Investigation of the T helper cell response against Epstein-Barr virus

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A. Abstracts

A.1 Zusammenfassung

Das Epstein-Barr-Virus (EBV) ist mit einer Vielzahl maligner Erkrankungen assoziiert. Nach Primärinfektion persistiert das Virus lebenslang im Wirt. Während dieser Phase der klinischen Viruslatenz wird die Virusproduktion zyklisch in latent infizierten B-Zellen reaktiviert. Bei ausbleibender immunologischer Kontrolle, zum Beispiel im Rahmen einer Organ- oder Stammzell-Transplantation, kann die Infektion disseminieren und eine lymphoproliferative Erkrankung auslösen. Durch den adoptiven Transfer von EBV-spezifischen T-Zellen, die durch wiederholte Stimulation mit autologen lymphoblastoiden Zelllinien (LCL) *in vitro* generiert werden, können lymphoproliferative Erkrankungen nach Transplantationen (PTLD) geheilt werden. Allerdings ist die Herstellung solcher T-Zell-Vakzine noch so aufwendig und langwierig, dass sie derzeit nur prospektiv ausgewählten Risiko-Patienten zur Verfügung stehen. Von einer Identifikation der relevanten T-Zell-Antigene des EBV wird eine Verbesserung der Therapieansätze erwartet, in Richtung breiter Anwendbarkeit bei Patienten mit drohender oder manifester PTLD. Während die EBV-spezifische Antwort von CD8+ T-Zellen bereits gut charakterisiert worden war, waren die Zielstrukturen der EBV-spezifischen CD4+ T-Zell-Antwort zu Beginn der vorliegenden Arbeit weitgehend unbekannt.

Ziel der vorliegenden Arbeit war es deshalb, die Spezifität und Breite der EBV-spezifischen Antwort von CD4+ Helfer-T-Zellen zu untersuchen. Zu diesem Zweck wurden zwei parallele Ansätze gewählt. Zum einen wurden von 23 verschiedenen, EBV-positiven und –negativen Spendern LCL angelegt, durch repetitive Stimulation mit diesen LCL autologe, CD4+ T-Zelllinien und –Klone generiert und deren Spezifität mit Hilfe von Protein-gepulsten Zielzellen näher charakterisiert. Zum anderen wurde mit Hilfe von Protein-gepulsten peripheren mononukleären Blutzellen (PBMC) nach EBV-spezifischen CD4+ Helfer-T-Zellvorläufern bei gesunden, EBV-positiven oder -negativen Probanden gesucht, und so die Breite der Immunantwort charakterisiert.

Zusammenfassend reagierten die CD4+ T-Zellen EBV-negativer Spender nur mäßig auf LCL und wiesen keine EBV-Spezifität auf. Dagegen waren alle von gesunden EBV-positiven Spendern etablierten CD4+ T-Zelllinien EBV-spezifisch. Die CD4+ T-Zelllinien von frisch infizierten Patienten mit Symptomen einer typischen Infektiösen Mononukleose (IM) waren je zur Hälfte EBV-spezifisch oder autoreaktiv. Überraschenderweise erkannten die EBV-spezifischen T-Zelllinien nicht, wie bisher allgemein angenommen, die in allen LCL exprimierten Latenzgene, sondern verschiedene Proteine des lytischen Zyklus von EBV.

Letztere waren vornehmlich Strukturproteine des Viruspartikels, mit einer dominanten Rolle des Tegument-Proteins BNRF1.

Die Frequenzanalyse von EBV-spezifischen Helfer-T-Zellvorläufern bestätigte eine zentrale Bedeutung von CD4+ T-Zellen mit Spezifität für verschiedene Antigene des lytischen Zyklus für das EBV-spezifische immunologische Gedächtnis. Bemerkenswerterweise erkannten die Viruspartikel-spezifischen CD4+ T-Zellen die EBV-positiven Zellen zum einen direkt und zum anderen unabhängig von der Expression von Proteinen des lytischen Zyklus. Dieser *bystander*-Effekt konnte durch einen effizienten, Rezeptor-vermittelten Transfer von Antigen zwischen Viruspartikel freisetzenden und Nachbarzellen erklärt werden. Von großer therapeutischer Bedeutung war die Beobachtung, dass die T-Zell-Erkennung von EBV-Partikel-aufnehmenden Zellen bereits bei Beladung mit nur einem Viruspartikel pro Zelle und noch vor Etablierung von Viruslatenz oder –Produktion stattfand. Klinisch bedeutsam war darüber hinaus der Befund, dass die Viruspartikel-spezifischen CD4+ T-Zellen nach Antigen-Kontakt über Sekretion von Perforin und Granzym B zytolytisch auf ihre Zielzelle wirkten und so die Proliferation und das Auswachsen von LCL aus frisch mit EBV infizierten B-Zellen verhindern konnten.

Diese Ergebnisse weisen erstmals auf eine zentrale Rolle von Virushüllproteinen in der MHC Klasse II-restringierten immunologischen Kontrolle von EBV-Infektionen hin und damit auf ihre hohe Relevanz für neue immuntherapeutische Ansätze. Die Befunde sind möglicherweise nicht nur für die Therapie von EBV-assoziierten Erkrankungen interessant, sondern könnten darüberhinaus im Kontext von Infektionen mit anderen Viren wichtig sein, die eine Virushülle tragen und über diese MHC Klass II-positive Zellen infizieren.

A.2 Summary

The Epstein-Barr virus (EBV) is associated with a number of human malignancies. Following primary infection, the virus persists lifelong in the infected host by latently infecting B cells and occasional cycles of reactivation, virus production and re-infection. Adoptively transferred EBV-specific T cells, generated by repeated stimulation with autologous lymphoblastoid cell lines (LCL) *in vitro*, are able to cure post-transplant lymphoproliferative disease (PTLD). However, the generation of these vaccines is labor and cost intensive precluding their general availability for all patients at risk. Novel insights into the mechanisms of protective antiviral immunity is expected to provide a better understanding of the pathogenesis of EBV-associated diseases and to facilitate the development of novel and generally available immunotherapeutic options.

The aim of this work was to assess specificity and breadth of the EBV-specific T helper cell response, using two different experimental strategies.

To define specificity, LCL-stimulated CD4+ T cell lines were established from 23 EBV-negative and -positive donors. The T cell lines generated from EBV-negative donors responded poorly against LCL and failed to show EBV-specificity. By contrast, all T cell lines established from healthy virus carriers were EBV-specific. Half of the lines from acutely EBV-infected patients with infectious mononucleosis (IM) were also EBV-specific, while the other half recognized EBV-positive and EBV-negative target cells. Unexpectedly, the EBV-specific T cell lines did not recognize latent antigens of EBV expressed in all LCL. Instead, these lines were specific for lytic cycle antigens predominantly derived from virion proteins. Several of the T cell lines recognized BNRF1, a viral tegument protein. Most T cell lines, however, recognized different virion antigens, suggesting that the family of virion proteins forms the immunodominant targets of the EBV-specific T helper cell response.

Studies on the breadth of the EBV-specific T helper response demonstrated that all healthy virus carriers maintain CD4+ T cell memory to lytic cycle antigens. T cells specific for virion antigens recognized EBV-positive cells directly and, surprisingly, a much higher percentage of target cells than those expressing lytic cycle proteins. Antigen was efficiently transferred to bystander B cells by receptor-mediated uptake of released virions, resulting in recognition of target cells incubated with less than one virion per cell. T cell recognition did not require productive infection and occurred early after virus entry before latency was established. By secreting perforin and granzyme B upon antigen recognition, virion-specific T helper cells inhibited proliferation of LCLs and suppressed the outgrowth of LCLs following infection of primary B cells with EBV.

These results established a novel role for virion-specific T helper cells in the control of EBV infection, and identify virion proteins as important immune targets. The findings have implications for the treatment of diseases associated with EBV and potentially other coated viruses infecting MHC class II-positive cells.

B. Abbreviations

µg	microgram
µl	microliter
APC	antigen presenting cell
BATDA	bis(acetomethyl) 2,2´:6´,2´´–tetrapyridine-6,6”dicarboxylate
BL	Burkitt’s lymphoma
cDNA	complementary deoxyribonucleic acid
CO_2	carbondioxide
DAB	diaminobenzidine
DC	dendritic cells
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
E.coli	Escherichia coli
EBER	Epstein-Barr virus encoded RNA
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot assay
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
X g	times the relative centrifuge force (gravity)
g	gram
GM-CSF	granulocyte-monocyte colony-stimulating-factor
Gy	Gray
h	hour(s)
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
IM	infectious mononucleosis

IPTG	isopropyl-1-thio-β-D-galactoside
kb	kilobases
l	liter
LCL	lymphoblastoid cell line
LMP	latent membrane protein
M	molar
mg	milligram
MHC	major histocompatibility complex
min	minute(s)
ml	milliliter
mm	millimeter
mM	millimolar
mRNA	messenger ribonucleic acid
ng	nanogram
NK cell	natural killer cell
OD	optical density
ORF	open reading frame
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PG-E2	prostaglandin E2
PHA	phytohemaglutinin
PI	propidium iodide
PTLD	post-transplantation lymphoproliferative disease
RNA	ribonucleic acid
rpm	rotations per minute
RT-PCR	reverse transcription-polymerase chain reaction
SOT	solid organ transplantation
TAE	Tris acetate-EDTA
TE	Tris-EDTA
TGF-β	Transforming growth factor-beta
Th	T helper
TNF-α	tumor necrosis factor-alpha

TR	terminal repeat
Tween 20	polyoxyethylene sorbitane monolaurate
U	unit
VCA	viral capsid antigen
X-gal	5-Bromo-4-Chloro-3-Indoyl-b-D-galactopyranosid
^{o}C	degree centigrade

1. Introduction

1.1 The Epstein-Barr virus

In the 1950s, the Irish missionary surgeon Dennis Burkitt observed and treated African children with previously undescribed extranodal lymphomas, known today as Burkitt's lymphoma (BL) (Burkitt, 1958). The high incidence of this tumor in regions of equatorial Africa led Burkitt to postulate that an infectious agent was involved in the tumor's etiology, prompting attempts by Tony Epstein to identify agents associated with the disease. Epstein (and his co-workers including Yvonne Barr) identified in 1964 particles of herpesviral morphology in the cell line EB1 that they had successfully established from a BL (Epstein et al., 1964). This virus proved to be biologically and antigenically distinct from other members of the human herpesvirus family, thereby identifying the Epstein-Barr virus (EBV) as the first human tumor virus.

1.1.1 Classification and structure

EBV is the prototype of the *gamma-herpesviridae* subfamily of herpesviruses. Because EBV preferentially infects B lymphocytes, the virus is classified under the genus *lymphocryptovirus* (Kieff and Rickinson, 2001).

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 and 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 EBV genome is a linear double stranded DNA of 184 kb that consists of repeats and unique sequences (Baer et al., 1984). Variable numbers of reiterated, 0.5 kb direct repeats called terminal repeats (TR) are located at both ends of the genome. The number of these repeats can vary among EBV strains and progenies of EBV replication. In latently infected cells, the number of TR in the viral genome usually remains constant. Analysis of TR, therefore, is conducted to determine whether a group of latently infected cells arose from a single progenitor. Between the TR, two largely unique sequences that contain almost all of the genome coding capacity are separated by a stretch of a variable number of 3 kb repeats (called internal repeats). The unique sequences also include perfect and imperfect tandem repeats of variable lengths, mostly within the nearly hundred open reading frames (ORFs) identified in the EBV genome (Kieff and Rickinson, 2001).

The EBV genome was sequenced from a BamHI fragment library. 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 **B**amHI **L** fragment, **l**eftward OR**F** number **1**. Such nomenclatures are still widely used. Well characterized gene products are more popular with alternative names. For example, the EBV proteins expressed during viral latency are called Epstein-Barr nuclear antigens (EBNAs) or latent membrane proteins (LMP).

1.1.2 Epidemiology and clinical manifestations

Two types of EBV are found in humans (Zimber et al., 1986). Type 1, previously called type A, is far more common in most populations. Type 2 (previously type B) is nearly as common as type 1 in equatorial Africa and Papua New Guinea, and is frequently encountered in human immunodeficiency virus (HIV) infected individuals.

The two types differ mainly in the genes that code for EBNA2, EBNA-leading protein (EBNA-LP; also known as EBNA5) and the three proteins of the EBNA3 family (EBNA3A, -3B and -3C; alternatively known as EBNA3, -4 and -6, respectively). Individual isolates of type 1 or type 2 EBV from widely different geographic areas can also differ by single base changes and repeat reiteration frequency. Persistent infection with more than one EBV type or strain is not unusual, particularly for more promiscuous and immunocompromised people.

Two distinct epidemiological patterns of EBV infection can be observed in different populations. These appear to be closely related with the socioeconomic status (Crawford, 2001). In developed countries, two peaks of infection are seen: the first in very young pre-school children up to six years of age, the second in young adults 14 to 20 years of age. Eventually, up to 95% of all adults worldwide are infected. In developing and underdeveloped societies, infection occurs at a much earlier age. Serological surveys for immunoglobulin G (IgG) antibodies against the viral capsid antigen (VCA) have shown that in the developing world most children become infected with the virus by three years of age. Almost 100% become EBV-seropositive within the first decade of life (Evans and Niederman, 1989).

Primary infection with EBV during early childhood is mostly non-clinical with minor common cold like symptoms. Rare cases of primary infection in early childhood and about half of those with delayed primary infection, however, manifest with the clinical syndrome of infectious mononucleosis (IM) (Papesch and Watkins, 2001; Khanna et al., 1995). IM is an acute febrile illness characterized clinically by pharyngitis, lymphadenopathy, splenomegaly, and hematologically by lymphocytosis with more than 10% atypical mononuclear

lymphocytes. Serologically, IM is characterized by elevated heterophile antibody titers and the development of anti-EBV antibodies (Niederman et al., 1968). EBV achieves its broad distribution in the population through multiple modes of transmission of which parent-to-child salivary transmission appears to be the major route. The practice of chewed feeding and thus early salivary contact within families is generally thought to be the cause of very early exposure to the virus in the developing world. In the Western world, the second peak of infection is perhaps a reflection of the salivary exchange during early adolescence. Hence, acute EBV infection is popularly known as “kissing disease”.

Besides salivary transmission, EBV can potentially be transmitted through other routes. In seropositive individuals the virus has been reported to be able to replicate in cervical epithelia, implicating that EBV may be transmitted sexually (Naher et al., 1992; Portnoy et al., 1984). Further, pregnant woman shed virus in their milk, prompting the hypothesis that mammary transmission also contributes to the early life acquisition of virus by babies and toddlers. EBV can also replicate in peripheral blood cells and thus can also be transmitted through transfusions and transplantations (Alfieri et al., 1996; Cen et al., 1991).

Disease	Population at high risk	EBV association
Infectious mononucleosis	Young adults in the West	Majority
X-linked lymphoproliferative disease (XLP)	Males with mutations in the *XLP* gene	Majority
B lymphoproliferative disease	Post transplantation HIV infection	90% 100%
Burkitt’s lymphoma	African children (endemic) HIV infection/ sporadic	97-100% 25%
Hodgkin’s lymphoma	Children in developing countries Young adults with history of IM	40-80%
Nasal T cell lymphoma NK lymphoma	Asian populations Chronic active EBV infection/ HIV infection	100% 10-100%
Primary effusion lymphoma	HIV infection	70-80%

Table 1. EBV-associated diseases of lymphoid origin (adapted from Williams and Crawford, 2005).

Besides IM, EBV is associated with an increasing list of illnesses (Kawa 2000; Young and Rickinson, 2004). Many of these EBV-associated diseases are malignancies, and EBV has been implicated in the etiology of at least some of these tumors. Since EBV is a ubiquitous virus, however, causal associations are difficult to demonstrate. Some diseases of lymphoid origin associated with EBV are listed in Table 1.

1.1.3 Latent and lytic infection

EBV preferentially infects B cells. This tropism is at least partly a reflection of the high level expression of CD21, the principal receptor for EBV, on B cells. CD21 on B cells serves as cellular receptor for the viral ligand, the glycoprotein gp350 (Fingeroth et al., 1984; Tanner et al., 1987). Gp350-deficient EBV poorly infects B cells although some infection seems to occur (Janz et al., 2000).

Two distinct types of EBV infections can be detected in cells that the virus can infect: (i) lytic replication of the virus which leads to production and eventually release of infectious viral particles, and (ii) latent infection of cells in which the viral genome circularizes upon entry into the nucleus, maintains itself usually as an episome, and is propagated to progeny of the infected cell (Adams and Lindahl, 1975; Hurley and Thorley-Lawson, 1988). Infection of primary B cells with EBV *in vitro* causes growth-transformation of the infected cells and the outgrowth of lymphoblastoid cell lines (LCLs). Under favorable conditions, virus in latently infected cells can reactivate and enter the lytic cycle (Bornkamm and Hammerschmidt, 2001) which involves lytic viral promoter activity and expression of lytic cycle genes (almost all of the genes encoded by the viral genome). Depending upon the chronology of their transcription during lytic replication, EBV genes are identified as immediate early, early and late genes (Rickinson and Kieff, 2001). The viral transactivators BZLF1 and BRLF1 are the only currently known immediate early genes. Their products initiate lytic replication (Chevallier-Greco et al., 1986). At least thirty EBV genes are grouped as early genes and another thirty as late genes. The products of early genes act as transactivators for the expression of other genes or are involved in viral DNA replication. Late genes are expressed temporally later but are formally categorized as late based on the marked reduction in their mRNA abundance in the presence of viral DNA replication inhibitors. In general, late genes are those that code for structural components including viral glycoproteins.

As opposed to lytic replication, a maximum of nine gene products, known as EBV latency proteins or antigens, can be detected during latency along with two untranslated RNAs (called EBERs for Epstein-Barr encoded RNAs) (Kieff and Rickinson, 2001). The latency antigens of EBV include six nuclear proteins, designated EBNA1, -2, -3A, -3B, -3C, and –LP, as well as three integral membrane proteins, namely the latent membrane proteins LMP1, -2A and -2B.

Among different latently-infected cells the spectrum of latency proteins expressed can differ. Depending on the expression profile, latently-infected cells are said to be in different types of latency (Kuppers, 2003). Originally three types of latency programmes were described. However, recent studies have proposed the possibility of additional patterns of gene

expression during latency. The viral products detectable in the different forms of latency and their occurrence are shown in Table 2.

Latency programme	*EBERs*	*EBNA1*	*LMP1*	*LMP2A*	*EBNA2*	*EBNA3s & EBNA-LP*	Occurrence
0	+	+/-	-	+/-	-	-	Peripheral blood memory B cells
I	+	+	-	-	-	-	Burkitt's lymphoma Primary effusion lymphoma
II	+	+	+	+	-	-	Hodgkin's lymphoma
III	+	+	+	+	+	+	Post transplantation lymphoproliferative disease

Table 2. EBV gene latency programmes (adapted from Küppers, 2003).

1.1.4 EBV infection in the natural host

In the most common mode of transmission, viral entry into the oropharynx of a naïve host is followed by the appearance of symptoms after an incubation period of four to six weeks. By that time, cell free virus can be obtained from throat washings (Sixbey et al., 1984). In which cells of the oropharynx EBV replicates after primary infection is still debated. The absence of detectable EBV DNA in the throat and peripheral blood of patients with X-linked agammaglobulinemia that lack B cells provides circumstantial evidence to the argument that B cells infiltrating the oral epithelium are the sites of primary virus replication (Faulkner et al., 1999).

Besides the shedding of infectious virus in salivary secretions, primary infection is also marked by a generalized infection of the circulating B cell pool in which virus colonization in progress can in fact be witnessed before the onset of symptoms. During acute disease 0.1 to 1% (in exceptional cases up to 10%) of circulating B cells stain positive for EBNAs or EBERs, which are reliable markers of EBV latent infection (Tierney et al., 1994). The inability to detect virus in freeze-thaw extracts of these cells suggests that these B cells are in fact latently infected *in vivo*, although some lytic cycle gene transcription activity can be detected in freshly isolated B cells from IM patients (Prang et al., 1997). Also, transcription profiling of freshly isolated B cells from IM patients shows mRNA expression characteristic of latency III, suggesting that these B cells are growth-transformed (Laytragoon-Lewin et al., 1997). Promoter activity studies in the circulating B cell pool of IM patients have, however, also detected cells with other forms of latency. EBER-positive B cells in IM patients have been found to range from large blast like cells similar to the Reed Sternberg cells in Hodgkin's lymphoma (similar also in the viral gene expression pattern: negative for EBNA2 and positive for LMP1) to small cells that are negative for EBNA2 and LMP1. Medium sized lymphoblastoid cells with a LCL-like latency III expression profile are also among the EBER-

positive cells along with intermediates that express EBNA2 and no LMP1 (Niedobitek et al., 1997; Kurth et al., 2000). These are thought to be recently infected cells that are progressing to the LMP1 positive state. It is unclear how these different forms of infection interrelate in time but from analysis of the immunoglobulin (Ig) gene loci of B cells it has been shown that different cell phenotypes can be generated within the same EBV-infected B cell clone (Kurth et al., 2000). Ig gene loci analyses have also demonstrated that the B cell clones expanding after EBV infection carry preferentially, if not exclusively, somatically mutated Ig genes, indicating that they are memory B cells (Kurth et al., 2000). This preferential expansion or survival of infected memory as opposed to infected naïve B cells is yet to be understood.

With subsidence of the symptoms of IM, virus replication in the oropharynx decreases. However, healthy virus carriers, including those that did not develop IM, continue to shed low levels of the virus from the oropharynx (Ling et al., 2003). Replication of virus in other mucosal sites has been reported, suggesting that once the virus has maintained a latent infection, reactivation can occur at any mucosal site with a B cell infiltrate (Sixbey et al., 1986; Egan et al., 1995). Terminal differentiation of infected B cells into plasma cells has been shown to trigger the initiation of lytic replication of EBV *in vivo* (Laichalk et al., 2005).

EBV-infected B cells can be detected in the peripheral blood as well as lymphoid tissues of healthy carriers throughout life. The frequencies are much lower than in acute IM. Based on polymerase chain reaction (PCR) of viral genes on limiting numbers of cells, the frequency of EBV-infected B cells in the peripheral blood has been determined to range from 1 in 10^4 to 1 in 10^6 B cells (Babcock et al., 1998). EBV-positive B cells in healthy carriers differ phenotypically from those in IM in that the former are not activated lymphoblasts. EBV-positive B cells in healthy carriers are resting IgD-and IgM-negative memory B cells (Babcock et al., 1998). The sequestration of EBV in the memory B cell compartment in healthy carriers is in accordance with the observation that predominantly B cells with a memory Ig genotype expand during primary infection.

The EBV gene expression profile of B cells in healthy carriers is also different from that seen in IM. The narrowing of the viral gene expression profile from primary infection to the carrier state is consistent with the reversion of the infected B cell from an activated to a resting memory phenotype. In the carrier state, viral antigen expression is often abrogated and viral transcription is limited to the non-coding EBERs (Miyashita et al., 1995). This type of latency is designated latency 0.

EBV-positive B cells in the lymphoid tissues of healthy virus carriers are found at frequencies similar to those in peripheral blood. However, the types of latency observed in lymphoid

tissue B cells are more heterogeneous than those detected in peripheral blood B cells (Rickinson and Kieff, 2001).

1.1.5 EBV infection *in vitro*

B lymphocytes derived from peripheral blood, tonsils or fetal cord are readily infected with EBV *in vitro*. Earlier stage B cells like those from the bone marrow or from fetal blood are also permissive for infection but the infection efficiency is about ten percent of that of peripheral blood B cells. The same applies for leukemic B cells and EBV-negative BL cells (Tatsumi et al., 1986; Calender et al., 1987; Doyle et al., 1993; Avila-Carino et al., 1994). EBV can also infect T cells, NK cells, some epithelial cell lines, and some carcinoma cell lines *in vitro* with even lower efficiencies (Kanegane et al., 1996; Yoshiyama et al., 1997; Imai et al., 1998).

About 10% of primary B cells infected with EBV *in vitro* become latently infected and give rise to a growth-transformed LCL (Nilsson et al., 1971). LCL typically expresses all nine latency proteins of EBV. Thus, unlike latently infected B cells in the healthy virus carriers where the viral genome is nearly silent, the viral genome in LCL is not inactive. It is latent only in the sense that lytic replication rarely occurs.

The viral latency gene products maintain the latent infection and induce continuous proliferation of the previously resting B cell. B cells growth-transformed by EBV are similar to B cells stimulated by antigen, mitogen, or interleukin-4 (IL-4) and CD40 ligation, express similar cell surface markers and adhesion molecules, secrete Ig, and grow in clumps (Vyth-Dreese et al., 1995). EBV-immortalized B cells may continue to secrete Ig even after years in culture and some of them undergo class switching *in vitro* when exposed to IL-4 (Steel et al., 1974; Litwin et al., 1974).

Wild-type EBV for *in vitro* use can be obtained from virus carriers (who intermittently shed EBV in the saliva) and from EBV-positive cell lines in culture. Although the outcome of *in vitro* infection of primary B cells is the conversion of infected cells to continuously proliferating LCLs which are in general non-permissive for lytic replication, few cells in LCL cultures may spontaneously become permissive for lytic replication. The rate of spontaneous lysis in cell lines known to be good virus producers is around 1% (Kieff and Rickinson, 2001). The phenomenon of few cells going into spontaneous lysis also holds true for EBV-positive BL cells (Kieff and Rickinson, 2001). Cell lines derived from EBV-positive BL are often more permissive for lytic replication than LCL. However, EBV-positive BL cell lines established from EBV-negative BL tumors by infection with EBV *in vitro* are less permissive

than LCLs (Kieff and Rickinson, 2001). The marmoset B cell line B95.8 infected with wild-type EBV is commonly used as a source of virus for *in vitro* studies (Miller et al., 1974).

Lytic replication can be induced in permissive cell lines through a variety of means, for example exposure to chemicals (protein kinase C stimulators and DNA methylation inhibitors among others), starvation, and surface Ig crosslinking (Khanna et al., 1995). Expression of the viral lytic transactivator BZLF1 can also be used to induce lytic replication, although not all latently infected cells in which this protein is expressed enter the lytic cycle (Gradoville et al. 1990). Lethal irradiation also induces lytic viral replication in latently infected cells. Virus production peaks four to five days after induction of lytic replication (Kieff and Rickinson, 2001).

1.2 Immune responses to EBV

1.2.1 Humoral immune response

Infection of the human host with EBV elicits antibody responses against a variety of viral antigenic determinants. Classically, the humoral response to EBV is divided into four distinct reactivities composed of multiple single antigens (Evans and Niederman, 1989).

a) *EBNA reactivity* dominated by anti-EBNA1 antibodies,

b) *early antigen (EA) reactivity* comprising responses against early lytic cycle proteins including BZLF1, the transactivator of lytic replication,

c) *viral capsid antigen (VCA) reactivity* which includes responses to the nucleocapsid, and

d) *membrane antigen (MA) reactivity* dominated largely by anti-gp350 responses and correlating with virus neutralizing activity.

Information on primary antibody responses to EBV is derived largely from IM patients but appears to be similar in asymptomatic infections. At the time of development of the symptoms, IM patients have substantial titers of anti-VCA IgM antibodies and rising IgG titers to VCA and EA. Over the ensuing months the anti-VCA IgM response disappears. The anti-VCA IgG response falls to a stable steady level after an initial peak while the anti-EA IgG response falls faster and further than the anti-VCA IgG response to become either undetectable or to stabilize at very low levels. Anti-MA antibodies are usually of low titer during IM and anti-MA IgG titers rise relatively late in the disease but persist lifelong. A transient IgA response to gp350 is also detectable during IM (Sixbey and Yao, 1992).

Of the nuclear antigens, an IgG response to EBNA2 (and probably to the EBNA3s) is detectable during IM whereas IgG responses to EBNA1 are usually not detectable until

convalescence (Henle et al., 1987). EBV LMP-specific antibody responses have not been detected in IM.

The role of the humoral immune response in the control of EBV infection is unclear. The virus neutralizing antibody response could be involved in limiting spread of the virus. There have, however, also been reports that the anti-gp350 IgA antibodies may actually help virus spread to epithelial cells (Sixbey and Yao, 1992). The discrepancy in the time of development of anti-EBNA1 and -EBNA2 antibodies probably reflects the predominance of different types of latently infected cells at different times during and after IM (Rickinson and Kieff, 2001).

Besides anti-EBV responses, the early phase of IM is also marked by elevated titers of serum IgM, IgG and IgA antibodies, probably as a result of the virus-driven polyclonal activation of the B cell system (Henle and Henle, 1979). Concomitant with this general increase is the transient appearance of a range of heterophile and autoantibodies, mostly of IgM class, which may also be products of virus-infected B cells (Garzelli et al., 1984). Of these various reactivities, heterophile antibodies with the capacity to agglutinate sheep and horse erythrocytes form the basis of the Paul-Bunnell-Davidsohn test that is diagnostic of EBV-associated IM (Henle and Henle, 1979).

Although healthy carriers are consistently positive for IgG antibodies against VCA, anti-gp350 neutralizing antibodies and anti-EBNA1 antibodies titers can differ significantly between individuals. Many carriers also possess further reactivities like those to EA or to other EBNAs (Evans and Niederman, 1989). Again, the roles of these antibodies during persistent infection are not known, except that the virus neutralizing antibodies could contribute to overall control of the infection by preventing potential outbreaks of viremia.

1.2.2 The CD8+ T cell response to EBV

With the realization that humoral immune responses alone are not sufficient to control virus infection, the focus has shifted towards T cell-mediated responses to EBV.

IM is marked by large numbers of atypical, mononuclear, predominantly CD8+ T lymphoblasts in the peripheral blood (Yata et al., 1973). The nature of the highly expanded CD8+ T cell response has long been debated. Findings of markedly skewed TCR Vβ subset distributions seen within CD8+ T cells of IM patients have been interpreted as bystander T cell activations or superantigen-induced expansions (Sutkowski et al., 1996).

In healthy virus carriers CD8+ T cells specific for latent cycle antigens were detected and the epitopes were found to be predominantly derived from the EBNA3 family of proteins. Subsequently, similar studies were performed in patients with acute infection. *In vitro*

expanded CD8+ T cells from the peripheral blood of these patients also responded against latent antigens of EBV even at the height of primary infection (Steven et al., 1996). In addition, responses against allogeneic LCL were observed with these T cells, suggesting that additional specificities were present in these lines. Studies of the cytotoxic T lymphocyte (CTL) response in young infants within months of primary infection led to similar results (Tamaki et al., 1995). Precursor frequency analyses of T cells specific for a given latent cycle antigen using major histocompatibility complex (MHC) tetramers, however, demonstrated that these T cells accounted for only a small percentage of the total CD8+ T cell population *in vivo* (Callan et al., 1998). When lytic cycle antigens were included in such assays it became clear that multiple lytic cycle antigen specific T cells expand during IM, and that these are clearly more abundant than latent cycle antigen specificities (Callan et al., 1998). The most dominant lytic cycle antigens were identified to be derived from the immediate early proteins BZLF1 and BRLF1 and the early proteins BMLF1, BMRF1 and BALF2 (Bogedain et al., 1995; Callan et al., 1998). Depending on the human leucocyte antigen (HLA) class I haplotypes, up to 50% of all CD8+ T cells in the peripheral blood of IM patients can be directed against a single epitope from a single lytic cycle antigen (Steven et al., 1997; Callan et al., 1998). These studies also revealed that the T cell reactivities against HLA-mismatched LCL reported in earlier studies are actually the result of cross-reactivity of certain EBV-specific T cells against allo MHC-peptide complexes (Gaston et al., 1983; Burrows et al., 1997).

Similar, albeit less abundant, T cell reactivities were observed in healthy virus carriers. While CD8+ T cells with specificity for a single latent cycle antigen were shown to account for up to 1% of the peripheral CD8+ T cell pool, T cells specific for a single lytic cycle antigen were found to be about five times more frequent (Tan et al., 1999).

Most CD8+ T cells in patients with IM show an activated phenotype. During convalescence, most of these T cells die, most likely because antigen becomes limiting. As determined by MHC tetramer staining, both lytic and latent antigen specificities contract massively after IM, but with different kinetics (Silins et al., 1996). The remaining cells revert to a resting phenotype, although cells with memory or effector cell phenotypes have been detected. EBV-specific T cell memory is best identified in regression assays (Moss et al., 1978). Unlike peripheral blood mononuclear cells (PBMCs) from EBV-seronegative donors, *in vitro* EBV-infected PBMC from seropositive individuals fail to form LCLs unless T cells are depleted or inhibited. LCL-stimulated T cell memory against EBV is dominated by reactivities against the EBNA3 family of proteins followed by subdominant epitopes derived from LMP2 (Rickinson

and Moss, 1997). Responses to EBNA2, EBNA-LP, LMP1 and EBNA1 are rarely observed, and only weak responses to the BARF0 encoded polypeptides have been described. This marked hierarchy of immunodominance among latent cycle antigens is only partly understood. In the case of EBNA1, an internal glycine-alanine repeat prevents proteasomal degradation, and hence the generation of MHCI peptides (Levitskaya et al., 1995). Nevertheless, EBNA1-specific CD8+ T cells do exist at least in EBV-positive donors with certain MHC haplotypes, providing space for the argument that EBV-specific CD8+ responses might be primed by cells other than EBV-infected B cells (Munz, 2004).

1.2.3 The CD4+ T cell response to EBV

CD4+ T cells recognize peptide/MHCII complexes presented on the cell surface of professional antigen presenting cells, e.g. B cells, DCs, and macrophages. MHCII molecules are heterodimers that bind peptides of variable lengths (Engelhard et al., 1994). MHCII molecules are synthesized into the lumen of the endoplasmatic reticulum where they associate with the invariant chain. This chaperone prevents binding of peptides to MHCII molecules and escorts newly synthesized MHCII molecules into the endosomal/lysosomal compartment (Cresswell, 1996). Within this increasingly acidic vesicular system, invariant chain is degraded, allowing access of peptides to the peptide binding groove (Hsing and Rudensky, 2005). These peptides are mostly derived from endocytosed proteins that are degraded in the lytic compartment. Exchange of CLIP, the cleavage product of invariant chain occupying the peptide binding groove, for peptides from endocytosed proteins is catalyzed by HLA-like molecules (Denzin et al., 2005). Vesicles with peptide-loaded MHCII molecules eventually traffic to the cell membrane where they can be recognized by CD4+ T cells.

In contrast to MHCI molecules which present peptides derived mostly from intracellular proteins, MHCII molecules present peptides predominantly derived from extracellular and cell membrane proteins (Watts, 2004). This dichotomy of antigen presentation, however, is not as absolute as originally thought. Peptides derived from exogenous proteins can be presented on MHCI and this “cross-priming” appears to be essential for immune responses against viruses and tumors (Chen et al., 2004; Otahal et al., 2005). Likewise, endogenous presentation of antigens synthesized within the antigen presenting cell on MHCII has been implicated in the induction of tolerance and anti-viral immunity (Rudensky et al., 1991; Dongre et al., 2001). Recently, autophagy has been proposed as mechanism responsible for the endogenous presentation of cytosolic and nuclear antigens on MHCII (Nimmerjahn et al., 2003; Paludan et al., 2005; Tewari et al., 2005; Zhou et al., 2005). Autophagy involves the

random sequestration of cytoplasm into membranous vesicles called autophagosomes which eventually fuse with endosomes/lysososomes thereby delivering their content into the MHCII processing and loading compartment (Yorimitsu and Klionsky, 2005) (Figure 1.1). Thus, T helper cells specific for an intracellular antigen may recognize target cells directly.

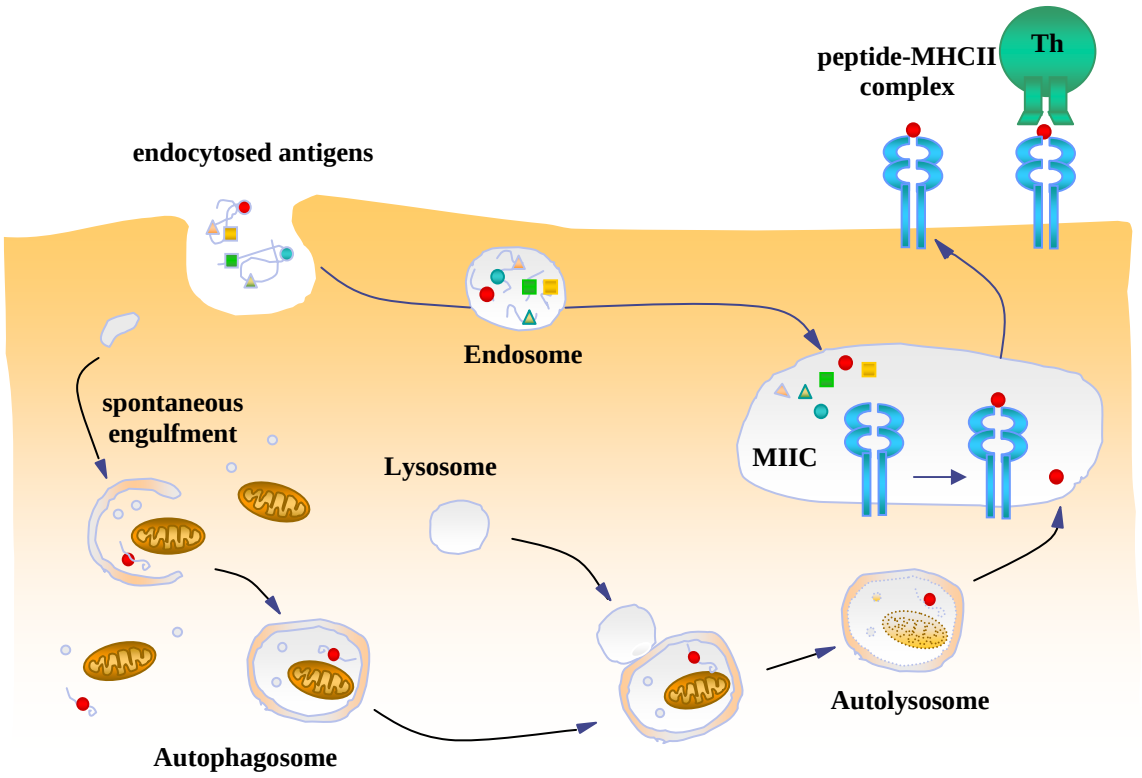


Figure 1. Peptides presented on MHCII molecules are derived from extracellular and intracellular proteins.
Endocytosed antigens follow the classical pathway of MHCII presentation. Endosomes carrying the endocytosed antigens fuse with MHCII antigen presentation compartments (MIIC). Intracellular proteins enter the vesicular system during autophagy in which parts of the cytoplasm are spontaneously engulfed into membrane-bound organelles (autophagosomes) which subsequently fuse with lysosomes thereby delivering the proteins also to the MHCII antigen presentation pathway.

CD4+ T cells are classically designated T helper cells because B cells specific for a given antigen cannot be activated to proliferate, form germinal centers, or differentiate into plasma cells, until they encounter a helper T cell that is specific for one of the peptides derived from the antigen (McHeyzer-Williams and McHeyzer-Williams, 2005). Subsequent studies revealed that T helper cells are the source of interferon-gamma (IFN-γ) required to activate macrophages to destroy intracellular microorganisms more efficiently (Pace et al., 1983). More recent studies identified an important function of T helper cells in licensing DCs to prime CD8+ T cells (Smith et al., 2004). Thus, T helper cells play a central role in the orchestration of humoral and cellular immune responses. Following endogenous presentation

of intracellular antigens on MHCII, T helper cells may also exert direct effector functions on target cells.

The multiple effector functions of CD4+ T cells appear to correlate with the pattern of cytokines secreted upon activation. Accordingly, CD4+ T cells can be divided into several subtypes including T helper type (Th) 1 and 2 among others (Mosmann and Sad, 1996).

Th1 cells are best identified by their secretion of IFN-γ upon target cell recognition. IFN-γ is able to activate macrophages and to inhibit proliferation of different cell types. In addition, IFN-γ enhances antigen presentation by inducing expression of MHC molecules (Christensen et al., 1999; Robertson et al., 2001; Paludan et al., 2002).

Th2 type CD4+ T cells are the classical T helper cells. They produce IL-4 and IL-13, but little or no IFN-γ, and help naïve B cells to proliferate and secrete Ig (Parker, 1993).

The term Th3 has been used to describe unique cells that produce mainly transforming growth factor-ß (TGF-ß) in response to antigen. Because of the T cell inhibitory function of TGF-ß, these T cells are also called regulatory T cells or T_R1 cells and appear to play an important role in preventing responses to food antigens (Faria and Weiner, 2005). More recently, a different type of regulatory T cells, characterized by the expression of CD4 and CD25 has been identified (Sakaguchi, 2004). These regulatory T cells are able to suppress T cells responses by an IL-10- and TGF-β–independent, but cell contact-dependent mechanism(s). These T cells seem to play an important role in preventing autoimmune responses (Lan et al., 2005). In addition, these regulatory T cells have been shown to prevent or ameliorate other immunopathologic syndromes such as graft-versus-host disease and graft rejection. There is even evidence that normal immune responses can be inhibited by these T cells, so they may have a role in controlling the extent, duration, and perhaps even the quality of normal as well as pathologic immune responses (O`Garra and Vieira, 2004). A recent report on regulatory T cells specifically recognizing epitopes derived from the EBV nuclear antigen EBNA1 suggested that such regulatory T cells may play a role in virus persistence (Voo et al., 2005).

Knowledge on the CD4+ T cell response to EBV is still scarce. In patients with IM, CD4+ T cells do not expand to the same degree as CD8+ T cells, and only few EBV-specific CD4+T cells have been described. Information on the CD4+ T cell memory to EBV in the healthy carrier is also still rudimentary despite recent suggestions that CD4+ T cells can recognize EBV-infected cells directly, and, more importantly, that they are pivotal in the maintenance of long term cellular immunity (Callan, 2004). The first CD4+ T cells reactive against EBV were identified as rare clones in LCL-stimulated CTL preparations (Rickinson and Moss, 1997). In recent years, the CD4+ memory to latency antigens has been analyzed more systematically

(Munz et al., 2000; Mautner et al., 2004; Long et al., 2005). Some reports suggest a hierarchy of immunodominance among the latent cycle antigens with EBNA1 and EBNA3C being the dominant ones (Leen et al., 2001). Further studies are needed to verify this. The recognition of endogenous EBNA1 on EBV-infected cells by CD4+ T cells is also controversially discussed. (Munz et al., 2000; Mautner et al., 2004). Thus, important issues regarding the CD4+ T cell response to EBV are still unresolved.

1.3 EBV in the immunocompromised

Immune impairment is typically associated with reduced EBV-specific CTL responses, increased virus shedding, increased viral genomic load, increased numbers of infected B cells in the blood, and also altered anti-EBV antibody profiles. These phenomena have been observed partly or wholly in long term low level immunosuppressed, solid organ transplant (SOT) recipients and also in HIV-infected individuals (Riddler et al., 1994; Baldanti et al., 2000).

As noticed first in bone marrow and cardiac transplant recipients, and later also in HIV-infected individuals, immunocompromised patients also differ from immunocompetent individuals in the number of viral strains they carry (Yao et al., 1996a). While simultaneous infection with multiple viral types and strains is rarely seen in immune competent hosts, HIV-infected individuals with detectable T cell impairment are frequently co-infected with type 1 and type 2 EBV strains (Yao et al., 1996b).

The effects of immune impairment are often most pronounced in bone marrow transplant (BMT) recipients in whom T cell function is more strongly suppressed, and in seronegative transplant recipients who often acquire their primary infection iatrogenically through the transplant or through transfusions. In BMT recipients, EBV DNA load in PBMC can reach very high levels in the first three to six months after transplantation which correlates with the risk of developing B cell post transplant lymphoproliferative disease (PTLD) (Gottschalk et al., 2005).

The link between sustained immunosuppression of transplant recipients and increased incidence of lymphoma, especially PTLD, has long been apparent and the association with EBV is now widely recognized. One major risk factor for PTLD development is the intensity of T cell suppression. Thus, patients receiving renal and liver transplants have overall incidence rates of 1% to 2%, versus 3% to 8% for lung and heart transplantations where higher levels of immunosuppression are necessary (Gottschalk et al., 2005). In BMT settings, the overall incidence rates are less than 1%. However, T cell depletion of the marrow graft can increase incidence rates to 12% to 24% (Nalesnik, 1998). Because in most BMT protocols

host lymphoid cells are totally ablated, PTLDs usually arise from infected donor B cells. Consequently, it is now a standard practice to treat high EBV load following stem cell transplantation with the monoclonal anti-CD20 antibody Rituximab which rapidly lowers the viral load by eliminating circulating B cells and has been shown to reduce the incidence of PTLD (van Esser et al., 2002).

The patient's EBV status at the time of transplantation is also an important risk factor for PTLD. A survey of SOT recipients showed a 20-fold increased incidence in seronegative recipients, explaining why younger children are at increased risks (Ho et al., 1985). The viral genomic load in transplant recipients has also been used to assess risk and in deciding prospective patients for adoptive T cell therapy (van Esser et al., 2001). The limited success of acyclovir in the prevention of PTLD has raised the question to what extent ongoing virus replication might increase the risk for PTLD development.

Classic PTLDs mostly arise within the first two years of transplantation. At least 90% of such lesions are EBV-associated and are either oligoclonal or monoclonal based on EBV TR and Ig gene analysis (Chadburn et al., 1997). By immunostaining, most EBV-associated PTLD lesions are dominated by EBNA1-, EBNA2-, and LMP1-positive cells. A minority of these tumor cells are EBNA1-positive with low or undetectable EBNA2 and LMP1. A small proportion of PTLDs have acquired mutations in the p53 and c-myc gene loci. PTLDs showing complex chromosomal aberrations often display more restricted forms of latency (Locker and Nalesnik, 1989; Knowles et al., 1995).

Many PTLDs were observed to regress when immunosuppressive therapy was reduced probably due to a re-emergence of the patient’s anti-EBV T cell response (Khatri et al., 1999). Therefore, a reduction in immunosuppression is the first treatment option for PTLD.

Similar to transplant recipients, HIV-infected individuals are at increased risks of EBV-associated B cell lymphoma development. The spectrum of tumor types in HIV positives is more diverse than that seen in the transplant setting (Gaidano et al., 1998). At least one major category of EBV-associated B cell lymphoma is essentially analogous to PTLD lesions and appears in end-stage AIDS when patients are profoundly immunocompromised. Most of the AIDS lymphomas presenting in the central nervous system are of this type.

1.4 Immunological approaches to control EBV infection

The identification of the major envelope glycoprotein gp350 as the dominant target of the virus neutralizing antibody response in the 1980s fuelled attempts to develop a prophylactic EBV vaccine (Epstein 1986; Morgan and Wilson, 1997). It is now clear that neutralizing

antibodies alone or in combination with cell-mediated responses to a single envelope component are not likely to offer long lasting sterile immunity, although such an approach could be expected to sufficiently reduce the free viral load which might be sufficient to prevent primary infections from leading to IM or, in immunocompromised patients, to PTLD. The recognition of the importance of CD8+ T cells in the control of primary infection have opened up possibilities of inducing CD8+ responses by priming naïve individuals through the use of synthetic peptides or polyepitope vaccine constructs (Moss et al., 1996). Novel therapeutic approaches to tackling EBV-associated tumors are constantly tested. Considerable progress has been made in the treatment of EBV-associated lymphoproliferative disorders (Gottschalk et al., 2005).

1.4.1 T cell therapy of EBV-associated lymphomas

In the SCID mouse model, EBV-positive human B cell lymphomas can be induced by reconstituting the mouse with PBMC from EBV-seropositive individuals and can be cured or growth impaired by the infusion of T cells from the autologous donor (Donjon et al., 1983). An analogous strategy in BMT recipients showed that the transfusion of small numbers of PBMC from the healthy seropositive donor can reverse PTLD growth. Although this regimen led to dramatic increases in the EBV-specific T cell frequencies, the incidence of graft-versus-host disease also went high. Subsequent studies demonstrated that LCL-stimulated PBMC could mediate equally effective tumor control without causing graft-versus-host disease (Rooney et al., 1995; Heslop and Rooney, 1997). This approach could also be used to reduce high EBV genome loads in the blood of patients at risk. In the SCID mouse model infused T cells were found to home preferentially to tumor sites (Lacerda et al., 1996). This together with the finding that non-specific CTLs fail to reduce viral load implied that EBV-specific T cells are indeed the effector cell population that serve the clinical purpose. This was further underlined by the observation that a tumor growing in face of T cell therapy had lost EBV epitopes against which the infused T cell populations were predominantly directed (Gottschalk et al., 2001). PTLD in SOT recipients are usually derived from host B cells rather than donor cells and thus pose a logistic problem. Although active T cell populations can still be generated from post transplantation bleedings when the patient is already immunosuppressed, alternative strategies like using allogeneic or partially MHC-matched donor-derived T cell populations are being explored (Haque et al., 2001). Such latter T cell populations would not be expected to stay long-term but could provide the immediate rescue,

at least in patients who underwent primary infection during transplantation, and thus have not developed anti-EBV T cell immunity yet.

Most EBV-associated malignancies other than PTLD show more restricted patterns of latent antigen expression and, therefore, are believed to be difficult to target through CTL therapy (Rickinson and Kieff, 2001). Recent studies, however, have shown that an EBV-specific T cell population can be generated by stimulating PBMC from patients with Hodgkin's disease with autologous LCL, that these T cells persist for several months after infusion and that they cause a reduction in the EBV genomic load (Bollard et al., 2004a).

CD8+ T cells recognizing latent antigens are thought to be the major effectors against PTLD. However it is usually acknowledged that CD4+ T cells are important in facilitating CD8+ T cell expansion and survival or as effectors in their own right (Kalams et al., 1998; Hung et al., 1998).

Due to the often rapid progression of PTLD, T cell lines for therapy have to be prepared prophylactically for patients at risk. This precludes a broader clinical application of this labor and cost-intensive adoptive T cell therapy. The development of alternative protocols that permit the generation of EBV-specific T cell lines more rapidly even after tumor manifestation is dependent on the knowledge of viral antigens and epitopes.

1.5 Aim of the work

The aim of this work was to characterize the EBV-specific CD4+ T cell response in EBV-negative (cord blood donors and EBV-negative adults) and EBV-positive individuals (during the acute and persistent phase of infection) and to identify the immunodominant antigens. The characterization of the immunodominant MHCII restricted EBV antigens should not only provide insight into how protective immunity is established but should also help in the identification of immunotherapeutic strategies for tumors with a more restricted viral expression profile than PTLD. Taken together the findings of this work were expected to contribute to a better understanding of the host-pathogen interaction as well as to novel therapeutic approaches against aggressive EBV-associated diseases.

2. Materials and Methods

This chapter describes the materials and methods used in this work. Where materials were bought from commercial manufactures, the manufacturer is indicated in parentheses after the material name where it appears first. For materials obtained as gifts the source is similarly indicated in parentheses. Where the source of commercial material has not been indicated they were bought from one of the following: Amersham, Becton Dickinson, BioRad, Calbiochem, Corning, Dianova, Eppendorf, Greiner, Integra, Invitrogen, MBI-Fermentas, Merck, Millipore, NEB, Neolab, Nunc, PAA, Packard Instruments, Perkin Elmer, Promega, Qiagen, Roche/Boehringer, Sartorius, Sigma-Aldrich, Stratagene.

2.1 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 (Haereus)
Bacteria shaker (New Brunswick Scientific)
Cell counting chamber (GLW)
Elektroporation 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 (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)

2.2 Cell biology materials and methods

2.2.1 Eukaryotic cell culture materials

The recipes used for the preparation of different cell culture media and solutions are as follows:

T cell media (for culture of T cells)
500 ml AIM-V (Invitrogen)
10% heat-inactivated pooled human serum (preparation described below)
2 mM L-glutamine (PAA)
10 mM HEPES (Invitrogen)
50 µg/ml gentamicin (Invitrogen)

LCL media
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 (PAA)
50 µg/ml gentamicin (Invitrogen)

Insect cell media
500 ml Grace's insect media (Invitrogen)
10% FCS
1% fungizone ((Invitrogen)
50 µg/ml gentamicin

Supplemented DMEM media
500 ml DMEM (PAA)
10% FCS
50 µg/ml gentamicin
2 mM L- glutamine

PBS

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.

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)

2.2.2 General conditions for eukaryotic cell culture

All cells were incubated in a cell culture incubator with 5% CO_2 at 37°C except for insect cells (described separately below). All media for cell culture were stored at 4°C for no longer than three months, and were used within two weeks following addition of serum. Serum was stored at -20°C for no longer than six months. Other supplements were kept at temperatures recommended by the manufacturer. Cell manipulations were performed in a biological containment cabinet (hood) under strict aseptic techniques using disposable plastic pipettes or autoclaved glass pipettes with cotton plugs. Cells in culture were refed with fresh growth medium as necessary, depending upon the growth and media consumption. Cells were ordinarily expanded and frozen to maintain a frozen inventory. Freezing of cells was done in 1.8 ml cryotubes (Nunc) with 5 X 10^6 to 1 X 10^7 cells in 1.8 ml of freezing media consisting of 10% dimethylsulfoxide (DMSO, Merck) in fetal calf serum (FCS). For allowing slow freezing, tubes with cells to be frozen were put in propanol compartments (Nunc) in -80°C

freezers. Within a week the tubes were transferred to liquid nitrogen tanks for long term storage.

Frozen cells were allowed to thaw rapidly at 37°C and transferred into a 15 ml Falcon tube in which they were washed in RPMI 1640 twice by centrifuging the cells at 1000 X g for 5 min in between washes. The cells obtained thereafter were maintained in fresh warm media. (Unless otherwise stated all centrifugation steps described below used to pellet cells were performed at 1000 X g for 5 min at room temperature). Thawed cells were treated for two weeks with mycoplasma removal agent (MP) to avoid mycoplasma contamination.

EBV-negative BL cell lines, their EBV-positive convertants, and the marmoset B cell line B95.8 were maintained in suspension culture in cell culture flasks in LCL media.

The adherently growing human fibroblast cell line Wi38 and the human embryonic kidney cell line 293T were cultivated in cell culture plates in supplemented DMEM media. They were split 1:4 every two to three days as necessary using 10 ml (or 5 ml) of 1 X Trypsin-EDTA (PAA) to detach cells by incubation at 37°C for 5 min. The detached cells were then transferred to a 15 mlFalcon tube along with supplemented DMEM media, pelleted by centrifugation, resuspended in fresh supplemented DMEM media and brought out in new plates at desired cell densities.

The insect cell line Sf9, also growing adherently, was maintained in a separate incubator at 27°C in cell culture plates and cultivated in insect cell media.

2.2.3 Separation of peripheral blood mononuclear cells from whole blood

PBMC used in this work were obtained either from buffy coats or from freshly obtained peripheral blood. Peripheral blood was obtained from EBV-positive and -negative healthy donors, IM patients and cord blood donors. Peripheral blood of IM patients were obtained from the Children's Hospital of the University of Technology, Munich. Cord blood samples were kindly provided by Dr. Andreas Moosmann (Clinical Cooperation Group "Molecular Oncology", GSF). Studies on material of human origin were approved by the Ethical Board of the University of Technology, Munich.

PBMC were isolated by density gradient purification. For this peripheral blood or buffy coats were collected in 50 ml Falcon tubes and treated with 0.5 mM EDTA to prevent coagulation. Samples were then diluted 1:1 in RPMI 1640, underlayed with 10 to12 ml Ficoll-Paque (Amersham) per 50 ml Falcon tube and centrifuged at 2000 X g without brakes for half an hour. This led to the collection of red blood cells at the bottom of the tube and white blood cells and platelets on top of the Ficoll phase. The white cell phase was carefully collected with

a pipette and transferred to a new Falcon tube diluted with RPMI 1640 and then centrifuged at 1000 X g for 10 min. After the centrifugation, the supernatant was discarded and the cellular pellet washed twice in RPMI 1640. Cells pelleted by centrifugation were then resuspended in 2 ml media, counted, and the cell suspension diluted with fresh media as required for subsequent applications.

PBMC derived from buffy coats were used as feeder cells for the stimulation of T cell clones. Equal numbers of PBMC, derived from at least three different buffy coats, were mixed on ice for half an hour after which they were irradiated (40Gray), washed with RPMI 1640, and resuspended at 10^5 cells /µl. These were then used in T cell cloning media at 2.5 X 10^5/ml.

2.2.4 Preparation of pooled human sera for T cell media

For T cell media, sera prepared from voluntary donors were used. Whole blood obtained from peripheral veins was collected in 50 ml Falcon tubes and allowed to coagulate. The coagulated blood was centrifuged at 3000 X g for 15 min and the clot-free supernatant transferred to new tubes which were then centrifuged in the same conditions as before to get rid of any remaining cells. The clear serum was heat-inactivated at 56°C for half an hour. Sera obtained from multiple (at least three) donors were pooled and the pooled sera used for the preparation of T cell media.

2.2.5 Establishment and culture of lymphoblastoid cell lines

Several LCLs were established during this work. For this purpose PBMC obtained after density gradient centrifugation were resuspended at 1-2 X 10^6 cells per 100 µl in LCL media. 100 µl of the cellular suspension were brought out per well of a 96-well flat bottom plate. Infection with EBV was performed by adding 100 µl of virus suspension in the presence of cyclosporine A at a final concentration of 0.5 mg/ml.

Two types of LCLs were generated by using two different viral strains. The B95.8 wild type virus was obtained by filtration of cell-free supernatant of densely grown B95.8 cells through 0.45 µl filters. The second EBV strain used was a laboratory derivative of the wild type virus. This laboratory strain called miniEBV differs from the wild type virus in that it is unable to undergo lytic replication. MiniEBV was kindly provided by Dr. Andreas Moosmann (Clinical Cooperation Group "Molecular Oncology", GSF). After 24 hours half the medium was removed and replenished with cyclosporine-supplemented fresh LCL media. Infected PBMC were regularly inspected for media exhaustion and were replenished with cyclosporine-A-supplemented LCL media as necessary. 10^4 cells/well of the human fibroblast cell line Wi38

were provided as feeders to cultures that failed to show B cell colonies within two weeks. Infected cells that started proliferating were transferred to 48-well and then subsequently to 24-well plates. From there on, expanding cell lines were transferred to plastic flasks in which the B cell lines were maintained as long as used.

EBV-immortalized cells were split regularly depending on proliferation. For this, the cells were resuspended by pipetting, half of the resuspension removed and replaced with fresh media.

In this work wild type EBV-immortalized cell lines are called LCL and miniEBV-immortalized cell lines are called miniLCL.

LCLs were HLA class I and II typed at the Laboratory of Immunogenetics, LMU, Munich.

2.2.6 Generation and maintenance of T cell lines and clones

EBV-specific T cells lines were generated either by using autologous LCL or recombinantly expressed EBV protein-pulsed autologous PBMC as stimulators of peripheral blood T cells.

LCL (or miniLCL)-specific T cell lines were generated by co-incubating 2 X 10^6 PBMC with 2 X 10^6 γ-irradiated (80 Gy) LCL (or miniLCL) in one well of a 24-well plates in 2 ml T cell media. Within 48 hours, cultures were supplemented with 10U/ml recombinant human IL-2 (Chiron). T cell lines were restimulated biweekly with irradiated LCL (or miniLCL) in the same fashion.

For establishing EBV protein-specific T cell lines, 2 X 10^6 PBMC were incubated in 2 ml T cell medium typically with 500 ng/ml recombinant EBV protein in one well of a 24-well plate. (Expression, purification and quantification of recombinant proteins is described below). IL-2 was added to the cultures within 48 hours. For the restimulation of T cell lines, 2 X 10^6 PBMC incubated with 500 ng/ml protein in one well of a 24-well plate for 24 hours were irradiated at 40 Gy and then co-cultured in 2ml T cell media in a well of a 24-well plate with 1 X 10^6 T cells from the last restimulation.

Between restimulations, T cell lines were expanded depending on proliferation. Expansion of T cells was done by transferring half of a proliferating culture to a new well and then replenishing the wells with T cell media supplemented with 10 U/ml IL-2.

In this work T cell lines have been qualified using the term passage (abbreviated p). This term followed by a number is used to identify the number of restimulations that the T cell line underwent. For example, a LCL-stimulated T cell line p10 indicates T cells obtained after 10 restimulations with irradiated autologous LCL.

To obtain CD4+ T cell lines, unselected T cells were subjected to magnetic sorting. CD4+ T cell enrichment was achieved either by positive or negative selection. Magnetic sorting was also used for the enrichment of B cells by sorting CD19+ PBMCs or CD4-CD8- PBMCs. Since the steps are identical, the general method of magnetic sorting is described.

Cells to be sorted were washed and resuspended at 10^7 cells in 80 µl MACS buffer (PBS with 1% bovine serum albumin and 2 mM EDTA). The cells were then incubated on ice for 20 min with 20 µl Ig microbeads (Miltenyi). After the 20 min, the beads-labeled cells were washed once with MACS buffer, pelleted by centrifugation and then allowed to run through a MACS column (Miltenyi) on a magnetic stand equilibrated with 3 ml MACS buffer. The flow through, which contains unlabelled cells, was collected when cell depletions were performed. For collecting the positively selected cells, the column was removed from the magnetic stand and its content was siphoned out into a 15 ml Falcon tube with 5 ml MACS buffer. Either fraction was pelleted by centrifugation, washed in RPMI 1640 media, and resuspended in media for further use.

T cell clones described in this study were derived from T cell lines by limiting dilution cloning. T cells at day 14 of the last restimulation were co-cultured with stimulator APC (prepared similarly as for the stimulation of the T cell lines) in cloning media consisting of T cell media supplemented with 10 U/ml IL-2, 250 ng/ml phytohemaglutinin (PHA) (Murex) and 2.5 X 10^5 /ml irradiated feeder cells consisting of PBMC of three donors at 0.3 to 3 T cells in 200 µl media/well in 96-well round bottom plates. After three days, most of the media was removed and replenished with IL-2-supplemented T cell media. After two weeks, outgrowing T cell clones were transferred to 48- or 24-well plates and restimulated in the same fashion.

2.2.7 Preparation of dendritic cells from PBMC

Myeloid DC were obtained by *in vitro* differentiation of precursors from peripheral blood. 100 mm petri dishes were coated with 10 ng/ml human IgG in 10ml PBS for 10 min at room temperature. The plates were washed once with PBS and 5-7 X 10^7 PBMCs in 10 ml LCL medium brought out per plate. The cells were incubated for 30 min at 37ºC after which non-adherent cells were removed by vigorous washing with PBS. The adherent remaining cells were exposed to 50 ng/ml GM-CSF (Berlex) and 100 ng/ml IL-4 (PAN) in 10 ml LCL media in order to induce DC differentiation. After two days at 37ºC, the cells were refed with fresh media supplemented with the same concentration of granulocyte-monocyte colony-stimulating-factor (GM-CSF) and IL-4. These cells were either used within two days as

immature DC or were subjected to maturation with the same concentration of GM-CSF and IL-4 along with 10 ng/ml TNF-α (R&D) , 1 μg/ml PG-E2 (Sigma-Aldrich) and 10 ng/ml IL-1β (R&D). After 24 hours of exposure to the maturation cocktail the cells were used as mature DC.

2.2.8 Methods for T cells phenotype and function analysis

2.2.8.1 Flow cytometric analysis of T cells

T cells were analyzed by two-color flow cytometry for surface CD4, CD8, TCRαβ and TCRγδ expression. All fluorescence labeled antibodies were bought from BD Biosciences.
For labeling the cells, approximately 3 X 10^5 cells were collected in a 1.5 ml Eppendorf tube, washed once with cold FACS buffer (PBS with 2% FCS) and incubated for 20 to 30 min on ice with an antibody against the surface molecule(s) in 100 μl FACS buffer. If the first antibody was not fluorescence-labeled, the cells were washed once with FACS buffer and then incubated for 20 min on ice with a fluorescence-labeled secondary antibody (specific for the first antibody) in a final volume of 100 μl FACS buffer. All incubations with fluorescence labeled antibodies were performed in the dark. After fluorescent labeling, cells were washed three times in 1 ml FACS buffer, pelleted and resuspended in 400 μl FACS buffer containing 0.1 mM propidium iodide. Cell samples prepared in the aforementioned way were analyzed on a FACScan flow cytometer with CellQuest software (Becton Dickinson).

2.2.8.2 Measuring T cell cytokine secretion by ELISA

T cells were assayed for target cell recognition by measuring specific cytokine release using ELISA kits (R&D). T cells to be assayed were co-cultured with appropriate antigen presenting cells in 96-well plates in 200 μl of LCL media. Where indicated, antigen presenting cells had been preincubated with recombinant protein (for at least 24 hours) or peptide (for at least two hours) in 200 μl LCL media per well in 96-well plates. Following incubation, the target cells were washed extensively to remove excess protein or peptide, and co-cultured with 10^5 T cells. In control wells, APC alone and T cells alone were assayed. After 20 to 24 hours of incubation, the cell culture supernatant was harvested and the cytokine content measured following the general ELISA protocol provided by the manufacturer. Xread Plus software was employed for quantification.

2.2.8.3 IFN-γ and perforin ELISPOT assays

IFN-γ ELISPOT assays were performed in 96-well nitrocellulose-lined microtiter plates (Millipore) principally following the protocols provided by the manufacturer. Briefly, plates were coated overnight at 4ºC with 100 µl of 10 µg/ml anti-IFN-γ mAb 1-D1K (Mabtech) in PBS. The following day, the plates were washed thrice with PBS and then blocked with T cell media at 37ºC for 1 h. After three washes with PBS, target and responder cells were added to the wells in a maximum total volume of 200 µl and the plate incubated at 37 ºC for 24 to 48 hours. The cells were then removed by washing the wells thoroughly three times with PBST (PBS with 0.05% Tween 20) (Sigma–Aldrich). Next, 100 µl of 1 µg/ml biotinylated anti-IFN-γ mAb 7-B6-1 biotin (Mabtech) in PBS were added per well and incubated for two hours at room temperature. Subsequently, the plates were washed six times with PBST and 100 µl of 1:1000 diluted ExtraVidin peroxidase conjugate (Sigma–Aldrich) were added per well. After an hour of incubation at room temperature and another round of six washes, 100 µl of freshly-prepared, 0.25 µm-filtered substrate solution were added per well. Depending on the development of color, the colorimetric reaction was terminated within 20 min by rinsing with tap water. The plates were air dried overnight and analyzed the next day under the light microscope for the number of spots in each well.

Perforin ELISPOT assays were performed similarly with modifications as suggested by the manufacturers (Mabtech and Millipore).

Substrate solution: 1 tablet each of Sigma FAST DAB and Sigma FAST Urea hydrogen peroxide (both from Sigma-Aldrich) in 10 ml deionised water.

2.2.8.4 T cell cytotoxicity assay

For measuring cytotoxicity of T cells, standard europium release assays were performed. Appropriate target cells were either left untreated or were labeled with 2 µM of peptides. After two hours of peptide labeling, target cells were washed thoroughly to remove excess peptide and labeled with BATDA reagent. 10^6 cells were labeled in 500 µl medium with 1 µl of BATDA reagent (8 mM, Perkin-Elmer) for 15 min at 37ºC. Labeled cells were run through a Ficoll gradient centrifugation step to remove dead cells and viable cells were washed, resuspended at 5000 cells/100 µl medium, and plated at 100 µl per well in 96-well V-bottom plates (Nunc). T cells were then added at different effector target ratios (10:1, 3:1 and 1:1). For measuring maximum and minimum release, 1% Triton X or medium only was added to the targets. All samples and controls were set in triplicates. The assay was incubated for three hours at 37°C after which 40 µl of the supernatant was carefully pipetted into 96-well flat-bottom plates. 200 µl of Europium solution were added to each supernatant sample, allowed

to shake gently at room temperature for at least 15 min and time-resolved fluorometry measured in a Wallac Victor2TM multilabel fluorescence plate reader (Perkin-Elmer).
Data were analyzed using Microsoft Excel. Means of triplicates were calculated and the specific lysis calculated using the following formula.
Percentage specific lysis = (mean release in the presence of T cells- mean minimum release)/ (mean maximum release-mean minimum release) X 100

2.2.8.5 Analysis of T cell receptor Vβ chain usage

cDNA was prepared from T cell lines or clones (RNA isolation and cDNA preparation described below) and used as template in 25 separate PCR reactions, which differed in the Vβ chain specific primers used. The primers were manufactured by Metabion with sequences described (Gussoni et al., 1997).
The PCR products were separated in an agarose gel, and the products of the PCRs visualized. Where required the PCR products were blotted from the gel onto a membrane and hybridized with a radioactive Vβ chain gene common region DNA-specific probe.

2.2.9 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 25,000 X g for three hours in a SW28 rotor. The supernatant was removed and the virus rich pellet resuspended in 1/20 volume of the original culture supernatant. The genomic copy number of this virus concentrate was determined by semi-quantitative real-time PCR using primers directed to the BALF5 gene. The virus concentrate used in this work contained 4.65 X 10^8 EBV genome equivalents (geq). For virus inactivation the EBV supernatant was heated to 56°C for one hour. Successful inactivation was verified by the inability of the heated virus supernatant to transform primary human B cells.

2.2.10 Fixation of cells

Paraformaldehyde (PFA) fixation was the method used in this work for immunofluorescence and antigen presentation studies. Briefly, cells to be fixed were harvested by centrifugation, washed three times thoroughly with PBS and then fixed in 0.5% PFA in PBS at room temperature for 10 min. Fixed cells were washed at least three times in PBS before subsequent use.

2.3 Bacteriology and biochemistry materials and methods

2.3.1 Nutrient media for bacterial culture

The chemicals were bought from Roche or Merck.

LB media

10 g NaCl, 10 g tryptone and 5 g yeast extract dissolved in 1 l deionised sterile water, pH adjusted to 7.0, autoclaved.

SOB media

20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, and 10 mM $MgSO_4$ per liter deionised water, autoclaved.

SOC media

SOB media with 0.2% Glucose.

Super broth media

35 g tryptone, 20 g yeast extract and 5 g NaCl dissolved per liter deionised water, pH adjustment to 7.5, autoclaved.

LB agar

10 g NaCl, 10 g tryptone, 5 g yeast extract and 20 g agar dissolved in 1 l deionised water, pH adjusted to 7.0, autoclaved and poured into petri dishes (~25 ml/100-mm plate).

2.3.2 Storage of bacteria

For storage, bacteria were frozen at -80°C in 10% glycerol (in LB media).

2.3.3 Preparation of electrocompetent bacteria

The electrocompetent bacterial strain used in this work was the XL1-Blue MRF (Stratagene). Stocks of this strain were plated onto LB agarose plates with tetracycline (working concentration of 10 µg/ml) and incubated overnight at 37°C to obtain single colonies. 400 ml of LB medium was inoculated with a single colony and incubated in a bacterial shaker to an optical density (OD) of 0.8. Bacteria were then centrifuged at 4°C at 2800 X g for 5 min, the supernatant discarded and the pellet resuspended in 400 ml of ice cold 10% glycerol. This resuspension was centrifuged for 10 min at the same speed and temperature. This wash step with 10% glycerol was repeated and the bacterial pellet resuspended in 2 to 4 ml of ice cold 10% glycerol. In 1.5 ml Eppendorf safe lock tubes, 50 µl aliquots/tube of this final resuspension were frozen down by immediately exposing the tubes to liquid nitrogen, and stored to -80°C.

2.3.4 Bacterial transformation with DNA

Bacteria were transformed with DNA by electroporation. Appropriate mixtures of electrocompetent bacteria and the DNA of interest were pipetted into a 1 mm cuvette and pulsed with 1500 V, 25 µF and 100 Ω. After the pulse, bacteria were transferred into a 1.5ml Eppendorf tube by pipetting with 1 ml of SOC media. After incubation at 37°C for at least 30 min the bacteria were pelleted by centrifugation at 2800 X g for 5 min, most of the supernatant discarded carefully and the bacterial pellet resuspended in the remaining media. This suspension was plated onto LB agarose plates with antibiotics as indicated.

2.3.5 Small scale plasmid preparation

Small scale plasmid preparation was performed to check single bacterial colonies for the plasmid they carried. 4 ml of LB media with appropriate antibiotics were inoculated in snap caps with single colonies of bacteria and incubated overnight in a shaker. The next day plasmid was extracted from the bacteria using the Jetstar miniprep kit (Genomed). 1 ml of an overnight culture was transferred into a 1.5 ml Eppendorf tube and the bacteria pelleted by centrifugation on a table centrifuge at 2,800 X g for 5 min. The supernatant was discarded, and the pellet resuspended in 100 µl solution E1 (supplemented with RNAse A). Bacterial lysis with 100 µl E2 followed after which the whole mix was neutralized with 100 µl neutralization solution E3. The mix was then centrifuged at 16,000 X g for 10 min after which the clear supernatant was carefully transferred into a new 1.5 ml Eppendorf tube, and the DNA precipitated with 70% ethanol. The DNA was pelleted at the bottom of the tube by centrifugation (16,000 X g for 15 min). The supernatant was discarded, the DNA pellet air dried and subsequently dissolved in an appropriate volume of water or 1 X TE as required.

1X TE: 10 mM Tris-HCl pH 7.5; 1 mM EDTA in deionised sterile water.

2.3.6 Large scale plasmid extraction

The basic protocol applied for large scale preparation of plasmid DNA was essentially similar to the one used for small scale plasmid DNA preparation. The same solutions were used. Larger volumes (400 to 500 ml) of bacterial cultures were grown overnight in a shaker. When the $OD_{\lambda 600}$ reached between 0.8 and 1.0, the bacteria were pelleted by centrifugation at 2,800 X g for 10 min and the supernatant discarded. The bacteria were then resuspended in 10 ml of E1 with RNAse A, lysed with 10 ml of E2 and the lysate neutralized with 10 ml of E3. A step of centrifugation allowed precipitates to settle down and low density dregs to float upon a clear supernatant. The supernatant was carefully poured onto pre-equilibrated (with 15 ml solution E4) maxi-prep columns through a cloth filter in order to avoid floating dregs onto the

column. The flow through was discarded and the column washed three times with 20 ml of wash solution E5. Following the wash the DNA was eluted of the column with 15 ml of elution buffer E6. DNA in the eluate was precipitated with 40% isopropanol final concentration and pelleted by centrifugation at 9,300 X g for 15 to 20 min. The pellet was washed with 70% ethanol and allowed to air dry after which it was dissolved in an appropriate volume of water or 1 X TE as necessary.

2.3.7 Verification of plasmid DNA

Identity of the plasmid DNA was verified by restriction endonuclease digestion or by sequencing. Sequencing was performed by Sequiserve.

2.3.8 RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated from cells using the RNeasy -Mini-Kit (Qiagen) according to the manufacturer's protocol. Isolated RNA was resuspended in RNAse free water and aliquots were frozen at -80°C.

cDNA was prepared from RNA by using the MuMLV reverse transcriptase (NEB). Reverse transcription reactions were set up typically in 25 µl volumes. In an Eppendorf tube 1 µl oligo (dT) (Roche) primer was added to 1 to 3 µg total RNA and filled up to 18 µl with RNAse-free water. The mix was then heated to 70°C for 10 min and chilled on ice for 30 sec. Subsequently, 5 µl 5 X reverse transcriptase buffer, 1 µl 10 mM dNTP mix (Amersham) and 1 µl reverse transcriptase were added to the tube, mixed by pipetting and incubated at 37°C for 1 hour.

PCRs were performed to amplify EBV genes and to amplify T cell receptor Vβ chains expressed in T cell lines and clones. As template for the TCR Vβ chain analysis, cDNA prepared from T cell lines and clones was used. For the amplification of EBV genes, the plasmid 2089 containing the whole B95.8 wild type viral DNA sequence (kindly provided by Prof. Wolfgang Hammerschmidt) was used as template. PCR reactions were set up according to the suggestion of the manufacturer of the DNA polymerases. If PCR products were to be used in subsequent cloning steps, Pfu polymerase was used. The number of PCR cycles varied between 20 and 45. PCR reactions consisted of an initial denaturation step at 95°C for 5 min followed by the cyclic reactions. Each cycle consisted of a denaturation step at 95°C for 1 min followed by a primer annealing step for 1 min at the annealing temperature between 56 and 62°C depending on the primer pairs used, and an extension step at 72°C 1 to 5 min depending on the expected length of the PCR product. Primer pairs used to amplify the coding sequences

of defined EBV genes carried recognition sequences for restriction enzymes to facilitate cloning of the PCR products into expression plasmids. The sequences of the primers used for EBV gene amplification and cloning are shown in the annex. PCR products were separated in agarose gels and the PCR products purified out of the gel using the Qiaex II Gel Extraction kit (Qiagen).

2.3.9 Separation of DNA fragments by agarose gel electrophoresis

Separation of DNA fragments was performed in 0.8% to 1.2% agarose gels with 0.4 µg/ml ethidium bromide in 1 X TAE by electrophoresis. The agarose content of the gel was determined by the size of the DNA band expected; the larger the DNA fragment, the lower the agarose content. DNA samples were mixed with 1/5 volume of DNA gel loading buffer (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol, 30% v/v glycerol) and loaded into the gel slots. Electrophoresis was performed in the horizontal position with 1x TAE running buffer. 1 kb DNA ladder (MBI Fermentas) was used as size standard. For the isolation of DNA fragments from agarose gels, the desired fragments were cut out from the gel with a clean scalpel and DNA isolated using a Qiaex II Gel Extraction kit (Qiagen) following the manufacturer's instructions.

50 x TAE: 2M Tris base and 50mM EDTA in deionised sterile water; pH adjusted to 8.5.

2.3.10 Phenol-chloroform extraction and precipitation of DNA

To remove contaminants from DNA, an equal volume of a mixture of phenol, chloroform and isoamyl alcohol (proportions 25/24/1) was added to the DNA solution. The mixture was centrifuged for 10 min at 13000 X g to separate the phases. The upper aqueous phase was transferred with a pipette into a new 1.5 ml Eppendorf tube, and the DNA in the aqueous phase precipitated by the addition of 2 volumes of 100% ethanol and 0.1 volume 3 M sodium acetate. After centrifugation at 13000 X g the pellet was washed in 70% ethanol, dried and dissolved in deionised sterile water. DNA concentration was measured by UV-spectrophotometer.

2.3.11 DNA restriction

For DNA restriction 2 to 4 U of the desired restriction enzymes were added to 1 µg of DNA in the appropriate restriction buffer. Commercially available buffers and enzymes were used and reactions set up as recommended by the manufacturers (NEB or MBI Fermentas). The total volume of digestion was 10 X the volume of enzyme added. The restriction mix was incubated for 1 to 2 h at the optimal working temperature for the restriction enzyme used.

2.3.12 Blunt ending of digested DNA

Where necessary, sticky ends of DNA products created by restriction enzyme digest were blunt ended. This was done by adding 10 U of T4 DNA polymerase (NEB) and 1 µl of 10 mM dNTP mix directly to the restriction enzyme digest and incubating the mix for 15 min at room temperature.

2.3.13 Ligation of DNA

DNA fragments with compatible ends were ligated with T4 DNA ligase (MBI Fermentas). A 3-fold molar excess of insert over vector was used. Typically, 1 U enzyme was used for the ligation of 200 ng DNA in a 20 µl reaction mix. Ligations were incubated overnight at 16°C.
When synthetic linkers were ligated to vectors, the oligonucleotides were first heated to 95°C and subsequently allowed to hybridize by slowly cooling the mix to room temperature.

2.3.14 Construction of EBV gene expression vectors and recombinant baculoviruses

The ORFs of EBV genes were amplified by PCR and cloned into the multiple cloning site (MCS) of the pCMV-CHis vector or a modification thereof. The primers were designed based on the published B95.8 wild type EBV sequence. In the sense primer the start codon of the genes was left out since the vector was prepared by digestion with NcoI and blunt ended with T4 polymerase. This created a blunt ended ATG sequence which provided the start codon for the inserted genes. The antisense primers contained at the 3`end a restriction enzyme recognition sequence not present elsewhere in the gene but present in the multiple cloning site of the vector. PCR products digested with this enzyme were purified by gel electrophoresis and ligated into the expression vector which had been digested with NcoI, treated with T4 polymerase, and digested with the same enzyme as the PCR product. The EBV genes cloned in this study are listed in table 3.2 of the results section.
In the pCMV-CHis vector the inserted gene is followed by sequences coding for the antibody epitope recognized by the EBNA1-specific monoclonal antibody 1H4 (kindly provided by Dr.

E. Kremmer) and for a tag consisting of six histidines. The histidine tag allows to purify the the gene products over nickel-agarose beads (described below), and the antibody epitope allows to visualize the proteins by Western blot.

Recombinant baculoviruses were created following the protocol of the manufacturer (BD Pharmingen). Briefly, the gene of interest was first transferred from the pCMV-CHis vector into a baculovirus transfer vector. This construct was transfected into Sf9 cells along with Baculogold DNA to generate recombinant baculoviruses by homologous recombination. The plaque assay was used to obtain individual recombinants. Amplified stocks derived from different plaques were used to infect Sf9 cells. Histidine-tagged proteins were purified from Sf9 cells four days after infection and analysed by Western blot. Virus stocks that led to high level expression of the inserted gene were selected, amplified, and used for large scale protein production.

2.3.15 Protein expression in eukaryotic cells

For protein expression in insect cells, approximately 70% confluent Sf9 cells were infected at an MOI of 3 to 10. The virus containing medium was replaced with fresh medium after 12 hours and the cells were allowed to produce protein for 72 to 96 hours. The cells were then harvested by centrifugation.

Transfection of 293T cells with plasmid DNA for protein expression was done using the $CaPO_4$ method. For transfection 60-70% confluent cells grown in 140 mm plates were used. For one such plate, the cells were first supplemented with 32 ml fresh media and then 8 ml of the transfection mix was dropped over the whole surface area of the plate. The 8 ml transfection mix consisted of 4 ml 2 X HBS. The rest was a mixture of DNA (80 µg per plate), $CaCl_2$ (400 µl of a 2.5 M sterile filtered solution) and chloroquine (40 µl of a 0.5 M solution) filled up to 4 ml with water. The transfection mix was prepared by slowly dripping the DNA-containing solution into the 2 X HBS while constantly bubbling air with a pipette through the HBS solution. The transfection mix was added to the cells within 5 min of preparation. 6 to12 hours after transfection, the media was removed and replenished with fresh media. The cells were harvested 48 to 72 hours after transfection and pelleted by centrifugation.

2X HBS: 11.9 g HEPES, 16.4 g NaCl and 0.21 g Na_2HPO_4 in 1 l deionised water; pH adjusted to 7.05; sterile filtered.

2.3.16 Lysis of protein expressing cells and purification of recombinant protein

The protein-expressing cells were harvested by centrifugation and 10^8 cells lysed in 50 ml urea lysis buffer. The lysate was centrifuged in order to pellet cell debris and DNA complexes, and the clear lysate in the supernatant was transferred to a new 50 ml Falcon tube. Next, 300 µl NiNTA agarose beads (Qiagen) were added, and the tubes allowed to rotate overnight at 4°C in an overhead rotator. The following day, the tubes were centrifuged, and the supernatant discarded. The beads with the bound proteins were washed once with lysis buffer. Subsequently, the proteins were eluted using elution buffer. The beads were resuspended with 300 µl elution buffer, centrifuged (10,000 X g for 2 min), and the supernatant transferred to a new tube. This procedure was repeated twice, and the collected supernatant dialysed against PBS and the protein content determined using the Bradford reagent. For quantification, known concentrations of bovine serum albumin were used as standards.

Urea lysis buffer: 8 M Urea, 0.1 M NaH_2PO_4, 0.01 M Tris base, 0.05% Tween 20, 20 mM imidazole dissolved in deionised sterile water; pH adjusted to 8.0.

Elution buffer: 0.5 M imidazole in Urea lysis buffer.

2.3.17 Western blot analysis of proteins

Eluted proteins were mixed with 1/5 volume of SDS loading buffer and heated to 95°C for 5 min. Subsequently, the samples were loaded onto 10% polyacrylamide gels. Usually two gels were run in parallel of which one was stained with Coomassie blue stain to detect all proteins in the eluate. A similar second gel was blotted onto a Hybond P membrane (Amersham). The membrane was blocked by letting it swim in Blocking Buffer for at least one hour and then incubated in Western buffer with the tag specific antibody (anti-EBNA1 antibody 1H4, kindly provided by Dr. Elisabeth Kremmer). After at least two hours of incubation, the primary antibody was removed, the membrane washed three times in Western buffer and then incubated with a peroxidase conjugated secondary antibody (goat anti rat) again in Western buffer. After two hours, the membrane was washed thrice and developed using the ECL plus developing solution (Amersham). For size approximations, a pre-stained protein marker (Fermentas) was run in at least one lane of the gel.

SDS loading buffer: 50 mM Tris-HCl (pH6.8), 2% SDS, 0.1% Bromophenol Blue, 10% glycerol.

SDS running buffer: 3 g Tris base, 14 g glycine and 10 ml 10% SDS filled to 1 l with deionised water.

Transfer buffer: 4.5 g Tris base, 21 g glycine in 1.5 l of 20% methanol in deionised water.

Western buffer: 1% skimmed milk powder in TBS (20 mM Tris-Cl, 150 mM NaCl in deionised water; pH 7.5).

Blocking Buffer: 3% skimmed milk powder in Western buffer.

3. Results

To investigate breadth and specificity of the EBV-specific T helper cell response, two experimental approaches were pursued (Fig 3.1).

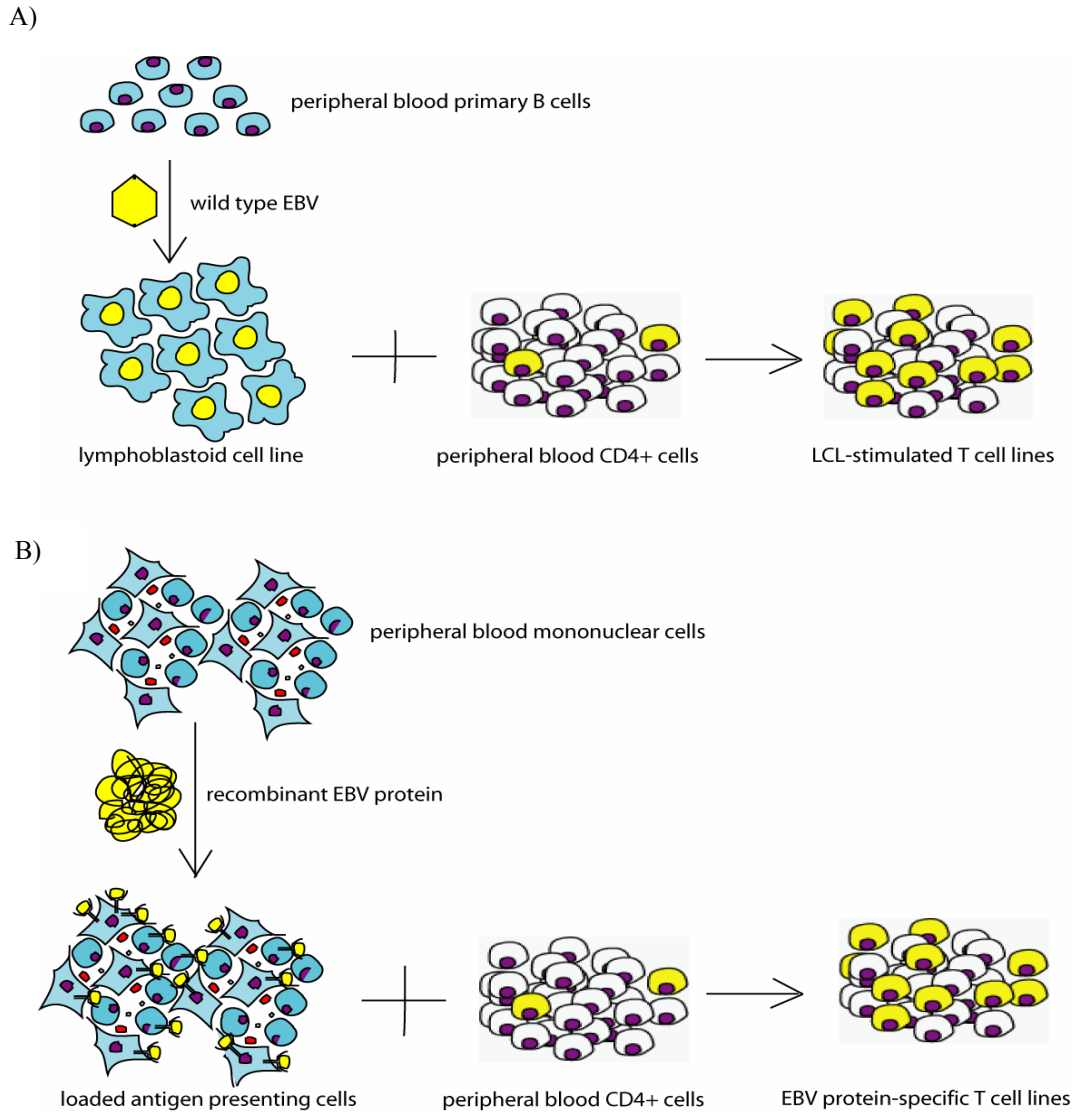


Figure 3.1. Schematic depiction of the two approaches used to generate EBV-specific CD4+ T cells *in vitro*.
CD4+ T cells from the peripheral blood of donors were stimulated every two weeks with either A) autologous LCLs generated by EBV infection of B cells *in vitro* or B) recombinant EBV protein-pulsed autologous PBMC.

First, CD4+ T cell lines were generated by repeated stimulation with the autologous LCL respectively. Because LCL express all nine latency proteins of EBV, this approach was expected to reactivate T cells with different specificities, and to eventually select T cells specific for immunodominant antigens. Second, CD4+ T cell lines specific for individual EBV antigens were established by repeated stimulation with protein-pulsed PBMCs. By this approach, the breadth of the T helper response against EBV could be analyzed.

3.1 MHCII-restricted CD4+ T cell lines were generated by stimulation with autologous LCL

LCL express high levels of MHCI, MHCII, and co-stimulatory molecules. Stimulation of peripheral blood T cells with LCL causes massive expansion of reactive CD4+ and CD8+ T cells. In order to obtain EBV-specific CD4+ T cell lines, PBMCs were repeatedly stimulated with irradiated autologous LCL, and the resulting T cell lines enriched for CD4+ cells by magnetic cell sorting (MACS), either by depleting CD8+ cells or by positively selecting CD4+ cells. This enrichment was performed after the fifth stimulation, and was repeated at least twice within the next five passages. The resulting T cell lines contained more than ninety percent CD4+, CD8-, and TCRα/β+ cells (Figure 3.2).

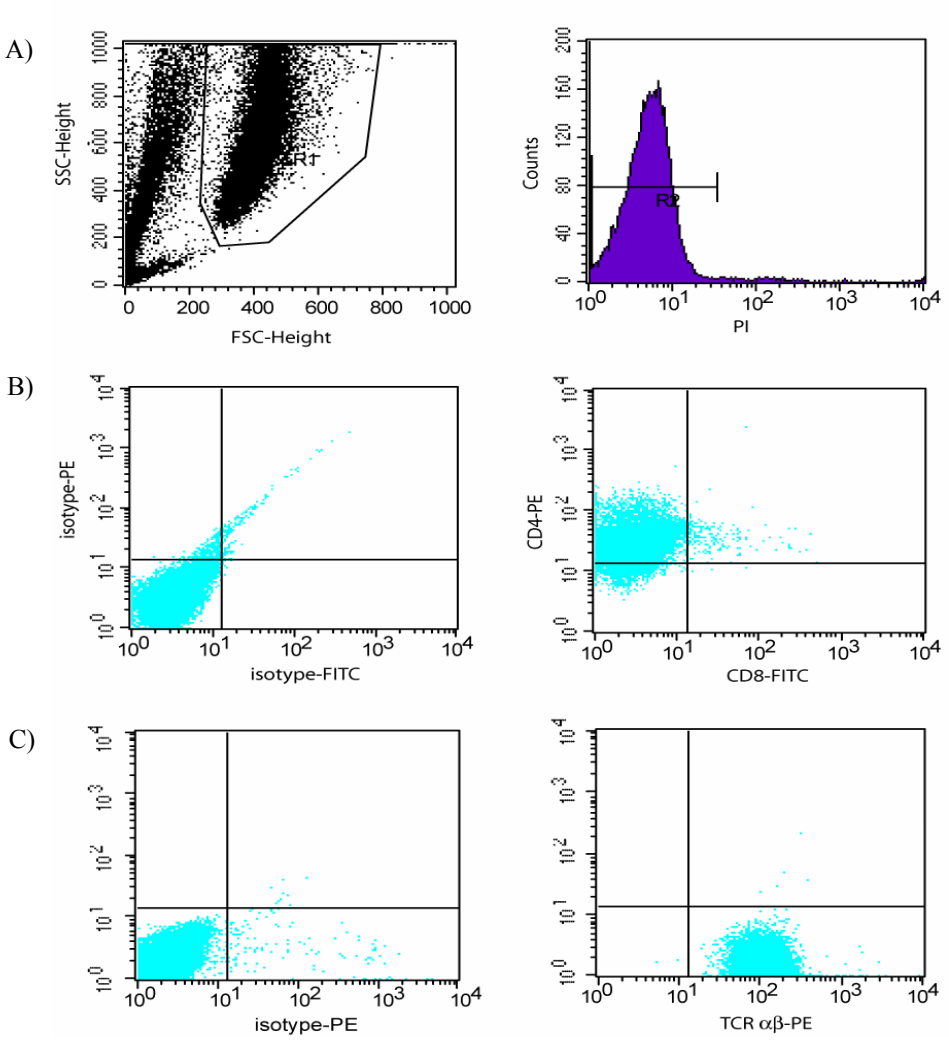


Figure 3.2. FACS analysis of a LCL-stimulated CD4+ T cell line.

A) Forward and sideward scatter analysis (left) and staining of dead cells with propidium iodide (right). B) Analysis of CD4 and CD8 expression by the T cells. The cells were stained with anti-human CD4-PE and anti-human CD8-FITC (right) or isotype control antibodies (left). C) Staining with anti-human TCRα/β-PE (right) or isotype control (left) antibodies. More than 90% of the T cells were CD4 positive, CD8 negative and TCR αβ positive.

LCL-stimulated T cell lines were generated from 23 individuals including eight IM patients, nine healthy virus carriers, one EBV-seronegative adult, and five cord blood donors. The MHCII genotypes of the donors and other cell lines used in this work are shown in Table 3.1.

Designation	HLA-DRB1	HLA-DQB1	HLA-DPB1	HLA-DRB3/4/5
Ag876	0701, 1101	0202, 0502	0101, 1301	DRB3*0202, DRB4*0101
AR	0801, 1401	0402, 0503	0401	DRB3*0202
BL30	0301, 1301	0201, 0603	0101, 1401	DRB3*0101
BL31	0401, 0402	0302, 0304	0401, 0501	DRB4*0103
BL41	0101/07, 1401	0501, 0503	0201, 1901	DRB3*0202
BL70	1301, 1501	0602, 0603	0401, 0402	DRB3*0101, DRB5*0101
BU	0407, 0701	0202, 0301	0301, 1701	DRB4*0103
DA	1502	0503, 0601		DRB5*0102
DG75	0404, 1301	0402, 0603	0401	DRB3*0101, DRB4*0103
EK	1101, 1201	0301	0401, 0402	DRB3*0202
GB/ KS	1101, 1301	0301, 0603	0401, 0402	DRB3*0202
IM1	1104, 1301	0301, 0603	0401	DRB3*0202
IM2	0101, 1501	0501, 0602	0201	DRB5*0101
IM3	0401, 1501	0302, 0602	0301	DRB4*0103, DRB5*0101
IM4	1103, 1501	0301, 0602	0402, 0501	DRB3*0202, DRB5*0101
IM5	1301, 1501	0602, 0603	0402	DRB3*0202, DRB5*0101
IM6	0701	0202, 03032	0401, 0402	DRB4*0103, DRB4*0201
IM7	0701, 1501	0202, 0602	0401, 0402	DRB4*0103, DRB5*0101
IM8	0701, 1302	0202, 0604	0201, 0301	DRB3*0301 DRB4*0103
JM	0801, 1301	0402, 0603	0401, 1301	DRB3*01011
KO	0701, 1302	0303, 0604	0301, 0402	DRB3*0301, DRB4*0103
LA	0701, 1501	0303, 0603	1301, 1401	DRB4*0103102, DRB5*0101
LEN	0101, 0701	0303, 0501	0401, 1001	DRB4*0103
MA	0801, 1502	0402, 0601	0201, 0401	DRB5*0102
RI	0701, 1302	0202, 0604	0402, 1301	DRB3*0301, DRB4*0103
MS	0401, 1104	0301, 0302	0401	DRB3*0202, DRB4*0103
SM	1501	0602, 0603	0401	DRB5*01011
TK	0301, 1301	0201, 0603	0201, 0401	DRB3*0101, DRB3*0202
UB	0701, 1101	0301, 0303	0201, 0301	DRB3*0202, DRB4*01031
VU	1401, 1501	0503, 0602	0401	DRB3*0202, DRB5*0101

Table 3.1. The HLA-DR, -DQ, -DP genotype of the donors and cell lines used in this work.

CD4+ T cell lines established by LCL stimulation were continuously cultivated for more than sixty passages (more than two years). This long term culture of the T cells *in vitro* permitted to analyze them in detail over multiple passages of stimulation.

3.2 LCL stimulation led to expansion of CD4+ T cells with different specificities

To assess MHCII restriction, the different T cell lines were co-cultured with target cell lines of known MHCII haplotypes and cytokine secretion by the T cells was measured by ELISA. T cell lines from all nine EBV-positive healthy donors and seven out of eight IM patients responded consistently against autologous and MHCII-matched, but not against MHCII-mismatched allogeneic LCL, suggesting that the T cell lines were restricted by self-MHCII molecules. Early passage T cell lines usually recognized target cells expressing non-overlapping sets of self-MHC molecules, indicating that these lines were polyclonal. MHC restriction of the same T cell lines at later passages was focused on one or two MHC alleles, suggesting that the T cell lines became oligoclonal with repeated stimulations. Figure 3.3 shows the cytokine response of several T cell lines against autologous and MHCII-matched as well as mismatched allogeneic LCL targets.

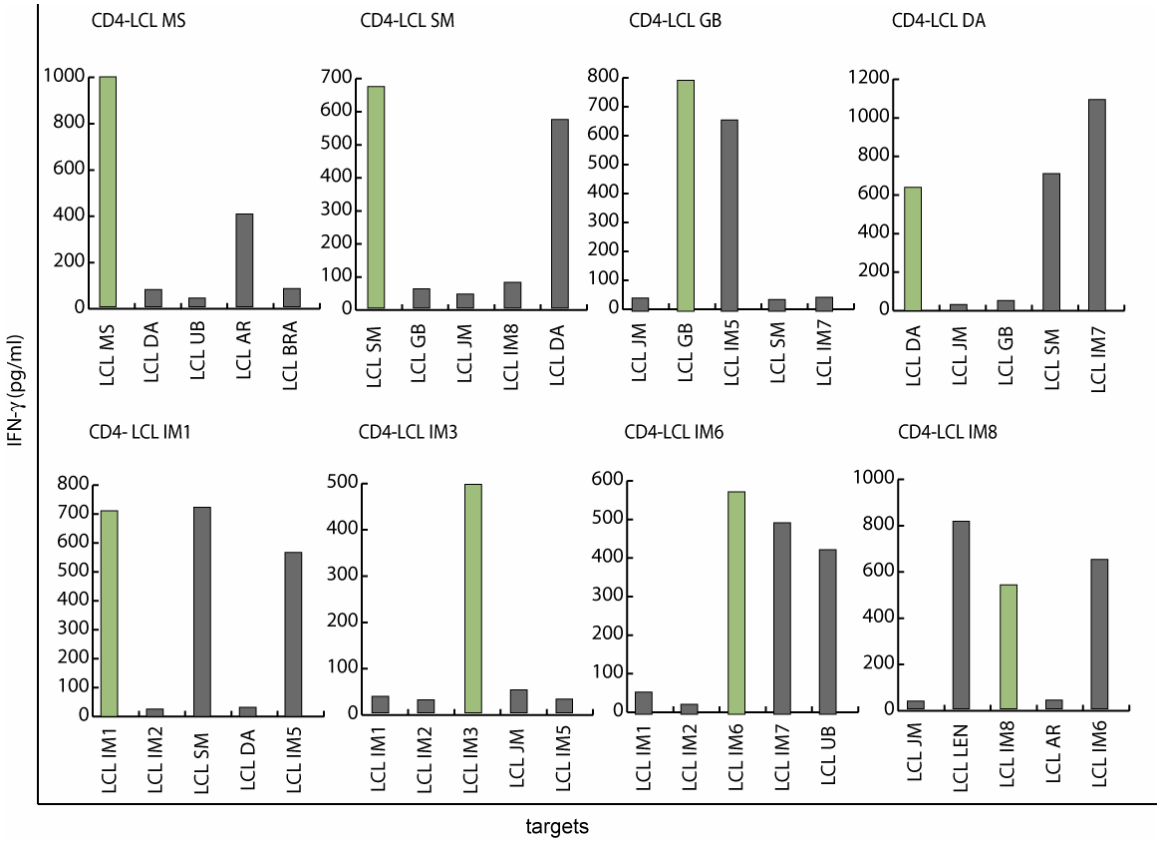


Figure 3.3. LCL-stimulated CD4+ T cell lines were MHCII-restricted.

The T cell lines were tested for target-specific IFN-γ secretion using autologous, MHCII-matched and mismatched LCL as targets. The T cell lines responded only against autologous (bars shaded light green) and MHCII-matched but not MHCII-mismatched allogeneic LCLs.

No MHCII restriction could be assigned to the T cell line IM7 established from IM patient 7 (IM7). This line failed to show any target-specific cytokine secretion.

Cord blood-derived T cell lines from five donors failed to show specific and consistent responses against autologous and MHCII-matched LCL. These lines, however, responded vigorously against some MHCII-mismatched LCLs, suggesting that these T cells were alloreactive. The T cell line established from the EBV-negative healthy adult AR displayed a similar pattern of recognition (Figure 3.4) which indicated that LCL-stimulated CD4+ T cell lines from EBV-negative donors poorly respond against EBV-positive autologous target cells but show a high degree of alloreactivity.

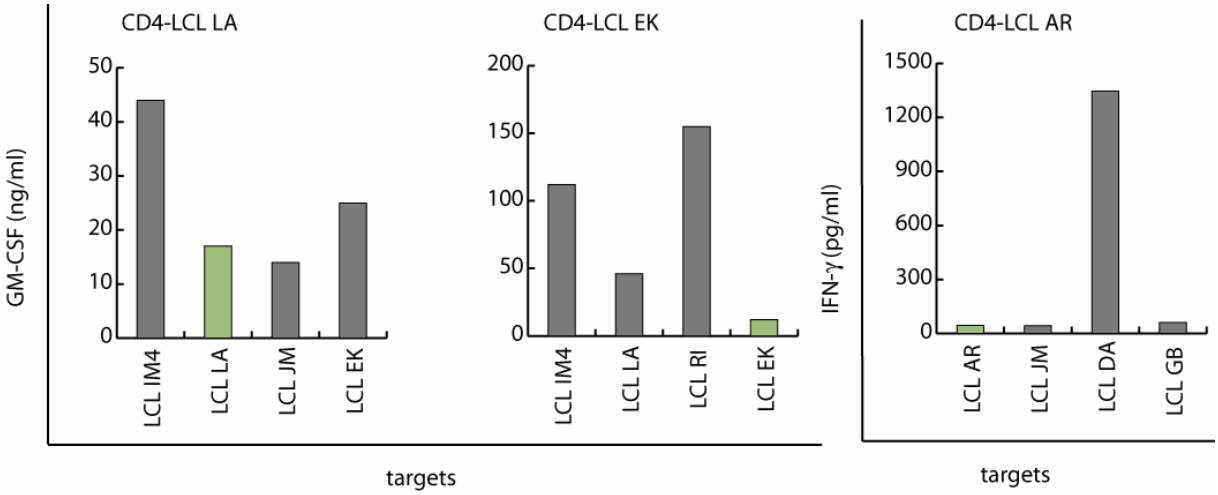


Figure 3.4. CD4+ T cell lines from EBV-negative donors fail to show consistent responses against autologous LCL.

T cell lines from two cord blood donors (LA and EK) and from the EBV-negative adult donor AR were tested against different targets for cytokine response. The T cell line from donor LA secreted insignificant amounts of GM-CSF (<50 ng/ml). The T cell lines from donors EK and AR failed to show autologous LCL-specific responses, but recognized MHC-mismatched allogeneic LCLs. Responses against autologous LCLs are shown by bars in green.

To assess EBV specificity of the T cell lines established from EBV-seropositive donors, partly MHCII-matched EBV-negative BL cell lines and when available *in vitro* EBV-infected convertants of the BL cell lines, were included as target cells in cytokine secretion assays. T cell lines from all healthy EBV-positive donors recognized MHC-matched EBV-positive but not EBV-negative BL lines, indicating that LCL-stimulated CD4+ T cells from EBV-positive individuals are specific for EBV antigens. Representative experiments with T cell lines derived from the healthy virus carriers GB and MA are shown in Figure 3.5. The T cell lines from four out of seven IM patients behaved similarly in such experiments. Surprisingly, T cell lines derived from three IM donors that had also recognized autologous LCL in an MHCII-restricted fashion in initial assays failed to show EBV-specific responses. These T cell lines responded equally well against partly MHCII-matched EBV-positive as well as EBV-negative

targets, suggesting that the dominant T cell populations in these T cell lines are directed against self-antigens (Figure 3.5).

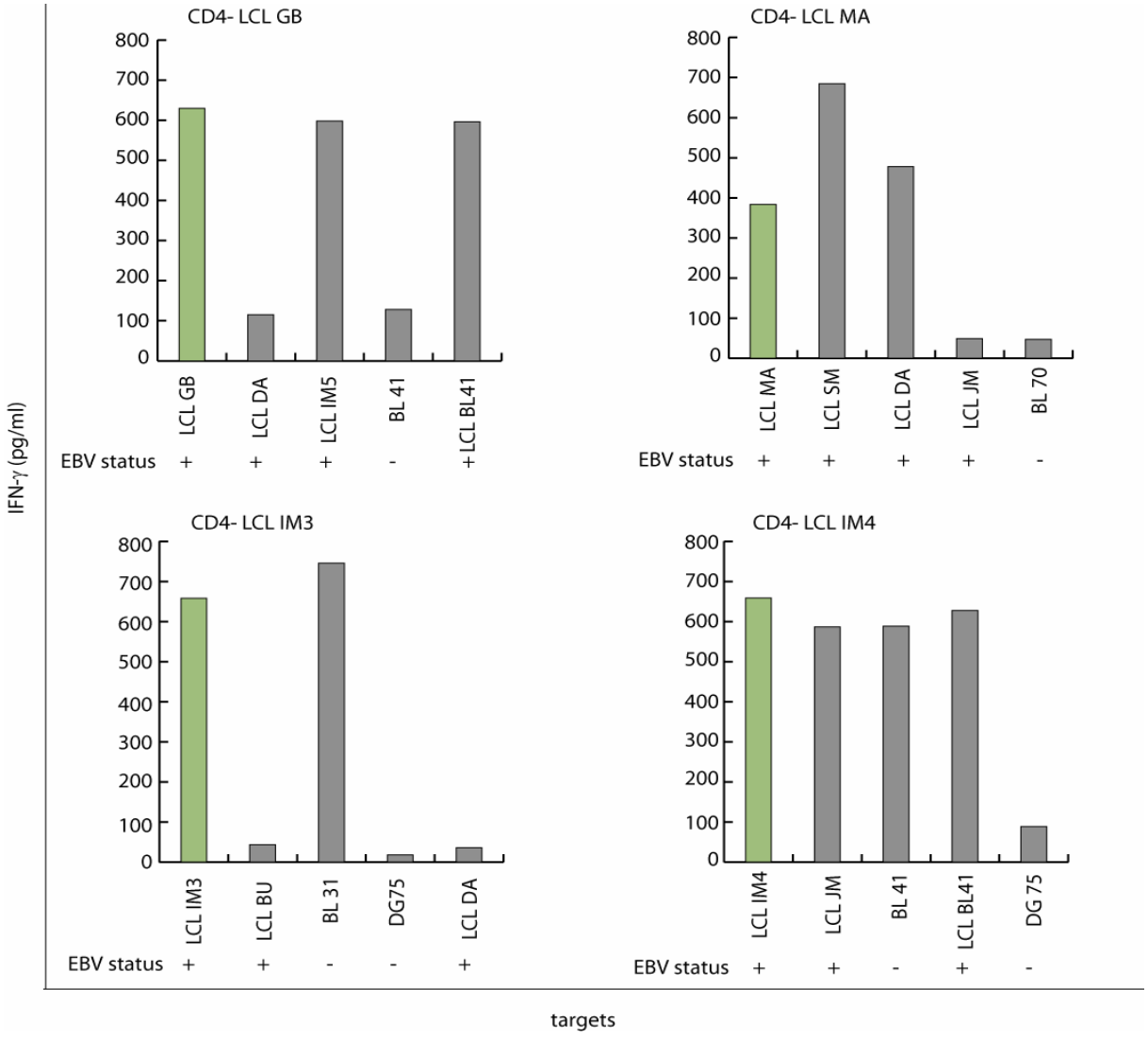


Figure 3.5. All LCL-stimulated CD4+ T cells from EBV-seropositive healthy individuals, but only half of the T cell lines from IM patients were EBV-specific.

T cells from different donors were tested for specific cytokine secretion against autologous (bars in green) and allogeneic, MHCII-matched and mismatched EBV-positive and -negative target cells.

3.3 LCL-stimulated, late passage CD4+ T cell lines were oligoclonal

The recognition of different LCL targets that shared no MHCII molecules by early passage T cell lines had indicated that LCL-stimulated T cell lines contained T cells of more than one specificity. Even T cell lines that had been stimulated more than 20 times still recognized allogeneic EBV-positive target cell lines with non-overlapping sets of MHCII alleles. To investigate how many different specificities were still present in late passage T cell lines, T cell receptor beta chain (Vβ) expression was analyzed. RNA was extracted from T cell lines

at different passages of stimulation, reverse transcribed, and the Vβ chains amplified by PCR using sequence-specific primers for the different human Vβ chains. The PCR products were separated in an agarose gel, blotted onto a nylon membrane, and hybridized with a Vβ common region-specific radioactive probe.

T cell lines stimulated less than 20 times usually expressed multiple Vβ chains. This pattern changed gradually with further rounds of stimulation. After 50 rounds of stimulation, a few T cell lines expressed only one, while most of the T cell lines still expressed at least two different Vβ chains, indicating that LCL-stimulated T cell lines remained oligoclonal even after almost two years of continuous *in vitro* culture. The results of such studies on two T cell lines are shown in Figure 3.6.

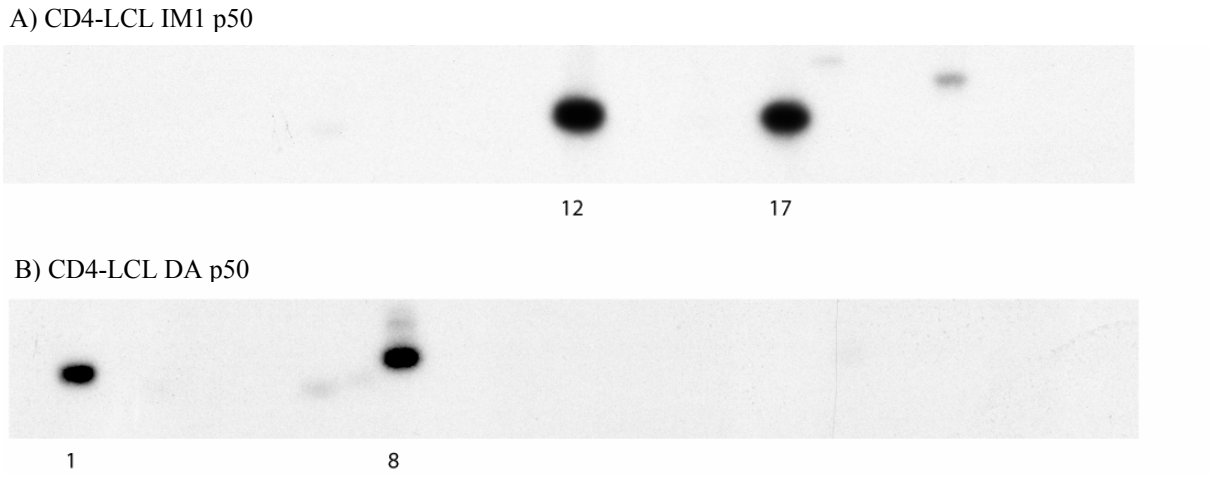


Figure 3.6. TCR Vβ chain analysis of late passage T cell lines.

RNA extracted from T cell lines established from IM patient 1 (IM1) (A) and the healthy EBV-seropositive donor DA (B) was reverse transcribed, and TCR Vβ chains amplified by PCR using primer pairs specific for the various human Vβ chains. The resulting PCR products were separated in an agarose gel, Southern blotted, and hybridized with a TCR Vβ-specific radioactive probe. The different Vβ chains expressed by the T cell lines are indicated beneath the autoradiographs. Even after 50 rounds of stimulation (p50), these LCL-stimulated T cell lines expressed more than one T cell receptor Vβ chain.

3.4 EBV latency antigens were not the targets of LCL-stimulated CD4+ T cell lines

LCL express up to eight antigenically distinct EBV latency proteins (Küppers et al., 2003). To test which of the latent viral antigens was recognized by the LCL-stimulated EBV-specific T cell lines, the different latency proteins were recombinantly expressed in a baculovirus expression system as histidine-tagged fusion proteins, and purified from virus-infected cell lysates over nickel columns. PBMCs of the donors were incubated separately with the different recombinant EBV latency proteins. After 24 hours, excess protein was removed by washing, and the protein-pulsed PBMCs probed with the autologous LCL-specific CD4+ T

cells. As shown in Figure 3.7, all of the T cell lines responded against autologous LCL targets, but not against autologous, untreated PBMCs, demonstrating that these lines were EBV-specific. Surprisingly, none of the T cell lines responded against any of the latency proteins.

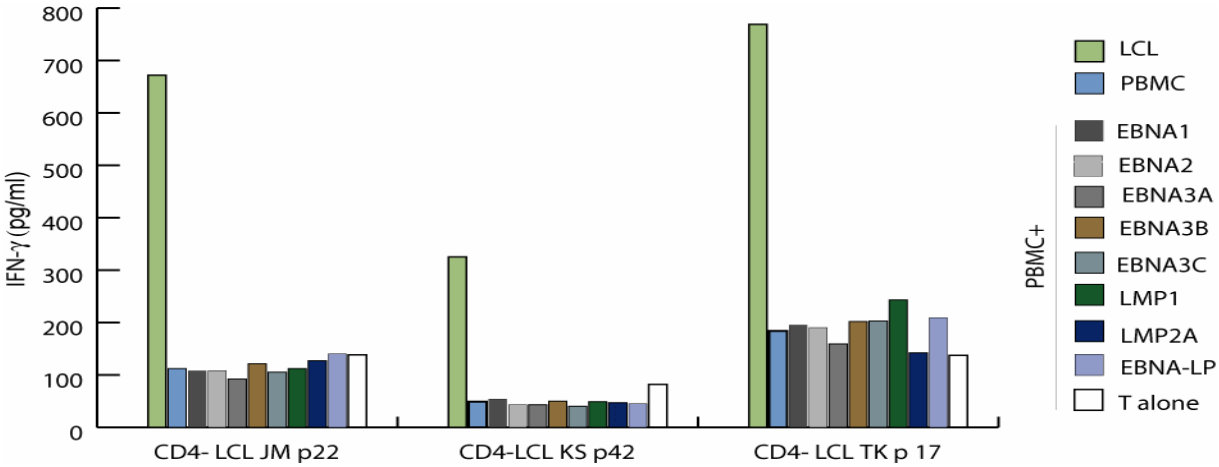


Figure 3.7. Latent antigens of EBV were not the targets of LCL-stimulated CD4+ T cell lines.

LCL-stimulated T cells were tested for recognition of latent antigens using PBMCs pulsed with latent proteins as targets in cytokine secretion assays. Although all three representative T cell lines responded against autologous LCL, no recognition of PBMCs alone, or PBMCs pulsed with any of the latent proteins of EBV was observed.

In control experiments of our group, the protein-pulsed PBMCs were probed with latent antigen-specific T cells that had been isolated from peripheral blood of healthy virus carriers. These T cells efficiently recognized protein-pulsed PBMC, demonstrating that antigen was efficiently presented by the APC (Mautner et al., unpublished data). Thus, although T cells specific for latent antigens of EBV are readily detectable in the peripheral blood of healthy virus carriers and have been isolated and characterized by several groups (Mautner et al., 2004; Long et al., 2005), LCL-stimulated EBV-specific T cell lines failed to recognize latent antigens of EBV. This might have been either due to inefficient presentation of endogenous latency proteins on MHCII or because latent antigen-specific T cells were too few in numbers and were therefore dominated by other specificities. These results implied that these T cell lines recognized either cellular antigens induced by EBV or viral antigens other than latent antigens.

3.5 Late passage LCL-stimulated CD4+ T cells of seropositive donors failed to respond against miniLCL

To discriminate between these possibilities, a genetically engineered EBV strain called miniEBV, was used. This viral mutant infects and growth-transforms primary B cells as efficiently as the wild-type EBV (Kempkes et al., 1995), and the resulting so-called miniLCL

are phenotypically identical to regular LCL established by infection of B cells with wild-type virus (Moosmann et al., 2002). MiniLCLs, however, are deficient in genes and genetic elements that are essential for lytic replication of the virus. Consequently, this viral mutant cannot reactivate from latency and enter the lytic cycle.

Since miniLCL present antigen as efficient as regular LCL and express all latency proteins of EBV (Moosmann et al., 2002), using them as targets in T cell assays should allow determining whether the LCL-stimulated T cell lines were specific for cellular antigens induced by the latency proteins of EBV, or recognized viral antigens expressed during lytic replication. Therefore, miniLCLs were generated from those donors from which PBMCs were available, and probed with the autologous T cell lines.

Early passage T cell lines responded similarly against LCLs and miniLCLs. Which antigens these T cells recognized was not known, but FCS could be excluded as potential antigen because miniLCLs grown in human serum were recognized equally well by these T cells. Since previous experiments had revealed that LCL-stimulated T cell lines did not recognize latent antigens of EBV, the miniLCL-reactive component in earlier passage T cell lines was probably recognizing a cellular antigen.

This pattern of recognition changed profoundly when later passage T cell lines were tested. T cell lines stimulated more than 10 to 15 times still responded strongly against autologous LCL, but progressively lost miniLCL-reactivity (Figure 3.8). Thus, later passage T cell lines turn specific to lytic antigens of EBV or cellular antigens induced by EBV replication.

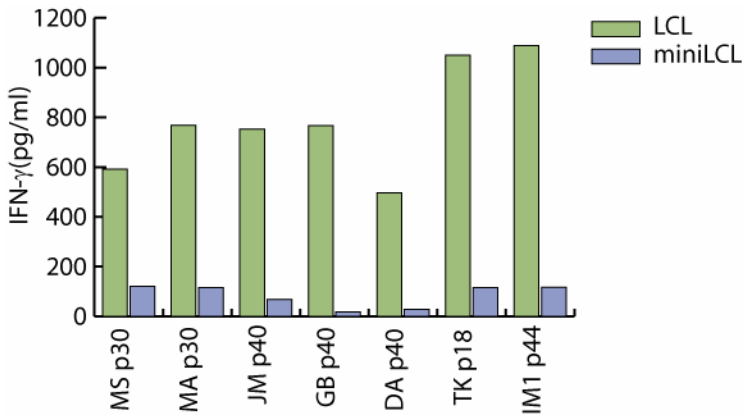


Figure 3.8. Late passage LCL-stimulated T cell lines failed to respond against miniLCL.

T cell lines from different donors at different passages of stimulation (indicated on the X-axis) were tested against the autologous LCL and miniLCL. In the case of IM patient 1 (IM1) from whom no autologous miniLCL were available, a MHC-matched allogeneic LCL and miniLCL were used.

To quantify changes in the reactivity of T cell lines against miniLCL over different passages, ELISPOT assays were performed. As shown in Figure 3.9, the T cell line from donor GB at

passage 9 responded to the LCL and miniLCL to similar levels while at passage 19 the miniLCL-reactivity of the T cell line had vanished.

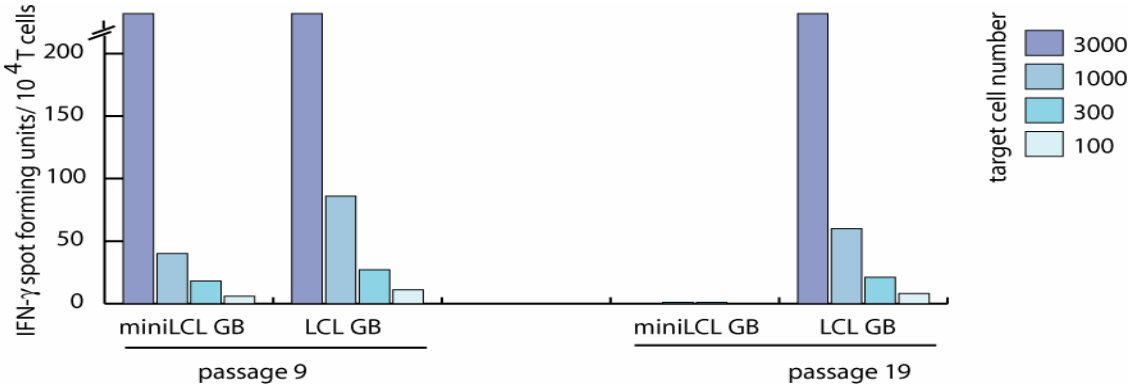


Figure 3.9. LCL-stimulated CD4+ T cell lines lost miniLCL reactivity upon repeated stimulations.

The response of the T cell line from donor GB against the autologous miniLCL and LCL were quantified in IFN-γ ELISPOT assays. A fixed number of 10^4 T cells at passage 9 and 19 of stimulation were co-cultured with titrated numbers of miniLCL or LCL GB cells. The number of target cells recognized were enumerated as spot forming units per 10^4 T cells. At passage 9, the response against the miniLCL and LCL was similar. At passage 19, however, the response against the LCL was unaltered but the response against the miniLCL had disappeared.

By contrast, the three T cell lines established from IM patients that had failed to show EBV-specificity in initial assays consistently responded similarly against miniLCLs and LCLs even upon long-term culture (data not shown). Again, latent antigens of EBV were not the targets of the T cell response, because PBMCs from IM patients pulsed with latency proteins were not recognized, suggesting that these lines predominantly reacted against cellular autoantigens rather than viral antigens.

Taken together, these results suggested that LCL-stimulation of CD4+ T cells led to the expansion of T cells specific for non-latent EBV or autoantigens. In T cell lines established from latently infected healthy virus carriers, the EBV-specific T cells expanded more vigorously than autoreactive T cells and dominated the T cell cultures over time. For reasons unknown, the proliferative capacity of EBV- and auto-reactive T cells from patients with IM appeared to be similar, and the T cell lines were eventually dominated by one or the other reactivity.

3.6 MiniLCL-stimulated CD4+ T cells responded against EBV-positive and -negative B cell lines to similar levels

The failure of all LCL-stimulated T cell lines to respond against latent antigens was surprising, because all cells in an LCL culture express latency proteins, and CD4+ T cells specific for latent antigens of EBV had been described by different groups (Munz et al., 2000; Leen et al., 2001; Mautner et al., 2004; Long et al., 2005). Furthermore, these T cells had been

shown in some cases to recognize LCLs directly, implying that LCLs should be able to expand such reactivities. One possible explanation for these unexpected findings was that latent antigen-specific T cells became activated and were induced to proliferate by LCL-stimulation, but were then overgrown by T cells specific for antigens associated with viral lytic replication. According to this scenario, LCL-stimulated T cell lines should display latent antigen-specificity if miniLCL were used as stimulators, since the latter do not express antigens associated with lytic replication of the virus. To address this model experimentally, miniLCL were used to reactivate specific CD4+ T cell memory from the peripheral blood of an EBV-seropositive donor from whom latent antigen-specific CD4+ T cells had been isolated and characterized in our laboratory (unpublished data). The stimulations with miniLCLs were performed as with LCLs. After several rounds of stimulation, the T cell line was tested for recognition of the autologous miniLCL, LCL and EBV-negative target cells. As shown in Figure 3.10, the T cell line recognized the autologous miniLCL and LCL to similar levels and also responded against EBV-negative BL cell lines. This pattern of recognition did not change with further rounds of stimulation, indicating that this T cell line was specific for autoantigen(s).

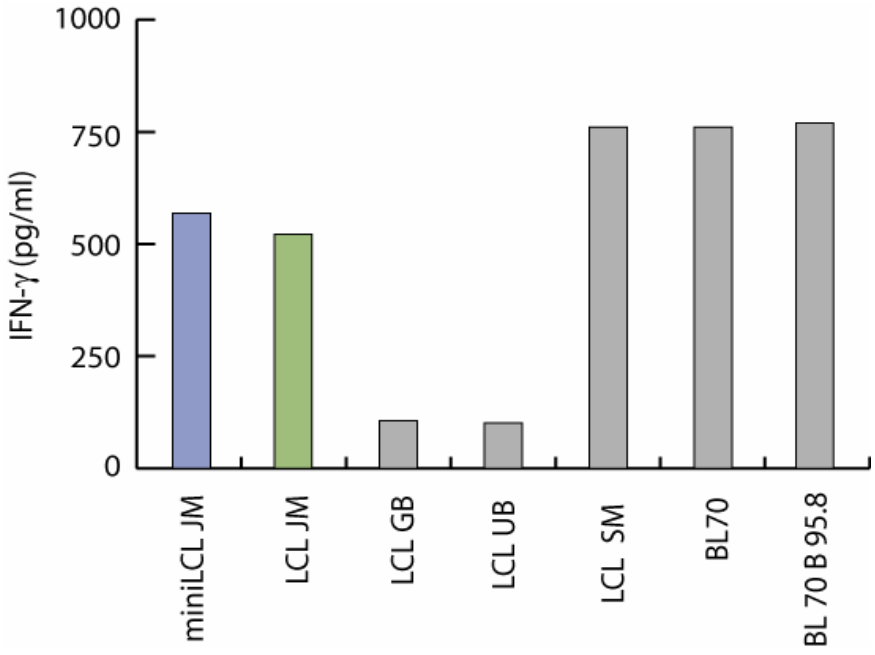


Figure 3.10. MiniLCL-stimulated CD4+ T cells were specific for autoantigens.

CD4+ T cells from donor JM were repeatedly stimulated with autologous miniLCL, and the T cells tested at passage 15 against autologous miniLCL and LCL, allogeneic LCL and the partly MHCII-matched EBV-negative BL cell line BL70 as well as its EBV-positive convertant BL70-B95.8. The T cell line recognized autologous and MHCII-matched LCL, autologous miniLCL and EBV-negative and -positive BL cell lines with similar efficiency.

These results demonstrated that T cells specific for latent antigens of EBV were not efficiently expanded by LCL- or miniLCL- stimulation, possibly because peptides derived from latent antigens were insufficiently presented on MHCII.

3.7 The targets of LCL-stimulated EBV-specific CD4+ T cells were lytic cycle antigens of EBV

The non-recognition of latent antigens by LCL-stimulated T cell lines and the differences in the recognition of LCLs versus miniLCLs suggested that lytic cycle antigens of EBV, or cellular antigens induced by lytic replication, were the targets of LCL-stimulated, EBV-specific CD4+ T cell lines. In fact, CD4+ T cell responses against lytic cycle antigens of EBV in the peripheral blood of healthy carriers had been reported before (Landais et al., 2004; Wallace et al., 1991). The EBV genome contains more than 90 putative ORFs, but the corresponding proteins have not been identified in all cases. In order to identify the antigens recognized by the EBV-specific T cell lines, an LCL-stimulated CD4+ T cell line was cloned and one of the EBV-specific clones tested by our group in a novel assay developed for the identification of antigens recognized by CD4+ T cells (Milosevic et al., unpublished data). Using this approach, the antigen recognized by the T cell clone was identified as BNRF1, a lytic cycle protein of EBV. These results for the first time provided experimental proof that lytic cycle proteins may be the targets of the LCL-stimulated T cell lines. In order to characterize the antigens recognized by the LCL-stimulated T cell lines in detail, 35 ORFs of lytic cycle genes of EBV (listed in table 3.2) were cloned into a mammalian expression vector.

Designation	ORF length (bp)
BALF2	3390
BALF4	2590
BALF5	3048
BBLF4	2430
BBRF1	1842
BBRF3	1218
BcLF1	4146
BcRF1	1730
BCRF1	513
BDLF1	906
BFLF2	957
BFRF1	1011

Designation	ORF length (bp)
BFRF3	531
BGLF5	1413
BHRF1	573
BILF2	747
BKRF2	414
BKRF3	768
BLLF1a	2724
BMLF1	1317
BMRF1	1230
BNRF1	3957
BOLF1	3720
BORF1	1095

Designation	ORF length (bp)
BORF2	2481
BPLF1	9450
BRLF1	1830
BSLF1	2625
BVRF1	1713
BVRF2	1818
BXLF1	1824
BXLF2	2121
BXRF1	747
BZLF1	738
BZLF2	672

Table 3.2. The ORFs of EBV lytic cycle genes cloned and expressed in this work. Shown are the gene designations and the lengths of the ORFs.

The EBV ORFs were amplified by PCR from B95.8 viral DNA and cloned into the MCS of the plasmid pCMV-CHis. The region immediately downstream of the MCS coded for the EBNA1 antibody epitope recognized by the monoclonal antibody 1H4, followed by six histidines. Expression of the inserted gene was controlled by a cytomegalovirus (CMV) promoter/enhancer. After transient transfection of the plasmids in HEK293T cells, fusion proteins were created that consisted of an EBV protein followed by the EBNA1 antibody epitope and a histidine tag (schematically depicted in Figure 3.11A). This permitted to purify the histidine-tagged recombinant proteins over nickel columns and to verify identity and integrity of the purified proteins by Western blotting using the EBNA1-specific antibody (Figure 3.11B).

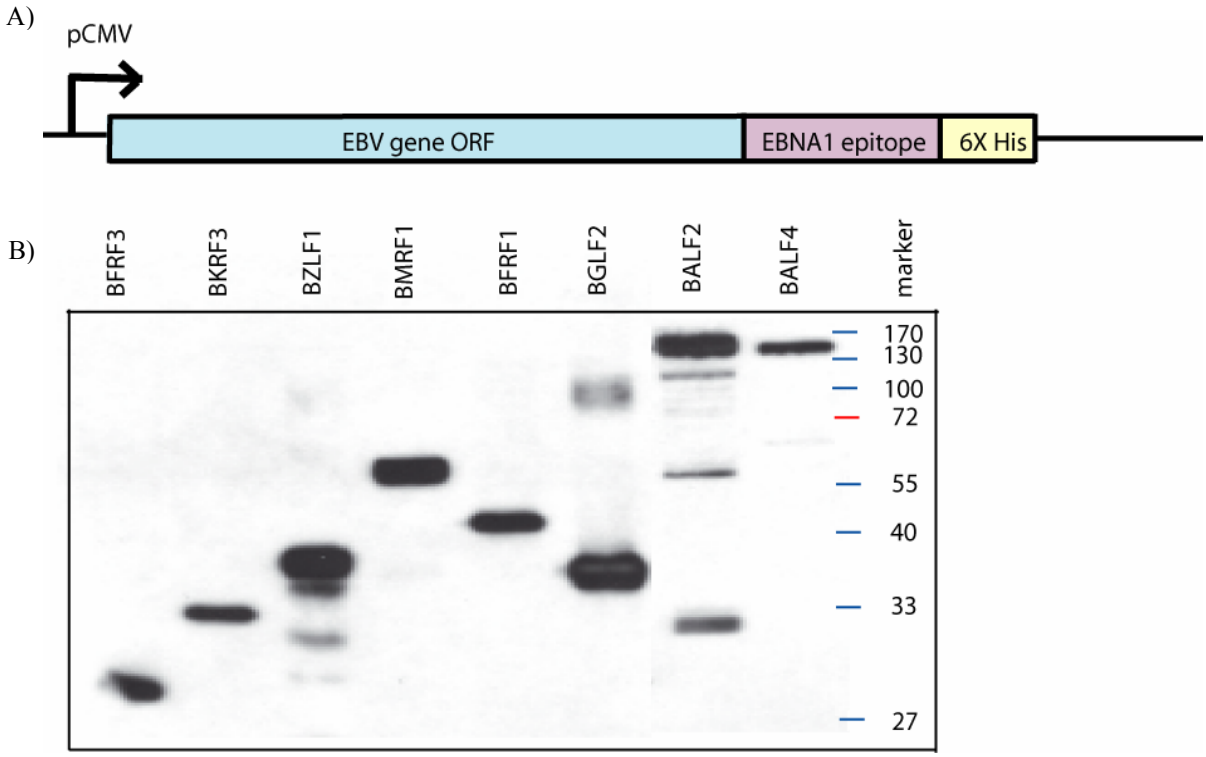


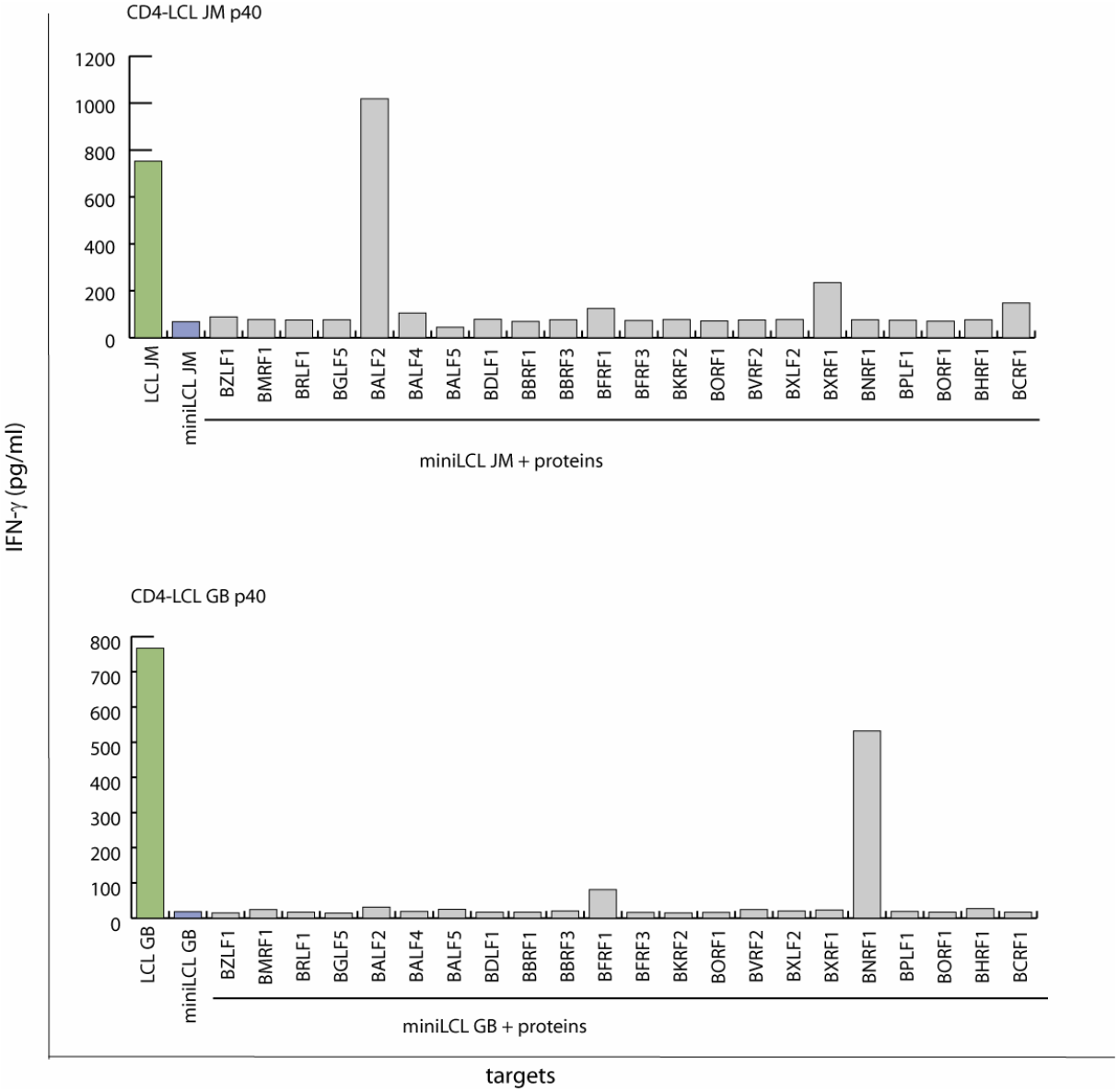
Figure 3.11. Cloning and expression of EBV lytic cycle genes.

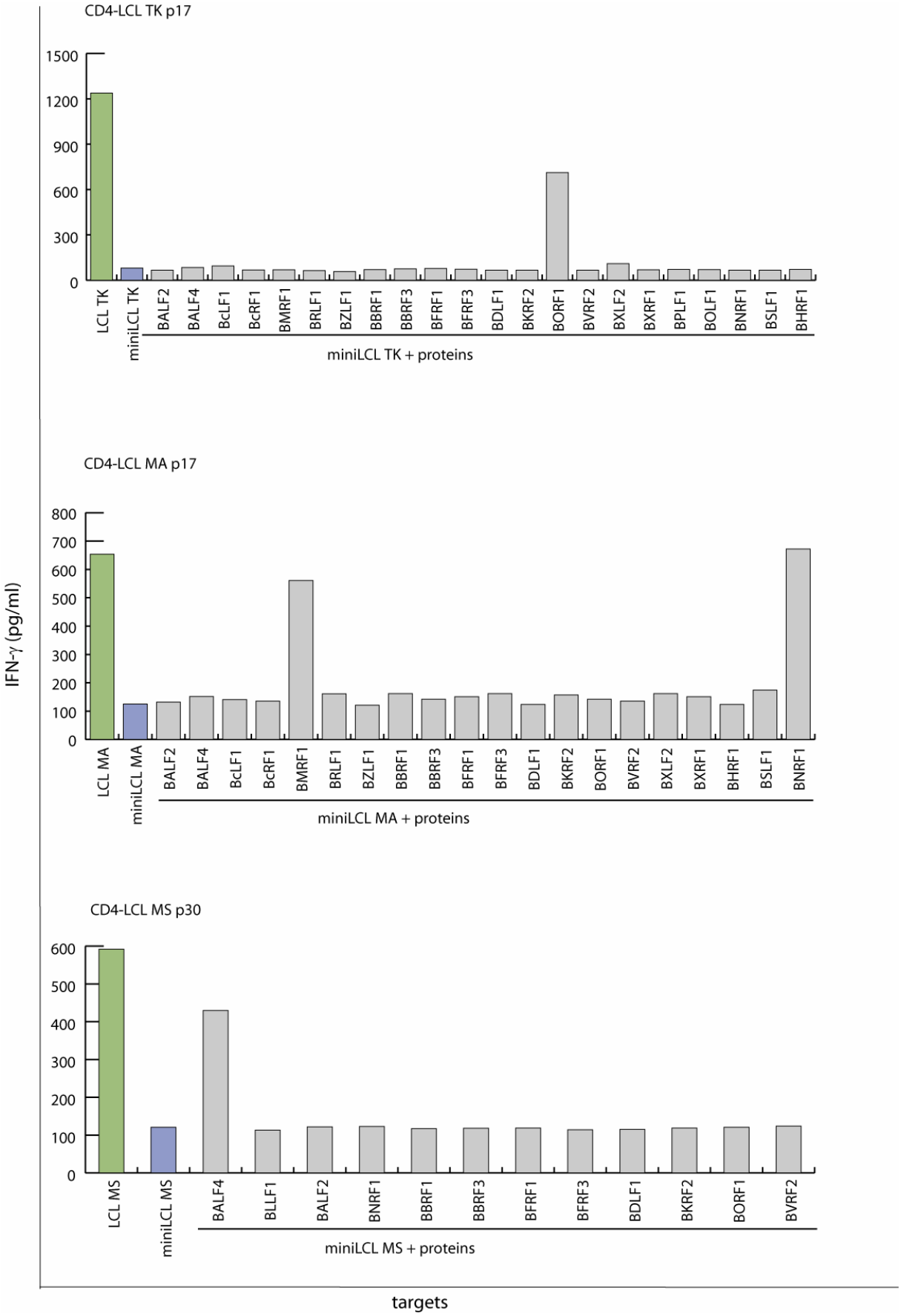
A) The ORFs of 35 EBV lytic cycle genes were cloned into the mammalian expression vector pCMV-CHis. Expression of inserted genes was controlled by a cytomegalovirus promoter/enhancer (pCMV) which leads to high level expression of the inserted genes in eukaryotic cells. The EBV proteins were C-terminally tagged with an EBNA1 antibody epitope followed by six histidines (6X His) that allowed purification of the fusion proteins over nickel-agarose beads. B) Eight purified lytic cycle proteins were analyzed by Western blot using the anti-EBNA1 antibody 1H4.

Appropriate APCs were pulsed with the recombinantly-expressed EBV lytic cycle proteins and subsequently probed with the T cell lines. In most experiments, autologous miniLCLs were used as APC because earlier experiments had shown that these cells were barely recognized by the T cell lines. In those cases where no autologous miniLCLs were available,

miniLCLs from partly MHCII-matched donors were used, provided that the LCL of this donor was efficiently recognized by the T cells.

Using this approach, several antigens recognized by the EBV-specific T cell lines were identified. While most of the T cell lines recognized one of the lytic cycle antigen tested, some late passage T cell lines recognized more than one antigen. This was in accordance with results obtained by Vβ chain analyses in earlier experiments showing that LCL-stimulated CD4+ T cells lines were oligoclonal. In the case of the T cell line IM1, none of the recombinantly expressed proteins was recognized. Because these T cells responded against MHC-matched LCL but not miniLCL of the same donor this T cell line most likely recognized (an) additional lytic cycle protein(s) that had not been included in this study. Representative results of these studies are shown in Figure 3.12.




CD4-LCL TK p17
1500
1200
900
600
300
0
LCL TK
miniLCL TK
BALF2
BALF4
BcLF1
BcRF1
BMRF1
BRLF1
BZLF1
BBRF1
BBRF3
BFRF1
BFRF3
BDLF1
BKRF2
BORF1
BVRF2
BXLF2
BXRF1
BPLF1
BOLF1
BNRF1
BSLF1
BHRF1
miniLCL TK + proteins
CD4-LCL MA p17
800
700
600
500
400
300
200
100
0
LCL MA
miniLCL MA
BALF2
BALF4
BcLF1
BcRF1
BMRF1
BRLF1
BZLF1
BBRF1
BBRF3
BFRF1
BFRF3
BDLF1
BKRF2
BORF1
BVRF2
BXLF2
BXRF1
BHRF1
BSLF1
BNRF1
miniLCL MA + proteins
IFN-γ (pg/ml)
CD4-LCL MS p30
600
500
400
300
200
100
0
LCL MS
miniLCL MS
BALF4
BLLF1
BALF2
BNRF1
BBRF1
BBRF3
BFRF1
BFRF3
BDLF1
BKRF2
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BVRF2
miniLCL MS + proteins
targets

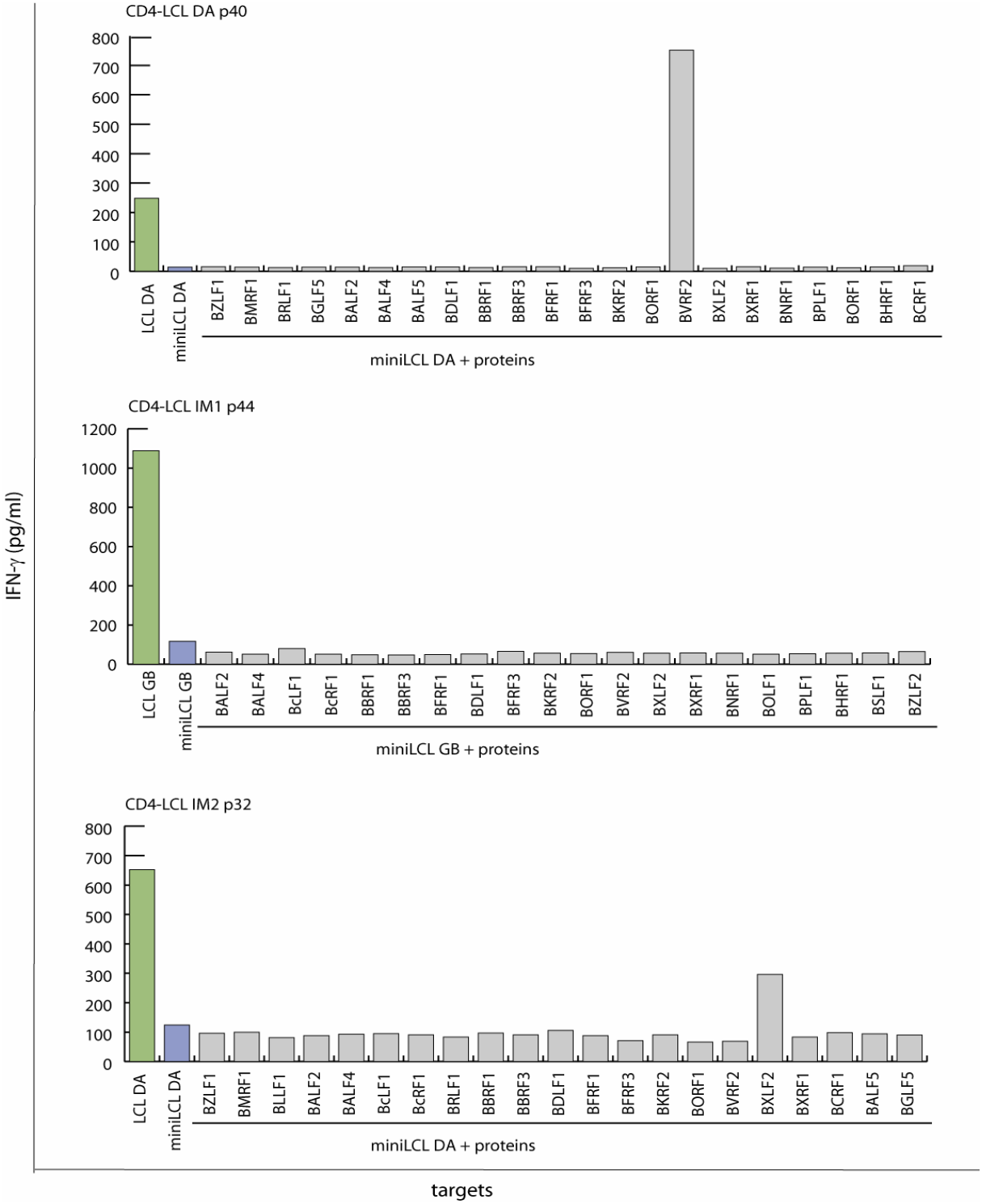

Figure 3.12. The targets of LCL-stimulated CD4+ T cells are lytic cycle proteins of EBV.

T cell lines from different donors at different passages (indicated as CD4-followed by the autologous stimulator LCL and the passage number) were tested in IFN-γ-specific ELISA for recognition of the LCL (green bars), miniLCL and miniLCL pulsed with different recombinantly expressed and purified EBV lytic cycle proteins. When an autologous miniLCL was not available (IM1 and IM2), a miniLCL derived from a partly MHCII-matched donor was used provided that the LCL of the donor was recognized by the T cells. For reasons of lucidity, only responses against a limited set of proteins are shown.

3.8 LCL-specific CD4+ T cell clones were generated and analyzed for their specificity

To analyze composition and specificity of the LCL-stimulated, oligoclonal EBV-specific T cell lines in more detail, several of the T cell lines were single cell cloned by limiting dilution, and the specificity of the outgrowing clones analyzed. Initially, outgrowing clones were tested against LCLs, miniLCLs, and miniLCLs pulsed with lytic cycle proteins identified as the antigen recognized by the parental T cell lines. In Figure 3.13, representative results obtained with T cell clones from two T cell lines are shown.

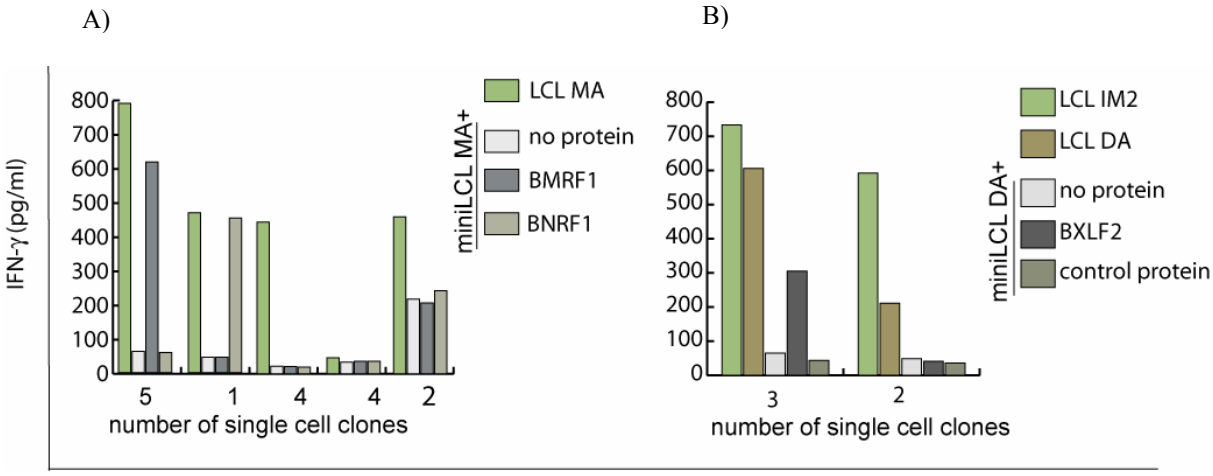

Figure 3.13. Analysis of single cell clones derived from autologous LCL-stimulated CD4+ T cell lines from healthy donor MA (A) and IM patient IM2 (B) in IFN-γ secretion ELISAs.

The T cell clones were tested for recognition of LCLs, miniLCLs and miniLCLs pulsed with the EBV lytic cycle protein identified as targets of the parental T cell line. For testing specificity of the T cell clones from IM2, the partly MHC-matched LCL and miniLCL from donor DA were used. 19 clones from the T cell line MA and five from the T cell line IM2 were analyzed. The numbers on the X-axis indicate the number of clones showing the pattern of recognition.

The parental T cell line MA p17 had responded against the BMRF1- and BNRF1-pulsed miniLCL. Consequently, the clones were tested against the LCL MA, miniLCL MA and miniLCL MA pulsed with BMRF1 or BNRF1 proteins. Among 19 clones that were analyzed from this line in IFN-γ secretion assays, five distinct patterns of recognition were observed. Eight of the clones recognized BMRF1, and one recognized BNRF1. Of the remaining clones, four responded exclusively against the LCL, and four responded neither against the LCL nor against the protein-pulsed or untreated miniLCL. Two T cell clones responded against the LCL and also significantly against the miniLCL, whether untreated or pulsed with recombinant protein. These results demonstrated that the LCL-stimulated line from donor MA contained at least five different specificities. Of these, two were directed against BMRF1 and BNRF1. The third that responded against the LCL but not against the miniLCL probably

recognized lytic cycle antigen(s) other than BMRF1 and BNRF1. For the fourth that secreted no IFN-γ in response to either the LCL or the miniLCL, the target antigen was not known (see section 3.9). The clones that recognized LCL and miniLCL probably represented autoantigen-specific T cells. These specificities were probably responsible for the background activity of the parental line against the miniLCL observed in experiments depicted in Figure 3.12. Since the antigens recognized by the last three types of T cells were unknown, it remained unclear whether the clones within the same group recognized one or several antigens.

Essentially similar observations were made with T cell clones obtained from another T cell line. Results on the clones obtained from the IM line IM2 are summarized in Figure 3.13b. Of the five clones tested, three recognized BXLF2, and the other two most likely recognized (an) other lytic cycle antigen(s) because these T cells recognized the MHCII-matched LCL but not the miniLCL.

Taken together, these results suggested that late passage LCL-stimulated T cell lines were predominantly, but not exclusively directed against lytic cycle antigens of EBV. Moreover, the T cell lines established from different donors recognized different antigens, suggesting that the targets of the EBV-specific T helper cell response lack a hierarchy of immunodominance.

Four of the T cell clones generated from donor MA had not secreted cytokines in response to autologous LCL, the stimulator cells for the parental T cell line. A similar pattern of apparent unresponsiveness had already been observed with the T cell line from IM patient 7, suggesting that T cells with this phenotype are not infrequent within LCL-stimulated CD4+ T cell lines. Because such T cells have not been described before, neither in the context of EBV infection nor in other conditions, attempts were made to characterize these T cells in more detail.

3.9 LCL stimulation also expanded CD4+ T cells failing to secrete prototype Th cytokines

To further characterize the non-IFN-γ-, non-GM-CSF-secreting T cells that grew out upon LCL stimulation, experiments with the T cell line IM7 and with a clone from the line DA were conducted. These T cells could be maintained in culture by stimulation with the autologous LCL and IL-2. The T cell line IM7 retained the same phenotype until passage 62 when further culture of this line was discontinued, suggesting that T cells with such specificity may dominate LCL-stimulated bulk cultures. The absence of any detectable IFN-γ secretion by these T cells could indicate that the T cells were of Th2 or T_R1 subtypes,

characterized by the secretion of IL-4 and transformimng growth factor-beta (TGFβ) plus IL-10, respectively. Therefore, secretion of these cytokines upon co-culture with autologous LCL was measured, but again, no target specific secretion of these cytokines could be detected (Figure 3.14).

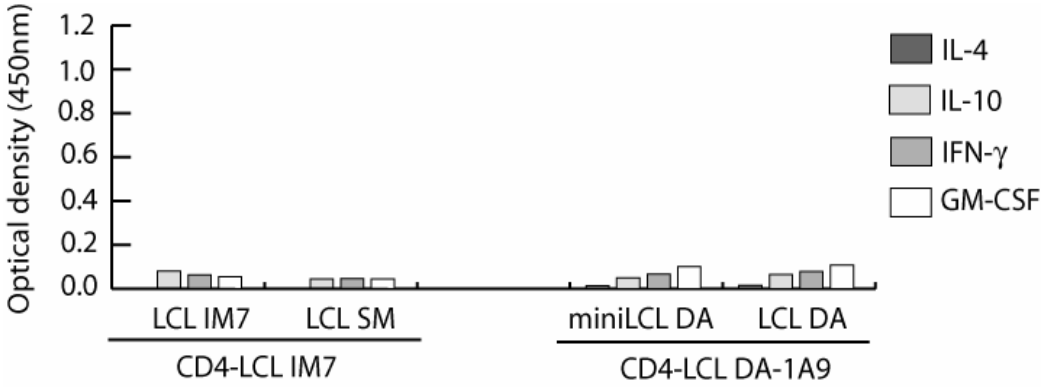

Figure 3.14. LCL-stimulation of CD4+ T cells expands T cells that fail to secrete prototype Th cytokines.

T cells of this type failed to show cytokine responses against autologous and allogeneic LCL and miniLCL targets. The response of the T cell line IM7 against autologous LCL 7 and allogeneic LCL SM, and of clone 1A9 derived from the T cell line DA against the autologous LCL and miniLCL are shown.

Even upon non-specific T cell activation with PHA, the T cells failed to secrete any of the cytokines tested. In an additional set of experiments, the T cells were studied in proliferation assays. A constant number of $1x10^5$ T cells was brought out per well in a 96-well plate, either alone, with 10 U/ml IL-2, with PHA and IL-2, or with 10^5 irradiated autologous or allogeneic LCL in the presence or absence of PHA and/or IL-2. After 72 hours incubation at 37°C, the supernatant was removed and fresh medium with (^{3}H)-thymidine added to the wells. After 24 hours, the wells were washed and (^{3}H)-thymidine incorporation measured with a scinticounter. The T cell line IM7 as well as the clone 1A9 proliferated best in the presence of IL-2 and proliferated only minimally in response to either the autologous or allogeneic LCL in the absence of IL-2. PHA alone or in the presence of autologous or allogeneic LCL did not induce proliferation (data not shown).

These results indicated that CD4+ T cells obtained by LCL stimulation might comprise a T cell population that does not belong to the classical T helper cell types. Further studies are required to characterize these T cells and identify their role(s) in the immune response to EBV.

3.10 LCL-specific CD4+ T cells were detected at low precursor frequencies in peripheral blood

The results of the previous experiment raised the question as to how many CD4+ T cells in the peripheral blood were specific for EBV lytic cycle antigens. Since lytic cycle antigens and not latent antigens of EBV were the major targets of CD4+ T cells obtained by LCL stimulation, this question could be addressed by comparing responses against LCL versus miniLCL. As suggested by previous experiments, LCLs stimulate autoantigen- and lytic cycle antigen-specific T cells whereas miniLCLs activate only autoantigen-specific T cells. Therefore, IFN-γ ELISPOT assays were performed on T cells from the peripheral blood of available donors from whom miniLCLs as well as LCL had been established. First, PBMC were enriched for CD4+ T cells by depleting CD8+ and CD19+ cells and subsequently added to titrated numbers of LCL and miniLCL targets. In order to exclude the detection of FCS-reactive T cells, targets grown in human serum were used. By subtracting the miniLCL-reactive from the LCL-reactive T cell population, an average of 0.5% to 1% of the peripheral blood CD4+ T cells were calculated to be specific for lytic cycle antigens of EBV.

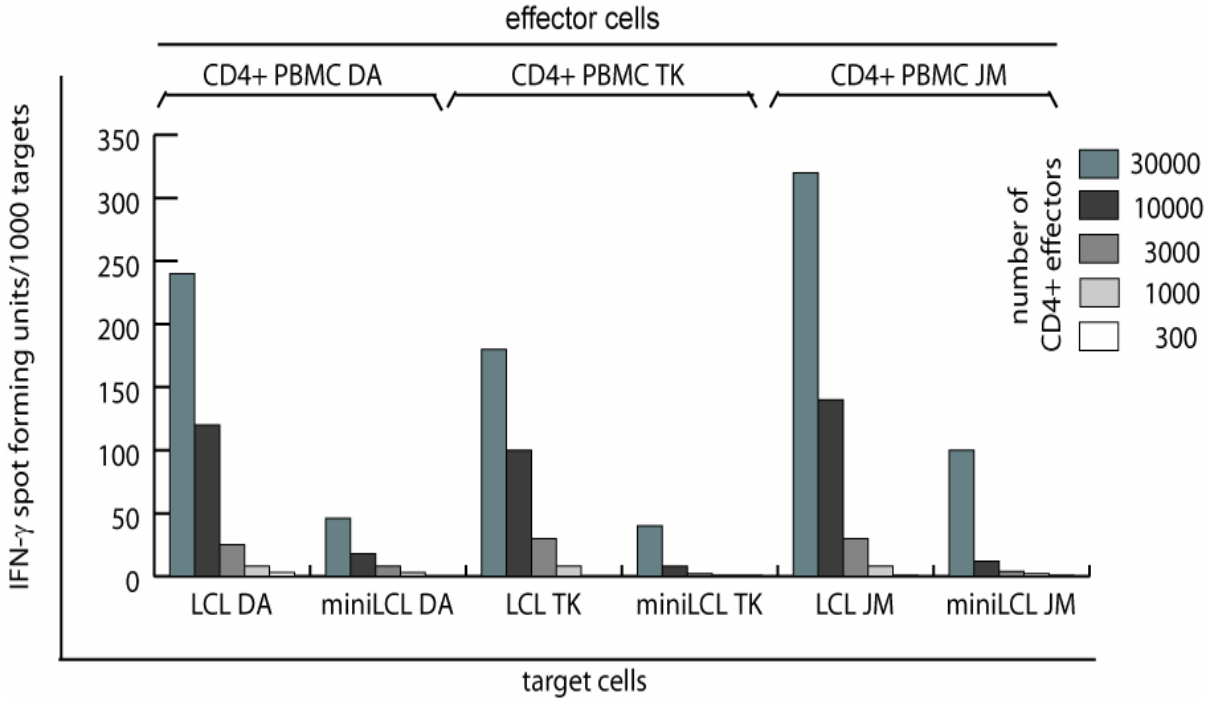

Figure 3.15. Quantification of peripheral blood CD4+ precursor frequencies reactive against lytic cycle antigens of EBV.

Titrated numbers of CD4-enriched PBMCs were tested in IFN-γ ELISPOT assays against a fixed number of 10^3 autologous LCL or miniLCL targets cultivated in human serum. The results of experiments with CD4+ cells from the peripheral blood of the three EBV-positive healthy donors DA, TK and JM are shown.

3.11 The lytic antigen-specific CD4+ T cell response was directed against several antigens

The apparent lack of any pattern of immunodominance among lytic cycle antigens together with the observations that about 0.5% to 1% of peripheral blood CD4+ T cells were specific for EBV lytic cycle antigens, while individual lytic antigen-specific T cells were present at much lower frequencies (data not shown), suggested that the EBV-specific T helper response might be directed against several lytic cycle antigens simultaneously. To investigate whether healthy virus carriers maintain memory responses against several lytic cycle antigens of EBV, the CD4+ T cell response against the EBV immediate early protein BZLF1 and two EBV late lytic cycle proteins, BLLF1 and BALF4, was studied. BLLF1 was chosen since EBV-infected individuals are consistently positive for antibodies to this membrane antigen, also referred to as EBV glycoprotein gp350/220 (Thorley-Lawson et al., 1982). BALF4 is another membrane glycoprotein (gp110) known to elicit an antibody response (Jilg et al., 1994). BZLF1 is a lytic cycle transactivator that has been shown to induce CD8+ T cell responses (Elliott et al., 1997). While BALF4 and BZLF1 recombinant proteins were expressed in and purified from 293T cells, BLLF1 was expressed in and purified from Sf9 cells by baculovirus infection because this protein was poorly expressed in 293T cells. Purified protein was then used to reactivate specific T cells from the peripheral blood of five healthy EBV carriers. Starting at passage four, T cells were tested for specificity in GM-CSF ELISAs. After six rounds of stimulation, the CD4+ T cell lines showed reactivity against the protein against which they had been raised, but not against control proteins which had been purified under identical conditions (Figure 3.16).

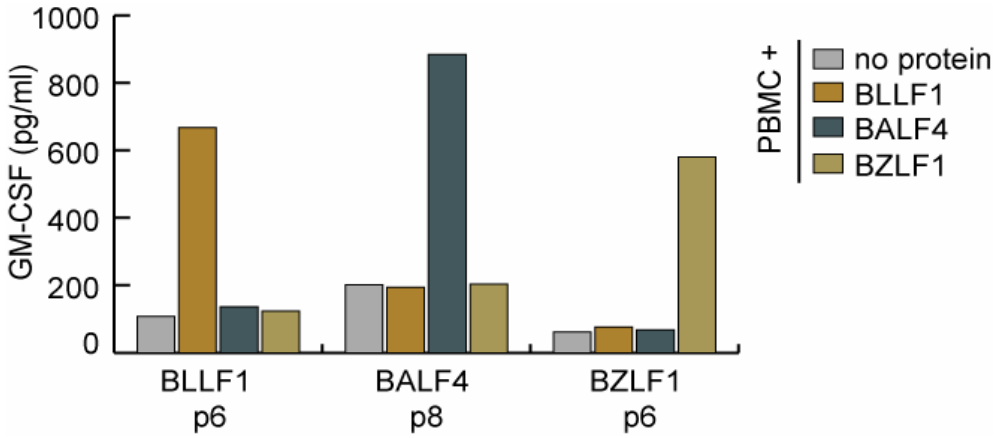

Figure 3.16. Peripheral blood of healthy EBV carriers contained CD4+ T cell memory against more than one EBV lytic cycle proteins.

Specificity of the CD4+ T cell lines established from donor JM was tested after six to eight rounds of stimulation by GM-CSF ELISA. PBMCs pulsed for 24 hours with different recombinant proteins were used as target cells. The T cell lines responded selectively against PBMCs pulsed with the stimulator but not irrelevant protein.

3.12 BLLF1-, BZLF1- and BALF4-specific T cell clones were generated from T cell lines and characterized

To characterize these T cells in more detail, the T cell lines from donor JM were cloned by limiting dilution. All outgrowing T cell clones were found to be CD4-positive, CD8-negative, TCRα/β-positive and TCRγ/δ-negative (data not shown). RT-PCR analysis in combination with Southern blot hybridization of the variable part of the TCR Vβ chain confirmed that the single cell outgrowths were monoclonal CD4+ T cell cultures (data not shown). The T cell clones obtained from each of the three T cell lines expressed two different Vβ chains, indicating that the clones derived from two different precursors.

To identify the restricting MHCII molecules of the different clones, PBMCs from various donors sharing different MHCII alleles with donor JM were pulsed with recombinant proteins and probed with the T cells. The antigenic epitopes recognized by the T cells were identified using a novel method for direct epitope identification (DEPI) established in our laboratory (Milosevic et al., 2005).

Not unexpectedly, all T cell clones specific for a given protein expressing the same Vβ chain recognized the same epitope presented on the same MHCII molecule. Six representative clones specific for two different epitopes in each of the three antigens were chosen for further analysis. These clones and their characteristics are summarized in Table 3.3.

T cell clone	Epitope	Restriction	TCR-Vβ
BLLF1-1H2	BLLF1 $AA_{130-144}$ -VYFQDVFGTMWCHHA-	HLA-DQB1*0402	Vβ5.2
BLLF1-1D6	BLLF1 AA_{65-79} -FGQLTPHTKAVYQPR-	HLA-DRB1*1301	Vβ21
BZLF1-3E4	BZLF1 $AA_{207-221}$ -KSSENDRLRLLLKQM-	HLA-DQB1*0402	Vβ21
BZLF1-3H11	BZLF1 $AA_{174-188}$ -ELEIKRYKNRVASRK-	HLA-DRB1*1301	Vβ4
BALF4-B5	BALF4 $AA_{482-496}$ -AWCLEQKRQNMVLRE-	HLA-DPB1*1301	Vβ6
BALF4-A9	BALF4 $AA_{575-589}$ -DNEIFLTKKMTEVCQ-	HLA-DRB1*0801	Vβ19

Table 3.3. Characterization of six EBV lytic cycle antigen-specific CD4+ T cell clones established from donor JM.

In order to define the affinities of the T cells for their cognate antigen, peptides spanning the epitopes were synthesized and pulsed at various concentrations onto autologous PBMCs, and recognition by the T cell clones were assayed in cytokine secretion experiments. As shown in

Figure 3.17, the various clones recognized target cells pulsed with cognate peptide at concentrations as low as 1 to 3 nM, while responses to control peptides were not observed even at much higher concentration.

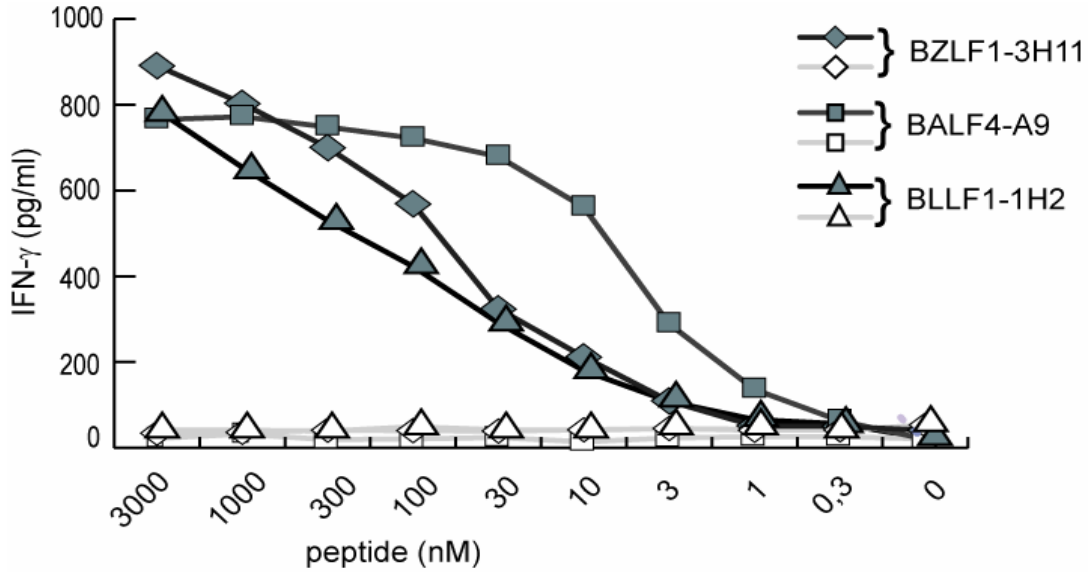

Figure 3.17. The T cell clones recognized antigen with high specificity.

Autologous PBMCs were pulsed with the indicated concentrations of cognate (shaded forms) and control peptides (open forms) for two hours. Subsequently, unbound peptides were removed by washing, and the peptide-pulsed PBMCs probed with the T cell clones. After 24 hours, IFN-γ secretion by the T cells was measured by ELISA.

In addition to GM-CSF, all lytic cycle antigen-specific CD4+ T cell clones secreted IFN-γ but no IL-4 upon specific target recognition. This pattern of cytokine secretion implied that the T cell clones were Th1 type CD4+ T cells.

To determine the precursor frequencies of these T cells in peripheral blood, IFN-γ ELISPOT assays were performed. PBMC of donor JM were enriched for CD4+ T cells by magnetic depletion of CD8+ T cells. Subsequently, 10^5 CD4+ enriched PBMCs were incubated with 2 µM of cognate or control peptides and the number of IFN-γ secreting cells determined by ELISPOT. No specific signals above background were detected with any of the peptides, suggesting that the frequency of these T cells in the peripheral blood is less than 1 in 10^4, which is considered the detection limit of this assay. Earlier experiments had shown that approximately 0.5% to 1% of all peripheral blood CD4+ T cells in healthy virus carriers are specific for lytic cycle antigens of EBV. The successful reactivation of memory T cell responses against three of three lytic cycle antigens in one donor further underlined that T

helper cells specific for many different lytic cycle antigens probably existed in the peripheral CD4+ T cell pool.

3.13 BLLF1- and BLLF4-specific CD4+ T cell clones responded against LCLs and EBV-positive BL cell lines, but not against miniLCLs

To test whether the EBV lytic cycle protein-specific T cell clones recognized EBV-infected cells autologous LCL and miniLCL were probed with the T cells. All four T cell clones specific for peptides derived from the two viral glycoproteins BLLF1 and BALF4, but not the two BZLF1-specific T cell clones recognized autologous and MHCII-matched allogeneic LCL (Figure 3.18).

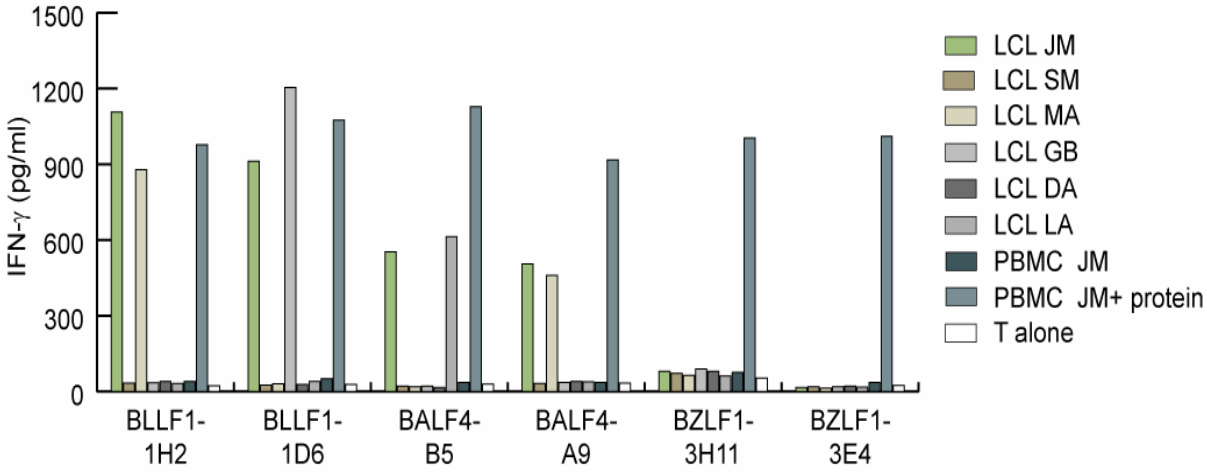

Figure 3.18. EBV glycoproteins BLLF1- and BALF4-specific but not BZLF1-specific CD4+ T cells recognized EBV-positive target cells directly.

The six different lytic cycle antigen-specific T cell clones were probed with autologous and partly MHC-matched allogeneic LCLs and IFN-γ secretion was subsequently measured by ELISA. The glycoprotein-specific T cells recognized LCLs in a MHCII-restricted manner, whereas BZLF1-specific T cells failed to recognize target cells directly. PBMCs pulsed with recombinant protein were used as positive controls in these experiments.

None of the T cell clones responded against autologous or MHCII-matched allogeneic miniLCLs (Figure 3.19A), indicating that lytic viral replication is necessary for the recognition of EBV-infected cells by lytic cycle antigen-specific T cells.

EBV-positive BL cells are more permissive for lytic replication than LCL (Rickinson and Kieff, 2001). To test whether glycoprotein-specific T cells also recognized these tumor cell targets, EBV-positive BL cell lines expressing the restricting MHC molecules were probed with the glycoprotein-specific T cell clones. As shown in Figure 3.19B, EBV-positive BL cell lines were recognized as efficiently as LCL by BLLF1- and BALF4-specific T cells.

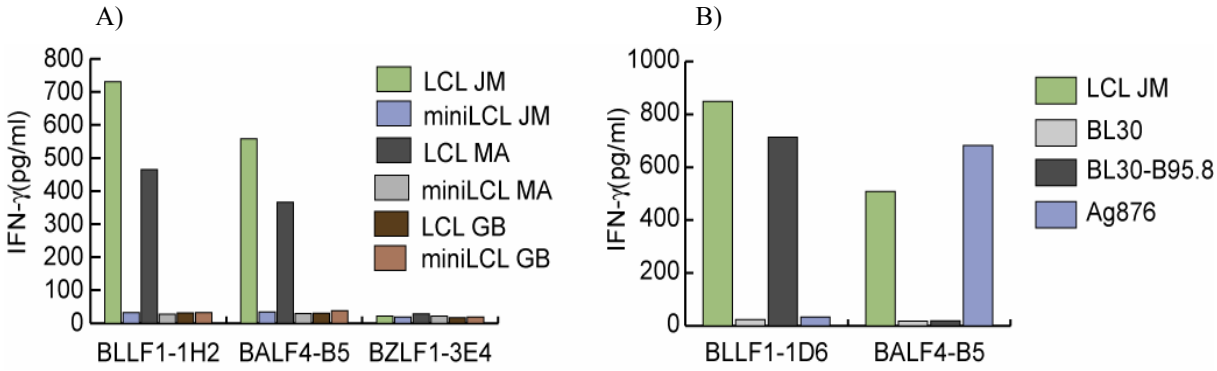

Figure 3.19. Recognition of EBV-positive target cells by glycoprotein-specific CD4+ T cells was dependent on viral lytic replication.

A)Lytic cycle protein-specific T cells were tested for recognition of autologous and allogeneic LCL and miniLCL pairs. The BLLF1- and BALF4-specific T cell clones recognized autologous and MHCII-matched LCLs, but not miniLCLs. The BZLF1-specific T cells recognized neither of the targets. B) Glycoprotein-specific T cells also recognized MHC-matched EBV-positive (BL30 B95.8, Ag876) but not -negative BL cell lines (BL30). Autologous LCLs served as positive controls while EBV-negative or MHCII-mismatched BL cell lines served as negative controls.

3.14 Antigen was transferred between cells in culture

The difference in the recognition of LCLs and miniLCLs by the T cells implied that recognition of EBV-infected cell lines by lytic cycle protein-specific T cells is dependent on lytic replication. Recognition of LCL cultures by BLLF1-specific CD4+ T cells had been noted before, and was found to be mediated by endogenous and exogenous presentation of the antigen following spontaneous lytic cycle replication in a small proportion of cells in LCL cultures (Lee et al., 1993). However, the strength of the signal detected in cytokine secretion experiments was surprising not only in view of the low percentage of usually less than 1% of cells in an LCL culture that spontaneously become permissive for lytic replication (Rickinson and Kieff, 2001), but also of the fact that cells undergoing lytic replication show greatly reduced expression of MHC molecules and secrete gp42, an EBV glycoprotein that has been shown to inhibit T helper cell recognition (Keating et al., 2002; Ressing et al., 2005). To gain further insight into antigen expression, processing and presentation of glycoproteins, the level of spontaneous lysis in the LCL JM was investigated. 10^5 LCL or miniLCL JM were fixed and labeled with monoclonal antibodies directed against BZLF1 and BLLF1. Expression of both proteins was indicative of active lytic replication. After staining of the cells with a fluorescence-labeled secondary antibody, the cells were inspected under an UV-fluorescence microscope. No staining was seen with the miniLCLs JM, whereas less than 1% of the cells in LCL JM were positive for expression of both proteins (data not shown).

To quantify the number of cells in LCL cultures recognized by the T cells, a fixed number of 10^5 per well antigen-specific T cells were used in IFN-γ ELISPOT assays using serial dilutions of autologous LCL, miniLCL or MHCII-mismatched LCL (control) targets. As shown in Figure 3.20A, a surprising 20% of LCL cells were spotted out by glycoprotein-specific T cells. MiniLCL and control LCL-containing wells showed background number of spots. No spots above background were detected with BZLF1-specific T cells, irrespectively of the target cells used (data not shown).

The discrepancy in the number of cells that stained positive for BZLF1 and BLLF1 in the immunofluorescence studies and the number of cells positive in the ELISPOT experiments suggested that antigen was transferred between cells, and peptides presented by bystander cells. To address this possibility, mixing experiments were performed. The autologous miniLCL was co-cultured with a MHCII-mismatched allogeneic LCL for 24 hours and then probed with the T cells. Neither the autologous miniLCL, nor the MHCII-mismatched LCL was recognized on its own. However, the cell mix was recognized by all glycoprotein-specific T cell clones but not by the BZLF1-specific T cell clones (Figure 3.20 B).

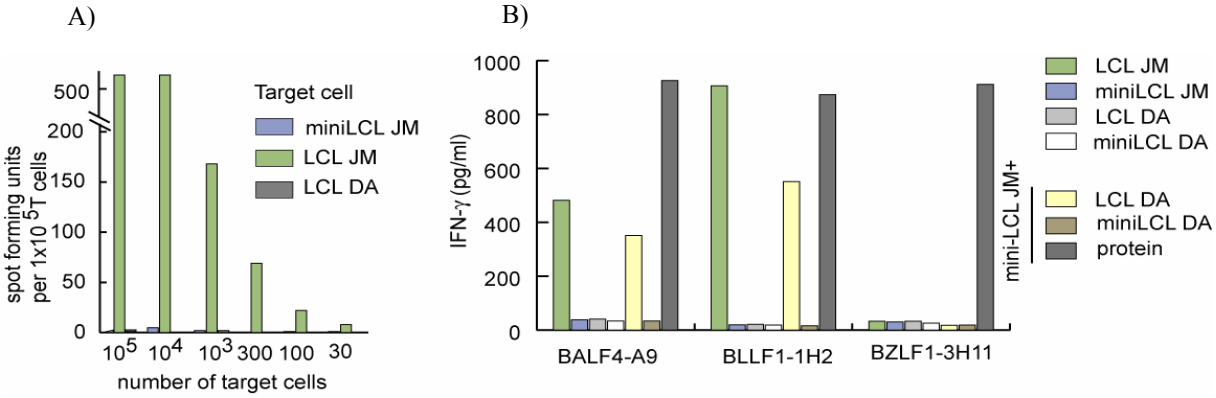

Figure 3.20. EBV glycoprotein antigens were transferred between cells in culture.

A) Serial dilutions of autologous LCL, miniLCL, and MHC-mismatched LCL cells were incubated with 10^5/well BLLF1-1H2 T cells, and the number of target cells recognized by the T cells was determined by IFN-γ ELISPOT. The T cells recognized nearly 20% of autologous LCL cells while addition of miniLCL or allogeneic LCL cells resulted in background numbers of spots. B) MiniLCL JM co-cultured with the MHC-mismatched allogeneic LCL DA, but not miniLCL DA was recognized by glycoprotein-specific but not BZLF1-specific T cells, indicating that a transfer of glycoprotein antigens has occurred between these cell types in culture. MiniLCL JM incubated with the cognate antigen was included as specificity control.

3.15 Released viral particles efficiently transferred antigen between cells

The sources of the transferred antigens were either fragments of cells in which EBV had replicated or released virus particles. To test the latter possibility, virus purified from supernatant of the B95.8 marmoset cell line by ultracentrifugation was pulsed onto the miniLCL JM that was subsequently probed with the T cells. Virus-pulsed miniLCL targets

were efficiently recognized by the BLLF1 and BALF4-specific T cells (data not shown), demonstrating that viral particles were capable of transferring antigen.

Viral particles could have transferred antigen in at least two different ways: (i) indirectly by superinfecting miniLCL followed by lytic replication in infected cells, and (ii) directly by transferring glycoproteins present in the virions. Expression of glycoproteins after induction of lytic replication requires 48 to 72 hours (Kieff and Rickinson, 2001). Because in the above mentioned experiments, glycoprotein-specific T cells had recognized miniLCL pulsed with viral concentrate for only 12 hours, superinfection as the mode of antigen transfer appeared unlikely. Even by taking into account that the virus-pulsed target cells were co-cultured with the T cells for additional 24 hours, this did not provide enough time for synthesis, processing and presentation of glycoproteins.

To assess the kinetics of antigen presentation in more detail, the miniLCL was pulsed with virus supernatant for various periods of time. Subsequently, the virus-pulsed cells were fixed with paraformaldehyde to terminate antigen presentation, and probed with the glycoprotein-specific T cells. These experiments demonstrated that miniLCL cells incubated with viral supernatant for only 12 hours were already efficiently recognized by the T cells (Fig. 21). Thus, viral particles appeared to transfer antigen directly and superinfection of target cells was not required. These findings were corroborated in additional control experiments where the viral concentrate was first heat inactivated at 56°C for half an hour, and then used to pulse the miniLCL. MiniLCL cells pulsed with heat-inactivated virus were recognized as efficiently as miniLCL cells pulsed with untreated virus, ruling out superinfection as mode of antigen transfer. The efficiency of this virus inactivation protocol was verified by its inability to immortalize primary B cells.

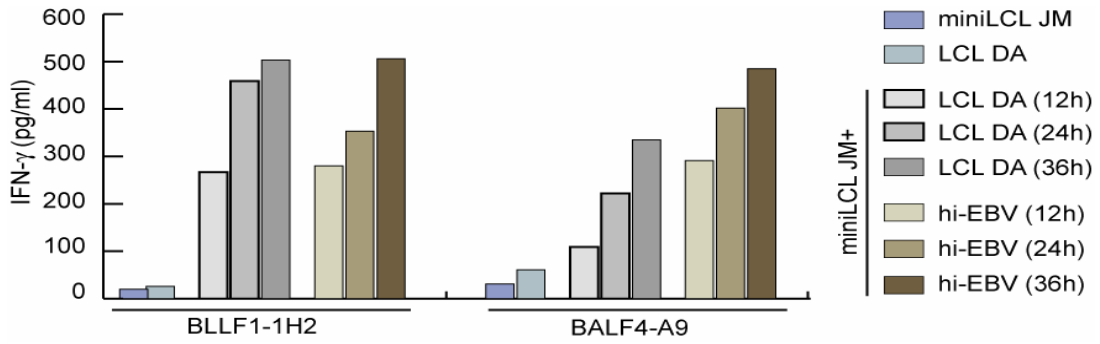

Figure 3.21. Viral particles transferred EBV glycoprotein antigens between cells.

The autologous miniLCL was pulsed with heat-inactivated purified EBV particles (hi-EBV), or was mixed with MHCII-mismatched LCL for different periods of time as indicated. Glycoprotein-specific T cells recognized the miniLCL pulsed with virus and co-cultured with a MHC-mismatched LCL with similar kinetics.

The miniLCL co-cultured with the MHC-mismatched LCL DA in cell mixing experiments was recognized by the T cells with a similar kinetics (Figure 3.21), providing further evidence that viral particles transfer antigen between cells in the form of structural proteins present in virions.

The efficient recognition of the miniLCL co-cultured with allogeneic LCL, however, was surprising, because LCL supernatant is known to contain few infectious virus particles. To assess efficiency of this antigen transfer, the concentration of EBV genome equivalents (geq) in the viral supernatant was determined. The miniLCL was pulsed with increasing amounts of geq and subsequently probed with glycoprotein-specific T cells.

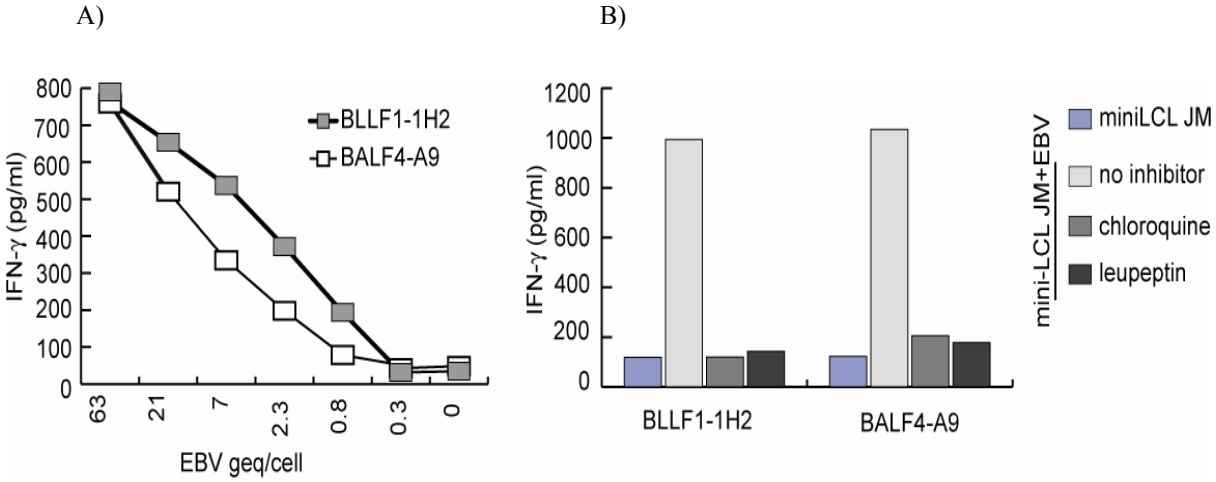

Figure 3.22. Presentation of virus supernatant-derived antigen was a very efficient process and involved processing in the endosomal /lysosomal compartment.

A) To determine the efficiency of the presentation of virus-derived antigens, miniLCL JM were pulsed with limiting concentrations of virus supernatant as indicated on the X-axis and probed with glycoprotein-specific T cells. BLLF1- as well as BALF4-specific T cells recognized the miniLCL pulsed with as little as 1 geq of virus per cell. B) MiniLCL JM was pulsed with virus for twenty-four hours in the absence or presence of chloroquine or leupeptin, fixed with paraformaldehyde, and then probed with glycoprotein-specific T cells (BLLF1-1H2 and BALF4-A9). MiniLCL cells incubated alone and incubated with virus supernatant in the absence of inhibitors served as negative and positive controls, respectively. Chloroquine and leupeptin both blocked the presentation of virus supernatant-derived antigens.

As shown in Figure 3.22A, miniLCL incubated with as little as 0.8 geq per cell were recognized by BLLF1- and BALF4-specific T cells. This demonstrated that the uptake and subsequent presentation of virion antigens is extremely efficient. When target cells were treated with chloroquine or leupeptin during incubation with viral supernatant, T cell recognition was almost completely abrogated, indicating that antigen processing occurred in the lysosomal compartment (Figure 3.22B). Chloroquine and leupeptin both inhibit lysosomal degradation (Honey and Rudensky, 2002).

3.16 Presentation of virion proteins was depending on receptor-mediated uptake of viral particles by B cells

Infection of B lymphocytes by EBV is receptor-mediated, and involves adsorption of gp350/220 to CD21, followed by aggregation of CD21 on the plasma membrane and the internalization of EBV into cytoplasmic vesicles (Carel et al., 1990; Tanner et al., 1987). To test whether this receptor-mediated uptake of EBV was essential for efficient presentation of virion antigens, EBV supernatant was incubated with the anti-gp350/220 monoclonal antibody 72A1. This antibody had been shown to prevent infection of B cells by EBV (Janz et al., 2000). Preincubation of viral concentrate with this antibody almost completely abrogated subsequent recognition of virus-pulsed miniLCL cells by glycoprotein-specific T cells. T cell recognition was not affected when miniLCL cells were pulsed with a viral supernatant that had been preincubated with a control isotype antibody (Figure 3.23A).

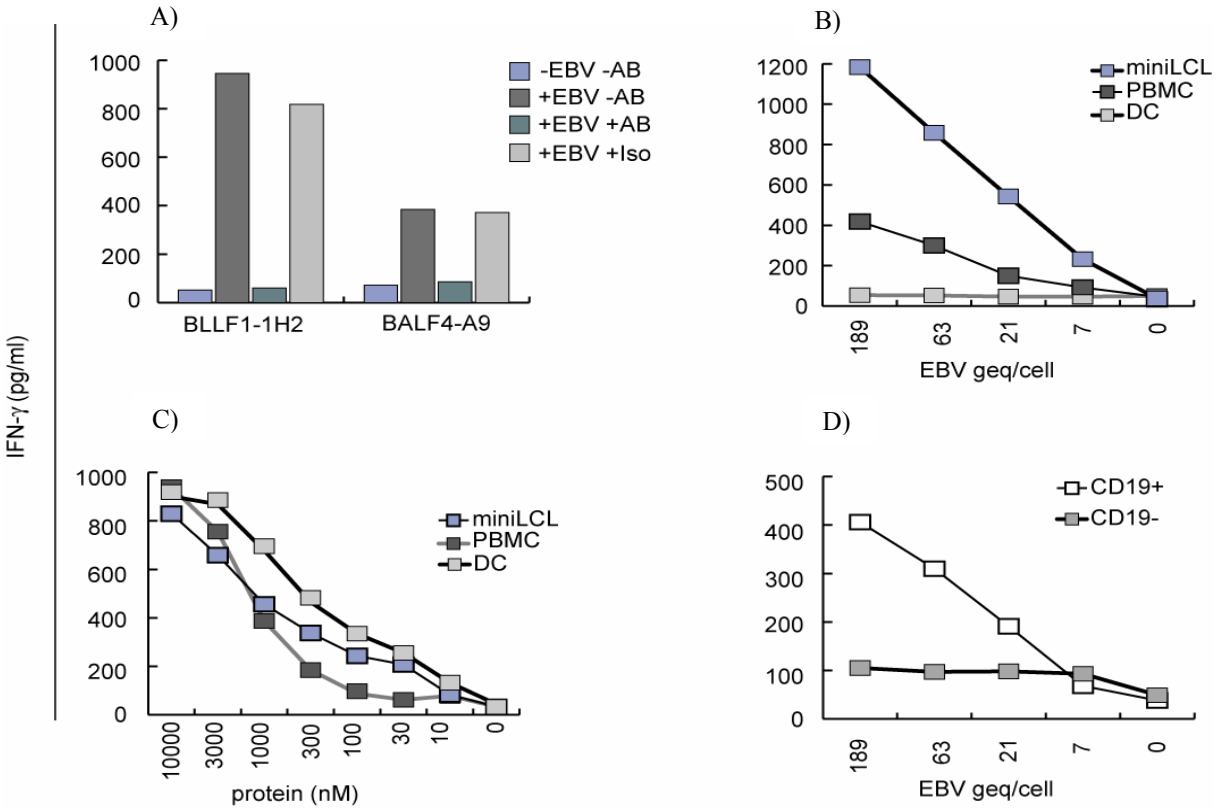

Figure 3.23. Presentation of virion-derived antigens was receptor-mediated.

A) MiniLCL JM were pulsed with untreated or pretreated (with an anti-gp350 antibody (AB) or an isotype control antibody (Iso)) virus supernatant and probed with glycoprotein-specific T cells. Anti-gp350 antibody pretreatment of virus supernatant abrogated the T cell response. B) MiniLCL, DC and PBMC JM were pulsed with virus supernatant (B) or recombinant BLLF1 protein (C) as indicated on the X axes for 24 hours and then probed with BLLF1-1H2 T cells. MiniLCL cells and PBMCs, but not dendritic cells were able to present virion antigens to T cells, but all three APC types presented exogenous antigen on MHCII with similar efficiency. D) PBMC JM were separated into CD19+ and CD19- populations, and the two populations pulsed separately with virus supernatant. Only the CD19+ population was able to present virion-derived antigens to BLLF1-1H2 T cells.

These results suggested that antigen presentation was dependent on receptor-mediated virus uptake. Consequently, only antigen presenting cells expressing CD21 should have been able to present virion antigens on MHCII. To test this supposition, EBV supernatant was pulsed onto the autologous miniLCL, monocyte-derived DCs and PBMCs. As shown in Figure 3.23B, miniLCL cells and PBMCs, but not DCs were able to present virion antigens to T cells under these experimental conditions. Of note, DCs efficiently stimulated the T cells when pulsed with BLLF1 peptides and, importantly, when incubated with purified BLLF1 protein, demonstrating that uptake, processing and presentation of exogenous antigens was not impaired in these cells. In fact, of all cell types tested, DCs presented exogenous antigens most efficiently on MHCII (Figure 3.23C). To investigate which cell population within PBMCs was able to present virion-derived antigens, B cells were isolated from PBMCs using anti-CD19 microbeads and magnetic sorting. CD19+ and the CD19-depleted PBMC fractions were then incubated with virus supernatant and probed with T cells. As shown in Figure 3.23D, the CD19-positive population, but not the CD19-depleted cell fraction was able to present virion antigens to T cells.

3.17 Cell fragments and proteins released through lysis of cells barely contributed to the amount of antigen transferred

These experiments illustrated that virus particles could transfer antigen, but did not exclude that under *in vitro* culture conditions, cells undergoing lytic replication or their remnants were a major source of antigen. Although the lack of LCL recognition by BZLF1-specific T cells suggested that the amount of antigen released by lysed cells was insufficient for T cell stimulation, the levels of expression of the virion proteins BALF4 and BLLF1 were likely to exceed that of the transcription factor BZLF1. Therefore, released virion proteins might have reached levels sufficient for T cell detection. Because DCs were incapable of receptor-mediated uptake of virion-derived antigens, DCs were used to assess to what extent released proteins and cell debris contributed to the transfer of antigen. Autologous DC were co-cultured with MHC-mismatched LCL cells and then probed with the lytic antigen-specific T cells None of the T cell clones recognized the DCs even after extended periods of co-culture, demonstrating that cells debris or released proteins contributed insignificantly, if at all, to the transfer of antigen (Figure 3.24).

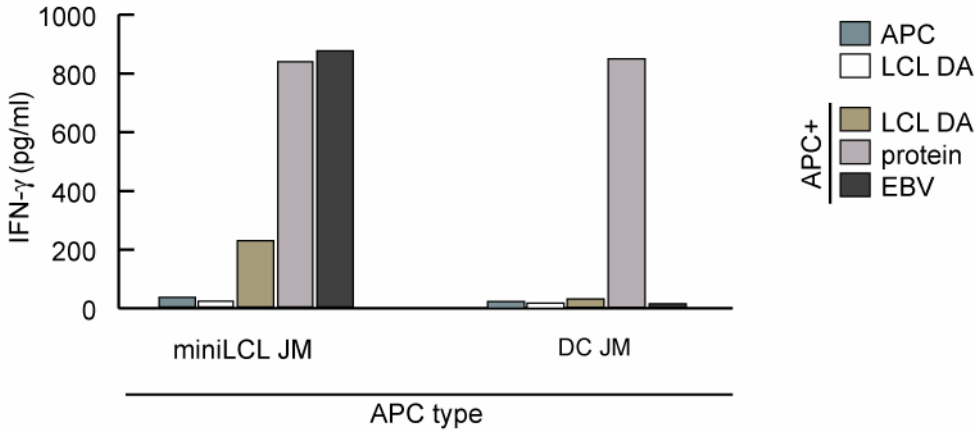

Figure 3.24. The amount of antigen released from lytically infected cells was insufficient for T cell recognition.

MiniLCL JM and DC JM were cultured either alone or together with the MHCII-mismatched LCL DA (ratio 1:1) for 24 hours. After the co-culture, the cells were probed with the BLLF1-1H2 T cells and cytokine secretion determined 24 hours later by ELISA. MiniLCLs JM and DCs JM pulsed with the cognate protein were included as controls.

3.18 EBV glycoprotein-specific CD4+ T cells inhibited the proliferation and generation of LCL *in vitro*

To assess direct effector functions of the virion-specific T cells on the growth of EBV-infected target cells, LCL JM and miniLCL JM were brought out in round-bottom 96-well plates in serial dilutions from 10^4 to 30 cells/well, with or without 10^4 monoclonal T cells, and proliferation of the cultures followed over time. The GFP-specific CD4+ T cell clone 3A2, also established from donor JM, was included as a control (Nimmerjahn et al., 2003). Wells were checked weekly for media exhaustion and were refed as necessary. After four weeks, the cultures were assayed microscopically for the outgrowth of cells, and CD19 staining demonstrated that the outgrowing cells were exclusively B cells (data not shown). As compared to irrelevant GFP-specific T cells, proliferation of LCL JM but not miniLCL JM was strongly impaired in the presence of glycoprotein-specific T cells (Figure 3.25A). Approximately 10-fold higher numbers of LCL were required to achieve continuous proliferation of the cells than in control wells. By contrast, proliferation of miniLCL was not affected by the glycoprotein-specific T cells. These experiments suggested that virion-specific T cells may play an important role in limiting virus spread by inhibiting or eliminating B cells that have become infected by EBV.

The next set of experiments therefore sought to investigate whether glycoprotein-specific T cells could hinder the generation of a LCL *in vitro*. PBMCs from donor JM were depleted of CD4+ and CD8+ T cells by magnetic sorting and were subsequently infected with B95.8 virus. After 24 hours, the cells were washed to remove free virus, and plated at serial dilutions

in 96-well microtiter plates together with 10^4 glycoprotein-, BZLF1-, or GFP-specific T cells. Cultures were maintained by weekly refeeding and were assayed for LCL outgrowth after four weeks. By that time, outgrowth was immediately apparent from microscopic inspection of the wells, and CD19 staining confirmed that the outgrowing cells were of B cell origin and not surviving T cells. Figure 3.25B presents the results of one such experiment, expressed as the minimum input cell number at which outgrowth of LCL occurred. Compared to control wells to which GFP-specific T cells had been added, approximately tenfold higher numbers of T cell-depleted PBMC were required to obtain LCL outgrowth. By contrast, addition of BZLF1-specific T cells had no inhibitory effect.

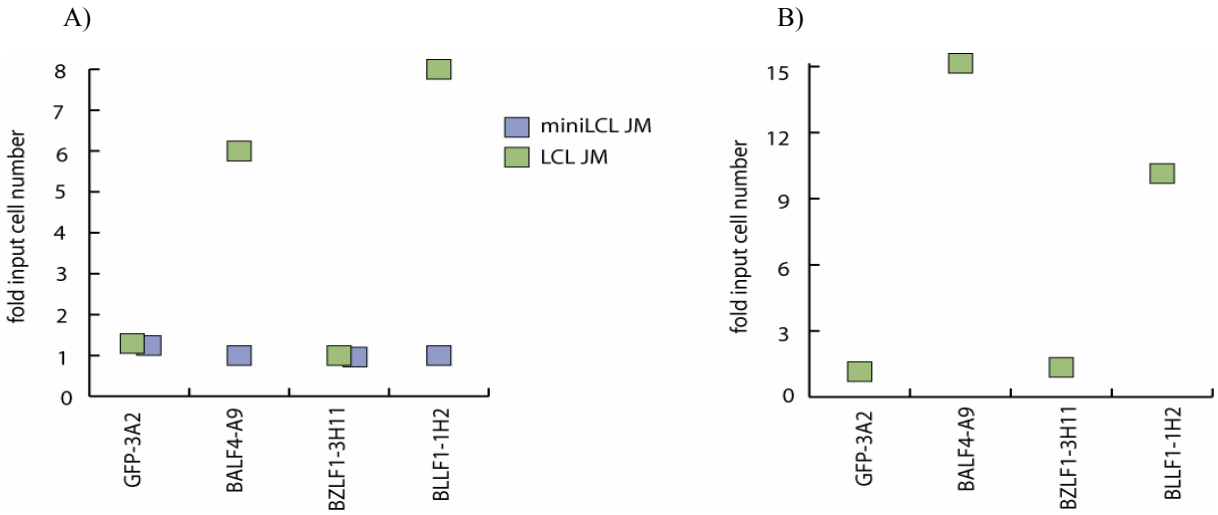

Figure 3.25 EBV glycoprotein-specific but not the transcription factor BZLF1-specific CD4+T cells inhibited the proliferation of autologous LCL cells and prevented the outgrowth of a LCL.

A) Decreasing numbers (10^4 to 30) of autologous LCL or miniLCL JM were plated in 96-well plates in the absence or presence of a fixed number (10^4) of BALF4-, BZLF1-, BLLF1- or GFP-specific CD4+ T cells from donor JM. After one month of culture, the wells were assayed for B cell proliferation by staining for CD19. The Y-axis shows the minimum starting number of LCL cells required to achieve a proliferating culture in the presence of T cells as x-fold the minimum number required in the absence of T cells. Co-culture of LCL JM, but not miniLCL JM, with BALF4- or BLLF1-specific T cells increased the minimum number of cells necessary for proliferation five- to tenfold. BZLF1- and GFP-specific T cells neither affected the proliferation of LCL nor miniLCL cells. B) 10^6 CD19+ primary B cells from donor JM were incubated with wild-type EBV for two hours. Subsequently, excess virus was removed by washing and the cells plated in 96-well round bottom plates in decreasing numbers starting form 10^4 down to 30 in the presence or absence of a fixed number of 10^5 T cells. The Y-axis depicts the x-fold minimum number of B cells plated to obtain a proliferating LCL culture with reference to the number of B cells required in the absence of T cells. GFP- and BZLF1-specific T cells did not inhibit LCL outgrowth, while in the presence of BALF4- and BLLF1-specific T cells ten- to fifteen fold higher numbers of B cells were required.

3.19 EBV glycoprotein-specific CD4+ T cells were lytic and used the granule exocytosis pathway to exert their effector functions

The inhibitory effect of the glycoprotein-specific T cells on proliferation and outgrowth of LCL raised the question by which mechanism the T cells exerted that function.

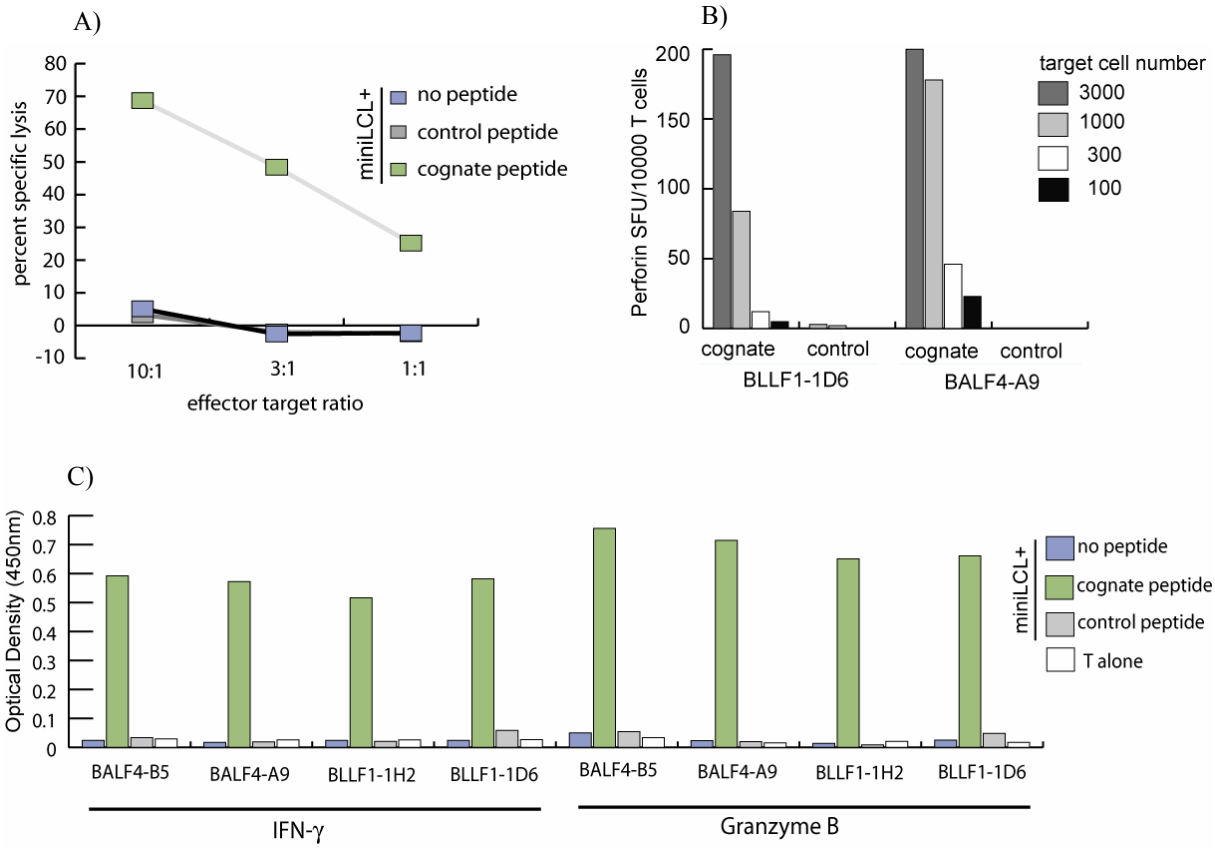

Figure 3.26. EBV glycoprotein-specific CD4+T cells were cytolytic by the granule exocytosis pathway.

A) MiniLCL JM incubated with no peptide, with the cognate peptide, or a control peptide, were probed with BALF4-B5 T cells in an europium release cytotoxicity assays. The X-axis shows the different effector to target (E:T) ratios used in the assays and the Y-axis the specific lysis at the corresponding ratios. Antigen-specific killing of up to 70% was observed at an E:T ratio of 10:1. B) BLLF1- and BALF4-specific T cells were assayed for target-specific perforin release in ELISPOT assays against miniLCL JM pulsed with 2 µM cognate or control peptides. A fixed number of 10^4 BLLF1-1D6 or BALF4-A9 T cells were co-cultured with titrated numbers ($3x10^3$ to 10^2) of peptide-labeled miniLCL. Between 8% and 15 % of cognate peptide-labeled but not control peptide-labeled targets were recognized by glycoprotein-specific T cells. C) Target-specific granzyme B release by BALF4- and BLLF1-specific T cells as measured in ELISA. MiniLCL JM labeled with either cognate or control peptides were used as targets. The glycoprotein-specific T cells secreted IFN-γ as well as granzyme B specifically upon antigen recognition.

To test whether the T cells were able to lyse target cells upon recognition, cytotoxicity was measured in europium release assays. 10^6 miniLCL cells were either left untreated or labeled with 2 µM of different peptides for two hours. The cells were then washed to remove unbound peptides and labeled with BATDA reagent. After 15 minutes at 37ºC, the cells were washed thoroughly and dead cells removed by Ficoll gradient centrifugation. $5x10^3$ live cells

in 100 µl culture medium were plated per well of a V-bottom plate. Subsequently, T cells in 100 µl culture medium were added at effector to target ratios from 10:1 to 1:1. After three hours incubation at 37ºC, 40 µl of the supernatant was collected and Europium solution added. Time-resolved fluorometry was measured and the specific lysis calculated as described (section 2.2.8.4). As shown in Figure 3.26A, the BALF4-B5 T cells killed targets pulsed with the cognate but not control peptide.

The rapid cytolysis by the T cells implied that granule exocytosis was involved in this process (Lieberman, 2003). Therefore, perforin ELISPOTs and granzyme B ELISAs were performed, using miniLCL cells labeled with cognate and control peptides as targets. For ELISPOT assays, decreasing numbers of target cells were co-incubated with a fixed number of 10^4 T cells. For the granzyme B ELISA, 10^5 T cells were incubated with the same number of target cells, and the granzyme B in the supernatant measured after 24 hours. As shown in Figure 3.26B and 26C, glycoprotein-specific T cells released granzyme B and perforin upon specific target recognition. These experiments demonstrated that the glycoprotein-specific T cells lysed target cells by the granule exocytosis pathway.

4. Discussion

Improved immunotherapeutic approaches against EBV-associated PTLD depend on the identification of the relevant immunodominant T cell antigens

In infected B cells, EBV is able to establish different types of latency characterized by the expression of different sets of latent cycle proteins (Rickinson and Moss, 1997). Infected B cells in immune competent hosts express only few or no latent viral antigens at all which allows the virus to evade immune recognition and elimination and to persist for life in the infected host (Williams and Crawford, 2005). During primary infection and in immunocompromised patients, eight antigenically distinct latent cycle proteins are expressed which cause growth-transformation of the infected B cell (Gottschalk et al., 2005). Uncontrolled proliferation of these cells in immunosuppressed transplant patients may lead to the development of EBV-associated PTLD (Moss and Rickinson, 2005).

The adoptive transfer of donor-derived EBV-specific T cell lines generated by repeated stimulations of peripheral blood T cells with autologous LCL cells has been shown to prevent and cure PTLD in BMT patients (Heslop et al., 1996; Bollard et al., 2004). With current protocols, the generation of sufficient numbers of EBV-specific T cells for clinical application takes at least a few months (Gottschalk et al., 2005). Since PTLDs often progress rapidly, such T cell lines would have to be prepared prophylactically for all patients at risk which is not possible on a routine basis. To attain a broader clinical application of this T cell therapy, alternative approaches have to be developed that allow to establish EBV-specific T cell lines faster, even after tumor manifestation. The direct isolation of specific T cells from peripheral blood by recently developed methods such as MHC tetramer technology and cytokine secretion and capture assay (Bakker and Schumacher, 2005; Mathioudakis et al., 2002) would obviate lengthy procedures of LCL generation and repeated rounds of T cell stimulation and shorten the time required to prepare such T cell lines. Such approaches, however, are dependent on the knowledge of the relevant immunodominant T cell antigens.

The immunodominant targets of the CD8+ T cell response to EBV are well characterized (Rickinson and Moss, 1997). Although CD4+ T cells are recognized as important component of the antiviral immune response, the EBV-specific T helper cell response is still poorly defined (Khanna and Burrows, 2000). By using two complementary approaches, this work attempted to investigate breadth and immunodominance of the EBV-specific T helper cell response. In the first approach, the immunodominant antigens recognized by repeatedly LCL-stimulated, oligoclonal EBV-specific T cell lines were defined. In the second approach, the

breadth of the EBV-specific T helper cell response was assessed by stimulating CD4+ T cells with autologous PBMCs pulsed with single recombinant proteins of EBV.

CD4+ T cell lines generated from EBV-negative donors failed to show EBV-specificity

LCL-stimulated CD4+ T cell lines were established from twenty-three individuals and maintained in culture for more than fifty passages. Cord blood-derived T cell lines and the T cell line from an EBV-negative adult failed to show EBV-specificity even at late passages. Initially, these T cell lines responded against autologous and allogeneic LCL without displaying a clear pattern of MHCII-restriction. With further rounds of stimulation, responses against autologous LCL disappeared and erratic responses against allogeneic LCL suggested that the T cell lines were predominantly alloreactive. In attempts to characterize LCL-reactive cord blood leucocytes, Moretta et al. had previously identified CD4+ T cells that participate in the innate response against EBV (Moretta et al., 1997). When cord blood leucocytes were stimulated *in vitro* with LCL, the resulting cell lines contained NK cells and CD4+ T cells that secreted IL-2 upon LCL stimulation, but the specificity of these T cells had not been examined. Non-EBV-specific CD4+ T cell lines generated by LCL stimulation of PBMC from EBV-seronegative children had also been reported by others (Savoldo et al., 2002). These authors also described a CD4+ T cell line that they had established from an EBV-negative adult by four rounds of LCL stimulation. The T cells specifically lysed autologous LCL but not PHA T cell blasts. The authors argued that the cytolytic effect is possibly mediated by cross-reactive T cells. Viral antigen-specific T cells that recognize antigens from other viruses by molecular mimicry had indeed been described (Welsh et al., 2004). Notably, the T cell line from the EBV-negative adult included in our study also responded against autologous LCL at early passages. After additional rounds of stimulation, however, this line became alloreactive, indicating that T cells putatively cross-reacting against LCL were lost from LCL-stimulated T cell cultures over time.

CD4+ T cell lines established from EBV-seropositive donors recognized EBV and autoantigens

Unlike T cell lines from EBV-negative donors, T cell lines established from the peripheral blood of all EBV-positive healthy donors and seven out of eight IM patients were MHCII-restricted, and all T cell lines from EBV-positive healthy adults were EBV-specific as demonstrated by their response against EBV-positive, but not against EBV-negative targets.

Of the seven MHCII-restricted T cell lines from patients with IM, four were EBV-specific whereas three responded equally well against EBV-positive and EBV-negative targets.

This high prevalence of non-EBV-specific responses was surprising because MHC tetramer analyses have demonstrated that up to 50% of the CD8+ T cells in peripheral blood of IM patients are specific for a single EBV antigen (Callan et al., 1998), and antibody responses against viral proteins also peak during acute infection (Rickinson and Kieff, 2001). Although CD4+ T cells do not expand as dramatically as CD8+ T cells during acute infection (Macallan et al., 2003), these results implied that a robust EBV-specific T helper cell response is induced in IM patients. Since humoral responses depend on T cell help, EBV-specific T helper cells must have developed at the time when the patients presented in the hospital and when IM was diagnosed serologically. Furthermore, the strong antiviral antibody response observed in IM patients implied that the magnitude of EBV-specific T helper cell response during the acute phase of infection is probably similar to or even exceeds the response during the persistent phase of infection. If this was indeed the case, then the lack of EBV-specificity observed in about half of the T cell lines established from patients with IM could either be due to a more vigorous expansion of autoreactive than EBV-specific CD4+ T cells during acute infection, or to the expansion of T helper cells specific for EBV antigens that are not presented by LCL. CD4+ T cells specific for BZLF1, for example, have been detected in three of three healthy virus carriers and these T cells failed to recognize LCL. Furthermore, BZLF1-specific T helper cell have also been described in patients with IM (Precopio et al., 2003), indicating that BZLF1-specific T cells expand during primary infection. Moreover, BZLF1 appears to be an immunodominant target of the CTL response during IM (Steven et al., 1997). Because all LCL-stimulated T cell lines from latently-infected healthy virus carriers recognized LCLs but not miniLCLs, both scenarios imply that T helper cells specific for EBV antigens presented on LCLs either expand less vigorously during acute infection or contract less dramatically during convalescence than T cells specific for autoantigens or EBV antigens that are not efficiently presented by LCLs. Consequently, the relative frequencies of autoreactive and EBV-specific T cells in the peripheral blood of IM patients should vary during the course of the illness, and the time of blood drawing may impact on the specificity of LCL-stimulated T cell lines established from IM patients.

An overproportional expansion of autoantigen-specific T cells during acute infection might also explain the appearance of heterophile antibodies and raises the intriguing possibility that these T cells might also contribute to the control of the viral infection. To gain further insight

into the role of the various CD4+ T cell specificities in establishing protective immunity against EBV, experiments were performed to define the antigens recognized by these T cells.

EBV-specific T cell lines failed to recognize latent cycle antigens of EBV

Although LCLs express all latent cycle antigens of EBV, none of the LCL-stimulated T cell lines recognized any of these antigens. This was again unexpected because LCL-stimulated CD8+ T cell lines target latent antigens of EBV (Rickinson and Moss, 1997), and CD4+ T cells specific for latent antigens of EBV have been isolated from healthy virus carriers by different groups (Munz et al., 2000; Leen et al., 2001; Mautner et al., 2004; Long et al., 2005). These findings raised two questions. First, why did latent antigen-specific CD4+ T cells not expand upon stimulation with LCL cells and second, what if not the latent antigens were the targets of LCL-stimulated EBV-specific CD4+ T cells? The failure of LCL-stimulated T cell lines to recognize latent antigens of the virus either indicated that latent antigen-specific T cells were present at lower frequencies than other EBV antigen-specific T cells, or that latent antigen-derived peptides were presented less efficiently on MHCII. Since miniLCL-stimulated T cells from a healthy virus carrier failed to show EBV-specificity, the second possibility appeared more likely. This notion was further supported by experiments showing that most of the latent antigen-specific CD4+ T cells that had been isolated from this donor did not respond against latently EBV-infected cells (Mautner et al., 2004 and unpublished results). Whether the presentation of latency protein-derived peptides on MHCII is actively inhibited by the virus or whether the level of viral protein expression is too low for T cell recognition is not known and needs to be addressed in further studies.

Autoantigen-specific T helper cells are an integral part of the CD4+ T cell response to EBV infection

Since LCL-stimulated CD4+ T cell lines did not recognize EBV latency antigens, the antigens recognized by early passage T cell lines on miniLCLs were probably autoantigens. Although this reactivity declined over subsequent rounds of stimulation and became undetectable in late passage T cell lines, almost all LCL-stimulated CD4+ T cell lines showed miniLCL-reactivity after ten passages, i.e. five months of *in vitro* culture. The T cell lines used to treat PTLD patients have usually been stimulated less than ten times with the autologous LCL (Gottschalk et al., 2005). Therefore, such adoptively transferred T cell lines probably contained autoreactive T cells. What fraction of the T cell population is autoantigen-reactive appears to vary between individuals, as suggested by the variable responses of LCL-stimulated T cells

against miniLCL. The clinical significance of LCL-reactive, autoantigen-specific CD4+ T cells is not known. In a recent study, autoreactive CD4+ T cells were identified as important effectors in LCL regression assays (Gudgeon et al., 2005). In that study the authors infected unseparated, CD4- or CD8-depleted PBMCs with EBV *in vitro* and assessed LCL outgrowth from EBV-transformed B cells. Because the cells that mediated regression in CD8-depleted PBMCs were found to be CD4+ T cells that specifically recognized LCL and miniLCL cells, but not latency antigens of EBV, the authors inferred that these T cells recognized autoantigens. Whether and how autoantigen-specific T cells contribute to regression of EBV-positive lymphomas *in vivo* is not known. The failure of these putative autoreactive T cells in the study by Gudgeon et al. to recognize EBV-negative B cell blasts implied that the antigens recognized by these T cells are induced by EBV. However, the autoantigen-reactive T cell lines in our study also recognized EBV-negative BL cell lines, indicating that the transformed phenotype *per se* rather than EBV confered T cell recognition. Whether the antigens recognized by the T cells were ubiquitously or B cell-specifically expressed is currently under investigation. If the latter was the case, then such autoantigen-reactive T cells might not only have a protective role in EBV immunity, but might also play a role in the immune response against EBV-negative B cell malignancies.

EBV-specific T helper cell lines from healthy virus carriers responded predominantly against lytic cycle antigens

The autoreactive component present in all early passage LCL-stimulated T cell lines gradually declined over further rounds of stimulation, and late passage T cell lines from all healthy virus carriers and from about half of the IM patients responded only against LCL but not against miniLCL. This pattern of recognition indicated that the antigens recognized by late passage T cell lines were derived from lytic cycle proteins of EBV. During lytic replication, approximately 80 viral proteins are expressed (Kutok and Wang, 2006). To identify the antigens recognized by the EBV-specific T cell lines, 35 EBV lytic cycle proteins were recombinantly expressed, and miniLCL pulsed with the purified recombinant EBV proteins used to screen late passage LCL-stimulated CD4+ T cell lines. At least nine different lytic cycle antigens were recognized by the seven T cell lines analyzed. Most T cell lines responded strongly against one or two antigens. In addition, some lines also responded weakly against presumably subdominant antigens. Two cell lines neither responded against any of the 35 lytic cycle proteins, nor against any of the latent cycle proteins. Because both T cell lines responded against LCL but not miniLCL cells, the antigens recognized by these T cells were

probably other lytic cycle antigens that had not been included in this study, or autoantigens induced by viral replication.

Structural proteins were the immunodominant targets of the EBV-specific T helper cell response

The nine EBV antigens identified as dominant targets of the CD4+ T cell response from different individuals included the tegument protein BNRF1, the early proteins BALF2 and BMRF1, the viral IL-10 homologue BCRF1 and the glycoproteins BALF4, BXLF2, BVRF2, BXRF1, and BORF1.

Except for three targets, namely BMRF1, BALF2, and BCRF1, all other antigens were derived from structural proteins of the virus. Responses against each of the three non-structural proteins were detected once and in different T cell lines, and always in conjunction with a structural protein as a second target. These results indicated that perhaps structural antigens of the virus comprise the major group of targets of LCL-stimulated CD4+ T cells. The tegument protein BNRF1 was recognized by three of the EBV-specific T cell lines tested which raised the possibility that BNRF1 is a common and immunodominant target of the CD4+ T cell response to EBV. Indeed, CD4+ T cells specific for this antigen have been established in our laboratory from some more individuals in the meantime (Adhikary, unpublished data). Of note, the various BNRF1-specific T cells were restricted by different MHCII molecules, suggesting that BNRF1 is an immunodominant antigen over a broad range of HLA class II alleles. BNRF1 is not the most abundant viral protein expressed in lytically infected cells, and no CTL responses against this protein have been described. Why this protein elicits strong CD4+ T cell responses, therefore, remains to be determined.

Studies on single cell clones isolated from the LCL-stimulated T cell lines confirmed and extended the results of earlier experiments in which TCR Vβ chain expression was analyzed. These experiments not only demonstrated that late passage T cell lines were indeed oligoclonal, but also showed that T cells present in these lines recognized different antigens, and in some cases also displayed different phenotypes. Most of the single cell outgrowths recognized lytic cycle antigens, verifying the dominance of lytic cycle antigen specificities in EBV-reactive CD4+ T cell lines. Besides the lytic antigens recognized by the parental cell line, some of the isolated T cell clones responded against LCL but not against miniLCL or miniLCL cells pulsed with any of the recombinantly expressed EBV proteins, suggesting that these T cells were specific for additional lytic cycle antigens. Further studies with the complete set of lytic cycle proteins, or genetic approaches like the DANI method developed in

our group for the direct identification of MHCII-restricted antigens (Milosevic et al., unpublished results), are required to define the complete set of immunodominant targets of the EBV-specific T helper cell response.

LCL-stimulated CD4+ T cell lines included T cells of non-Th1 or Th2 subtype

Some T cell clones isolated from LCL-stimulated T cell lines, and the T cell line established from patient IM7 failed to secrete prototype Th1 or Th2 cytokines (IFN-γ and IL-4, respectively) in response to either autologous or allogeneic LCL. The T cell line IM7 proliferated in response to autologous LCL and IL-2 and retained this non-Th1/Th2 phenotype until *in vitro* culture was discontinued after more than 50 passages. Although the antigen(s) recognized by this T cell lines remained unknown, the frequent isolation of such T cells from various LCL-stimulated T cell lines indicated that LCL-stimulation promotes the expansion of T cells with this phenotype. Whether these T cells belong to the T_R1 suppressor subtype characterized by the secretion of TGF-β (Roncarolo et al., 2001) or to the recently identified T helper cell subtype with autoaggressive potential characterized by the secretion of IL-17 (Harrington et al., 2005) or to a completely new subtype of T helper cells, remains to be determined. As long as no effector functions have been defined, the role of these T cells in the immune response to EBV remains elusive.

In healthy virus carriers the CD4+ T cell response is directed against a broad set of lytic cycle antigens

To assess the breadth of the lytic cycle antigen-specific T helper cell response, protein-pulsed PBMC were used to reactivate T helper cells from peripheral blood of healthy virus carriers. BZLF1 and BLLF1 (also referred to as gp350) were chosen as target antigens because T helper cell responses against these antigens had been detected earlier in peripheral blood of patients with IM and healthy virus carriers, respectively (Precopio et al., 2003; Ulaeto et al., 1988). In addition, the glycoprotein BALF4 (also referred to as gp110) was included in this analysis because humoral responses against this antigen had been described (Jilg et al., 1994). T cells specific for these antigens were generated from PBMC of five out of five EBV-positive carriers but not from PBMC of an EBV-negative adult used as control. Of the three proteins tested, only BALF4 had been identified as target of LCL-stimulated T cell lines, indicating that the EBV-specific T helper cell response is directed against a broader set of lytic antigens than that identified by the LCL-stimulation approach. For the purpose of characterizing the BZLF1-, BALF4- and BLLF1-specific T cells in more detail, the T cell

lines from one EBV-seropositive individual were cloned by limiting dilution and six representative clones specific for two different epitopes in each of the three antigens were chosen for further analysis

Virion-derived antigens were efficiently presented on MHCII following receptor-mediated uptake of released viral particles

Although all clones recognized their cognate antigenic peptide with similar avidity (1 to 3 nM), only T cells specific for the glycoproteins BLLF1 and BALF4, but not for the transcription factor BZLF1, recognized autologous as well as MHCII-matched allogeneic EBV-infected target cells. MiniLCL were not recognized by any of the T cells, suggesting that T cell recognition was dependent on sporadic lytic replication occurring in a low percentage of cells in culture. However, the percentage of cells recognized by the glycoprotein-specific T cells was substantially higher than those positive for lytic cycle proteins in immunofluorescence studies. Co-culture experiments of HLA-mismatched LCLs and HLA-matched miniLCLs indicated that antigen was transferred from one cell type to the other. Additional experiments provided several lines of evidence that the transferred antigen was derived from released virions.

First, miniLCL cells pulsed with purified virus preparations were recognized by the T cells, demonstrating that virions can serve as source of antigen. Because miniLCL cells pulsed with heat-inactivated virus were still recognized, T cell recognition did not depend on productive infection of target cells. Of note, virus supernatant had been utilized before as source of antigen to reactivate and expand BLLF1-specific T cells. In fact, these were the first EBV-specific T helper cell clones isolated and characterized *in vitro* (Wallace et al., 1991). The authors showed that these T cells were able to recognize LCL presenting endogenous and exogenous antigen on MHCII, but further implications of such recognition in the control of EBV infection were not addressed.

Second, antigen was most efficiently transferred to B cells, and antigen transfer could be blocked by a BLLF1-specific monoclonal antibody which had been shown to prevent EBV infection of B cells (Wendtner et al., 2003), suggesting that antigen uptake was receptor-mediated. Importantly, this antibody treatment not only prevented subsequent recognition by BLLF1-specific but also by BALF4-specific T cells, further adding to the notion that the antigens presented were derived from virions and not from released proteins.

Third, the failure of BZLF1-specific T cells to recognize LCL cells, and the failure of BALF4- and BLLF1-specific T cells to recognize autologous DCs co-cultured with HLA-

mismatched LCL cells, demonstrated that the amount of antigen released by dead or dying cells was not sufficient for T cell activation. The failure of BZLF1-specific T cells to recognize endogenous antigen in cells that had become permissive for viral replication might be the consequence of inefficient access to the endogenous antigen presentation pathway(s) (Zimmerman et al., 2003), or might be related to immune evasion strategies evolved by EBV to prevent immune recognition of lytically infected cells (Keating et al., 2002; Ressing et al., 2005).

Interestingly, CD4+ T cells specific for the lytic cycle protein BHRF1 had been reported to recognize a similarly high percentage of cells in LCL cultures as the glycoprotein-specific T helper cells described here (Landais et al., 2004). However, pathway(s) of antigen presentation had not been investigated in that study, and a similar transfer of antigen by viral particles appears unlikely because BHRF1 is not a constituent of virions (Johannsen et al., 2004).

Virus uncoating renders newly infected B cells vulnerable to T helper cell recognition

The observation that mainly B cells, whether primary or EBV-infected, present virion proteins most efficiently implicated a protective role of such T cells in controlling spread of infection. Importantly, glycoprotein specific T cells recognized virus-pulsed target cells as early as twelve hours after incubation. This is before the EBV genome has circularized and before EBNA2, the protein essential for primary B lymphocyte growth transformation, is expressed (Alfieri et al., 1991). This implied that glycoprotein-specific T cells can recognize target cells that are internalizing virus.

EBV infection of B lymphocytes is initiated by BLLF1 adsorption to CD21 on the B cell plasma membrane, followed by BXLF2 mediated envelope fusion with the cell membrane and nucleocapsid exocytosis into the cytoplasm (Tanner et al., 1987; Carel et al., 1990). During this process of virus uncoating, proteins of the viral envelope are probably retained at the cell membrane, as indicated by the higher percentage of cells positive for BLLF1 than for BZLF1 in immunofluorescence experiments (Lee et al., 1993). Since cell membrane proteins efficiently access the MHC class II processing and loading compartment, T helper cells specific for envelope antigens might be able to detect EBV-infected cells before viral latency is established.

This receptor-mediated virion uptake and subsequent presentation of glycoprotein-derived peptides on MHC class II was extremely efficient. BALF4- and BLLF1-specific T cells were able to recognize miniLCL cells incubated with less than one EBV geq per cell. Thus, virion

proteins retained at the cell surface during virus uncoating render newly EBV-infected B cells vulnerable to immune attack by CD4+ T cells specific for virion proteins. This mechanism of immune surveillance may not be limited to EBV, but may also apply to other coated viruses infecting MHC class II-positive target cells.

Glycoprotein-specific T helper cells might play a role in preventing virus spread during persistent infection and in the control of EBV-positive tumors

The glycoprotein specific CD4+ T cells described here were of Th1 type and were able to inhibit the proliferation of LCL *in vitro*. Importantly, these T cells were also able to prevent the outgrowth of a LCL from primary B cells infected with EBV *in vitro*, implicating a role of such T cells in diminishing the pool of EBV-infected B cells *in vivo*. Consistent with this, subsidence of T cell surveillance following immunosuppression is often associated with increased viral shedding in the throat, and higher numbers of EBV-infected B cells in the peripheral blood (Babcock et al., 1999; Baldanti et al., 2000). In BMT and SOT recipients, PTLD incidence correlates with the severity of immunosuppression. In PTLD as well as in BL, lytic replication has been observed at the site of tumor development (Montone et al., 1996; Tao et al., 1998). Thus, glycoprotein-specific Th cells may not only aid in controlling persistent infection, but may also play a role in the immune control of EBV-associated malignancies.

As compared to blood or LCL cultures, diffusion is impeded in solid tumors leading to the sequestration and accumulation of viral progeny within tumors. Because the transformed cells in PTLD are considered as *in vivo* correlate of LCL, these cells are expected to take up released virions with similar efficiency. Also, BL cells express the principal EBV receptor CD21 and have retained the ability to process exogenous antigen through the MHCII pathway for presentation to CD4+ T cells (Khanna et al., 1997). Because antigen presentation on MHCI is severely impaired in BL cells (Staege et al., 2002), CD4+ T cells may play an important role in the immune response against this tumor.

In BL cells, the only viral latency protein expressed *in vivo* is EBNA1. Although EBNA1-specific Th1 type CD4+ T cells have been shown to kill type III BL cells (Paludan et al., 2002), it is not known whether type I BL cells are also recognized by these T cells. Furthermore, recognition of endogenous antigen by EBNA1-specific CD4+ T cell clones has been observed in some (Munz et al., 2000; Voo et al., 2002), but not in other studies (Khanna et al., 1997; Leen et al., 2001) and is still a matter of contention (Long, et al, 2004; Mautner,

et al, 2004). Therefore, the antitumoral efficacy of EBNA1-specific T helper cells is still unresolved.

This work provided first evidence for the recognition of type I BL cells by glycoprotein-specific CD4+ T cells in a MHCII-restricted manner. Therefore, glycoprotein-specific T helper cells may play a critical role in the immune response against this tumor. Evidences in support of this concept had been obtained in preclinical models. Injection with purified BLLF1 or infection with vaccinia virus expressing BLLF1 had been shown to protect cottontop tamarins against a lethal, lymphomagenic EBV challenge (Morgan et al., 1988; Morgan et al., 1997). Although BLLF1 had been identified as the dominant target of the neutralizing antibody response in humans (Thorley-Lawson et al., 1982), protective immunity in these and other studies did not always correlate with the presence of virus-neutralizing antibodies, inferring a role for BLLF1-specific cell-mediated responses in disease protection (Wilson et al., 1996). In fact, more recent studies in our group demonstrated that glycoprotein-specific CD4+ T helper cells can inhibit the proliferation of BL cells *in vitro*. T helper cell recognition of newly EBV-infected B cells following receptor-mediated cell adhesion, penetration and uncoating as described in the present study may provide a mechanistic explanation for these findings. Thus, the targets of the EBV-specific T helper cell response may not be limited to a small set of latent cycle proteins expressed in these tumors, but may also include antigens transferred by virions.

Upon antigen recognition, glycoprotein-specific T cells lysed target cells by secreting perforin and granzyme B

Cytotoxic CD4+ T cells specific for the viral protein EBNA1 have been reported to kill target cells by the Fas-FasL pathway (Nikiforow et al., 2003). The glycoprotein-specific T cells described here lysed their targets by using the granule exocytosis pathway. Previous studies had already suggested that this pathway is used by T helper cells specific for herpesviruses, including CMV and EBV (Khanolkar et al., 2001; van Leeuwen et al., 2004). More recent studies, however, implied that CMV-specific but not EBV-specific CD4+ T cells were able to use this pathway (Amyes et al., 2003; Appay, 2004). The detection of perforin and granzyme B-mediated killing of target cells by glycoprotein-specific CD4+ T cells in this work not only provided experimental evidence for the existence of EBV-specific cytolytic CD4+ T cells, but also added to the importance of CD4+ T cells as direct effectors in the control of EBV infection.

Non-structural proteins were presented on MHCII by different pathways

The finding that structural antigens of EBV were efficiently presented on MHCII following receptor-mediated virion uptake may explain why most LCL-stimulated T cell lines targeted structural proteins. Recent experiments of our group with T cell clones specific for additional virion proteins including BNRF1 confirmed that recognition of LCLs by these T cells is dependent on antigen transfer by virions (Mautner et al., unpublished results). The antigens recognized by LCL-stimulated T cell lines also included non-structural lytic cycle proteins of EBV. How these antigens were presented on MHCII is not known. In the case of BCRF1, the viral homologue of IL-10, efficient presentation is probably mediated by binding of BCRF1 to the IL-10 receptor, followed by the internalization and processing of the complex in the endosomal/lysosomal compartment. BALF2, a single-stranded DNA binding protein, and BMRF1, a DNA polymerase processivity factor, have also been detected in virions (Johanssen, et al, 2004), suggesting that the presentation of these antigens is also receptor mediated. However, preliminary experiments suggest that LCL-recognition by T cells specific for these antigens is not dependent on virion transfer. Whether these antigens were presented endogenously by cells that have entered the lytic cycle or exogenously by bystander B cells that took up released protein from dead cells is currently not known. The elucidation of the presentation pathway(s) will be important to understand why T cells specific for these antigens recognize LCL, while T cells specific for the transactivator BZLF1 fail to do so.

Whether lytically infected cells are recognized by glycoprotein-specific T helper cells is not known

EBV lytic cycle proteins are grouped into immediate early, early and late antigens depending on the time of appearance during lytic replication. Once late proteins start appearing the infected cells go into lysis (Kieff and Rickinson, 2001). Therefore, immediate early and early lytic cycle proteins should have access to the antigen presentation machinery.

This concept is supported by recent results on the CTL response to lytic cycle proteins of EBV (Pudney et al., 2005). In this work the authors compared the efficiency of recognition of LCL cells by CD8+ T cells specific for immediate early, early, and late antigens. Although the T cells responded similarly to peptide-pulsed target cells, the late antigen-specific CD8+ T cells responded only very poorly against LCL cells that had become permissive for lytic replication. These findings suggested that at the time when late antigens are expressed, the antigen presentation function of EBV-infected cells is impaired.

Whether CD4+ T helper cells specific for late antigens of EBV are able to recognize cells that entered the lytic cycle, and whether this recognition limits virus production is not known. The described reduction in MHCI and MHCII expression on lytically infected cells and the secretion of gp42, a glycoprotein interfering with antigen recognition by CD4+ Th cells (Keating et al., 2002; Ressing et al., 2003), suggest that CD4+ T cell recognition of cells in the lytic cycle is also impaired. Experimental access to this question is difficult, because antigen transfer following sporadic lytic replication in LCL leads to T cell recognition of latently infected bystander B cells, concealing any recognition of lytically infected cells. With the development of recombinant DNA technologies that allow to genetically manipulate EBV (Delecluse and Hammerschmidt, 2000), viral mutants can be created that may facilitate to address this important immunological question. For instance, LCLs established with a viral mutant that is unable to enter the lytic cycle spontaneously, but in which lytic replication can be induced, may be instrumental for clarifying this issue.

Approximately 1% of CD4+ T cells in the peripheral blood of healthy virus carriers are EBV-specific

The average precursor frequency of EBV-specific T cells in the peripheral blood of healthy virus carriers was found here to range between 0.5% and 1%. By using APCs pulsed with lysate from EBV-infected cells and measuring intracellular IFN-γ response in CD4+ T cells from peripheral blood, Amyes et al. had found that the frequency of EBV-specific CD4+ T cells was between undetectable to 1.26% (mean 0.34%) (Amyes et al., 2003). Although the results from both studies were similar, they probably underestimated the true precursor frequencies for different reasons.

First and foremost, composition and concentration of EBV proteins in the commercial lysate used by Amyes et al. had been poorly defined. Therefore, it remains unclear whether sufficient amounts of all EBV proteins were present in the lysate for subsequent detection by antigen-specific T helper cells. In our own experiments, DCs mixed for over 24 hours with allogeneic LCL cells failed to activate structural antigen-specific CD4+ T cells, indicating that the amount of protein released from dying cells was not sufficient to activate these T cells. Even though the percentage of lytically-infected cells in the commercial cell lysate was presumably much higher than the low percentage of LCL cells that had spontaneously entered the lytic cycle in our experiment, the concentration of late cycle proteins in cell lysates might be limiting, because structural proteins are rapidly incorporated into virions and released in the cell culture supernatant (Kieff and Rickinson, 2001).

Second, as the authors showed, the lysate contained distinct proteins in huge amounts (e.g. BMRF1, BZLF1 and LMP1). These amounts clearly exceed what is expected in the physiological setting and may thus lead to the activation of low-avidity and potentially cross-reactive T cells.

Third, although the same cellular source of antigen was used, the activation of alloreactive, autoreactive and cross-reactive T cells by the commercial lysate is expected to differ from donor to donor and therefore may add to the high variability in precursor frequencies.

Fourth, the contribution of FCS in the commercial lysate and consequently the activation of FCS-reactive T cells to the measurements is not clear. False positive FCS-, autoantigen-, allo-, and cross-reactive responses are excluded when LCLs and miniLCLs are used to measure the frequency of EBV-specific T helper cells in the peripheral blood. However, only T cells specific for antigens presented on LCLs will become activated with this approach which probably includes all structural and some non-structural proteins of EBV, but excludes most of the latency proteins, BZLF1, and probably the majority of early lytic cycle proteins.

Fifth, because both approaches used IFN-γ secretion as read-out, EBV-specific Th2 and Treg cells which had been detected in healthy virus carriers (Voo et al., 2005; Sun et al., 2002) were omitted. If the non-Th1/Th2 type of T cells described in this work also recognized EBV antigens, the average percentage of EBV-specific T helper cells in peripheral blood of healthy virus carriers is probably even more than 1%. This is probably still much lower than the EBV-specific CD8+ T cell response where single T cell specificities may already account several percent of the peripheral CD8+ T cell population (Tan et al., 1999). This imbalance in precursor frequencies perhaps contributes to the observed dominance of CD8+ T cells over CD4+ T cells in LCL-stimulated mixed T cell cultures.

CD4+ and CD8+ T cells may synergize in preventing virus spread during the persistent phase of EBV infection

The level of antigen expression and lytic replication vary during the lifetime of an EBV-infected individual. Following primary infection, infected B cells express all latency proteins of EBV, and some infected cells enter the lytic cycle and produce infectious virus (Rickinson and Kieff, 2001).

Immune control of these cells is believed to be mediated by CD8+ T cells. Probably due to immune pressure, infected cells switch off expression of most if not all viral proteins which allows the virus to hibernate in cells without being detected by CD8+ T cells. This is a state of compromise in which the virus-infected cell does not proliferate, and the immune system fails

to “see” the virus. Virus-positive individuals, however, do shed virus throughout life owing to occasional lytic replication. This is important for virus propagation and probably also for virus persistence. Released viral particles could potentially infect new B cells and drive them into proliferation. CD8+ T cells can sense this expansion of EBV infection only once the infected cells started expressing latent and lytic cycle antigens.

The finding that viral structural proteins dominate the CD4+ T cell response suggested that CD4+ T cells play an important role not only in the recognition of infected cells, but also in controlling the spreading of infection by eliminating B cells into which viral particles have recently gained entry. Thus, CD4+ T cells may contribute to the virus-host balance by limiting the number of newly infected cells and thereby reducing the reservoir from which EBV may reactivate.

This balance is disturbed during states of immunodeficiency where the virus produced by a basal rate of lytic replication is no more controlled, resulting in unrestrained viral replication, re-infection of B cells and increased viral load. Adoptively-transferred T cells restore the virus-host balance, presumably by interfering with the virus’ life cycle at more than one stage. Latent antigen-specific CD8+ T cells that are known to expand upon LCL stimulation are able to eradicate proliferating type III latency cells. CD8+ T cells specific for immediate early and early lytic cycle antigens prevent virus production by cells that entered the lytic cycle. CD4+ T cells specific for virion proteins which also expand by LCL-stimulation eliminate newly infected B cells and thereby diminish the viral reservoir.

Virion antigens represent promising targets for MHCII-restricted immunotherapy not only against PTLD but also against other virus-associated diseases

Adoptive T cell therapy has been successfully applied to prevent or cure PTLD in BMT patients. Overall, the rates of PTLD in allogeneic BMT are less than 1% but can rise dramatically to 12% to 24% among patients in whom donor T cells are depleted from the marrow graft. In SOT patients, PTLD incidence ranges between 1% to 8%, depending on the organ transplanted and the intensity of immunosuppression (Rickinson and Kieff, 2001; Gottschalk et al., 2005).

Due to the often rapid progression of PTLD, EBV-specific T cell lines have to be prepared in advance for patients at risk. Given the high number of transplantations performed, and the low percentage of transplant patients who have access to this adoptive T cell therapy, rapid protocols for the generation of EBV-specific T cells after PTLD manifestation are urgently needed. The time required to establish EBV-specific T cell lines could be dramatically

reduced if recently developed methods for the isolation of rare antigen-specific T cells from PBMC were combined with methods of antigen-independent T cell expansion *in vitro*. Such methods of T cell isolation, however, are dependent on the knowledge of the relevant T cell antigens and epitopes.

The identification of a previously unrecognized point in the life cycle of EBV that is subject to immune control by CD4+ T cells could have important clinical implications. Induction of lytic replication in EBV-infected B cells followed by antiviral therapy has been proposed as a possible strategy for eliminating EBV-associated tumors (Daibata et al., 2005). The combination of virion-specific CD4+ T cell therapy with the induction of lytic replication may even have synergistic effects. Induction of lytic replication would eventually lead to the destruction of the virus-infected cell, while virion-specific T cells would prevent new cycles of infection. Increased virus production inside of solid tumors may also render bystander tumor cells susceptible to T cell attack.

The identification of virion proteins as the prime targets of the EBV-specific CD4+ T cell response may facilitate the rapid generation of EBV-specific T cell lines for PTLD treatment, and complement the well characterized CD8+ T cell response to EBV. In addition, the identification of virus uncoating as a vulnerable step for the T helper cell control may fuel efforts to target additional EBV-associated diseases.

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C. Appendix

C.1Acknowledgements

When I left Nepal for Germany in February 2002, my parents were not the happiest parents on earth. I was leaving for three years (and it has been more than that!). A grown up son, in the Eastern Hindu society ought to stay by aging parents as a walking stick as they walk through the dusk of life. I left them, and they hardly complained. My dearest father and mother, I am proud to have you as my parents. All my thanks for letting me go my way. My brother Vinod, well I would do everything to have years with you (time willing). Thank you for being there, I love you.

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Dinesh.

C.2 Curriculum vitae

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