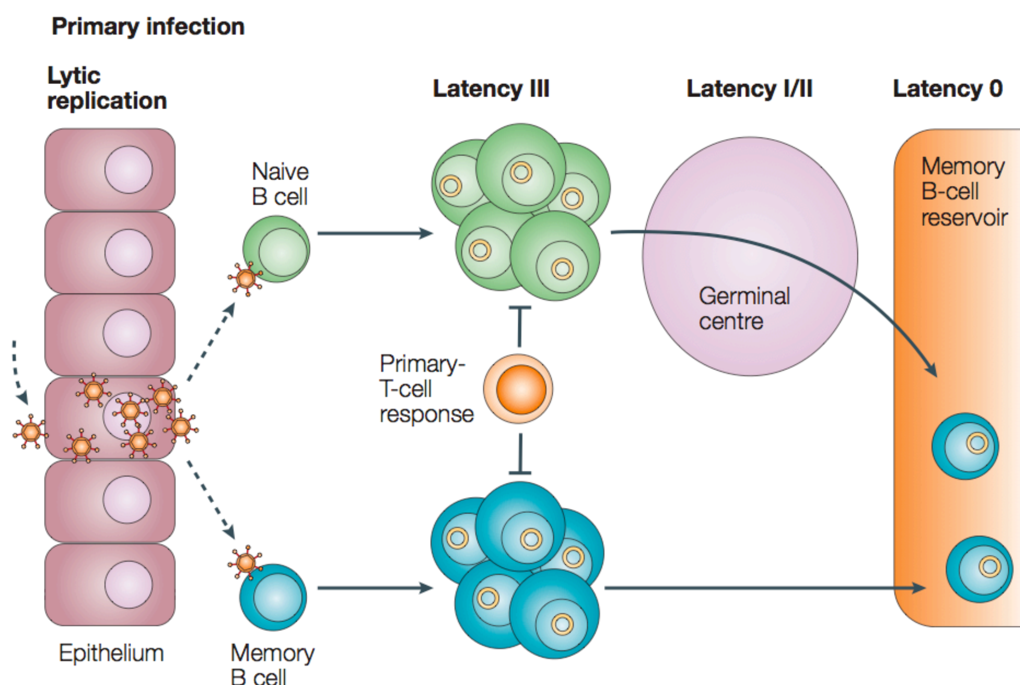


Fig. 3: Primary infection with EBV

Infection of stratified epithelium and B lymphocytes – naïve and memory B cells. Latency 0 is the longterm state of infection of the memory B cell reservoir. Source: Young, Rickinson, 2004.

The tropism of EBV for B cells is conferred by the viral glycoprotein gp350/220 which binds to the B cell-specific surface protein CD21. A second glycoprotein, gp42, binds to major histocompatibility complex class II (MHC class II) molecules, which serve as co-receptors (Borza et al., 2002). Binding to all other cell types is much less efficient and occurs through other, poorly understood pathways (*ibidem*).

Latent infection is confined to the IgD-, CD27+ memory B cell subset, leading to the notion that EBV drives – or at least exploits - the process of antigen-dependent differentiation to acquire memory status, but how EBV exactly manages to enter the B cell memory compartment is still a matter of debate. When entering the state of latent infection, cells downregulate most, if not all, viral proteins (Babcock et al., 2000) and thereby become invisible for cellular immune attacks.

There are at least four different types of viral latency distinguished by the expression of viral proteins. In type I only the virus nuclear antigen EBNA1 is expressed, in type II the latent membrane proteins, LMP1 and LMP2 are additionally expressed and in type III all nine latency-associated proteins are expressed, including the immunodominant EBNA3 family of proteins. In addition, the Epstein-Barr virus encoded small RNA (EBERs), non-coding viral small RNAs, are expressed abundantly in all types of latency. In the latency 0 state, non-coding EBERs are still transcribed, but no viral proteins are expressed (Rickinson and Kieff, 2006).

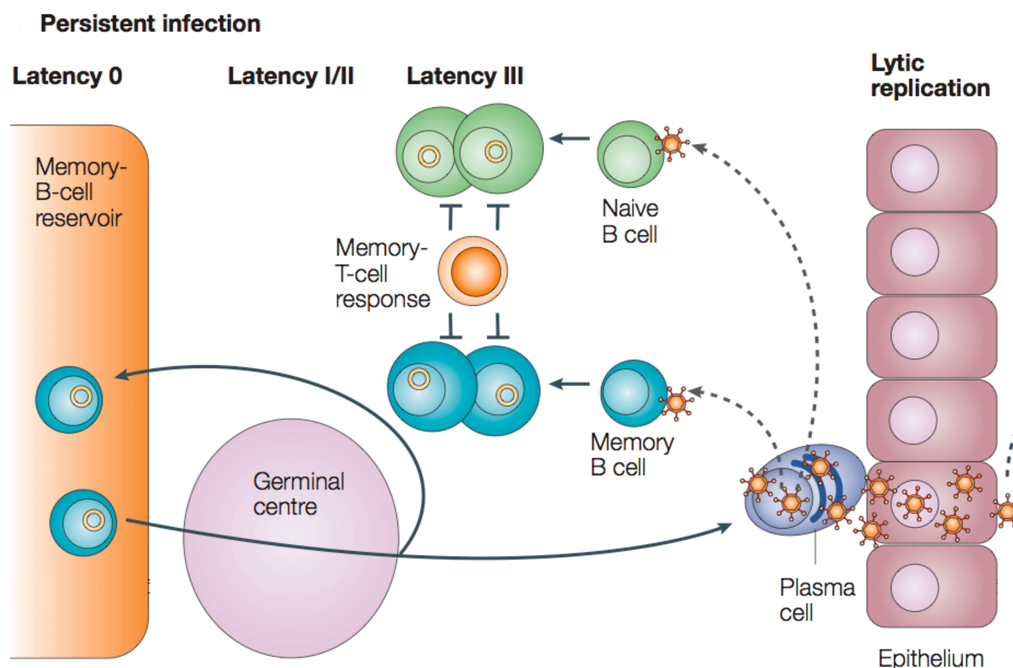


Fig. 4: EBV persistent infection

Memory B cells infected with EBV are subject to migration and differentiation processes. When getting into germinal centre reaction they might pass through different latency programs, differentiate into plasma cells or re-enter lytic cycle at mucosal sites. Source: Young, Rickinson, 2004.

The frequency of infected B cells in healthy, EBV-positive carriers, as determined by PCR, range from 1 to 50 per 10^6 B cells (Khan, 1996). During IM, these numbers are up to 1 000-fold increased.

A small percentage of latently EBV-infected B cells spontaneously become permissive for viral replication and release infectious viral particles that sustain the colonisation of the B cell compartment (Thorley-Lawson, 2001).

1.5.2 Infection of B cells *in vitro*

In vitro, the host cells that can be efficiently infected by EBV are restricted to primary human B cells. B lymphocytes from peripheral blood, tonsils and fetal cord blood can be easily infected. B lymphocytes from earlier stages of development, as from fetal or adult bone marrow or fetal liver, as well as leukemic, non EBV-infected (e.g. Burkitt's lymphoma) cell lines can be infected but with lower efficiencies.

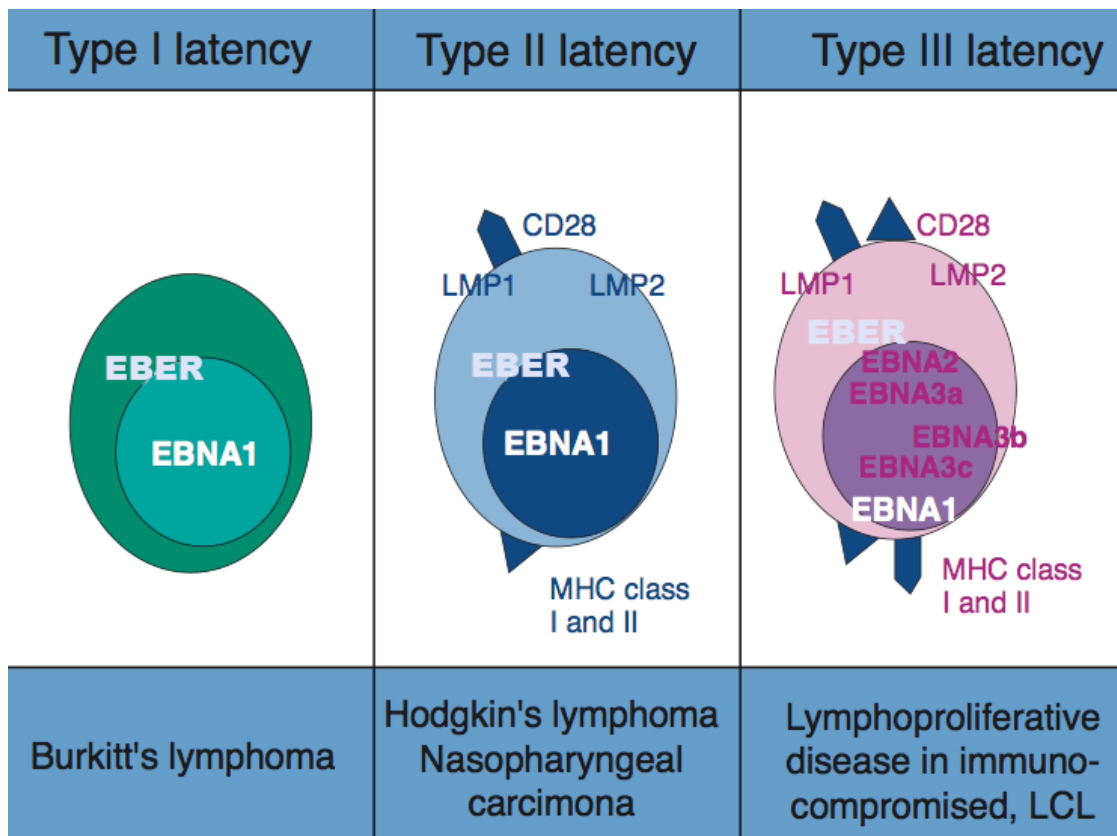


Fig. 5: EBV latency types and their antigen patterns

EBER and EBNA1 are expressed in all latency types. In Hodgkin's lymphoma and nasopharyngeal carcinoma, the EBV antigens LMP1 and LMP2 are additionally expressed as well as MHC class I and II molecules (flash-shaped in the picture) and CD28, a T cell costimulatory molecule (triangular in the picture). In post-transplant lymphoproliferative disease and lymphoblastic cell line (LCL) cells, a broader spectrum of EBV antigens is present. Source: Straathof et al., 2003.

Fully differentiated plasma cells cannot be infected. With a low efficiency EBV can also establish latent infections in T lymphocytes, natural killer (NK) cells, and in some carcinoma cell lines: kidney, biliary, laryngeal, hepatocellular, colon, bladder and gastric carcinoma cell lines are some examples. The efficiency of the infection is similar to that of EBV-negative BL cells.

When EBV-infected, peripheral blood B cells are transferred into culture, and T cell control is suppressed by the addition of cyclosporine A (CSA), they will grow out as huge immunoblastic cells and give rise to a so-called lymphoblastoid cell line (LCL).

LCL express the latency III program, i.e. all latent antigens: EBNA-LP, EBNA1, EBNA2, EBNA3A, -3B, -3C, LMP1, LMP2. In addition, they express the non-coding RNAs EBER-1 and -2, the BamA rightward transcripts (BART), and viral micro-RNAs.

1.6 Immune response to EBV

Like to other pathogens, the human immune system responds to EBV with a combination of innate and adaptive cellular and non-cellular attack mechanisms.

1.6.1 Innate cellular immune response

Like in other microbial infections, NK cells are key components of the first immunological answer after primary EBV infection. Studies have demonstrated that NK cells can inhibit the *in vitro* outgrowth of EBV-immortalized B cells, probably mainly because of the effect of IFNγ (Lotz et al., 1985). Their role *in*

vivo does not appear to be critical for the control of EBV infection: PTLD develop usually 3 to 6 months after transplantation, when the patients are T cell-depleted but have regained normal NK cell counts (O'Reilly et al., 1997).

1.6.2 Adaptive immune response

1.6.2.1 Humoral immune response

The seroepidemiology and the diagnostics used in IM rely on a set of immunofluorescence assays to detect antibody responses to viral proteins. The early antigen (EA) is expressed early, the viral capsid antigen (VCA) late in the lytic cycle. The membrane antigen (MA) is expressed on the surface of cells late in the lytic cycle. All of these antigens are actually a combination of several viral proteins. The accuracy of enzyme-linked immunosorbent assays (ELISA) to detect single proteins is still not high enough for diagnostic purposes.

IM patients show a rise in IgM against VCA and IgG to VCA and EA, the IgM anti-VCA response subsequently vanishes, whereas the IgG anti-VCA rises to a peak after about 10-14 months (Horwitz et al., 1985, Rea et al., 2002) and falls then over months to a stable level. The major target antigen for virus neutralizing antibodies – which tend to be at low titers – is gp350. Many patients show an IgG response to EBNA2 early during the acute phase of the disease, whereas IgG antibodies to EBNA1 usually are not detectable until convalescence (Rickinson and Kieff, 2006).

Additionally, EBV provokes a general activation of the B cell system, either by the direct infection of B cells (Garzelli et al., 1984) or by an aberrant T cell help (Recher et al., 2002), thereby eliciting a range of heterophile and autoreactive

IgM responses. These antibodies are responsible for the diagnostically used Monospot or Paul-Bunnell reaction, which is based on the agglutination of horse or sheep erythrocytes by these antibodies (Paul and Bunnell, 1932).

In healthy virus carriers, antibodies of IgG isotype that are directed against EBNA1, VCA and gp350 are consistently detected. Titers may differ interindividually but tend to be stable intraindividually. Which role antibodies really do play in the control of EBV-infection is not clear. Antibodies against viral glycoproteins such as gp350 may interfere with viral adhesion to target cells and thereby diminish viral spreading. In immunocompromised patients, a decline of anti-EBNA1 and an increase of anti-EBNA2 antibodies can be observed (Rickinson and Kieff, 2006). Elevated antibody titers against EBNA1 have been associated with an increased risk for developing multiple sclerosis (Levin et al., 2005).

1.6.2.2 CD8+ T cell immune response

During acute disease, IM patients show a remarkable increase in blood CD8+ T cell numbers, which are perforin-positive with cytotoxic functions *ex vivo* and display a markedly skewed Vβ chain usage (Hislop et al., 2007).

MHC multimer studies demonstrated that individual virus epitope-specific T cells can account for up to 40 % of the CD8+ T cell pool in peripheral blood (*ibidem*). The CD8+ T cells found during IM show an activated CD38+ Ki-67+, proapoptotic phenotype, whereas later on CD8+ memory T cells lacking activation markers like CD38 or CD69, form the main part of the EBV-specific CD8+ T cell pool, and may comprise up to 3 % of the total peripheral CD8+ T cell pool in the younger population. In the elder population this percentage can be increased to more than 10 % (Bihl et al., 2006).

During primary infection, CD8+ T cell responses against lytic cycle antigens are much more frequent than those against EBV latent cycle proteins (Scherrenburg et al., 2008). Remarkably, the main targets of the lytic cycle antigen-specific CD8+ T cells response are the immediate early antigens BZLF1 and BRLF1 and to a lesser extent some early lytic cycle proteins, e.g. BMLF1, BMRF1, BSLF2, and BALF5. Proteins expressed late during the lytic cycle are poor targets for the virus-specific CD8+ T cell response.

The main targets of the latent antigen-specific CD8+ T cell response are derived from the EBNA3 family of proteins and LMP2, whereas EBNA1, EBNA2, EBNA-LP and LMP1 are rarely targeted (Landais et al. 2005).

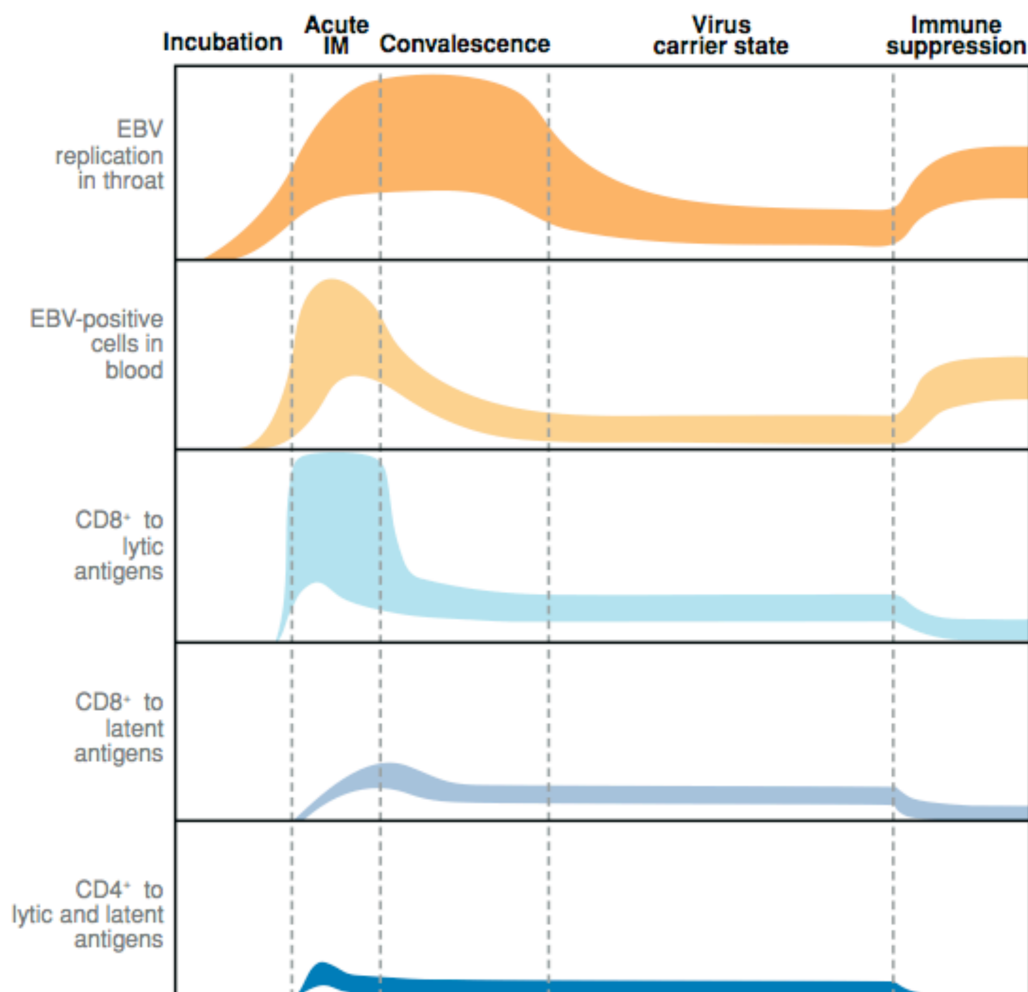


Fig. 6: EBV infection and its immune response over time.

Source: Hislop et al., 2007.

1.6.2.3 CD4+ T cell immune response

CD4+ T cells recognize antigens presented on MHC class II molecules that are expressed on macrophages, monocytes, dendritic cells – i.e. all professional antigen presenting cells (APC). They are subdivided in a T helper type 1- (Th1) and a type 2- (Th2) population. The Th1 group plays an important role in the defence against intracellular pathogens while Th2 cells are critical for fighting extracellular pathogens by regulating humoral immune responses (Janeway et al., 2005).

The CD4+ T cell response during primary EBV infection has been neglected until some years ago, possibly because of the relatively small size of the virus-specific CD4+ memory population (Precopio et al., 2003), the relatively small number of defined epitopes, as well as the lack of MHC class II tetramers that are instrumental for analyzing these cells (Hislop et al., 2007).

Both latent and lytic cycle antigen-specific CD4+ T cells have been detected in blood from IM patients (Amyes et al., 2003), but their frequencies are about 10- to 100-fold lower than the corresponding CD8+ memory T cell numbers (Hislop et al., 2007).

In vitro, EBV-positive PBMC grow out as LCL if the T cell control is abolished, e.g. by the addition of cyclosporine A. If only CD4+ T cells are removed, LCL will also grow out. But if CD4+ cells are added to these cultures, they inhibit the outgrowth better than unsorted PBMC (Heller et al., 2006), demonstrating that EBV-specific CD4+ cells can prevent B cell transformation *in vitro*.

During acute infection, the CD4+ T cell compartment barely expands and EBV-specific CD4+ T cells represent no more than 1 % of the whole CD4+ T cell pool. The antigens recognized by these CD4+ T cells are derived from both lytic and latent proteins (Precopio et al., 2003). After IM, the size of the EBV-specific

CD4+ T cell response is even smaller: it drops below 0.1 % after six months and is nearly undetectable one year post IM.

The cytokines produced by EBV-specific CD4+ T cells include INFγ and tumor necrosis factor α (TNFα), and, to a lower extent, interleukin 2 (IL-2) (Hislop et al., 2007). However, cell culture conditions may have influenced the cytokine profile and, therefore, no longer reflect the *in vivo* situation.

CD4+ memory T cells specific for latent EBV antigens respond against multiple EBNA- and LMP-derived epitopes and their frequencies can be measured in INFγ-Elispot assays (Hislop et al., 2007). Various LMP1 peptides seem to induce IL-10 production, suggesting that they play a role in the induction of regulatory T (Treg) cells (*ibidem*). Treg cells are a unique subset of T cells that express both CD4 and CD25 and that play an important role in preventing autoimmune diseases (Janeway et al., 2005). Treg cells are also supposed to attenuate the anti-tumour activity of CTL if infused in patients with EBV-associated malignancies (Li et al., 2009a). Thus, the induction of Treg cells may facilitate the outgrowth of EBV-associated tumors.

Regarding the CD4+ T cell response against viral antigens expressed during the lytic phase, CD4+ T cell clones specific for the immediate early, early, and late lytic cycle antigens have been isolated from peripheral blood of virus carriers (Hislop et al., 2007). Among these, CD4+ cells specific for virion antigens were shown to recognize EBV-infected target cells following receptor-mediated uptake of released viral particles (Adhikary et al., 2006). These T cells, that were detected in the peripheral blood of all healthy virus carriers examined, displayed a Th1 phenotype and were able to inhibit the outgrowth of newly EBV-infected B cells as well as the proliferation of BL cells and LCL *in vitro*. These findings suggested that virion antigen-specific CD4+ T cells play an important role in the control of EBV infection by keeping the number of EBV-infected B cells low.

1.7 Failure of immune response

Lethal EBV infections are seen in immunosuppressed patients who lack functional T cells. Disorders like fatal IM (FIM) or virus-associated hemophagocytic syndrome (VAHS) are fulminant EBV infections which develop mainly in Asian populations and in Caucasian patients with XLP, or rarely in patients with other kinds of immunosuppression like SLE (Young et al., 2008). FIM is associated with a defect in the T cell-mediated immune response, an aberrant T cell cytotoxicity, and an uncontrolled B cell proliferation.

In X-linked lymphoproliferative disease (XLP, Duncan's disease), affected boys develop an overwhelming IM-like disease, called hemophagocytic lymphohistiocytosis (HLH), after primary infection with EBV. They develop high fever, lymphadenopathy, and acute expansion of NK and T cell populations in blood, which often leads to a fatal aplasia, macrophage activation, hemophagocytosis, and eventually bone marrow failure (Chaganti et al., 2008). XLP is caused by mutations in the SH2D1A gene, which codes for SAP (SLAM-associated protein), an adaptor protein involved in the regulation of B cell/T cell interactions. CD8+ T cells from XLP patients are unable to efficiently kill EBV-transformed B cells *in vitro*, probably because of a defect in the polarization of the lytic machinery (Dupré et al., 2005).

Because of immunosuppressive therapy, solid organ and hematopoietic stem cell transplant recipients often develop EBV-associated PTLD. Depending on the level of immunosuppression, PTLD incidence ranges from less than 1 % up to 33 %, (Dolcetti, 2007). PTLD in bone marrow recipients usually are from donor cell origin, in SOT recipients mainly from recipient origin, although exceptions have been reported (Bollard et al., 2003). Main risk factors for developing PTLD are EBV seronegativity before transplantation, high levels of immunosuppression, the coeval presence of cytomegalovirus (CMV) disease, and young age (Dolcetti, 2007). Despite these common features, PTLD are a quite hetero-

geneous group of lymphoproliferative diseases that include polyclonal and polymorphic B cell proliferations as well as malignant B cell lymphomas, for example diffuse large B cell lymphoma, BL and BL-like lymphomas and HL (Harris et al., 2001).

Due to a progressive immunosuppression, patients with AIDS may develop different EBV-associated lymphomas at different time points after infection (Münz and Moormann, 2008). HIV coinfection promotes the development of three types of NHL. Firstly, diffuse large cell and immunoblastic lymphomas develop in severely immunosuppressed patients and cause the typical CNS lymphoma in AIDS. These lymphomas express all latent EBV antigens and thus resemble PTLD. Secondly, a coinfection by HIV, EBV and Karposi's sarcoma herpesvirus (KSHV), another human γ-herpesvirus (also called HHV-8), can lead to the development of primary effusion lymphoma (PEL). PEL is an extremely rare tumour in immunocompetent individuals. PEL are always KSHV-positive and in most cases also EBV-positive.

Several studies have indicated that CD4+ T cells play a protective role against EBV-associated lymphomas. First evidence was provided by Piriou et al. (2005) who demonstrated that loss of EBNA1-specific CD8+ and CD4+ T cell responses in patients with HIV/AIDS considerably increased the risk for developing EBV-associated NHL. Because CD4+ T cells disappear first in these patients and are required to maintain CD8+ T cell responses, CD4+ T cells appear to be the critical tumor-protective population. In a different study, only patients with HIV/AIDS that lacked EBV-specific CD4+ cells were found to develop EBV-associated primary central nervous system (CNS) lymphoma (Gasser et al., 2007). In addition, low endogenous CD4+ T cell numbers have been identified as important risk factors for the development of EBV-associated diseases in immunosuppressed patients (Sebelin-Wulf et al., 2007). Furthermore, patients with PTLD showed better clinical responses in a recent phase II trial when the infused T cell lines contained higher proportions of CD4+ T cells (Haque et al., 2007).

1.8 Therapeutic approaches

1.8.1 Therapy of infectious mononucleosis

IM is a self-limiting lymphoproliferation that does not require antiviral therapy. In the case of painful cervical swelling, severe pharyngitis, or airway obstruction by lymphadenopathy or tonsillar swelling, corticosteroids can be given to ameliorate the symptoms.

Some of the antiviral drugs used in other herpesvirus infections such as herpes labialis or herpes zoster, which show good efficacy in these diseases, are ineffective in IM. One reason is the long incubation period of EBV from about 4 to 6 weeks, and the late onset of symptoms, and with it, the late possibility of therapeutic intervention. Furthermore, the symptoms of IM are not caused by the virus directly, but are a consequence of the immunopathic effects of the immune response against EBV (Gershburg and Pagano, 2005).

In vitro, drugs that target viral DNA polymerase such as acyclovir, gancyclovir (acyclic nucleoside analogues) as well as cidofovir (acyclic nucleotide analogues) or foscarnet (pyrophosphate analogues) are potent inhibitors of EBV replication. Because clinical trials with these drugs have been disappointing and none of them has yet been licensed for the treatment of IM, new drugs still need to be developed (Gershburg and Pagano, 2005).

1.8.2 Treatment of EBV-associated malignancies

Treatment of PTLD involves the reduction of immunosuppression, chemotherapy, antiviral therapy, IFNα, monoclonal antibodies - especially the anti-CD20 antibody Rituximab – and rarely, in cases of localized PTLD, radiation or surgery. The reduction in immunosuppression is considered the first-line therapy and shows good response in cases with good prognostic factors and in the absence of organ failure (Svoboda et al., 2006). In aggressive PTLD, chemotherapy is used as the first-line therapy, yet is associated with a high toxicity-related mortality (25 %). Antiviral therapy with acyclovir or gancyclovir as a prophylactic treatment seems to reduce PTLD incidence (McDiarmid et al., 1998), but is ineffective for the treatment of manifest disease (Crumpacker, 1996).

CD20 is a surface molecule on B cells, its natural ligand and function is not known. It is present on all stages of B cell development, but not in the earliest form, the pro-B cells, neither on its latest form, the plasma cells (Janeway et al., 2005). It is also expressed on B lymphoma cells and, therefore, has gained significant relevance as a target of antibody therapy. In the case of EBV-induced PTLD, the chimeric monoclonal anti-CD20 antibody Rituximab is nowadays often used prophylactically and therapeutically (Milpied et al., 2000). Response rates up to 100 % have been reported, but as Rituximab does not restore cellular immunity, progressive disease after 12 months is frequently observed (Choquet et al., 2007).

Some trials are aiming at vaccinating actively against EBV - as it is known that a clinically symptomatic primary EBV infection as IM is a predisposing factor for the development of HL: it is associated with an up to fourfold higher risk with a median incubation time of four years after IM (Münz and Moormann, 2008).

Recombinant gp350 has been tried in phase I/II studies, but the efficacy of the vaccine remains unclear (Moutschen et al., 2007).

Given the existing burden of EBV-associated malignancies and the failure to develop prophylactiv vaccines, attention has turned to immunologic targeting of the tumor itself. In human stem cell transplantation (HSCT) patients, EBV-positive PTLD has been successfully treated by the infusion of donor lymphocytes (DLI), albeit at the risk of causing life-threatening graft-versus-host disease (GvHD). Therefore, this strategy was extended to EBV-specific T cells generated by repeated *in vitro* stimulation of peripheral blood T cells with autologous LCL as stimulators. Such *in vitro* pre-selected EBV-specific T cell preparations of donor origin have been successfully used to prevent and treat PTLD in HSCT patients without causing GvHD.

However, owing to the considerable technical requirements and financial implications of extensive *in vitro* T cell culture, adoptive T cell therapy still has a limited role in the management of virus-associated complications in transplant patients. A wider applicability of this approach is also hampered by two logistic difficulties. One is the problem of the transplant recipient who is still EBV-negative at the time of transplantion and who is at highest risk of developing PTLD. Efforts to generate clinically effective EBV-specific T cell preparation by *in vitro* priming have not been successful. The second is the time required to generate T cell preparations when the clinical need is urgent. The generation of specific CTL takes three to four months, while PTLD often progress to fatal disease within weeks. For that reasons efforts have been made to establish a bank of *in vitro* reactivated EBV-specific T cell preparations from healthy seropositive donors of known HLA genotype for adoptive transfer as allogeneic partially HLA-matched effectors. Although such allogeneic cells can only serve as first line defense and do not persist longterm, they have been used successfully in a number of patients with PTLD (Wilkie et al., 2004; Haque et al., 2007).

Apart from trials for developing a therapy of EBV-positive PTLD, adoptive CD8+ T cell therapy targeting EBV-positive tumours like HL, NHL or BL in immunocompetent patients has met with limited success. One potential explication is that these tumors express only a limited set of low- or non-immunogenic antigens. Yet, these studies mostly didn't include virus-specific CD4+ T cells that might be required to elicit sufficient antitumoral responses.

1.9 Aim of the work

This project aims to characterize the CD4+ T cell response to structural antigens of EBV in healthy EBV carriers and patients with IM. To this aim, two experimental approaches were pursued.

First, the frequencies of virion antigen-specific CD4+ T cells were investigated in healthy EBV carriers and in IM patients during disease and convalescence by IFNγ Elispot assays.

In a second approach, gp350- and BNRF1-specific CD4+ T cell lines were generated by repeatedly stimulating peripheral blood T cells from ten different EBV-seropositive donors with autologous PBMC that had been pulsed with these two proteins. In this approach we wanted to address the question whether CD4+ T cells specific for these antigens are consistently present in the peripheral T cell pool, and to identify immunodominant epitopes presented on the various HLA molecules expressed by the donors.

2 MATERIALS AND METHODS

2.1 Materials

Unless otherwise stated, all chemicals were bought from Roche or Merck, all enzymes from NEB and Fermentas.

2.1.1 Media for cell and bacteria culture

AIM-V medium (Invitrogen)	Base for T cell medium
Freezing medium for all kinds of cells	90 % FCS (Biochrom), 10 % DMSO (Merck)
HEK293T cell medium	500 ml DMEM (GIBCO), 10 % fetal calf serum (FCS) (Biochrom), 2 mM L-glutamine (GIBCO), 2.5 µg/ml amphotericin B fungizone (GIBCO), 50 µg/ml gentamicin (Invitrogen)
LB medium	10 g NaCl, 10 g tryptone and 5 g yeast extract dissolved in 1 l deionised sterile water, pH adjusted to 7.0, autoclaved
LCL medium	500 ml RPMI-1640 (PAA), 10 % fetal calf serum (FCS) (Biochrom), 1 % non-essential amino acids (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 2 mM L-glutamine (GIBCO

	25030 (100x) 200mM), 50 µg/ml gentamicin (Invitrogen), 2.5 µg/ml amphotericin B (GIBCO)
RPMI-1640 (PAA)	Base for LCL medium
T cell medium	500 ml AIM-V (Invitrogen), 10 % heat-inactivated pooled human serum, 2 mM L-glutamine (GIBCO 25030 (100x) 200mM), 10 mM HEPES (Invitrogen), 50 µg/ml gentamicin (Invitrogen), 2.5 µg/ml amphotericin B (GIBCO)
Wash medium	To wash all kind of cells: RPMI-1640 (PAA)

2.1.2 Chemicals and buffer

Elution buffer	0.5 M imidazole in Urea lysis buffer
FACS buffer	PBS with 2 % fetal calf serum
Loading buffer for gel electrophoresis	0.25 % w/v bromophenol blue, 0.25 % w/v xylene cyanol, 30 % v/v glycerol (6x concentrated)
Lysis buffer	100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, 10 mM imidazole, 0.05 % Tween 20, pH 8.0

MACS buffer	PBS with 1 % bovine serum albumin and 2 mM EDTA
PBS buffer	2 g potassium chloride (KCl), 2 g potassium dihydrogen phosphate (KH_2PO_4), 80 g sodium chloride (NaCl) and 14.3 g disodium hydrogenphosphate (Na_2HPO_4:$2H_2O$) dissolved to a final volume of 10 l in deionized sterile water, pH adjusted to 7.2 to 7.4, sterile filtered
SDS running buffer **for westernblot**	3 g TRIS/base, 14 g glycine, 10 ml SDS in 1 l H2O
TAE buffer (50x)	2 M TRIS/base and 50 mM EDTA in deionised sterile water; pH adjusted to 8.5; diluted 1:50 to prepare 1x TAE
TBS (1x)	20mM Tris-Cl, 150 mM NaCl in deionised sterile water; pH 7.5
TE buffer (1x)	10 mM Tris-HCl pH 7.5; 1 mM EDTA in deionised sterile water
Transfer buffer **for westernblot**	4.5 g Tris Base, 21 g Glycine in 1.5 l of 20 % methanol in deionised sterile water
TRIS/SDS (2x) pH8.8 **TRIS/SDS (2x) pH6.8**	22.68 g TRIS/base, 2.5 ml 20 % SDS, 250 ml H_2O, pH with 2 N HCl adjusted to 8.8 7.56 g TRIS/base, 2.5 ml 20 % SDS, 250 ml H_2O, pH with 2 N HCl adjusted to 6.8

Urea lysis buffer	8 M urea, 0.1 M NaH_2PO_4, 0.01 M TRIS/base, 0.05 % Tween 20, 20 mM imidazole, in deionised sterile water, pH adjusted to 8.0

2.1.3 Laboratory equipments

Cell culture CO_2 incubators (Heraeus; ThermoForma)

Centrifuges (Eppendorf; Sorvall; Beckman; Hettich)

- 80 °C freezers (Colora)

- 20 °C freezers (Liebherr)

- 4 °C refrigerators (Liebherr)

Bacteria incubator (Heraeus)

Bacteria shaker (New Brunswick Scientific)

Cell counting chamber (GLW)

Electroporation equipment Gene Pulser II (BioRad)

ELISA Reader (Tecan)

FACS Scan equipment (Becton-Dickinson)

Gel electrophoresis chamber (Invitrogen)

Light-optical microscope (Carl Zeiss)

Milli-Q water preparation equipment (Millipore)

pH-Meter (Knick)

Pipettes (10, 20, 200, 1000 µl) (Gilson)

Pipette Boy (Integra)

Power supply Power-Pac 300 (BioRad)

Spectrophotometer (Eppendorf)

Sterile bench (Bio Flow Technik)

UV-Transilluminator (UVP Inc.)

Vortex Genie 2 (Bender & Hobein)

2.1.4 Disposable cell culture material

140 mm and 100 mm cell culture plates (Nunc)

6-well, 12-well, 24-well, 48-well, 96-well flat bottom plates (Becton Dickinson)

96-well round bottom plates (Corning)

96-well V bottom plates (Nunc)

Cotton-stuffed plastic pipettes (2 ml, 5 ml, 10 ml, and 25 ml) (Corning)

1 ml, 200 µl, 20 µl and 10 µl stuffed pipette tips (Molecular Bioproducts)

10 ml, 200 ml and 400 ml cell culture flasks (Greiner)

15 ml and 50 ml Falcon tubes (Becton Dickinson)

1.5 ml and 2 ml Eppendorf tubes (Eppendorf)

500 µl PCR tubes (Sarstedt)

2.2 Methods for eukaryotic cell culture

2.2.1 General conditions for eukaryotic cell culture

All eukaryotic cells used for the experiments were incubated in cell culture incubators at 5 % CO_2 and 37 °C. All media for cell culture were stored at 4 °C for no longer than three months, after the addition of fetal calf serum they had to be used within two weeks. All other supplements were kept at temperatures and conditions recommended by the manufacturer. Handling with cell culture was exclusively performed in a biological containment cabinet (hood) under strict aseptic techniques using aseptic material. Depending upon the growth and medium consumption, cells in culture were split and refed with fresh growth medium. To maintain a frozen inventory, cells were expanded and frozen as soon as possible. Freezing of cells was done in 1.8 ml cryotubes (Nunc) with 5 x 10^6 to 1 x 10^7 cells in 1.0 ml of freezing medium (10 % dimethylsulfoxide (DMSO, Merck), 90 % fetal calf serum). The cryotubes were put in propanol compartments (Nunc) in -80 °C freezers, allowing a slow adaptation of the cells to the temperature. For long term storage, the tubes were transferred to a liquid nitrogen tank after 48 h.

The marmoset B cell line B95.8 was maintained as suspension culture in cell culture flasks in LCL medium.

The human embryonic kidney cell line 293T (HEK293T cells) and the adherently growing human fibroblast cell line Wi38 were cultivated in cell culture plates in HEK293T cell medium. They were split 1:4 every two to three days, using 1x Trypsin-EDTA (PAA) and following incubation at 37 °C for 5 min to detach the cells. The cells were then transferred to a Falcon tube along with HEK293T cell medium and centrifuged. The pellet was subsequently resus-

pended with fresh medium and the cells brought out in new plates at the desired density.

2.2.2 Isolation of PBMC from peripheral blood

20 ml freshly drawn blood was filled into 50 ml falcons containing 250 µl of 0.5 M EDTA for the prevention of coagulation and 15 ml of plain RPMI-1640 medium and underlaid with 10 ml Ficoll solution (GE Healthcare). For the intended density gradient purification, the blood was then centrifuged for 30 min at 750 x g without brakes at room temperature. In this way, the red blood cells were collected at the bottom of the tube and the white blood cells and platelets on the top of the Ficoll phase. The interphase, which contained the white blood cells, was transferred to new 50 ml tubes and washed three times with RPMI-1640 at 500 x g and room temperature for 10 min. The supernatant was discarded again and the pellet transferred to a 15 ml tube with 10 ml RPMI-1640. After resuspension, the cells were ready for counting and subsequent applications.

Peripheral blood was obtained from EBV-positive and -negative healthy donors, and IM patients from the Kinderklinik of the Technische Universität München. Studies on material of human origin had been approved by the Ethical Board of the Technische Universität München.

2.2.3 Thawing of cryopreserved cells

Frozen cells were supposed to be thawed rapidly in a water bath at 37 °C. After immediate transfer to 15 ml Falcon tubes filled with wash medium, they were washed twice with RPMI-1640. The cells obtained thereafter were maintained in fresh warm medium. (Unless otherwise stated all centrifugation steps described below used to pellet cells were performed at 500 x g for 5 min at room temperature).

2.2.4 Expression and purification of EBV proteins

The plasmids used for protein expression encode the EBV proteins gp350 and BNRF1. The genes were cloned into the CMV promoter/enhancer driven mammalian expression vector pCMV-EHis, tagging the EBV genes at their 3' end with sequences coding for the epitope recognized by the monoclonal anti-EBNA1 antibody 1H4, and a His-tag consisting of six consecutive histidines. For recombinant protein expression, the plasmids were transiently transfected into HEK293T cells by using the polyethylenimine method: 450 µg of DNA were mixed with 15 ml of Optimem and 675 µl of PEI. After 15 minutes of incubation at room temperature, 150 ml of HEK293T cell medium were added to transfect ten 140 mm diameter cell culture plates of HEK293T cells. After 6 to 12 hours, 25 ml of fresh medium were added to each plate, and after 48 to 72 hours the cells were harvested by scratching them off the plates and spinning them at 700 x g for 5 min. The supernatant was discarded and the cells were resuspended in urea lysis buffer. The lysate was centrifuged in order to pellet cell debris and

DNA complexes and the clear lysate in the supernatant transferred to a new 50 ml Falcon tube. After addition of 300 µl NiNTA agarose beads (Qiagen) per tube and rotation of the tubes in an overhead rotator for at least 6 hours, the tubes were centrifuged at 1000 x g for ten minutes and the supernatant discarded. The beads with the bound proteins were washed once with lysis buffer. Thereafter, the proteins were eluted using elution buffer, three elutions with 300 µl elution buffer each were performed. In order to get rid of excess urea, the eluate was dialyzed against PBS for 48 h. The protein content was quantified using the Bradford method, mixing the protein solution with Bradford dye (Bio-Rad) and measuring the optical density in a spectrometer. Known concentration of bovine serum albumin was used as a standard for the quantification.

2.2.5 Preparation of concentrated B95.8 culture supernatant

Supernatant from B95.8 cells was centrifuged at 1000 x g for 10 min to remove cells and cellular debris. Cell free supernatant was then filtered through a 0.8 µm filter and ultracentrifuged at 100 000 x g for 3 h. The supernatant was removed and the virus rich pellet resuspended in 1/20 volume of the original culture supernatant.

2.2.6 Generation of CD4+ T cell lines and clones

For the generation of CD4+ T cell lines, 2 x 10^6 PBMC were incubated in 2 ml T cell medium in one well of a 24-well plate with 200 µl of the recombinant EBV protein gp350 or 75 µl of BNRF1. After 24 h, 10 U/ml of recombinant human IL-

2 (Chiron) were added. When needed, the cells were split by resuspension, transfer of half of the cells to new wells and addition of T cell medium containing 10 U/ml of IL-2.

The restimulation of the T cell lines was done as follows: 2 x 10^6 PBMC were incubated with 200 µl of gp350 or 75 µl of BNRF1 recombinant protein for 24 h. After irradiation (40 Gy), the cells were harvested and washed three times. The T cells of the corresponding donor were then collected, mixed in a 1:1 ratio with the antigen presenting cells (APC) and centrifuged. The cell pellet of APC and T cells was then resuspended in 4 ml of T cell medium without IL-2 and brought out in 24-well plates. After 24 to 48 h, 10 U/ml of recombinant IL-2 were added. The T cells were restimulated every two weeks in the same way. In this work, the term passage (abbreviated p) is used to denote the number of restimulations that a T cell line underwent.

2.2.7 Establishment and culture of lymphoblastoid cell lines

PBMC were extracted from the blood of healthy donors following the description given above. One to two million cells were resuspended in 100 µl of LCL medium and brought out in a well of a 96-well flat bottom plate. B95.8 wild type EBV was obtained by filtration of the cell-free supernatant of densely grown B95.8 cells through a 0.8 µl filter. 100 µl of this virus supernatant was added to each well together with cyclosporine A at a final concentration of 0.5 mg/ml. Cyclosporine is needed to suppress the T cells via cyclophilin which inhibits calcineurin, an activator of IL-2 transcription.

For the generation of the so-called miniLCL, an EBV-knockout virus was used that lacks the gene for BZLF1 and so the ability to enter the lytic cycle.

After 24 h, half of the medium was removed and cyclosporine A-supplemented fresh LCL medium was added. When the growth of the cells did not advance as supposed, the human fibroblast line Wi38 (10 000 cells per well) was added as feeder cells. Once expansion of LCL was observed, cells were transferred to 48- or 24-well plates and ultimately to tissue culture flasks. Aliquots of the cells were frozen and stored in liquid nitrogen as back-up. The growing lines were split when necessary by removing half of the cell suspension and filling it up with fresh LCL medium.

2.2.8 Extraction of genomic DNA from eukaryotic cells

Approximately 2 x 10^8 LCL were collected from each of the 10 donors in four 50 ml falcons and washed once with PBS. The cell pellet in each tube was resuspended in 32.5 ml 1x TE. Then, 2.5 ml 10 % SDS and 2.5 mg RNAse A were added and the cell lysates incubated for 30 min in a 37 °C water bath. After addition of 3.75 mg proteinase K, the tubes were incubated at 60 °C overnight. The next day, 15 ml of 5 M NaCl were added to each falcon and the tubes then centrifuged for 30 min at 8000 x g. The supernatants were transferred into new tubes and the same volume of isopropanol was added. After centrifugation at 8000 x g for 30 min, the pelleted DNA was washed with 70 % ethanol and precipitated again by centrifugation at 8000 x g for 15 min. After removal of the supernatant, the pellet was air dried and then dissolved in 1 ml of 1x TE.

2.3 Methods for bacteriological cell culture and biochemical analysis

The *Escherichia coli* strain XL1-Blue MRF' (Stratagene) was used in all experiments. The following concentrations of antibiotics were used: 100 µg/ml ampicillin, 15 µg/ml tetracycline, and 30 µg/ml chloramphenicol. Protein expression was induced by the addition of iso-propyl-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM.

2.3.1 Large scale plasmid extraction

The *E. coli* strain XL1-Blue MRF' (Stratagene) was transformed with the plasmids pCMV-gp350-EHis and pCMV-BNRF1-EHis and stored at -80 °C. When needed, aliquots of the frozen stock were streaked out on agar plates containing ampicillin (100 µg/ml) and incubated at 37 °C. A single colony was picked and used to inoculate 400 ml of LB medium containing 100 µg/ml ampicillin in an Erlenmeyer flask.

After overnight incubation at 37 °C in the bacteria shaker, the now turbid fluid was transferred to centrifugation bottles, centrifuged at 300 x g for 15 min and the plasmids extracted using the JETstar 2.0 plasmid DNA extraction kit (Genomed). The supernatant was discarded and the bacteria resuspended in 10 ml of buffer E1 containing 100 µg/ml RNAse A. The bacteria were then lysed by adding 10 ml of E2 lysis buffer and mixing by gently flipping the tube. Subsequently, 10 ml of buffer E3 were added. Following centrifugation at 3400 x g for 15 min, the clear supernatant was loaded onto Maxi prep columns that had been

equilibrated with 15 ml of buffer E4. After washing the columns three times with 20 ml E5, the DNA was eluted using 15 ml of elution buffer E6.

The plasmid DNA was precipitated by adding 10.5 ml of isopropanol and pelleted by centrifugation at 8200 x g for 20 min. The supernatant was discarded and the air-dried pellet dissolved in 1 ml of 1x TE.

2.3.2 Phenol-chloroform extraction and precipitation of DNA

An equal volume of a mixture of phenol, chloroform and isoamyl alcohol (proportions 25/24/1) was added to the DNA solution in order to remove contaminants. The phases were separated by centrifugation at 13000 x g for 10 min. The upper aqueous phase containing the DNA was transferred into a 1.5 ml Eppendorf tube, the DNA could then be precipitated by the addition of 2.5 volumes of 100 % ethanol and 0.1 volume of 3 M sodium acetate and following centrifugation at 13000 x g. The DNA pellet was then washed in 70 % ethanol, dried and redissolved in deionised sterile water. The DNA concentration could then be measured using a UV-spectrophotometer.

2.3.3 DNA restriction

For DNA restriction, 2 to 4 units of the respective restriction enzymes were added to 1 µg of DNA in the appropriate restriction buffer, in a total volume corresponding to the 10x volume of enzyme added. Buffers and enzymes were bought ready-to-use and applied as recommended by the manufacturers (NEB

or MBI Fermentas). The restriction mix was incubated for 1-2 h at the optimal temperature for enzymatic activity.

2.3.4 Separation of DNA fragments by agarose gel electrophoresis

Gels with an agarose concentration between 0.8-1.2 % and with an ethidium bromide concentration at 0.4 µg/ml in 1x TAE were used for the separation of the DNA fragments. The agarose concentration of the gel was dependent on the expected size of the DNA fragment: the bigger the fragment, the lower the agarose content.

DNA samples were mixed with 6x concentrated gel loading buffer and loaded into the gel slots. Electrophoresis was performed in the horizontal position with 1x TAE running buffer, the voltage was applied at 5-10 V/cm. A 1 kb DNA ladder (MBI Fermentas) was used as a size standard.

2.3.5 Generation of gp350 protein pools

The frequently cutting restriction enzymes HaeIII, MnlI, AciI (Biolabs) and CviJI* (EurX) were used to digest the open reading frame encoding gp350. T4 polymerase served to generate blunt ends.

The expression vector mix contained equal amounts of the plasmids F315A, F315B, and F315C that had been cut with StuI and further with MscI, Eco47III, or SnaBI and had been treated with calf intestinal alkaline phosphatase for the prevention of the religation of the vector. The fragments of the ORF of gp350

were ligated to this expression vector mix. The isolation of the vector DNA was done by phenol-chloroform precipitation and following separation by agarose gel electrophoresis. The purification of the DNA was done with a gel extraction kit (Qiaex II, Qiagen). The efficiency of the whole process was tested by transformation of XL1-Blue MRF cells with an aliquot of the ligation mix, growing them on agar plates containing ITPG and chloramphenicol, and counting the colonies afterwards.

In the next step, 48 bacterial colony forming units were used to inoculate Superbroth medium cultures, containing ampicillin and tetracycline. The protein expression was induced by the addition of IPTG at a final concentration of 1 mM. When the cultures had reached an OD 600 of 0.8, 30 µg/ml chloramphenicol was added. After four hours, the bacteria were harvested by centrifugation (3000 x g for 15 min) and the bacterial pellet resuspended in 50 ml of lysis buffer.

After centrifugation (5000 x g, 15 min) the histidine (His)-tagged proteins were extracted by nickel-nitrilotriacetic acid agarose beads (Qiagen) and dialyzed in phosphate-buffered saline for 48 h. The protein concentration was measured using Bradford reagent (Bio-Rad).

Subsequently, the proteins were separated by SDS-PAGE. Coomassie staining and Westernblot (AntiXpress antibody, Invitrogen; ECL Plus detection system, GE Healthcare) were performed to prove the identity and purity of the proteins.

2.3.6 Western blot analysis

Recombinantly expressed and purified proteins were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The separation gel was prepared by mixing 7 ml of 30 % acrylamide

(Rotiphorese), 10 ml 2x TRIS/SDS pH 8.8, 2.8 ml H_2O, 167 µl freshly prepared ammonium persulfate (APS, 10 % in H_2O) and 17 µl tetramethylethylenediamine (TEMED). The solution was poured into a gel pouring cast and overlaid with n-butanol. When the gel had solidified, the stacking gel was prepared by mixing 2 ml 30 % polyacrylamide (PAA), 7.5 ml 2x TRIS/SDS pH 6.8, 5.4 ml H_2O, 90 µl fresh APS, and 10 µl TEMED. After removal of n-butanol, the separation gel was overlaid with the stacking gel. The chamber was filled with the running buffer. After loading the samples and a size standard, the gel was run for 1 h at 90 V.

Afterwards, the proteins were transferred electrophoretically (1 h at 120 V) from the gel onto a polyvinyl-difluoride (PVDF) membrane (Hybond P, GE Healthcare) using transfer buffer. The membrane was then blocked in 1x TBS with 5 % skim milk powder for at least 1 h and washed three times for 5 min with 3 % skim milk powder in 1x TBS. For immunodetection of the recombinant proteins, the membrane was incubated with diluted (1:10 in 3 % skim milk powder in 1x TBS) supernatant from a hybridoma producing the anti-EBNA1 antibody 1H4 (kindly provided by Dr. E. Kremmer, Helmholtz Zentrum München) for at least 4 h, washed three times, and then incubated with horse-radish peroxidase-coupled anti-rat IgG antibody (1:10 000 in 3 % skim milk powder 1x TBS) for 1 h. The membrane was washed again three times as before and once in H_2O.

For detection, the membrane was incubated with ECL Plus developing solution for 5 min, and exposed to autoradiography.

2.4 Methods for T cell function and phenotype analysis

2.4.1 Magnetic cell separation

To obtain CD4+ T cells, T cells from peripheral blood were subjected to magnetic cell separation (MACS). CD4+ T cell enrichment was achieved either by positive or negative selection.

Cells destined to be sorted were washed, resuspended at 1 x 10^7 cells in 80 µl MACS buffer and incubated with 20 µl of anti-CD4 Ig microbeads (Miltenyi) on ice for 20 min. After 20 min, the beads-labeled cells were washed once with MACS buffer, pelleted by centrifugation and then given onto a MACS column (Miltenyi). The column had previously been equilibrated with MACS buffer and been attached to a magnetic stand.

When a negative selection was performed, the flow-through with the unlabelled cells was collected. For a positive selection, the column was removed from the magnetic stand and its content was washed out into a 15 ml Falcon tube containing 5 ml MACS buffer. The desired cell fraction was then pelleted by centrifugation, washed and resuspended in medium for further use.

2.4.2 Flow cytometric analysis of T cells

T cells were analyzed by fluorescence activated cell scanning (FACS) for surface CD3, CD4, CD8, CD14, CD20 and CD56 expression. All fluorescence-labeled antibodies were bought from BD Biosciences.

Approximately 3 x 10^5 cells for each sample were collected in a 1.5 ml Eppendorf tube, washed once with cold FACS buffer. For labeling the cells, they were incubated for 30 min on ice with one or two antibodies in 100 µl FACS buffer. One sample without antibody for each series of measurements served as reference for the setup.

All incubations with fluorescence-labeled antibodies were performed in the dark, using light protection for all tubes containing labeled cells. Subsequently, the cells were washed 3 times in 1 ml FACS buffer, pelleted and resuspended in 400 µl FACS buffer containing 0.1 mM propidium iodide. The cell samples were then ready for the analysis on a FACScan flow cytometer with CellQuest software (Becton Dickinson).

2.4.3 Measuring T cell IFNγ secretion by ELISA

ELISA kits (R&D Systems) were applied to measure IFNγ release of the T cells to test their ability of target cell recognition.

The T cells were co-cultured with the appropriate antigen presenting cells (LCL, miniLCL) in 96-well plates and 200 µl of LCL medium per well. When specific reactivity had to be tested, antigen presenting cells had previously been incubated with their respective recombinant protein (during at least 24 h) or peptide (during at least 2 h). After the incubation, the target cells were washed thoroughly to remove excess protein or peptide, and then transferred to co-culture with 1 x 10^5 T cells per well. In control wells, APC and T cells were assayed separately (negative controls). After 20 to 24 h of incubation, the cell culture supernatant was harvested and the cytokine content measured following the general ELISA protocol as provided by the manufacturer. An ELISA Reader (Tecan)

with Xread Plus software was employed for measurement, fluorescence for the IFNγ ELISA was quantified photometrically at the wavelength of 450 nm.

2.4.4 IFNγ ELISPOT assay

The Elispot assay is widely applied for the investigation of specific immune responses in various diseases. In contrast to ELISA, antigen-specific responses can be detected at a single cell level.

A study performed on IFNγ responses to Varicella-zoster virus in Elispot (Smith et al., 2001) showed that IFNγ signals were derived almost entirely from CD4+ cells. In addition, it was shown that the results from assays with frozen cells did not differ significantly from those with freshly isolated cells.

Although often disputed, Elispot assays were recently shown to be reproducible under different laboratory conditions, even when Elispot-inexperienced researchers performed the experiments (Zhang et al., 2009).

ELISPOT assays were performed according to the guidelines of the manufacturer (Mabtech) to quantitate the number of EBV-specific IFNγ producing CD4+ cells in peripheral blood of seropositive healthy donors and patients with IM. Briefly, 96-well PVDF (polyvinyl-difluoride) membrane plates (Millipore Multiscreen®HTS) were treated with 70 % ethanol, then washed with PBS buffer and subsequently coated with 100 µl of 1:100 diluted (in PBS) mAb 1-D1K antibody per well and incubated overnight at 4 °C. After another washing step, 150 µl of T cell medium was added per well and the plates were incubated again at 4 °C overnight. On the third day, PBMC were extracted from fresh human blood of healthy donors and counted. The CD4+ fraction was separated by MACS. Both, the CD4+ and the CD4- fractions were counted. The Elispot plate was washed once again, and 100 000 CD4- cells were plated as APC in 100 µl AIM-V

medium. Virus and the CD4+ cells were plated following the test settings (3×10^5, 1×10^5, 3×10^4 or 1×10^4 cells in 100 µl AIM-V medium) and the plates were put to overnight incubation at 37 °C. The fourth day, after washing the plates with PBS + Tween-20, 100 µl/well of the detection antibody 7-B6-1-Biotin, diluted 1:1000 in 100 µl 1 % BSA in PBS, was added and the plates incubated overnight at 4 °C. The fifth day, the plates were washed again with PBS and detergent and then incubated with 100 µl of Streptavidin-ALP per well, diluted 1:1000 in 1 % BSA in PBS, for 1 h at room temperature. After a last washing step, 100 µl per well of filtered BCIP/NBT Plus substrate were added and incubated on the shaker for 10 minutes at room temperature. After apparition of dark blue spots, the reaction was stopped with tap water.

3 RESULTS

3.A Analysis of the frequency of EBV structural antigen-specific CD4+ T cells in the peripheral blood of healthy virus carriers and patients with infectious mononucleosis

The physiologic properties of virus-specific CD4+ T cells, their features as their reactivity, dynamics and their role in infectious and immunologic processes are still largely unknown. The following work aims at contributing to our knowledge on the role of CD4+ cells in EBV infection by analysing the frequency of EBV structural antigen-specific CD4+ cells in healthy EBV carriers and IM patients. To this aim, the percentage of virion-specific CD4+ T cells within peripheral blood was measured using IFNγ ELISPOT assay.

3.A.1 Separating cells by MACS: purity of the cell fractions

For all assays shown, CD4+ T cells from peripheral blood were isolated by magnetic cell separation using either positive or negative selection. For positive selection, peripheral blood mononuclear cells (PBMC) were incubated with an anti-CD4 antibody coupled with iron particles (microbeads) that allow sorting of bound cells in a strong magnetic field. The microbead is of a biodegradable matrix and thus does not have to be removed after cell separation.

For negative cell selection, antibodies specific for all other cell subtypes are used, i.e. anti-CD20 antibody for B cells, anti-CD14 antibody for monocytic

cells, anti-CD8 antibody for CD8+ lymphocytes and anti-CD56 antibody for NK cells. All cells except CD4+ cells will stick to the magnetic column, while CD4+ cells are contained within the through-flow. To evaluate separation efficiency, both positively and negatively separated cell fractions were analyzed by FACS. Although both methods yielded similar results (*data not shown*), positive selection proved to be faster and easier and thus better suited for routine application.

3.A.1.1 FACS analysis of the positively selected cell fraction

PBMC were incubated with anti-CD4 microbeads and then separated by MACS.

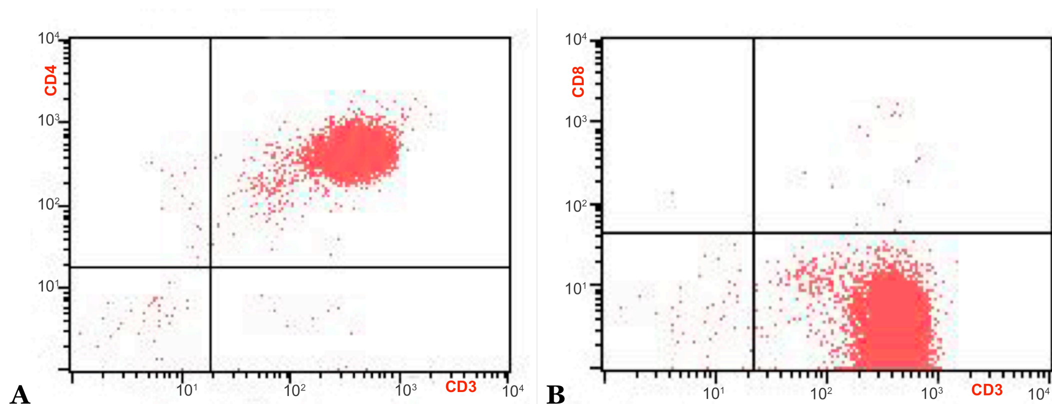


Fig. 7: FACS analysis of positively selected cells

Positively selected cells were stained with anti-CD3 antibody (CD3-FITC) and, in addition, with antibodies directed against CD4 (CD4-PE) (A) or CD8 (CD8-PE) (B), and analyzed by FACS. The selected cell population contained almost exclusively CD3+ CD4+ double positive cells. Only few CD3-negative or CD3+ CD8+ double positive cells were detected, indicating that an almost pure CD4+ T cell population was obtained by positive selection.

Positively selected cells were stained with an antibody directed against the T cell marker CD3 and, in addition, with antibodies specific for CD4 or CD8, and then analyzed by FACS (Fig. 7). The positively selected cell fraction consisted almost

exclusively of CD3 and CD4 double-positive cells. Thus, an almost pure CD4+ T cell population was obtained by this positive selection method.

3.A.1.2 FACS analysis of the flow-through fraction

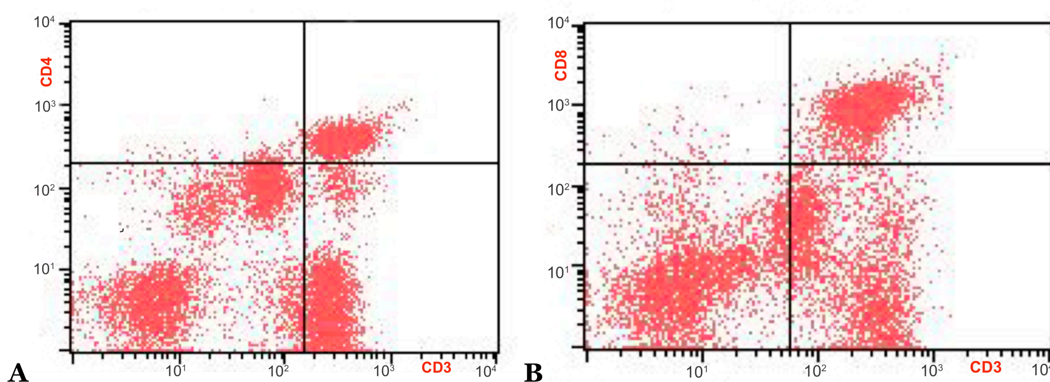


Fig. 8: FACS analysis of the flow-through fraction of CD4+ selected PBMC

The cells of the flow-through fraction were double stained with antibodies specific for the T cell marker CD3 (CD3-FITC) and, in addition, with an antibody specific for CD4 (CD4-PE) (A) or CD8 (CD8-PE) (B) and analyzed by FACS. A substantial amount of CD3/CD4 double positive T cells was still detected in this fraction, indicating that the separation was not complete.

As shown in Fig. 8A, a significant CD3+ CD4+ cell fraction – about 16 % of the total cell number - was still present in the flow-through fraction after MACS. This indicated that some cells had not been extracted efficiently. Most likely, the amount of anti-CD4 antibody added to the PBMC was limiting, leading to the loss of some CD4+ cells. By contrast, the CD3+ CD8+ T cell population was not diminished, demonstrating that selectively CD4+ T cells were separated by this method (Fig. 8B).

Therefore, increasing amounts of anti-CD4 microbeads were used to define the minimum concentration required to select all CD4+ cells. As shown in Fig. 9,

almost no CD3+ CD4+ double positive cells were found in the flow-through fraction under these conditions (20 µl of CD4 microbeads per 1 x 10^7 total cells).

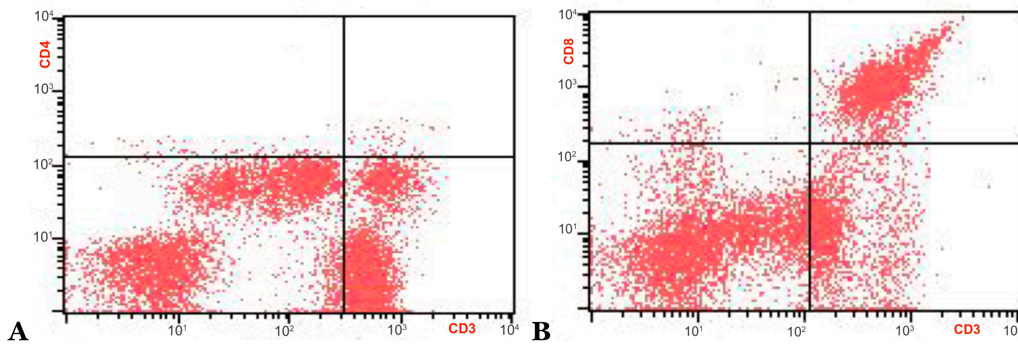


Fig. 9: Efficient separation of CD4+ T cells with higher amounts of CD4-microbeads

The flow-through fraction of PBMC selected with twice the amount of CD4-microbeads were stained with antibodies against CD3 (CD3-FITC), CD4 (CD4-PE) and CD8 (CD8-PE) and analyzed by FACS. Almost no CD3/CD4 double-positive T cells were detected in the flow-through fraction, indicating that the positive selection of CD4+ cells from PBMC worked efficiently when sufficient amounts of CD4-microbeads were used (A). By contrast, the CD3/CD8 double-positive T cell population remained unchanged (B), excluding that this increased antibody concentration resulted in a non-specific selection of cells.

3.A.1.3 Analysis of the cell subtypes present in the CD4- fraction after CD4+ MACS

In subsequent IFNγ ELISPOT assays, cells of the flow-through fraction were used as antigen presenting cells. As cytokines secreted by these cells could possibly influence and bias the results, it was important to characterize identity and proportion of cell subpopulations present in this fraction.

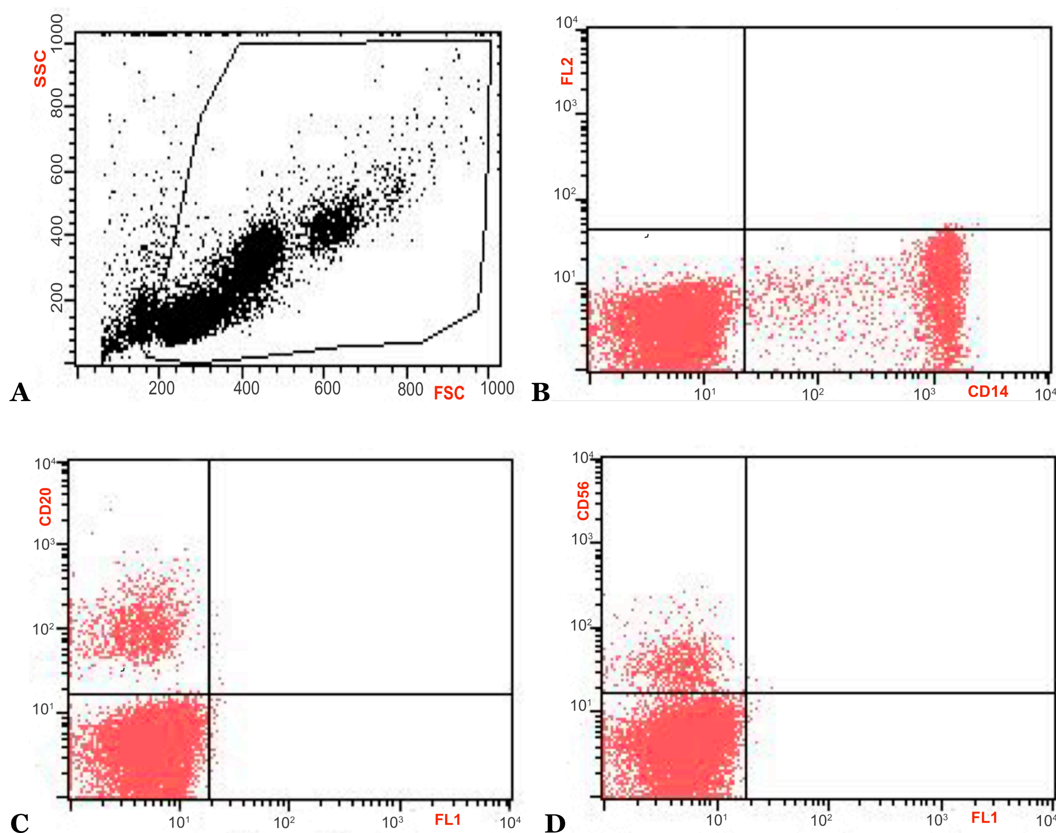


Fig. 10: FACS analysis of the cell types present in the CD4- fraction

(A) Forward and sideward scatter of the cell population. Living cells were gated and further analyzed for expression of CD14 (CD14-FITC) (B), CD20 (CD20-PE) (C), and CD56 (CD56-PE) (D). CD14+ monocytes represented about 30 % (B), CD20+ B cells about 11 % (C) and CD56+ NK cells about 7 % (D) of the gated cells in the CD4- fraction.

By using antibodies specific for cell surface markers expressed on monocytes (CD14), B cells (CD20), NK cells (CD56), and CTL cells (CD8), the presence and the proportion of these cell-types in the flow-through fraction was assessed by FACS. As shown in Fig. 10, the CD4- cell fraction contained approximately 30 % CD8+ T lymphocytes, 30 % cells of the monocytic lineage, 7 % NK cells, 11 % B cells, as well as some dendritic cells, residual CD4+ cells and some not further defined cell types (*data not shown*).

3.A.2 Optimization of the IFNγ ELISPOT protocol

The Elispot assay is used to detect cytokine secretion on the single cell level. The basic principle is the same as for ELISA assay: the plate, a polyvinylidene fluoride membrane, is coated with a cytokine-specific antibody, the cells are plated, stimulated with the antigen of interest, and then washed away. Subsequently, a biotinylated antibody recognizing a different epitope of the cytokine is added. After addition of streptavidin-coupled alkaline phosphatase and soluble colour substrate, insoluble colour precipitates are deposited on the membrane where cytokine has been captured. Thus, the quantification does not result from absorption measurement as in ELISA, but from counting the number of spots on the plate.

To assess the frequency of EBV structural antigen-specific CD4+ T cells, CD4- cells were pulsed with virus concentrate and then co-cultured with CD4+ T cells from the same donor. Depending on the number of PBMC available, Elispot assays were performed in duplicates or triplicates for each setting. To evaluate the basal level of IFNγ secretion and, hence, the activation state of the cells, each experiment was accompanied by a negative control in which no virus was added to the cells. In the same way, negative controls were included in each set-ting by omitting either CD4+ or CD4- cells.

Earlier studies had raised concerns that the reactivity of freshly isolated versus cryo-preserved and re-thawed cells yielded similar results in ELISPOT experiments. Own experiments indicated that the levels of IFNγ secretion, as measured by the Elispot assay, were about the same for both settings, yet there was less background activity in the assays with frozen cells (*data not shown*). Background activity may pose a severe problem for the evaluation of Elispot assays because the presence of many very small spots or artefacts produced by cell

clumps complicates the quantification of specific spots. Hence, all further assays were performed by using cryopreserved cells.

Furthermore, it was tested whether T cell function was affected by the virus preparation. For example, residual cytokines secreted by the virus producer cell line or other metabolites might still be present in the preparation and inhibit or activate the T cells.

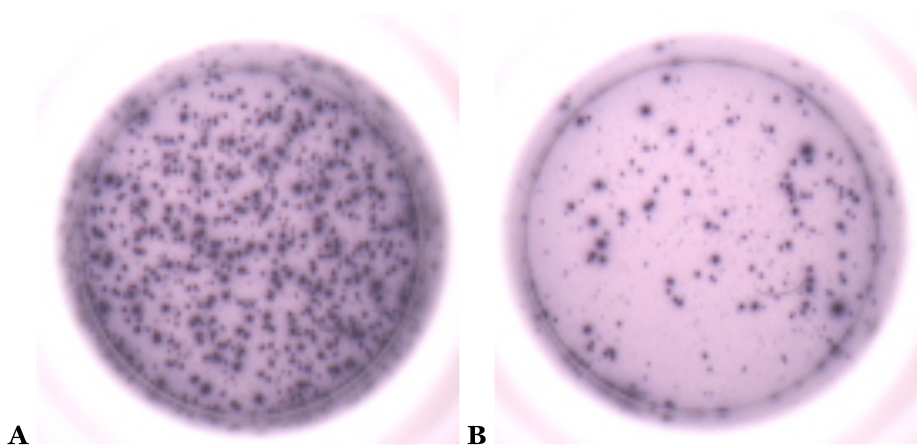


Fig. 11: Influence of cell medium on Elispot results

In the Elispot wells shown, 3 x 10^5 CD4+ cells, 1 x 10^5 CD4- cells and 10 µl virus had been plated in LCL medium (A), or AIM-V medium (B). The number of spots in wells without virus (negative control) was also higher in LCL as compared to AIM-V medium (*data not shown*). These results indicated that the higher number of spots in (A) might have been caused by xenogenic proteins present in bovine serum that are recognized by human T cells.

Therefore, increasing amounts of EBNA3C protein were loaded onto LCL and these protein-pulsed cells used as targets for EBNA3C-specific CD4+ T cells. To these cells, 1 or 10 µl of 20-fold concentrated virus supernatant, or 100 µl of non-concentrated virus supernatant in AIM-V medium only, or 100 µl of non-concentrated virus supernatant in AIM-V medium supplemented with bovine serum, were added. The different virus preparations had no significant effect on the number of spot forming units (SFU) (*data not shown*). Therefore, 10 µl of

20x concentrated virus supernatant in AIM-V medium were used per well in all further experiments.

To investigate which medium is best suited for the ELISPOT assay, CD4- antigen presenting cells and CD4+ responder cells were incubated with virus supernatant and either cultured in LCL medium or in AIM-V medium.

A significantly higher number of spots was obtained when LCL medium was used (Fig. 11A). However, the number of spots was also increased in control wells, suggesting that LCL medium led to a non-specific increase in background levels. Similar results were obtained when cells of different donors were used. Therefore, AIM-V medium was used in all following Elispot assays.

The Assay Working Group of the Cancer Vaccine Consortium has recently identified the choice of serum to be the leading cause for variability and suboptimal performance in large international Elispot proficiency panels. For this reason, a recently published multicenter study (Janetzki et al., 2009) addressed that question and showed that the use of serum-free medium does not cause cell loss or negative effects on cell viability, but that background activity is significantly reduced in comparison to serum-containing medium. Our results accord with these findings.

3.A.3 Measuring IFNγ secretion by EBV-specific CD4+ cells

To gain further insight into the EBV-specific CD4+ T cell response, we sought to investigate the frequency of virus antigen-reactive CD4+ T cells in peripheral blood of healthy virus carriers and patients with IM during acute infection and during convalescence. Because T cells secreting IFNγ are considered as main effector cells in the control of EBV infection and because previous results by our group and others had indicated that the frequency of individual viral epitope-

specific CD4+ T cells in peripheral blood is low (Rickinson and Kieff, 2006), we chose IFNγ ELISPOT to assess the frequency of these T cells.

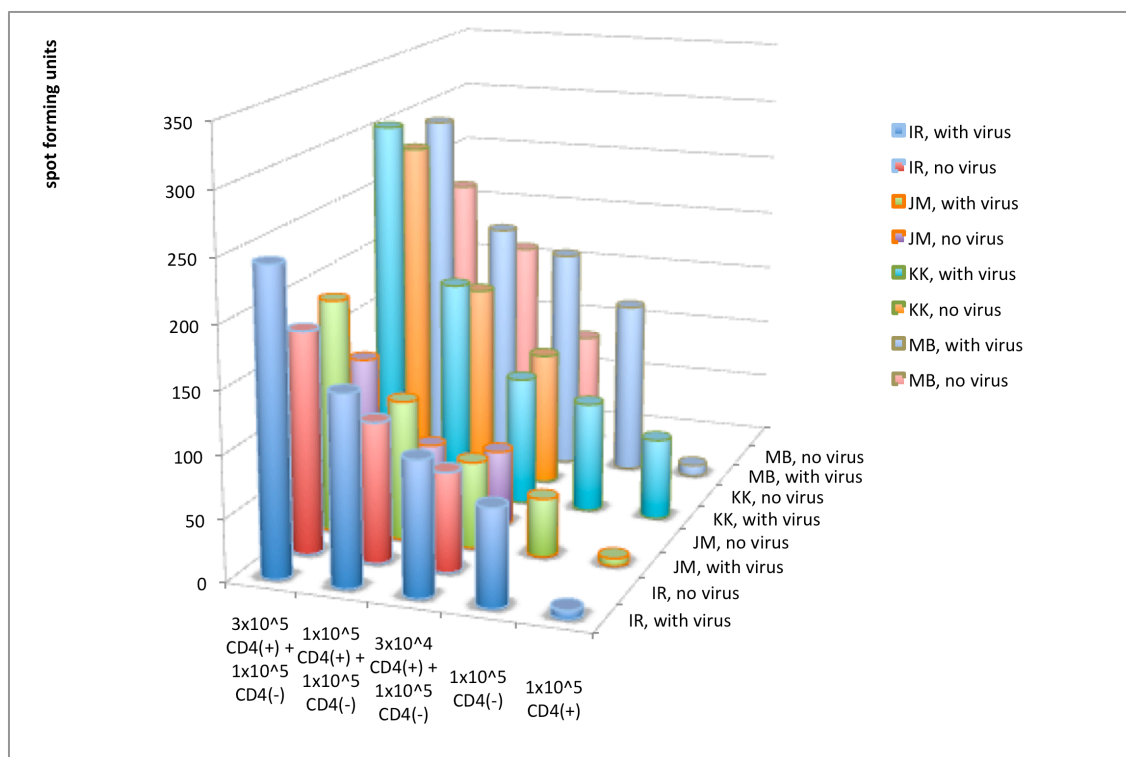


Fig. 12: Measuring the frequency of EBV-specific, IFNγ producing CD4+ cells in peripheral blood of four healthy virus carriers

Decreasing numbers of CD4+ T cells from four healthy EBV-seropositive donors (IR, JM, KK, MB) were probed with 1 x 10^5 autologous CD4- antigen presenting cells that had or had not been pulsed with EBV, and the number of reactive T cells determined by IFNγ Elispot. All measurements were performed in triplicates and the mean value is shown for each donor. The frequency of virus-specific CD4+ T cells corresponds to the number of spot forming units (SFU) in the presence of virus minus the number of SFU in the absence of virus. All samples with CD4+ and CD4- cells were done with and without virus. Two negative controls with only one cell group plus virus were additionally examined.

Cells were plated in triplicates in decreasing numbers: 300 000, 100 000, 30 000 and 10 000 CD4+ cells per well. To each well, 100 000 CD4- cells and 10 µl of 10-fold concentrated wild type virus were added.

As a control, the cells were plated without virus in parallel. IFNγ-secreting T cells in these wells were either autoreactive, or spontaneously activated under these experimental conditions. In addition, CD4+ cells plus virus and CD4- cells plus virus were included as controls to detect spontaneous IFNγ production by these cell populations in response to virus supernatant.

The number of SFU correlated with the number of CD4+ cells per well and depended on the presence of both cell fractions. In the absence of APC, IFNγ secretion was significantly reduced, and in the absence of CD4+ cells, nearly no SFU were detected. Thus, IFNγ was predominantly produced by CD4+ T cells and this production was dependent on contact with CD4- APC.

The virus-specific CD4+ T cell response was quantified by subtracting the number of SFU obtained in response to CD4- alone from the number of spots obtained in the presence of virus.

As shown in Fig. 12, the number of spots in wells with 3×10^5 CD4+ or 1×10^5 CD4+ cells was increased when virus was added.

On average, the presence of EBV increased the number of SFU by about 20 percent. Thus, between 0.3 to 1 virus-specific CD4+ T cells per thousand CD4+ T cells were detected in peripheral blood of healthy virus carriers with this method.

3.A.4 Assessing the frequency of virus-specific CD4+ T cells during infectious mononucleosis

The above experiments demonstrated that EBV-specific CD4+ T cells can be detected by IFNγ Elispot. However, the frequency of these T cells in peripheral blood of healthy virus carriers proved to be extremely low.

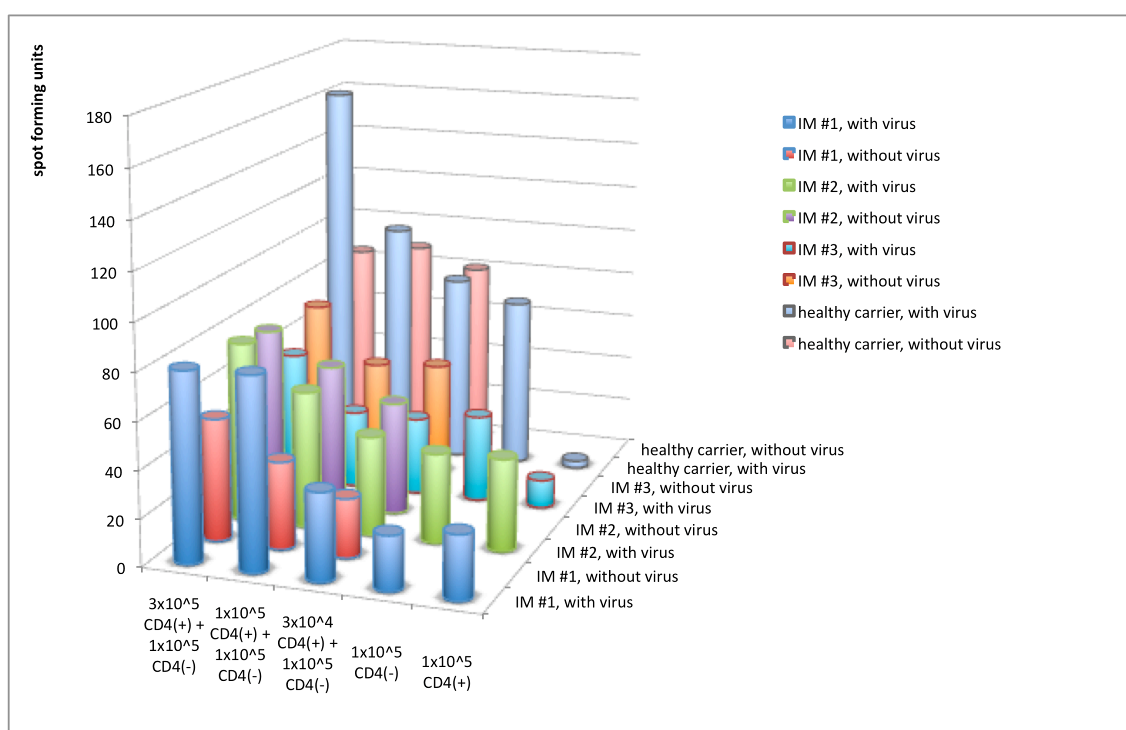


Fig. 13: Frequency of EBV-specific CD4+ T cells in peripheral blood of patients with acute infectious mononucleosis

Decreasing numbers of CD4+ T cells from three patients with IM and one healthy virus carrier were co-cultured with virus-pulsed or -unpulsed autologous CD4-depleted PBMC, and the number of IFNγ secreting cells (SFU) determined by Elispot. Numbers of SFU are the averages from duplicate determinations. In IM, lower numbers of virus-specific CD4+ cells were detected than in healthy virus carriers.

In patients with IM, the frequency of virus-specific CD8+ T cells increases dramatically; up to almost 50 % of all peripheral CD8+ T cells have been shown to respond against viral antigens. Whether the virus-specific CD4+ compartment expands comparably is not known. Therefore, we extended these analyses to patients with IM.

Surprisingly, compared to healthy virus carriers, patients with IM showed significantly lower numbers of spots, possibly indicating that fewer virion-specific CD4+ T cells were present in peripheral blood during acute versus persistent phase of infection. This might either indicate that these T cells have extravasated into lymphoid tissue, or that the development of the virus-specific CD4+ as compared to the CD8+ T cell response is inhibited or delayed. Alternatively, the specific CD4+ T cells, though numerous, are not yet stimulated sufficiently and do produce only small amounts of cytokines.

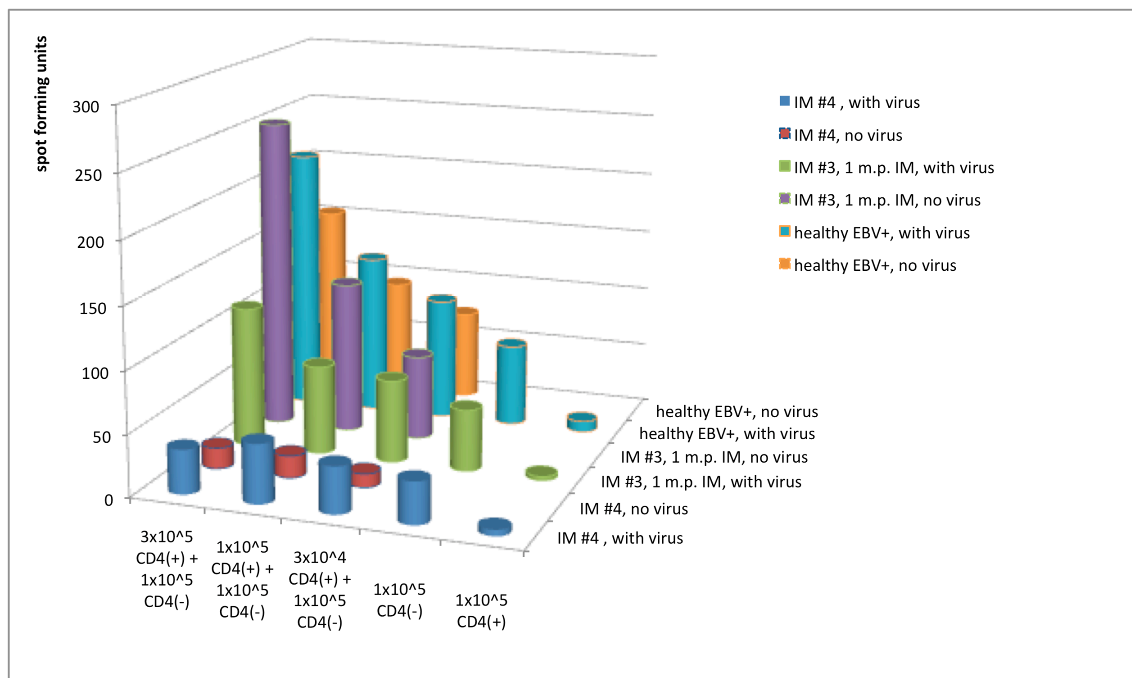


Fig. 14: The number of EBV-specific CD4+ T cells in a patients with infectious mononucleosis barely increases during convalescence

The frequency of virus-specific CD4+ T cells in peripheral blood of an IM patient was analyzed one month after acute infection by IFNγ Elispot, and compared to an IM patient during acute phase of infection and to a healthy virus carrier. The means of triplicate determinations are shown.

To differentiate between these possibilities, the frequency of EBV-specific CD4+ T cells was assessed one month after symptoms of acute IM had subsided.

As shown in Fig. 14, similar low numbers of virus-specific CD4+ T cells were detected in a patient with IM during convalescence as in a patient during acute phase of infection. The number of IFNγ secreting cells was even lower in samples to which virus had been added than in the controls. A non-specific inhibitory effect of the virus preparation could be excluded because the number of virus-specific CD4+ T cells in a healthy virus carrier and a patient during IM was as observed previously.

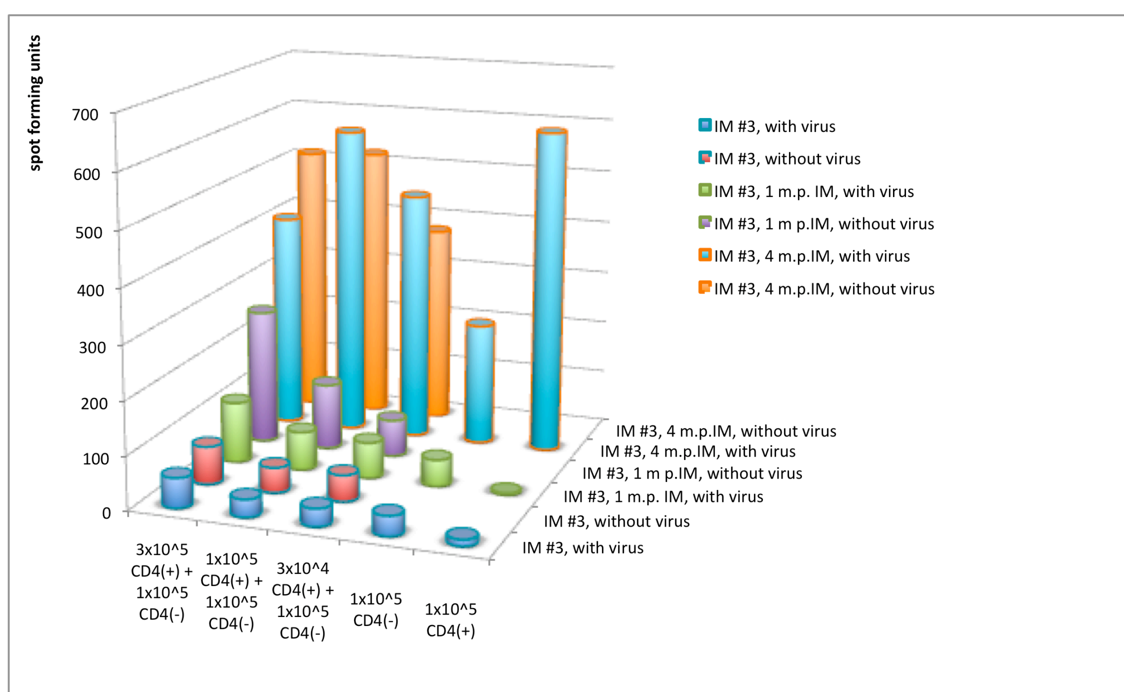


Fig. 15: Analysis of the virus-specific CD4+ T cell response in a patient during acute infection and at different time points post infection

The frequency of virus-specific CD4+ T cells in peripheral blood of a patient with IM was assessed by IFNγ Elispot during the acute phase of infection, as well as 1 and 4 months after clinical symptoms had subsided.

These results suggested that, compared to healthy virus carriers, lower frequencies of EBV-specific CD4+ T cells are present in pe-ripheral blood during acute

infection and during convalescence. To assess whether and when EBV-specific CD4+ T cells in patients suffering from IM reached levels comparable to healthy virus carriers, these analyses were ex-tended to later time points after acute infection.

Fig. 15 shows the data of one patient during acute and at different time points after infection. No virus-specific CD4+ T cells were detected in peripheral blood during or shortly after acute infection. However, 4 months after IM, an EBV-specific CD4+ T cell response became detectable.

These findings suggested that compared to virus-specific CD8+ T cells, there is a delay in the development of an EBV-specific CD4+ T cell response in patients suffering from IM. Whether this is also true when EBV is contracted asymptomatically, or whether the absence of a detectable CD4+ T cell response against EBV is responsible for the clinical symptoms, is currently not known. During convalescence, virus-specific CD4+ T cell counts in patients with IM slowly increase to levels observed in healthy virus carriers, which was found to be in the range of 1 in 10 000 to 1 in 100 000 PBMC (0.1-0.01 ‰), and thus approximately 100-fold lower than the corresponding CD8+ T cell response.

This low frequency of EBV-specific CD4+ T cells in the presence of a comparably strong autoreactive CD4+ T cell response precluded accurate measurements and impaired further analysis of the virus-specific T cell population.

Therefore, a second experimental approach was pursued. T cells from ten different EBV-positive donors were repeatedly stimulated in vitro with two EBV proteins that had been shown to elicit CD4+ T cell responses in healthy virus carriers. By this approach, larger quantities of EBV-specific cells should be obtained and further analyzed.

3.B Generation of gp350- and BNRF1-specific CD4+ T cell lines

To gain further insight into the antiviral immune response, we sought to study the CD4+ T cell response to the glycoprotein gp350 and the tegument protein BNRF1 in ten healthy virus carriers.

Gp350 is an outer EBV envelope glycoprotein and it is the most abundant virion protein (Kieff and Rickinson, 2006). The binding of gp350 to CD21 on the surface of human B cells is the first step in the infection of B cells and antibodies against gp350 abrogate EBV infectivity.

BNRF1 is the major EBV tegument protein (Feederle et al., 2006). It has been shown to be an important CD4+ T cell target antigen in EBV seropositive individuals (Adhikary et al., 2007).

3.B.1 Generation of antigen-specific CD4+ T cell lines

Peripheral blood samples were taken from ten healthy EBV-positive donors and PBMC isolated by Ficoll gradient centrifugation. PBMC from each donor were cryopreserved in fractions of 10 million cells per tube.

Table 2: HLA types of the ten PBMC donors used to generate EBV antigen-specific CD4+ T cell lines

Designation	HLA-DRB1	HLA-DQB1	HLA-DPB1	HLA-DRB3/4/5
AR (A)	0405, 1501	0302, 0602	0401	DRB4*0103 DRB5*0101
FL (B)	0301, 1101	0201, 0301	0101, 0201	DRB3*0101, 0202
IB (C)	0701,	0202, 0303	1101, 1301	DRB4*0103
IE (D)	0401, 1101	0301,	0401, 1601	DRB3*0202 DRB4*0103
KB (E)	0101,	0501,	0401, 0402	-
OW (F)	0803, 1301	0301, 0603	0101, 0201	DRB3*0202
SG (G)	0401, 1103	0301,	0401,	DRB3*0202 DRB4*0103
SH (H)	04, 1302	0301, 0604	0301, 0402	DRB3*0301 DRB4*0103
SL (I)	1302, 1501	0602, 0604	0201, 0401	DRB3*0301 DRB5*0101
TK (J)	0301, 1301	0201, 0603	0201, 0401	DRB3*0101, 0202

After thawing, cells were brought out in RPMI medium in two wells per protein in 24-well plates, four wells altogether per donor. These cells were incubated with 200 µl of gp350 protein or 75 µl of BNRF1 protein per well. The recombi-

nant proteins were purified from transiently transfected HEK293T cells, a highly transfectable human embryonic kidney cell line (Graham et al., 1977). Because of differences in the protein concentrations, different volumes of the protein solutions were used for T cell stimulation. Gp350 is a highly glycosylated protein and, compared to BNRF1, is produced in much lower amounts in HEK293T cells.

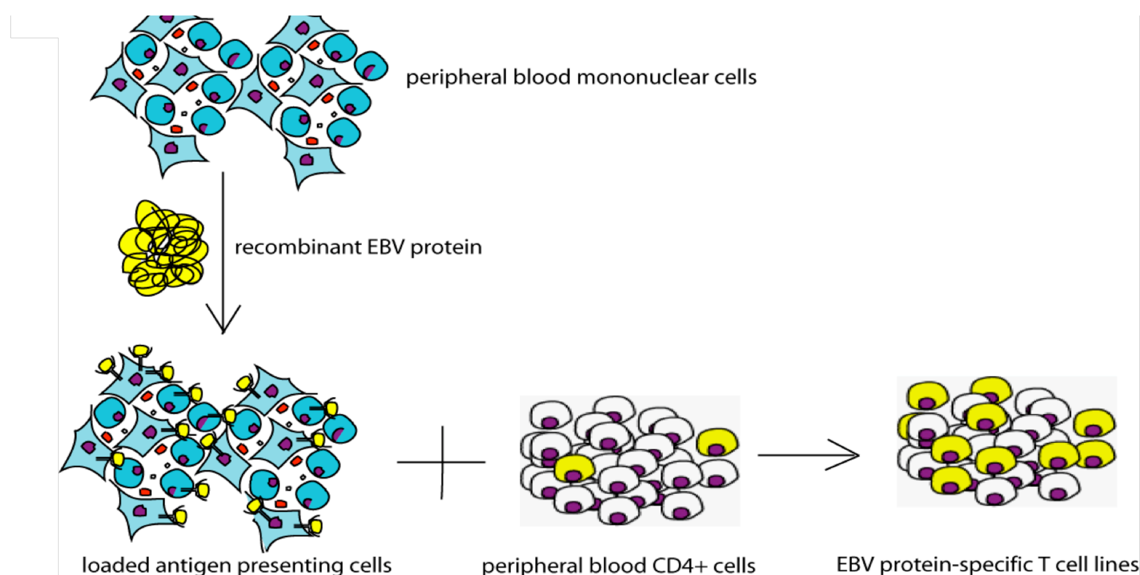


Fig. 16: Generation of EBV-specific CD4+ T cell lines

Outline of the experimental approach: EBV protein-specific CD4+ T cells were generated by repeated *in vitro* stimulation of CD4+ T cells from peripheral blood with protein loaded, autologous PBMC. Source: With friendly permission of Dr. Dinesh Adhikary.

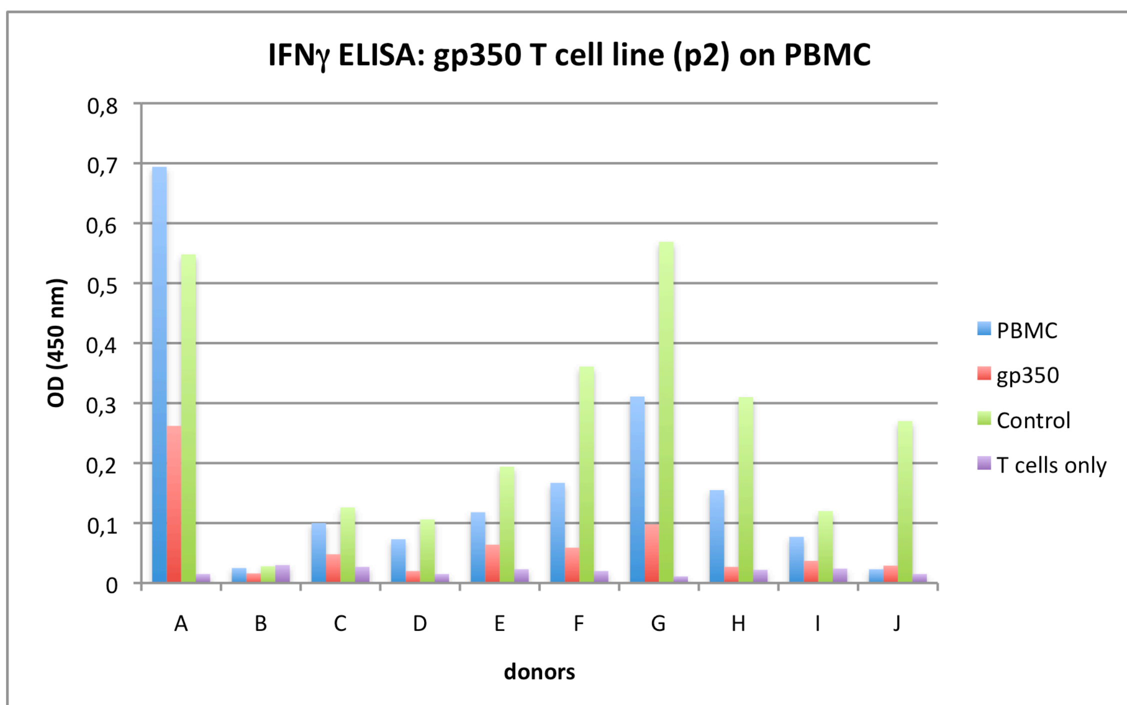
The cell lines were restimulated every other week, initially with protein-pulsed autologous PBMC, later, from the 6th stimulation onwards, with autologous, EBV-transformed LCL that had been incubated with the respective antigen. Before incubation with the CD4+ lines, APC were irradiated, LCL with 80 Gy and PBMC with 40 Gy, to preclude that they would overgrow the lymphocyte cultures. 24 h after stimulation, the T cell-stimulatory cytokine IL-2 (10 U/ml) was added to the cultures.

3.B.2 Specificity of T cell lines in IFNγ ELISA assays

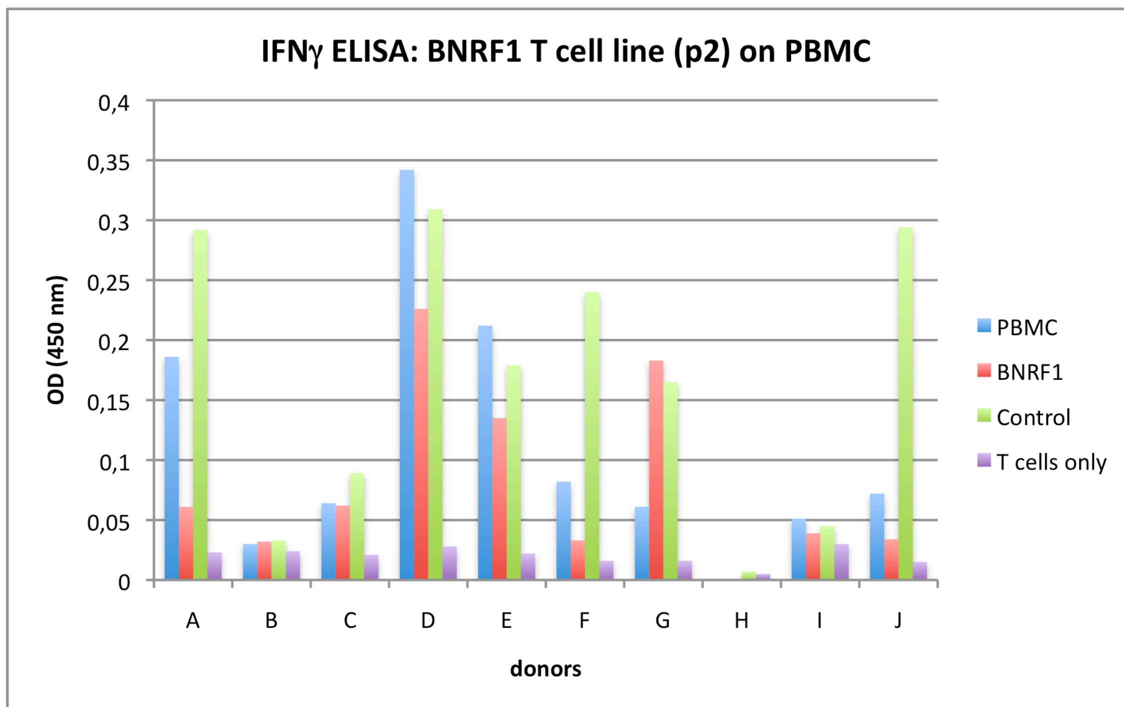
At the end of the stimulation period, specificity of the T cells was tested by incubating T cells with an equal number of target cells. APC were incubated with the target protein for 24 hours and then washed to remove excess protein. Subsequently, the cells were co-cultured at a 1:1 ratio with 1 x 10^5 T cells. Cytokine secretion was analyzed 20 h later by ELISA assay.

After the tenth stimulation, antigen-unresponsive lines were discarded while responding lines were followed up to 25 rounds of stimulation. The cell lines that secreted IFNγ in response to the target antigen were subjected to epitope mapping and restriction analysis.

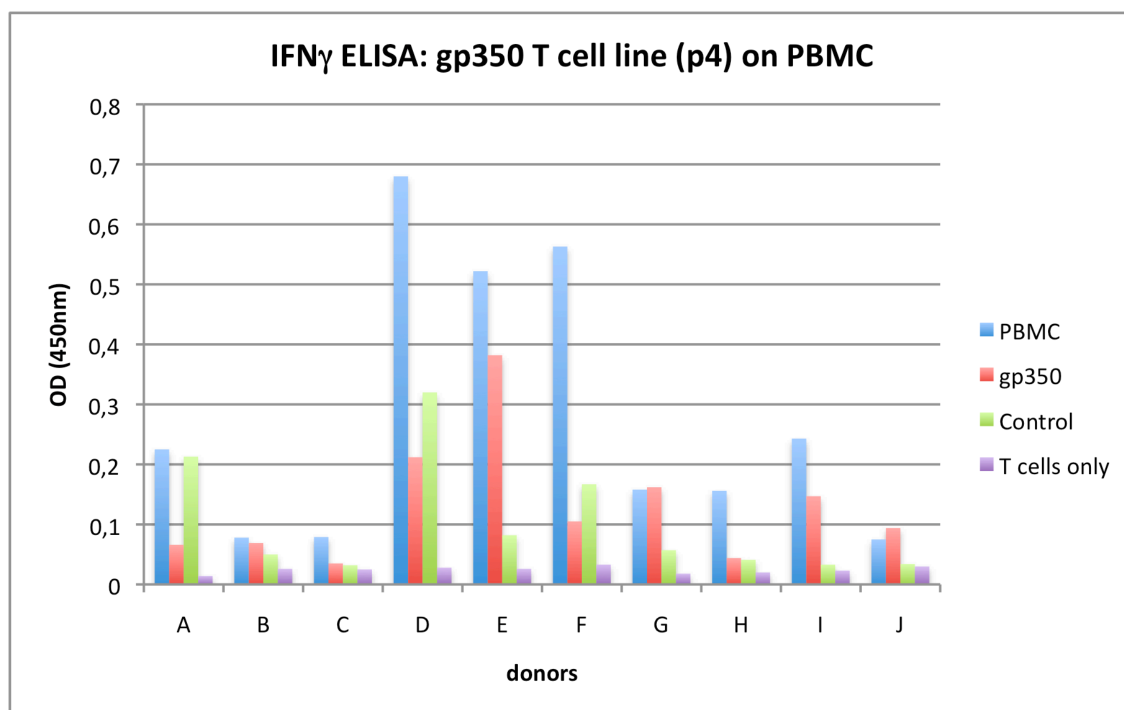
A



B



C



D

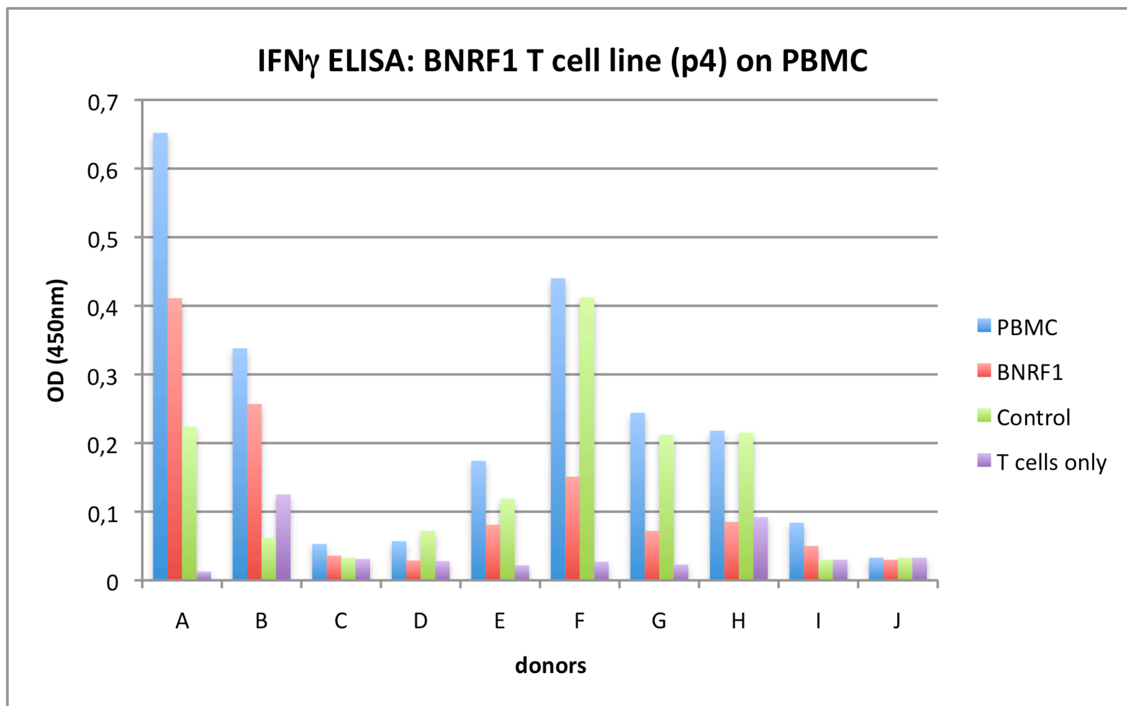
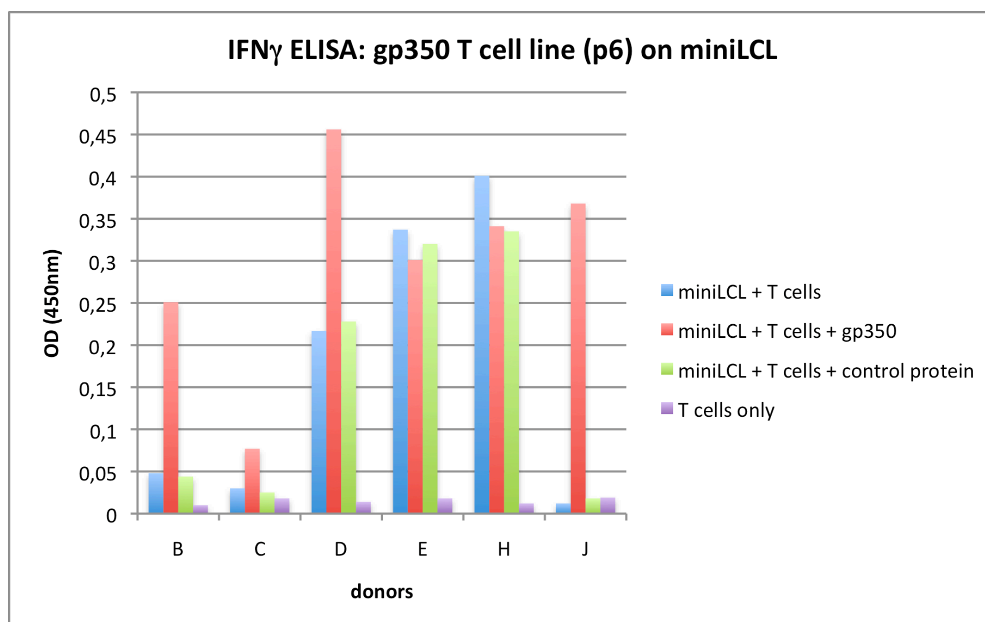


Fig. 17: Specificity analysis of the gp350 or BNRF1-stimulated T cell lines after two and four rounds of stimulation

Specificity of the gp350 and BNRF1-stimulated T cell lines were tested after two (A, B) or four (C, D) rounds of stimulation. Autologous PBMC pulsed with gp350 or BNRF1 proteins were co-cultured with the T cell lines. After 20 h, IFNγ secretion by the T cells was measured by ELISA. Unpulsed PBMC and PBMC pulsed with a control protein were used as controls. No antigen-specific IFNγ secretion was detected with these early passage T cell lines.

As expected from previous experiments of our laboratory, specificity against the proteins used for stimulation was not detected after two to four rounds of stimulation. The frequency of these cells in peripheral blood is usually very low and they need several rounds of stimulation *in vitro* to reach detectable levels.

A



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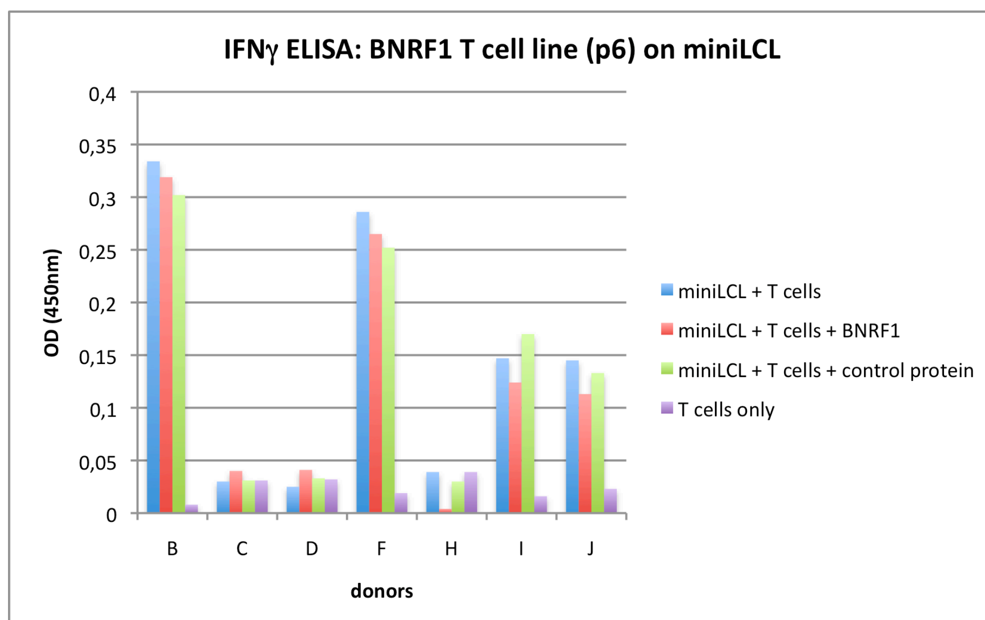


Fig. 18: Specificity analysis of the gp350 or BNRF1-stimulated T cell lines after six rounds of stimulation

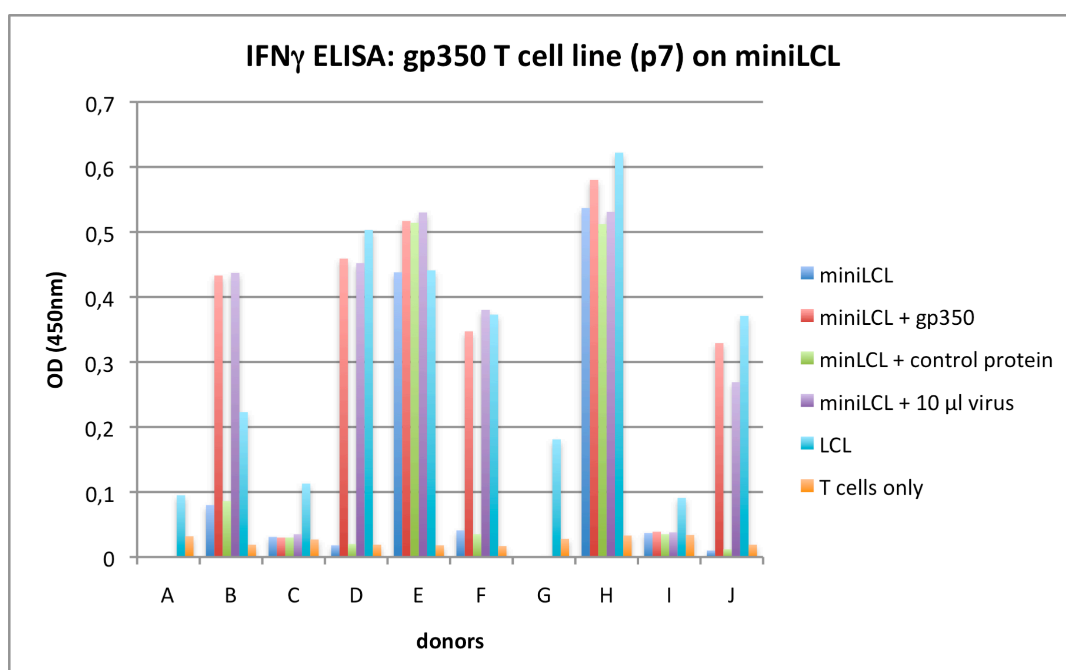
After six rounds of in vitro stimulation, gp350 (A) and BNRF1 (B) stimulated T cell lines were tested for reactivity against autologous miniLCL, miniLCL pulsed with the stimulator or a control protein by IFNγ ELISA. Four of the gp350-stimulated T cell lines specifically responded against target cells pulsed with gp350 protein (A). By contrast, none of the BNRF1-stimulated T cell lines specifically recognized target cells pulsed with BNRF1 protein (B).

After the sixth stimulation, four of the gp350 protein-stimulated T cell lines (lines B, C, D, J, see Fig. 18A) specifically secreted IFNγ upon co-culture with miniLCL pulsed with gp350 protein, suggesting that these lines became antigen-specific.

MiniLCL (Z-) are EBV-transformed B cells, generated by infection with a genetically engineered viral mutant that lacks the BZLF1 gene. LCL established by infection with this virus are phenotypically identical to wildtype LCL, but lack expression of the lytic cycle proteins gp350 and BNRF1.

By contrast, the T cell lines that had been established by repeated stimulation with BNRF1-protein pulsed target cells showed no enhanced secretion of IFNγ when probed with BNRF1 protein-pulsed APC. Instead, some of these lines responded vigorously against miniLCL, irrespectively of whether these cells had been pulsed with BNRF1 or a control protein or no protein at all. These T cell lines probably recognized a self antigen expressed by these cells.

A



B

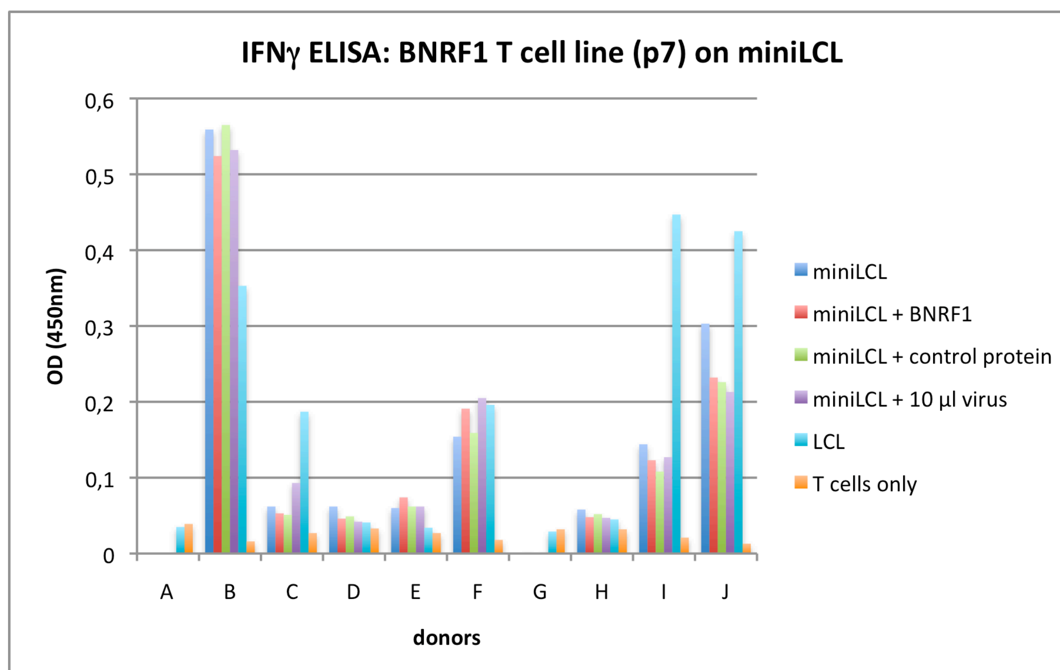


Fig. 19: Specificity analysis of gp350 and BNRF1-stimulated T cell lines after seven rounds of stimulation

After seven rounds of stimulation, the gp350 (A) and BNRF1 (B) T cell lines were probed with autologous miniLCL that had either been left untreated or pulsed with the stimulator or a control protein, and IFNγ secretion by the T cells measured by ELISA. In addition, autologous LCL as well as miniLCL pulsed with EBV were included as target cells. The T cell lines from donors A and G had expanded poorly, therefore these T cell lines were only tested against LCL.

After the seventh stimulation, responses of the T cells were also investigated upon co-culture with miniLCL that had been incubated with wild-type virus. Furthermore, the cytokine response after incubation with LCL was examined. LCL, in contrast to miniLCL, are able to express lytic cycle antigens.

In these experiments, antigen- as well as donor-dependent differences were observed. The gp350-stimulated T cell lines from donors B, D, F and J responded specifically against LCL as well as miniLCL loaded with gp350 protein or virus. These lines failed to recognize miniLCL alone or miniLCL pulsed with control protein, indicating that these lines had indeed become gp350-specific. By con-

trast, the lines from donors E and H responed against all target cells whether pulsed with gp350 protein or not. These lines probably recognized an autoantigen expressed by the APC. The T cell lines from donors C and I weakly responded against autologous LCL, but failed to recognize gp350-pulsed miniLCL. Which antigen(s) these T cell lines recognized remained unknown. The T cell lines A and G expanded poorly in culture and were only tested against autologous LCL. Some reactivity against LCL was observed, but it remained unknown whether these T cell lines had become gp350-specific.

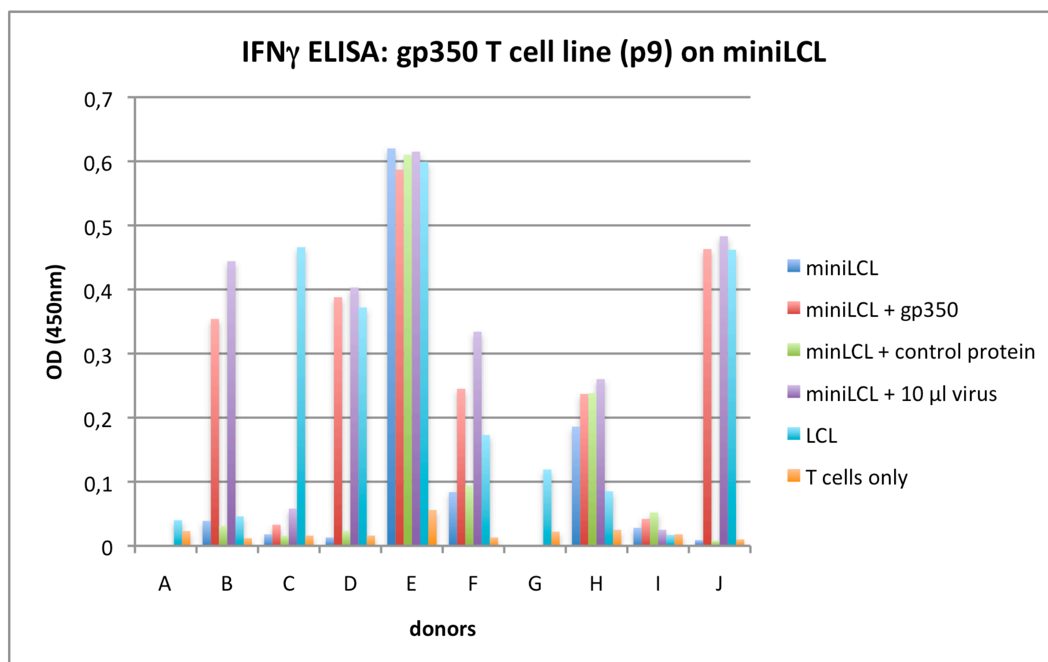


Fig. 20: Specificity analysis of the gp350-stimulated T cell lines after nine rounds of stimulation

After passage 9, the gp350 protein-stimulated T cell lines were probed with autologous miniLCL that had either been left untreated or pulsed with the stimulator or a control protein. In addition, miniLCL pulsed with EBV as well as autologous LCL were included as target cells. The T cell lines from donors A and G were tested against LCL only. IFNγ secretion of the T cell was measured in the cell culture supernatant by ELISA.

While about half of the gp350-stimulated T cell lines had become antigen-specific after seven rounds of stimulation, none of the BNRF1-stimulated T cell

lines from ten donors displayed BNRF1-specificity even after ten rounds of stimulation (*data not shown*).

Therefore, the BNRF1-stimulated T cell lines were discontinued and further experiments focused on the gp350 protein-stimulated T cell lines.

To test whether additional T cell lines became specific for gp350 when further stimulated *in vitro*, specificity of the T cell lines was assessed after nine rounds of stimulation.

As shown in Fig. 20, no further changes in the pattern of recognition were observed. Lines B, D, F and J showed strong reactivity against autologous LCL and against miniLCL pulsed with gp350 protein or virus supernatant. Lines E and H recognized miniLCL irrespectively of the added protein or virus, line C reacted only against LCL, lines A and I barely secreted any IFNγ in response to the various target cells, and line G recognized LCL specifically, but antigen specificity of this line could not be defined. These results suggested that no gross changes in T cell specificity would occur with further rounds of stimulation. Therefore, further analyses focused on the gp350-specific T cell lines lines (B, D, F, and J), and on the T cell line from donor C, which had responded specifically against LCL and miniLCL pulsed with virus, suggesting that it recognized a lytic cycle antigen of the EBV.

3.B.3 FACS analysis of an exemplary T cell line

Specificity of the T cell lines had been assessed by measuring IFNγ secretion by the T cells after co-culture with antigen presenting cells. Since IFNγ is also produced by CD8+ and NK cells, the composition of the T cell lines was analyzed by FACS. As shown in Fig. 21 for the gp350 protein-stimulated T cell line from donor D after the 8th stimulation, almost all cells were T cells (CD3-positiv) and

predominantly CD4-positive. CD8+ T cells formed only a minor proportion of the population and might at least in part stem from surviving PBMC used as APC. As the analysis was carried out just before the following stimulation, many dead cells were detected by FACS.

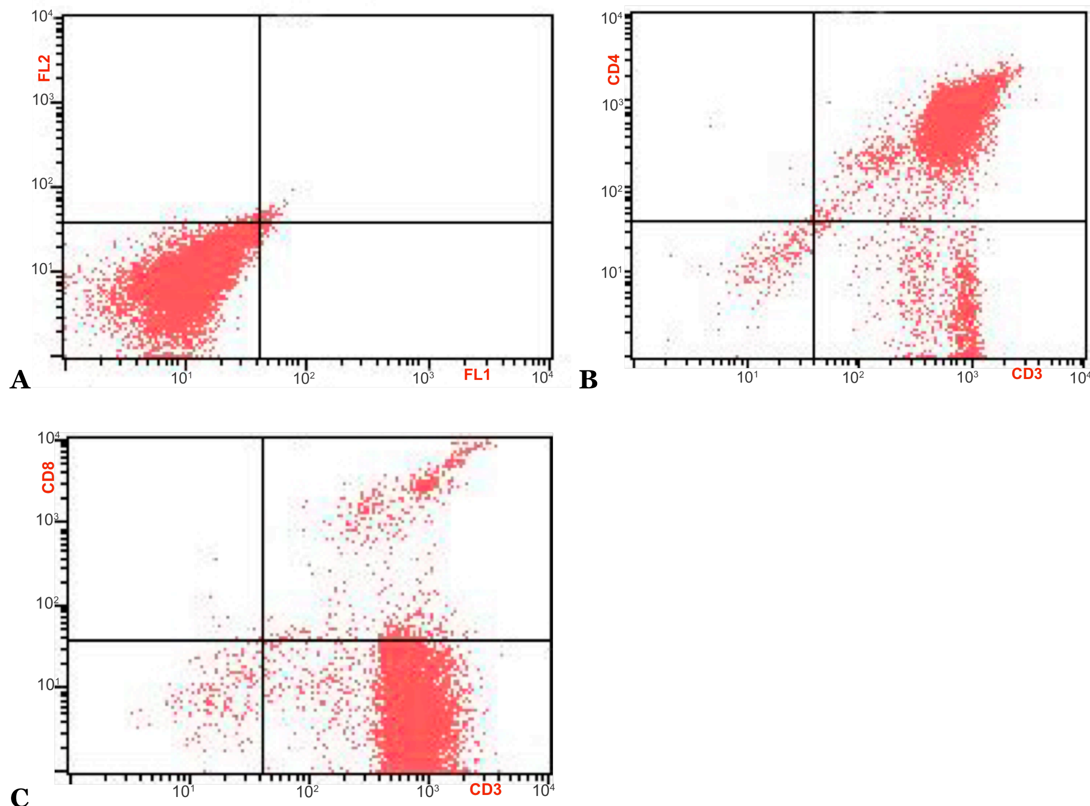


Fig. 21: FACS analysis of the cellular composition of T cell line D after the 8th stimulation

The cells from T cell line from donor D at passage 8 were stained with antibodies specific for CD3, CD4, and CD8 and analyzed by FACS. (A) unstained cells, (B) cells double stained with antibodies directed against CD3 (CD3-FITC) and CD4 (CD4-PE), (C) CD3 (CD3-FITC) and CD8 (CD8-PE) double-stained cells.

Thus, CD4+ and not CD8+ T cells or NK cells were the main source of IFNγ in the recognition assays.

3.B.4 Restriction element analysis of the gp350-reactive T cell lines

T cells recognize the complex of an antigenic peptide bound to the restricting HLA molecule. To define T cell restriction, the various gp350-specific T cells lines were tested in IFNγ ELISA for their reactivity against different allogeneic LCL with known HLA types that had been pulsed with antigen (10x concentrated virus supernatant). Previously, it had been shown that gp350 is the most abundant protein in the virion and incubation of B cells with viral particles leads to their uptake and to the presentation of virion antigens on MHC class II molecules (Adhikary et al., 2006).

Using the panel of miniLCL derived from the T cell donors, the restriction element could not be defined unambiguously in all cases. Therefore, additional cell lines with known HLA haplotype, that were available in the laboratory, were used to identify the restricting HLA alleles.

These target cell lines were either left untreated or treated with concentrated viral supernatant and then co-cultured with the T cells. Cytokine secretion by the T cells was measured 20 h later by ELISA (Fig. 22). In the case of the T cell line from donor D (HLA class II genotype: DRB1*0401, 1101; DQB1*0301; DPB1*0401, 1601; DRB3*0202; DRB4*0103), the target cell lines miniLCL from donor D, miniLCL from donor C, DG75, KMH2 and miniLCL Faka were recognized when these had been pulsed with virus. All these lines share the HLA DRB4*0103 allele, suggesting that this is the restriction element of these T cells. The target cells LCL7 and FB also share the DRB4*0103 allele and were recognized by the T cells even when no virus was added. In contrast to miniLCL, these LCL express lytic cycle antigens and can be recognized by the T cells directly, provided that they express the restricting HLA allele.

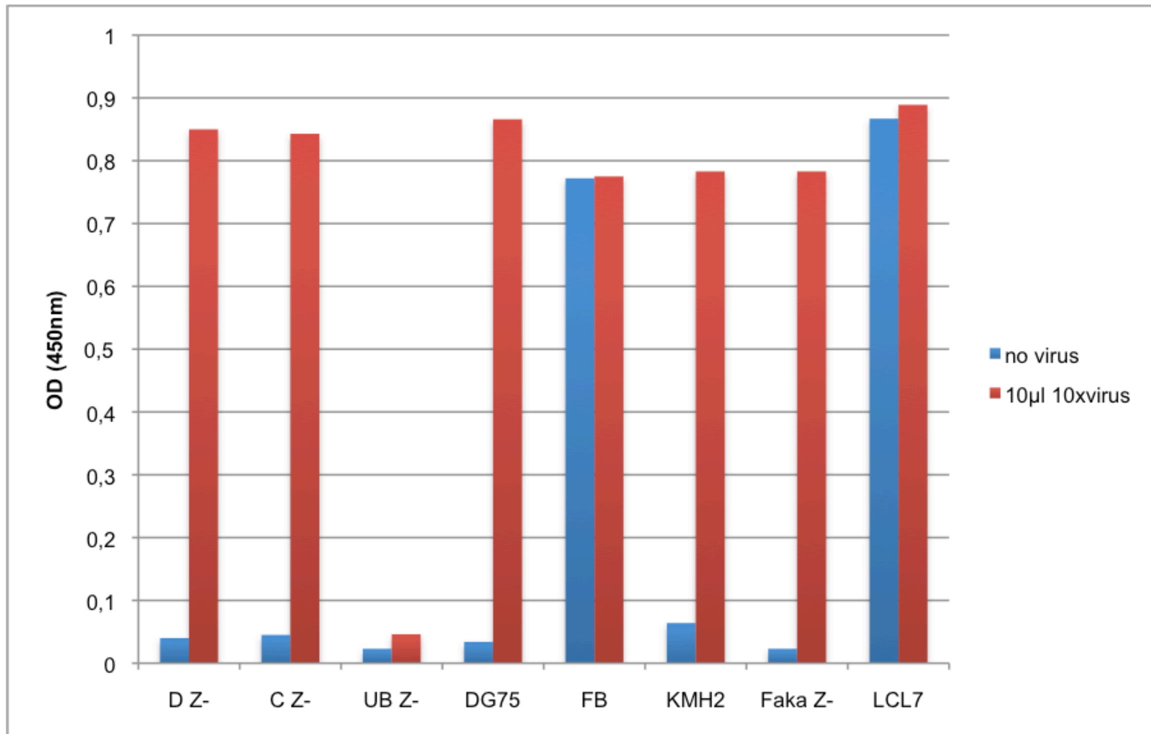


Fig. 22: Restriction element analysis of the gp350-specific T cell line from donor D

Various target cell lines with known HLA-haplotype, that had been left untreated or incubated with concentrated viral supernatant, were probed with the T cells. Cytokine secretion was measured 20 h later by IFNγ ELISA. The target cells Z- from donor D, Z- from donor C, DG75, KMH2 and Faka Z- only share HLA DRB4*0103, suggesting that this is the restriction element of these T cells. The target cells LCL7 and FB also express the DRB4*0103 allele and were recognized by the T cells even when no virus had been added. In contrast to miniLCL, these LCL express lytic cycle antigens and can be recognized by the T cells directly.

The gp350-specific T cell line of donor C (HLA class II genotype: DRB1*0701; DQB1*0202, 0303; DPB1*1101, 1301; DRB4*0103) displayed a similar pattern of recognition (Fig. 23). These T cells recognized the virus-pulsed miniLCL from donor D, donor C, and Faka Z-. In addition, these T cells recognized the virus-pulsed lymphoma cell lines DG75 and KMH2. The target cells FB and LCL7 were efficiently recognized even when no virus was added. All these lines share the DRB4*0103 allele, suggesting that this is the restriction element for these T cells.

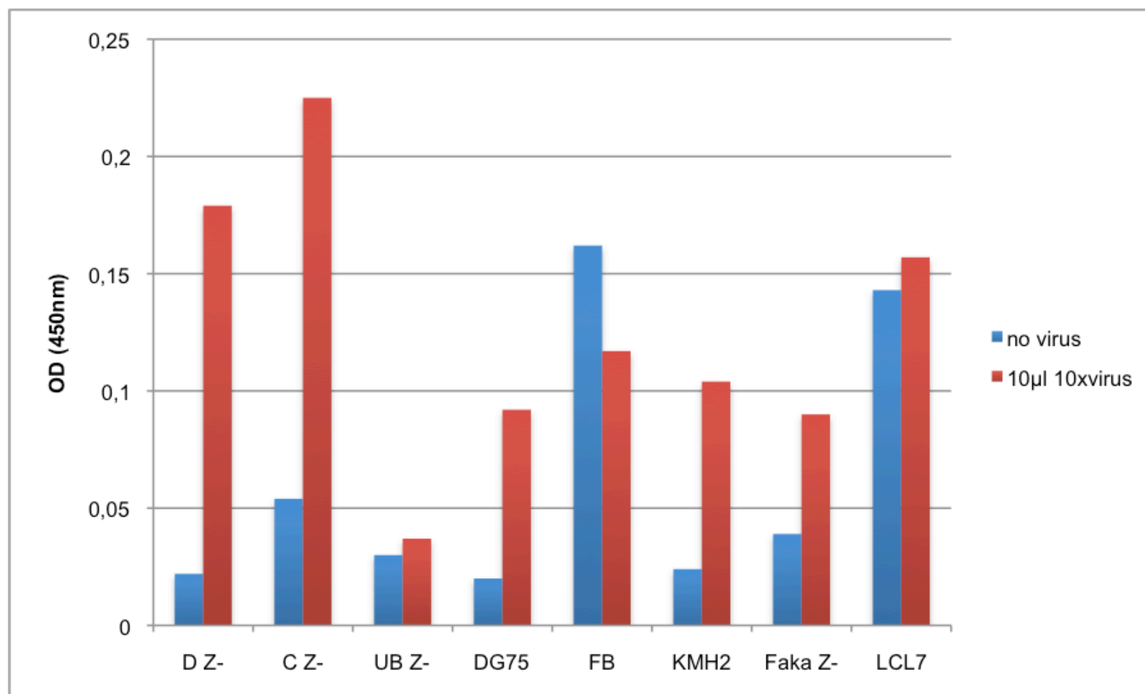


Fig. 23: Restriction element analysis of the gp350-specific T cell line from donor C

Various target cell lines with known HLA-haplotype, that had been left untreated or incubated with concentrated viral supernatant, were probed with the T cells of the line from donor C, IFNγ secretion was measured by ELISA. The target cells Z- from donor D, Z- from donor C, DG75, KMH2, and Faka Z- only share HLA DRB4*0103, suggesting that this is the restriction element of these T cells. The target cells LCL7 and FB also express the DRB4*0103 allele and were recognized by the T cells even when no virus had been added, as also seen in the specificity test for the T cell of the line from donor D. In contrast to miniLCL, these LCL express lytic cycle antigens and can be recognized by the T cells directly.

Thus, the CD4+ T cell lines of donor C and D showed the same pattern of recognition, indicating that these two T cell lines were restricted by the same HLA class II molecule and possibly recognized the same antigen.

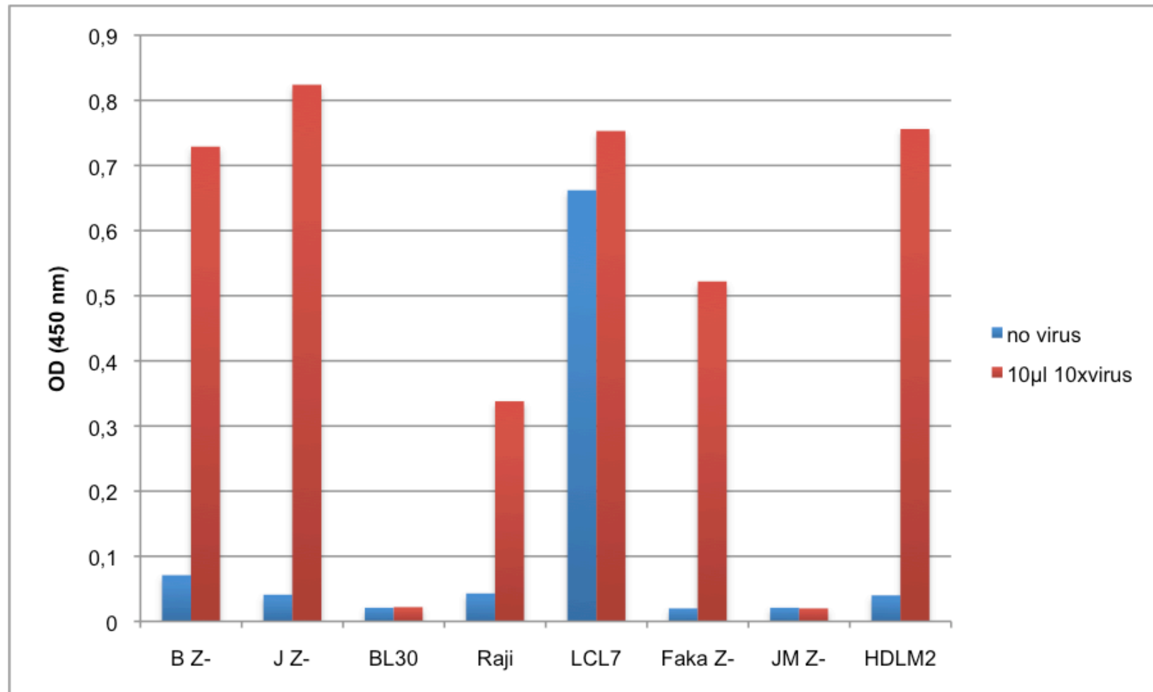


Fig. 24: Restriction element analysis of the gp350-specific T cell line from donor B

Various target cell lines with known HLA-haplotype, that had been left untreated or incubated with concentrated viral supernatant, were probed with the T cell line from donor B and cytokine secretion measured by IFNγ ELISA. This line recognized the virus-pulsed target cells Z- from donor B, Z- from donor J, Faka Z, Raji, and HDLM2, which only share the DQB1*0201 allele, suggesting that this is the restriction element. The target cells LCL7 also express this allele and were recognized by the T cells even when no virus had been added. LCL express lytic cycle antigens and thus can be recognized directly by the T cells.

The gp350-specific T cell line from donor B (HLA class II genotype: DRB1*0301, 1101; DQB1*0201, 0301; DPB1*0101, 0201; DRB3*0101, 0201) recognized the line J that shares DRB1*0301, and DQB1*0201 with the T cell donor, LCL Faka (DQB1*0201) and HDLM2 (DRB1*0301, DQB1*0201) when virus had been added. It reacted against LCL7 (DRB1*0701, 1501; DQB1*0202, 0602) irrespectively of the addition of virus, but did not recognize BL30 (DRB1*0301, 1301; DQB1*0201, 0603) or JM Z- (DRB3*0101). The HLA class II molecule shared by all cell lines recognized by the T cells is DQB1*0201/0202, suggesting that this is the restricting allele. However, BL30 also expresses DQB1*0201 and was not recognized in this assay. This might be due to the fact

that HLA class II molecules are heterodimers consisting of an α- and a β-chain. Since the DQα-chains expressed in these donors are not known, the failure of the T cells to recognize virus-loaded BL30 cells might have been caused by the formation of a DQB1*0201/DQA1 heterodimer that was either unable to bind the peptide or that was not recognized as restricting element by the T cells.

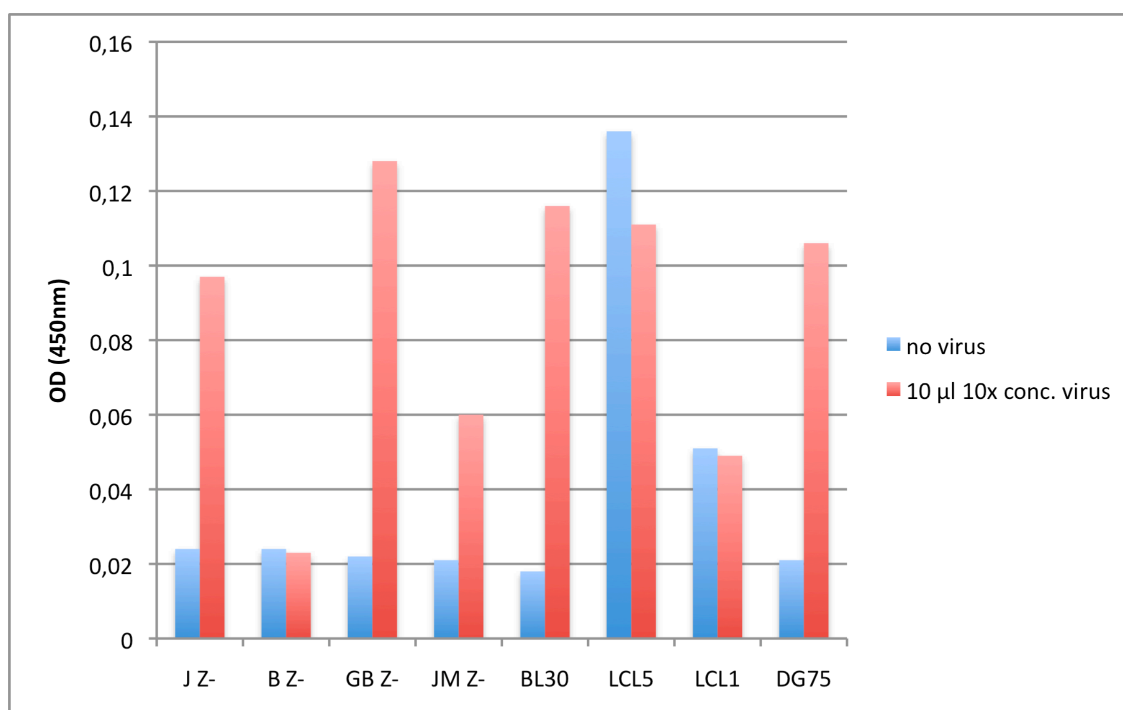


Fig. 25: Restriction element analysis of the gp350-specific T cell line from donor J

Untreated or virus-pulsed target cells were probed with the gp350-specific T cells from donor J and cytokine secretion measured by IFNγ ELISA. This T cell line recognized the virus-pulsed target cell lines Z- from donor J, Z- from donor GB, Z- from donor JM, BL30, and DG75. These lines share the DRB1*1301 and the DQB1*0603 alleles. The target cells LCL and LCL5, which express lytic cycle antigens and also express the DRB1*1301 and the DQB1*0603 alleles, were recognized irrespectively of the addition of virus.

The gp350 T cell line of donor J (DRB1*0301, 1301, DQB1*0201, 0603) recognized the virus-loaded GB Z- (DRB1*1101, 1301, DQB1*0301, 0603) and JM Z- (DRB1*0801, 1301, DQB1*0402, 0603), BL30 (DRB1*0301, 1301, DQB1*0201, 0603), and DG75 (DRB1*0404, 1301, DQB1*0402, 0603), but not B Z-

(DRB1*0301, 1101, DQB1*0201, 0301). It recognized LCL5 (DRB1*1301, 1501, DQB1*0602, 0603) and LCL1 (DRB1*1104, 1301, DQB1*0301, 0603) that express lytic cycle antigens even when no virus is added. The DQB1*0603 and DRB1*1301 alleles are common to all these lines, suggesting that one of them is the restriction element.

Table 3: HLA genotype of the cell lines used in these restriction element analysis experiments.

Designation	HLA-DRB1	HLA-DQB1	HLA-DPB1	HLA-DRB3/4/5
BL 30	0301, 1301	0201, 0603	0101, 1401	DRB3*0101
DG75	0404, 1301	0402, 0603	0401, 0402	DRB3*0101 DRB4*0103
Faka	0701, 1201	0202, 0301	0201	DRB3*0202 DRB4*0101
FB	0402	0302	0301, 1501	DRB4*0103
GB	1101, 1301	0301, 0603	0401, 0402	DRB3*0202
HDLM2	0301, 1401	0201, 0602	0401, 1101	DRB3*0101 DRB3*0202
JM	0801, 1301	0402, 0603	0401, 1301	DRB3*01011
KMH2	04, 1101	0301, 0302	0201, 0501	DRB3*0202 DRB4*0103
L428	1201	0301	0401	DRB3*0202
L540	0301, 1001	0201, 0501	0101	DRB3*0202

LCL1	1104, 1301	0301, 0603	0401	DRB3*0202
LCL5	1301, 1501	0602, 0603	0402	DRB3*0202 DRB5*0101
LCL7	0701, 1501	0202, 0602	0401, 0402	DRB4*0103 DRB5*0101
Raji	0301, 1001	0201, 0501	0101	DRB3*0202
UB	0701, 1101	0301, 0303	0401	DRB3*0202, DRB4*01031

3.B.5 Defining the epitopes recognized by the T cells

To map the specific epitope that was recognized by a particular T cell line, the direct antigen identification approach (DANI), patentet by our group was used (Milosevic et. Al, 2005 et 2006). The DNA coding sequence of gp350 was cleaved by frequently cutting restriction enzymes and inserted upstream of the open reading frame coding for the chloramphenicol acetyltransferase (CAT) gene in bacterial expression plasmids under the control of an IPTG (iso-propyl-b-D-thiogalactopyranoside) inducible promoter. Bacteria were transformed with these plasmids and protein expression induced by adding IPTG to the medium. In addition, 30 µg/ml chloramphenicol was added to the medium to select for those bacteria that expressed a functional fusion protein consisting of the antigenic fragment derived from gp350 and CAT (Milosevic et al., 2005 and 2006). Eight pools, each consisting of 48 separate chloramphenicol-resistant colonies were prepared and the recombinant proteins isolated.

MiniLCL were incubated with these proteins and subsequently probed with the respective T cell line. Except for the T cell line of donor E, all T cell lines showed an enhanced IFNγ secretion upon incubation with one or several of the pools.

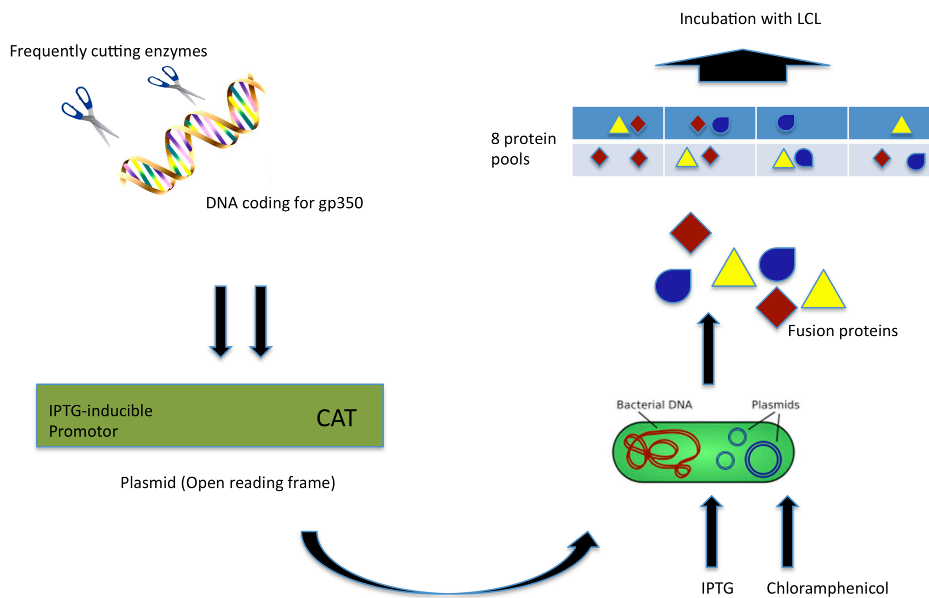


Fig. 26: Schematic depiction of the direct antigen identification strategy (DANI)

DNA coding for gp350 was digested with frequently cutting restriction enzymes and inserted into a bacterial expression plasmid, upstream of the chloramphenicol acetyltransferase gene which is under the control of an IPTG-inducible promoter. Eight pools each consisting of 48 chloramphenicol resistant bacterial colonies that expressed functional gp350-CAT fusion proteins were established. MiniLCL were incubated with the 8 pools for 24 h and then co-cultured with the gp350-specific T cells. Cytokine secretion by the T cells was subsequently measured by ELISA.

These experiments showed that the lines of the donors B and F reacted mainly to the protein pools IIA, IIB, IIIB, the lines of the donors C and D mainly to IIA, IIIB and the line of donor J only to IB. The T cell line from donor E responded strongly against unpulsed miniLCL, and this response was not further increased with any of the protein pools or viral supernatant, indicating that this line recognized an antigen expressed by the target cells which was not gp350.

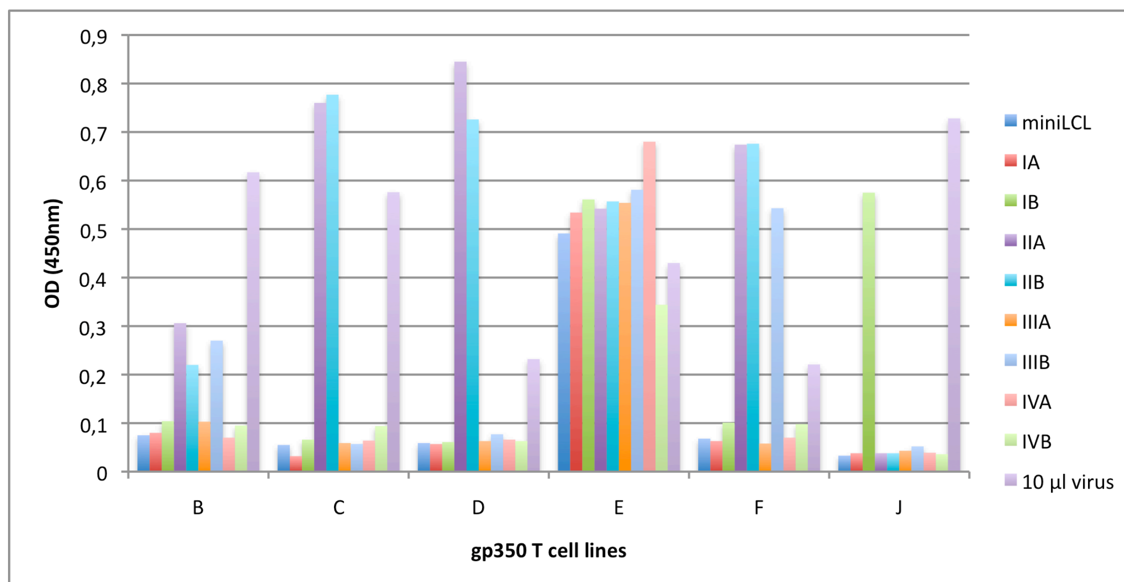


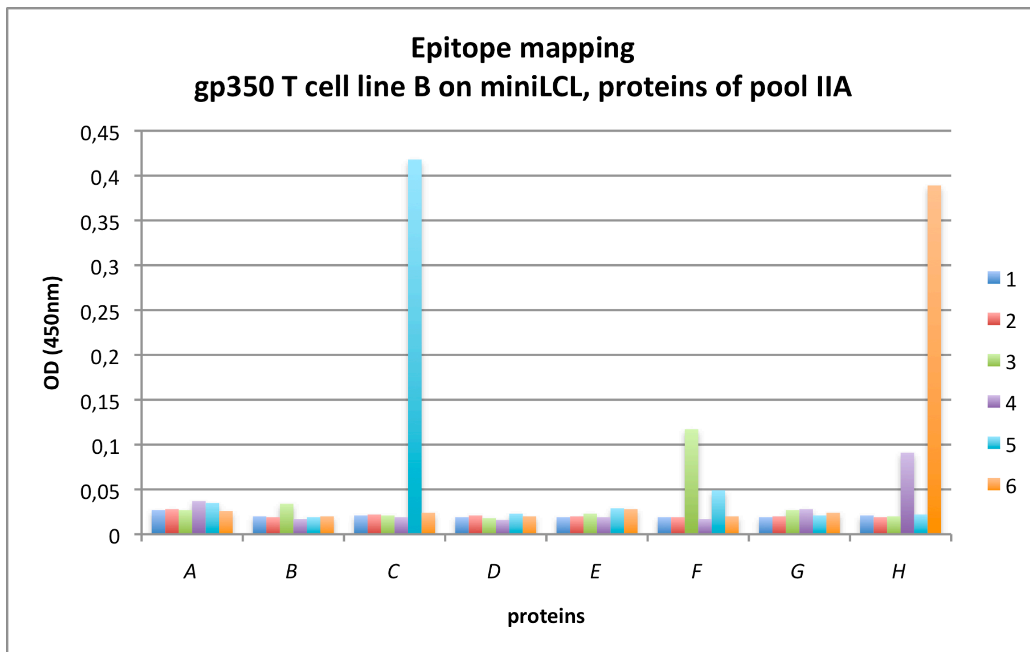
Fig. 27: Identification of the antigens recognized by the gp350-specific T cell lines

Autologous miniLCL were loaded with 8 different protein pools (IA – IVB), each consisting of 48 bacterially expressed proteins. Concentrated virus supernatant was used as positive control. After overnight incubation, these target cells were probed with the gp350-specific T cell lines and cytokine secretion measured 20 h later by IFNγ ELISA.

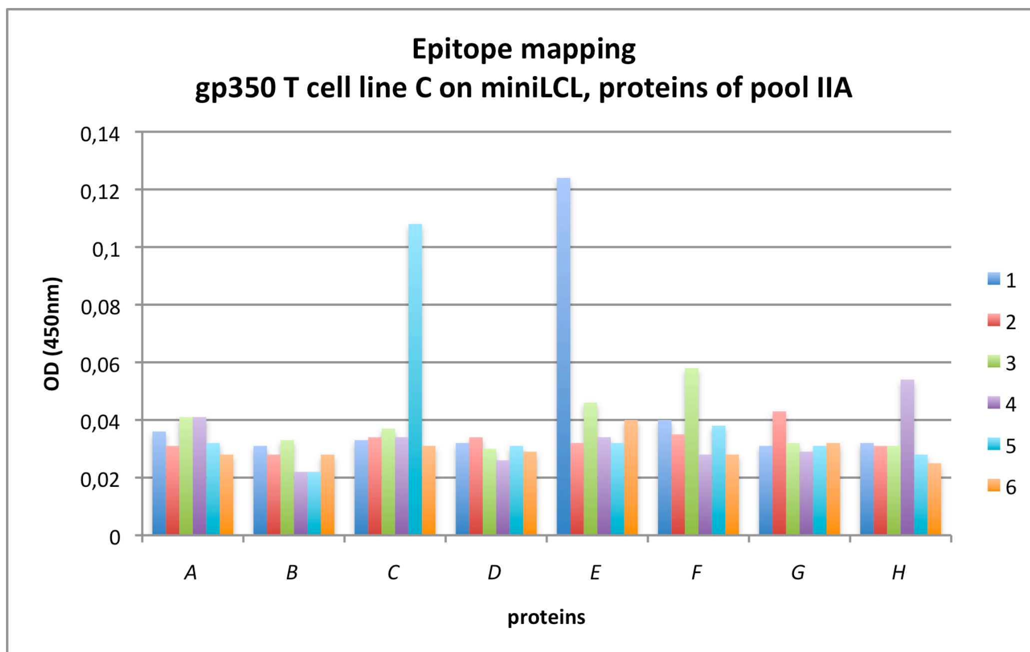
To define the epitopes recognized by the T cells, protein expression was induced in all 48 single colonies of each of the positive bacterial pools. Single recombinant proteins were again purified from these cultures over nickel columns and tested individually in T cell assays as described.

As shown in Fig. 27, the various T cell lines recognized target cells that had been pulsed with individual proteins.

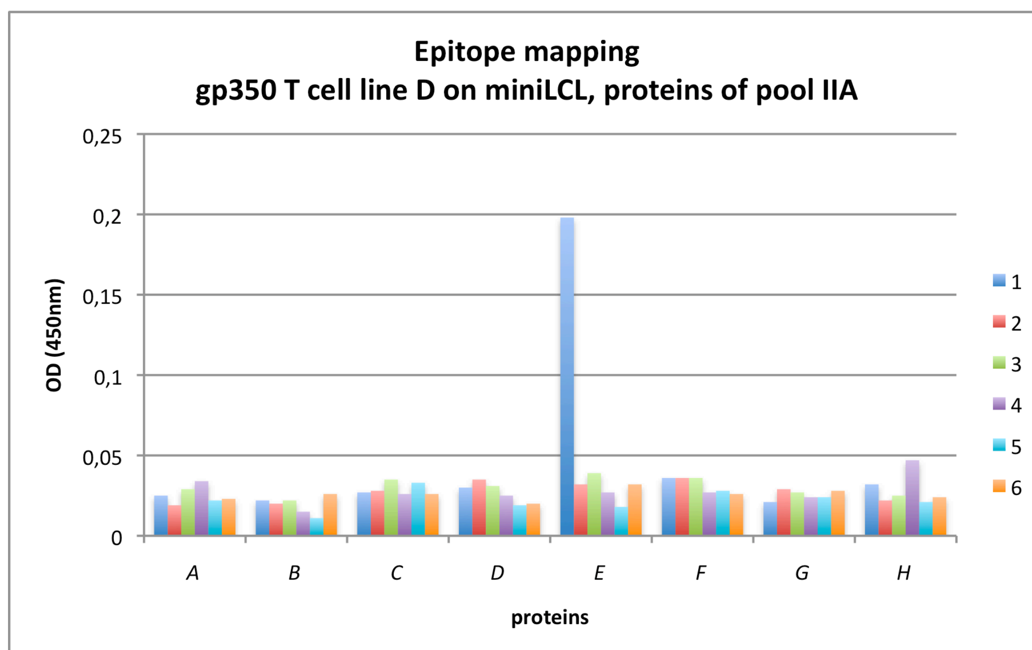
A



B



C



D

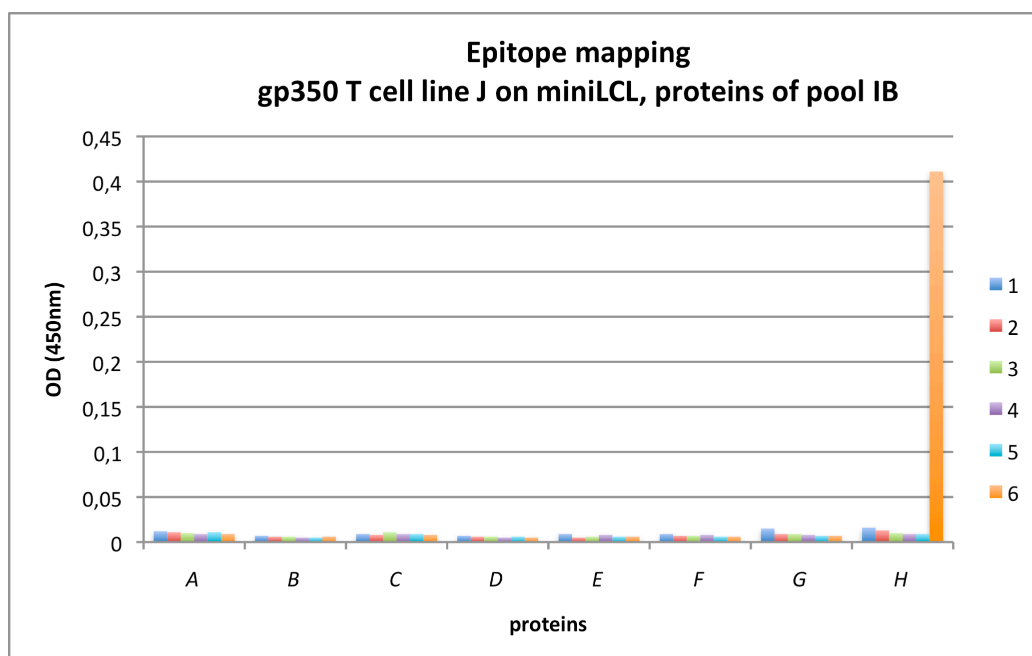


Fig. 28: Epitope mapping using single bacterial proteins

Autologous miniLCL were pulsed with 48 single bacterial proteins of the bacterial pools that had been recognized by the four gp350-specific T cell lines, and then tested for recognition by the T cells. Each of the T cell lines from the four different donors (A-D) recognized one or several of the bacterial proteins. (Roman letters in italic, as single proteins but not donors A-H are depicted).

The gp350-specific T cell line of donor B responded against the proteins IIA F3, E1 and C5, the lines from donor C and donor D to IIA E1, and the line from donor J to IB H6. The T cell line from donor line F could not be further analyzed because the cells proliferated poorly *in vitro*.

Subsequently, the plasmids encoding the proteins recognized by the T cell lines were extracted and sequenced. All carried short DNA fragments derived from the gp350 ORF of approximately 50-250 bp, encoding for peptides of 15-80 amino acids in length. Experiments to define the minimal epitopes recognized by the T cell are currently underway.

3.B.6 Defining the restricting elements of the epitope-specific T cells

Although the restriction elements for the different gp350-specific T cell lines had already been determined (see chapter 3-B.4), additional experiments were performed to investigate whether the identified epitopes were indeed presented on these HLA molecules. Even after ten passages, the T cell lines were usually oligoclonal and contained more than one T cell specificity. Thus, the T cell lines may have consisted of a mix of T cells recognizing different epitopes of gp350. If this was the case, then the restriction element identified by using whole gp350 protein (e.g. viral supernatant) might have differed from the one presenting the identified epitope.

To this end, autologous and allogeneic miniLCL were loaded with the bacterial proteins that contained the epitopes recognized by the T cells. Subsequently, T cells were added to these APC and IFNγ secretion measured by ELISA 20 h later.

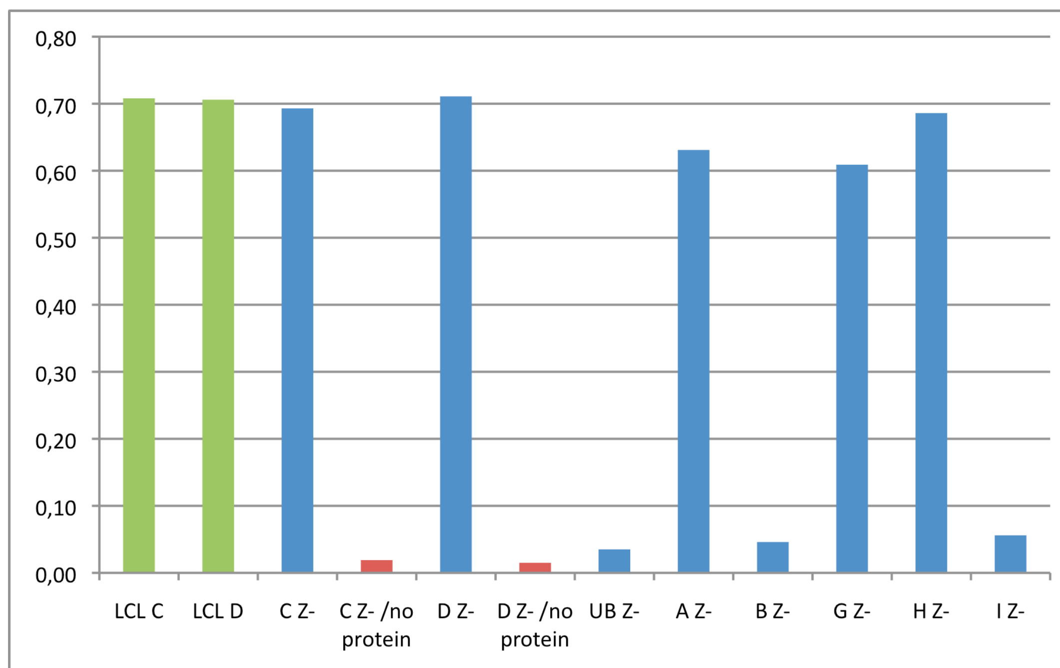


Fig. 29: Restriction analysis test for the CD4+ T cell line of donor D

To each of the indicated miniLCL (blue) 10 µl of IIA E1 protein were added for 24 h and the APC then co-cultured with T cells from line D. In addition, T cells were co-cultured with LCL C and LCL D (green) as well as miniLCL of these donors (C Z- and D Z-) (red), to which no protein had been added. The only HLA molecule expressed by all cell lines recognized by the T cells was HLA DRB4*0103, suggesting that this is the restricting element for these T cells.

The lines C and D both recognized the antigen epitope IIA E1. As shown in Fig. 29, the T cell line from donor D recognized LCL from the donors D and C directly, and several miniLCL incubated with IIA E1 protein. Identical results were obtained for the T cell line from donor C (*data not shown*). All target cell lines that were recognized by the T cells only share the DRB4*0103 allele, implying that the antigenic peptide in IIA E1 is presented on this HLA molecule. The same HLA allele had been identified as restriction element for these two T cell lines in earlier experiments using virus supernatant as source of antigen. Thus, these T cell lines seem to be dominated by T cells specific for the gp350 epitope present in protein IIA E1 and presented on HLA DRB4*0103.

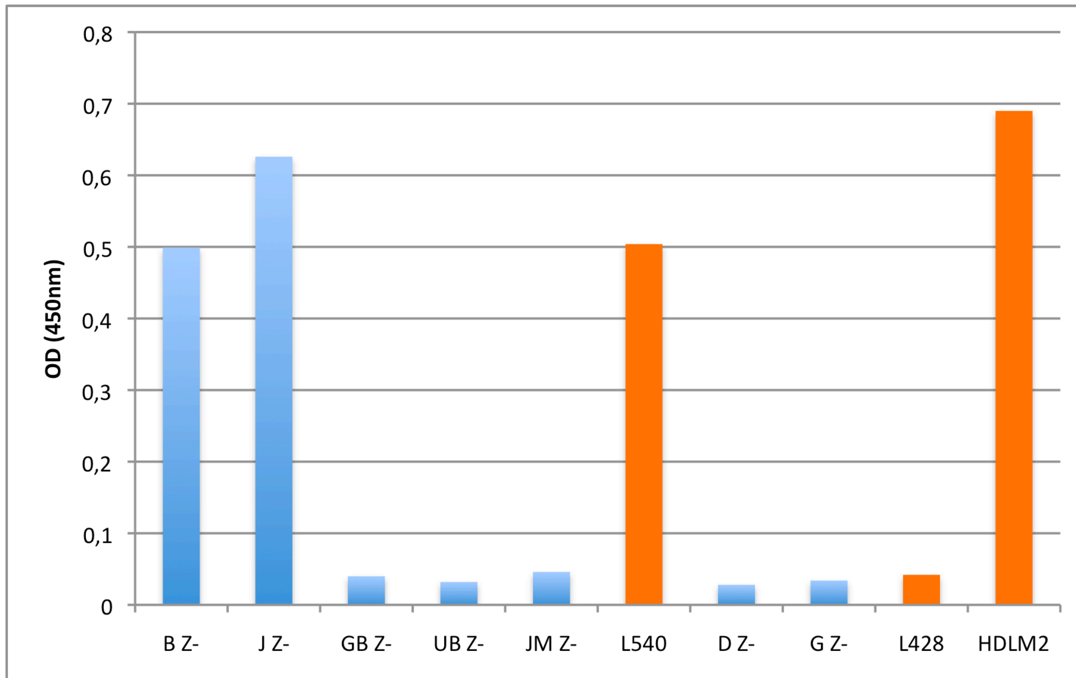


Fig. 30: Restriction element analysis of the IIA C5 epitope-specific T cells from the line of donor B (p14)

Several miniLCL (blue) and EBV-negative Hodgkin's lymphoma cell lines (orange), that were not recognized by the T cells, were loaded with 10 µl of protein IIA C5 and then probed with the T cell line from donor B. IFNγ secretion was measured 20 h later by ELISA.

The T cell line of donor B recognized miniLCL B and J and the EBV-negative HL cell lines L540 and HDLM2 after these target cells had been pulsed with IIA C5 protein. All these lines share the DRB1*0301, DQB1*0201 and DRB3*0202 alleles. Therefore, additional target cell lines were used to define the restricting element.

The T cell line failed to recognize protein-pulsed miniLCL from donors D, F and G that express DRB3*0202 (*data not shown*), so that this HLA allele can be excluded as restricting element. Thus, only the DRB1*0301 or the DQB1*0201 alleles remain as potential candidates for the presentation of the antigen in IB H6. Because target cell lines that would express only one or the other of these alleles

only were not available, it remained unknown which of these two alleles was the restricting element.

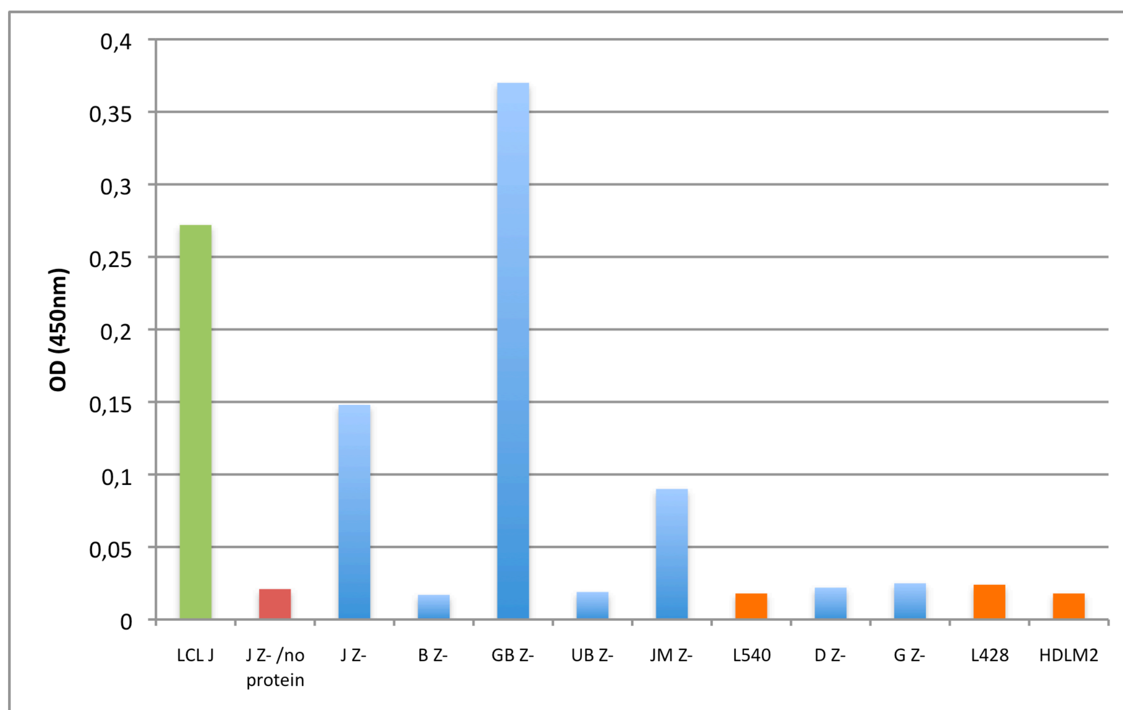


Fig. 31: Restriction element analysis of IB H6 epitope-specific T cells from donor J

Autologous LCL (green) and miniLCL (blue) as well as allogeneic miniLCL (blue) and EBV-negative Hodgkin's lymphoma cell lines (orange), that were either left untreated (red) or loaded with 10 µl IB H6 protein (all but red) were used as targets for the T cell line from donor J. IFNγ secretion was measured 20 h later by ELISA.

T cells of donor J recognized the IB H6 protein on miniLCL of the donors J, GB, and JM. These lines share the HLA molecules DRB1*1301, DQB1*0603 and DPB1*0401. Additional experiments with the target cell lines BL30 and LCL5 that share the DRB1*1301 and DQB1*0603 alleles but are DPB1*0401 negative (*data not shown*) indicated that either DRB1*1301 or DQB1*0603 was the restriction element for the T cells from donor J.

These results suggested that epitopes derived from gp350 can be presented on different HLA class II alleles. In addition, experiments using peptides spanning

currently known gp350 epitopes revealed that the T cells targeted novel epitopes (*data not shown*). Taken together, these findings indicated that the gp350-specific CD4+ T cell response is broad and frequent within healthy virus carriers, and that the virus-specific CD4+ T cell responses can be assessed with this approach despite their overall low magnitude.

4 DISCUSSION

4.A Frequency analysis of EBV-specific CD4+ cells

4.A.1 Frequencies of EBV-specific CD4+ T cells during acute and persistent phase of infection

The recent identification of low endogenous CD4+ T cell numbers as an important risk factor for the development of EBV-associated diseases in immunosuppressed patients (Sebelin-Wulf et al., 2007), and of better clinical responses in patients with PTLD receiving EBV-specific T cell lines that contained higher proportions of CD4+ T cells (Haque et al., 2007), imply an important role of CD4+ T cells in the control of EBV infection *in vivo*. EBV-specific CD4+ T cells in LCL-stimulated T cell preparations are mostly directed against virion antigens (Adhikary et al., 2007), which are efficiently presented on MHC class II following receptor-mediated uptake of released viral particles (Adhikary et al., 2006).

The aim of this study was to evaluate the frequencies of virion-specific CD4+ cells in peripheral blood of healthy, EBV-positive individuals and patients with IM. A better understanding of the EBV-specific CD4+ T cell response in health and disease may help to identify patients at imminent risk of developing EBV-associated disease and to design immunotherapeutic strategies for enhancing antiviral immunity.

Unexpectedly, these experiments demonstrated that the frequency of virus-specific CD4+ T cells is very low in healthy virus carriers, and barely detectable in patients with IM. As measured by IFNγ Elispot, the frequencies of virion-

specific CD4+ T cells in healthy EBV carriers ranged from 50 to 100 per 1 x 10^5 CD8+ depleted PBMC (0.05 % - 0.1 %). In patients with acute IM, even two- to fourfold lower frequencies (0.02 % - 0.025 %) were detected. In one patient with IM that was followed during convalescence, virus-specific CD4+ cell numbers had reached 30 cells per 1 x 10^5 PBMC (0.03 %) one month after acute infection, and 130 per 1 x 10^5 PBMC (0.13 %) four months after infection. These results suggested that EB virion-specific CD4+ T cell responses in patients with IM gradually increase during convalescence to similar levels as in the healthy, EBV-positive cohort.

This low number of virion-specific CD4+ T cells in healthy virus carriers and especially in patients with IM was unexpected, because virion antigens have been identified as immunodominant targets of the EBV-specific CD4+ T cell response, and higher frequencies of CD4+ T cells specific for single viral subdominant antigens had already been described in earlier studies (Scherrenburg et al., 2008; Marshall et al., 2007; Precopio et al., 2003; Leen et al., 2001).
For example, analysis of the CD4+ T cell responses against individual peptides derived from the latent cycle proteins EBNA1 and EBNA3C were shown to range from 50-280 IFNγ-producing cells per 1 x 10^6 CD8-depleted PBMC in Elispot experiments (Leen et al., 2001). Moreover, CD4+ T cells specific for different epitopes within a single latent cycle protein were often identified. Thus, in healthy virus carriers the frequencies of CD4+ T cells specific for EBNA1 and EBNA3C almost matched those recognizing virion antigens.
Paludan et al. (2002) measured EBNA1-specific CD4+ T cell numbers in a small number of healthy EBV carriers by intracellular cytokine staining. In this study, 0.24- 0.66 % of peripheral blood CD4+ T cells were found to be EBNA1-specific. Precopio et al. (2003) used short-term *in vitro* stimulation assays followed by intracellular cytokine staining to characterize the timing, magnitude, and antigen specificity of CD4+ T cells over the course of primary EBV infection. By measuring T cell responses against two lytic cycle (BZLF1 and BMLF1) and two latent cycle (EBNA1 and EBNA3A) antigens, they found that up to 2.7 % of all

circulating effector/memory CD4+ T cells were EBV-specific and that EBV-specific CD4+ T cell frequencies generally paralleled peripheral blood viral load. The frequency of BZLF1-specific CD4+ T cells was highest at presentation and then declined over the next six weeks. This was in contrast to the CD8+ T cell responses, which increased during the first six weeks after infection and eventually outnumbered CD4+ T cell frequencies. Scherrenburg et al. (2008) also analyzed the virus-specific T cell response during and after acute infection by intracellular cytokine staining. *Ex vivo* frequencies of 0.03-0.13 % for EBNA1 and 0.03-0.36 % for BZLF1-specific CD4+ T cells were detected. While CD4+ T cell responses against BZLF1 decreased during convalescence, responses against EBNA1 remained stable over the first six months after primary infection.

In all these studies, significant CD4+ T cell responses against diverse single EBV antigens were detected. When extrapolating these numbers to the almost 100 different viral proteins, a significant proportion of the CD4+ T cell compartment should be dedicated to EBV, unless the antigens investigated are the immunodominant antigens and additional viral proteins are barely targeted by CD4+ T cells. However, the latter possibility appears unlikely because earlier findings demonstrated that virion antigens are the immunodominant CD4+ T cell targets.

To test CD4+ T cell responses against the whole antigenic repertoire of EBV, Amyes et al. (2003) used APC pulsed with lysate from EBV-infected cells as target cells and measured the frequency of CD4+ T cells that responded to the EBV-infected B cell lysate in 36 patients with IM, in seven patients four months after primary infection, and in 28 healthy EBV-seropositive individuals. In these experiments, a mean of 1.4 % CD4+ T cells responded during the primary phase of infection, a mean of 0.22 % CD4+ T cells responded four months later, and a mean of 0.34 % CD4+ T cells responded during the persistent phase of infection. In addition, eight responses to peptides from latent proteins, four of these being specific for EBNA1, three for EBNA2, and one for EBNA3C, and six responses to peptides from lytic cycle proteins, one specific for BZLF1, two for

BMLF1, and three for gp350, were analyzed. In a representative example of results from one EBV-seropositive donor, 0.03 % CD4+ T cells responded to a peptide epitope from the latent protein EBNA3C and 0.16 % CD4+ T cells responded to a peptide from the lytic cycle protein gp350 (Amyes et al., 2003). Collectively, the results from this study indicated that EBV-specific CD4+ T cell responses (i) are increased during acute versus persistent infection, (ii) comprise a moderate proportion of the CD4+ T cell compartment, and (iii) appear to be focused on few viral epitopes.

Thus, different studies have yielded partially inconsistent results. Potential reasons for these discrepancies are the generally low numbers of cases analyzed and differences in the experimental approaches. Strikingly, high T cell frequencies were often detected when single peptides or proteins were used as antigen. However, high antigen doses may have led to false positive results by activating low-affinity or cross-reactive T cells, especially when derived from an inflammatory environment as in the case of IM.

With regard to EBV-specific CD4+ T cell responses in healthy virus carriers, higher frequencies (0.34 %) were detected in the study by Amyes et al. (2003) compared to our experiments (0.05 % - 0.1 %). These differences may have been caused by testing different sets of antigens. By using B cells pulsed with viral particles, CD4+ T cells were probed against virion antigens of EBV in our study. By contrast, Amyes et al. (2003) used infected cell lysate as source of antigen which additionally contains latent and non-structural lytic cycle proteins of EBV. Responses against these antigens may have augmented the overall virus-specific CD4+ T cell response. However, no non-infected control cell lysate was used, therefore it cannot be excluded that responses against auto- and alloantigens, or FCS components were responsible for this increased CD4+ T cell response.

Alternatively, the different magnitudes of the virus-specific CD4+ T cell responder population may be a reflection of the different experimental approaches used. Paradoxically, the number of IFNγ secreting cells in Elispot as-

says was lower in some IM patients when virus had been added than in the negative controls. To test whether the virus concentrate contained inhibitory molecules, non-virion-specific CD4+ T cells were probed with target cells in the presence and absence of virus concentrate. No differences in IFNγ release were detected, indicating that the virus supernatant itself did not contain T cell inhibitory factors (*data not shown*). However, LCL and not PBMC were used as target cells in these experiments. Since it has been demonstrated that EBV induces the production and release of chemokines in human neutrophils (McColl et al., 1997), it cannot be excluded that molecules are released from PBMC upon contact with EBV which dampen cytokine secretion by T cells.

4.A.2 Possible reasons for interindividual differences in EBV-specific CD4+ T cell responses

In all studies, the frequencies of CD4+ EBV-specific cells in healthy carriers appeared to vary greatly, in the study of Amyes et al. (2003) even more than hundred-fold (0.04 to 5.2 %). Several reasons may account for these interindividual differences.

First, as demonstrated in several studies, the magnitude of the EBV-specific CD4+ T cell response changes from acute to persistent phase of infection. Thus, the distance of time to primary infection may be responsible for inter- and intra-individual differences. Also, recent superinfections with a different EBV strain may cause fluctuations in the virus-specific immune response. Second, the status of an individual's immune system may affect EBV-specific CD4+ T cell responses. For example, acute infections with different viruses have been shown to trigger the expansion of EBV-specific CD8+ T cells, probably through infection-associated IL-15 release (Sandalova et al., 2010). Other pathogens and dis-

eases, drugs, as well as environmental factors affecting immune function may also alter EBV-specific T cell responses. Several studies have demonstrated that persistent infections with herpesviruses in general and CMV in particular have a profound influence on subset distribution, phenotype, and potentially also on the function of T cells (Karrer et al., 2009).

Third, to what extent immune responses against EBV are influenced by age, gender, ethnicity, provenance or social status is still unknown. However, the bell-shaped incidence curve of IM (Ebell et al., 2004) may indicate that the immune response against EBV changes with age.

These observations suggest that many factors impinge on the EBV-specific CD4+ T cell response. A simple measurement of the total virus-specific CD4+ T cell response, therefore, is unlikely to provide information on the patient's antiviral immune status. To identify patients at immanent risk of developing EBV-associated diseases, probably more specific parameters need to be defined.

4.B BNRF1- and gp350-specific T cell lines

4.B.1 Possible reasons for the failure to reactivate BNRF1-specific CD4+ T cells *in vitro*

In earlier studies, the EBV tegument protein BNRF1 has been identified as a major target of the EBV-specific CD4+ T cell response in different individuals with different HLA class II genotypes (Adhikary et al., 2007). Although identity and quantity of the purified BNRF1 protein used for T cell stimulation was verified by SDS-polyacrylamide gelelectrophoresis and Western-blot (*data not shown*), no T cell lines specific for BNRF1 could be generated in this study, even after repeated rounds of stimulation *in vitro*.

Several reasons may account for these negative results. First, the spectrum of HLA class II alleles able to present BNRF1 epitopes might be narrower than previously thought and all selected donors coincidentally expressed an unfavorable MHC haplotype. Second, the frequency of BNRF1-specific CD4+ T cells in peripheral blood of EBV-positive donors could be very low and/or the expansion of these cells *in vitro* dependent on factors provided by other immune cells. Of note, the BNRF1-specific CD4+ T cells described by Adhikary et al. (2007) had been established from T cell lines generated by repeated stimulation with autologous LCL and not purified BNRF1 protein. Stimulation of T cells with LCL leads to the expansion of T cells with many different specificities and phenotypes, which might be favorable for the expansion of BNRF1-specific CD4+ T cells. Third, a T cell inhibitory effect of purified BNRF1 protein, as described for LMP1 (Nitta et al., 2004), cannot be excluded at the moment.

For testing these hypotheses, a bigger cohort of donors with varying HLA haplotypes needs to be analyzed. If BNRF1-specific CD4+ T cell lines can be established from a low percentage of individuals, then immunogenicity of BNRF1 would probably depend on the expression of certain HLA class II alleles. If again no BNRF1-specific CD4+ T cell lines can be established, then the *in vitro* expansion of these T cells is compromised or the BNRF1 protein has an inhibitory effect on T cell growth. Depending on the outcome of these experiments, a reassessment of the BNRF1-specific CD4+ T cell response with modified stimulation protocols is warranted.

4.B.2 Gp350 as a major CD4+ T cell antigen

In contrast to BNRF1, CD4+ T cell responses against gp350 were detected in half of the donors analyzed. Gp350 is the major EBV membrane glycoprotein

and the most abundant protein in the viral envelope. Its binding to CD21 on human B cells initiates virus uptake and facilitates productive infection (Busse et al., 2010). Thus, viruses lacking gp350 barely infect B cells and gp350 has been identified as the dominant target of the neutralizing antibody response (Thorley-Lawson and Poodry, 1982). These *in vitro* observations formed the basis for the development of vaccines that aim at increasing anti-gp350 antibody titers to inhibit spreading of infection. First clinical evidence in support of this concept has recently been provided.

Following vaccination with recombinant EBV subunit gp350, virtually all EBV-seronegative, healthy, young adult volunteers showed seroconversion to anti-gp350 antibodies and remained anti-gp350 antibody positive for more than 18 months. Moreover, the vaccine had demonstrable efficacy in preventing the development of IM, but did not prevent asymptomatic EBV infection (Sokal et al., 2007).

In a recent phase I trial to prevent EBV-associated PTLD, EBV-negative children with chronic kidney disease awaiting transplantation were vaccinated with recombinant gp350 protein (Rees et al., 2009). Again, neutralizing antibodies were induced in some of the patients, but immune responses declined rapidly after vaccination and did not affect posttransplant events, indicating that vaccine dosage, time of treatment, and choice of adjuvants require further optimization (*ibidem*).

In virtually all healthy virus carriers, IgG antibodies against gp350 can be detected. Because immunoglobulin isotype switching and high titer antibody production usually requires CD4+ T cell help, IgG responses are often associated with CD4+ T cell responses against the same antigen.

In preclinical models, injection with purified, or infection with vaccinia virus expressing gp350, has been shown to protect cottontop tamarins against a lethal, lymphomagenic EBV challenge (Morgan and Wilson, 1997). Importantly, protective immunity in these and other studies did not always correlate with the

presence of virus-neutralizing antibodies, inferring a role for gp350-specific cell-mediated responses in disease protection (Wilson et al., 1996).

In earlier studies, gp350 had been identified as potential T cell target and the first EBV-specific CD4+ T cell clones isolated and characterized *in vitro* were found to recognize gp350 (Wallace et al., 1991). In a more recent analysis, CD4+ T cells specific cells for gp350 were shown to recognize and eliminate newly EBV-infected B cells and to prevent the outgrowth of LCL (Adhikary et al., 2006). These results suggested that besides providing help for the production of virus-neutralizing antibodies, gp350-specific CD4+ T cells may also play a direct role in controlling EBV infection *in vivo* by keeping the pool of EBV infected B cells low.

Thus, gp350 appears to be an important immune target and both, gp350-specific humoral and cellular immune responses may contribute to the control of EBV infection.

4.B.3 HLA-restriction of the gp350-specific CD4+ T cells

The restricting HLA class II molecules for the gp350-specific CD4+ T cells isolated from the various donors were DRB4*0103 (donor C and D), DQB1*0201/0202 (donor B), DQB1*0603 or DRB1*1301 (donor J). These results on a small number of donors suggest that different HLA class II alleles are able to present gp350 peptides. In order to define whether certain combinations of HLA class II alleles and gp350 epitopes elicit stronger CD4+ T cell responses than others, and whether HLA haplotypes unable to present gp350-derived peptides exist within the population, a larger panel of gp350-specific CD4+ T cells from different donors needs to be analyzed. The outcome of these experiments might have implications for vaccine design and for identifying those patients that would, or would not, profit from such a vaccine. In addition, defining im-

munogenic peptide/MHC combinations may provide mechanistic insights into the described association between the expression of certain HLA alleles and the risk of developing EBV-associated diseases.

Genetic markers closely linked to the HLA-A*01 allele were shown to be associated with the development of IM upon primary EBV infection (McAulay et al., 2007). In HL, regions within the HLA class I and class II loci are associated with susceptibility to HL, and the association with HLA class I is specific for EBV-positive disease, suggesting that the presentation of EBV-derived peptides is involved in the pathogenesis of EBV-positive HL. Besides, the association with certain HLA alleles may provide an explanation for the ethnic variation in the incidence of HL (Diepstra et al., 2005).
Such an association with certain HLA haplotypes is also well established for NPC, which is closely associated with EBV. Conceivably, some HLA alleles have weak viral antigen binding capacity which may result in diminished immune activation and increased disease susceptibility. Conversely, other alleles may have higher efficiency in eliciting an effective immune response, which may explain host resistance to disease. Alternatively, HLA may represent a genetic marker flagging the presence of a predisposition locus in close linkage disequilibrium with the HLA region (Li et al., 2009b; Pasini et al., 2009).
Association studies have identified certain HLA class I but not class II alleles as risk factor for developing EBV-associated disease, either because CD8+ but not CD4+ T cell responses are critically involved, or because HLA class II typing is still not routinely performed for all alleles, leaving potential associations unidentified.

This latter notion is supported by the identification of low endogenous CD4+ T cell numbers as important risk factors for the development of EBV-associated diseases in immunosuppressed patients (Sebelin-Wulf et al., 2007) and by the positive correlation of CD4+ T cell proportion and clinical response in patients with PTLD treated with EBV-specific T cell lines (Haque et al., 2007).

Conceivably, CD4+ T cells might play a crucial role in disease protection especially in EBV-associated disorders with HLA class I processing and presentation defects such as HL (Diepstra et al., 2007) and BL (Stäge et al., 2002). In fact, EBNA1-specific CD4+ cells were found to recognize and kill EBV-positive BL cell lines (Paludan et al., 2002), whereas virus-specific CD8+ T cells failed to do so (Stäge et al., 2002).

Interestingly, EBV has also been implicated in the etiology of several autoimmune diseases, e.g. multiple sclerosis (MS), and circumstantial evidence indicates an association of MS with certain HLA class II alleles. To date, the HLA DR15/DQ6 haplotype is the strongest genetic risk factor associated with MS susceptibility, while other HLA class II alleles, such as HLA-DR11, show a protective effect. Although it is currently not known whether the presentation of EBV-derived peptides on these HLA alleles confers disease susceptibility, the finding that EBNA1-specific CD4+ T cells from patients with MS cross-react with myelin antigens provides circumstantial evidence for a role of EBV-specific CD4+ T cells in disease pathogenesis (Lünemann et al., 2008).

Thus, an underrepresentation of HLA class II alleles presenting immunogenic gp350 epitopes in patients with EBV-associated disorders might indicate a disease-protective effect of CD4+ T cells specific for this antigen.

4.B.4 Immunotherapy of EBV-associated malignancies

The successful treatment of immanent and manifest PTLD in hematopoietic stem cell transplant recipients by the adoptive transfer of LCL-stimulated T cell preparations has provided an important proof of principle for this form of immunotherapy. However, owing to the often rapid progression of PTLD, which necessitates the prophylactic preparation of these T cell lines for HSCT patients, this form of adoptive T cell therapy has not become therapeutic mainstay.

Moreover, clinical responses against PTLD in solid organ transplant recipients and other EBV-associated malignancies, e.g. NPC and HL, are often partial and transient. Thus, generic and fast approaches for the generation of EBV-specific T cell lines enriched in disease-relevant specificities and with enhanced clinical efficacy need to be developed.

An alternative approach for generating EBV-specific T cell lines for the treatment of PTLD in HSCT patients has recently been described. Following overnight stimulation of PBMC from HSC donors with a cocktail of EBV peptides binding to different HLA class I and class II alleles, the responding T cell population were isolated by IFNγ surface capture and immunomagnetic separation. Infusion of a small number of these directly isolated T cells led to a rapid and sustained reconstitution of a protective EBV-specific T cell immunity (Moosmann et al., 2010). Incorporating additional peptides from immunogenic antigens like gp350 may not only increase the spectrum of different HLA haplotype covered by this peptide cocktail, but may also increase clinical efficacy of these T cell preparations.

In situations where the HSC donor is EBV-negative or not available, or in solid organ transplant recipients, EBV-specific T cells from third party donors can be adoptively transferred. To have these cells immediately available for patients in need, a frozen bank of EBV-specific T cell lines derived from HLA-typed, healthy blood donors has been established (Haque et al., 2002). Despite continuous immunosuppression, the infused T cells are quickly eliminated by the patient's immune system. Consequently, no long term reconstitution of antiviral immunity can be established with these partly HLA-matched T cell lines. However, allogeneic T cells can provide a first line therapy against PTLD (Wilkie et al., 2004; Haque et al., 2007).

Due to immediate elimination, allogeneic T cells cannot be used to treat EBV-associated tumors in immunocompetent individuals. Moreover, these EBV-associated malignancies, e.g. HL and NPC, respond poorly to LCL-stimulated T cell preparations, most likely because of immune evasion strategies by tumor

cells such as non-expression of the EBNA3 family of proteins, the immuno-dominant targets of the latent antigen-specific CD8+ T cell response. In these cases, T cells specific for the limited set of viral antigens expressed in these tumors need to be isolated, expanded *in vitro*, and reinfused into the patient. The isolation of these T cells can be performed in several ways, e.g. by cytokine capture assay or MHC multimers, but in all cases knowledge of the antigenic epitope is required.

Furthermore, redirecting T cell specificity to selected antigens by transferring T cell receptor genes into T cells has been shown to confer antiviral and antitumoral immunity (Kieback and Uckert, 2010). Thus, the identification of immunogenic CD4+ T cell epitopes and the definition of the restricting HLA class II alleles, as performed in this study for the EBV antigen gp350, in combination with T cell receptor Vα and Vβ chain sequence analysis of the gp350-specific CD4+ T cells may aid at improving T cell-based immunotherapy of EBV-associated diseases in several ways.

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