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Modulation of immunity by Epstein-Barr virus

von

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Erklärung

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- Herrn Prof. Dr. Wolfgang Hammerschmidt betreut
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July 16th-19th, 2014 Brisbane, Australia	16th Symposium on EBV and associated diseases Talk: "Immunomodulatory functions of LMP2A"
June 17th-18th, 2013 Amsterdam, The Netherlands	European EBV Meeting Talk: "Immunomodulatory functions of LMP1 and LMP2A"
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May 3rd-4th, 2012 Berlin, Germany	DFG SFB-TR36 Symposium "Adoptive T cell therapy" Poster: "Role of NKG2D ligands in the control of EBV infection"

Summary

Epstein-Barr virus is a herpesvirus that efficiently infects B cells. During infection, EBV takes advantage of the normal B cell differentiation processes to establish a persistent infection within the host. The present study investigates how EBV infection modifies and controls the immunogenicity of the infected B cells in order to minimize attack by immune cells.

To understand the effects of EBV infection on B cells, the immunophenotype of EBV-infected B cells was analyzed through a detailed investigation of the modulation of the surface expression of molecules serving as agonistic or antagonistic ligands for important receptors on antiviral immune cells. Expression of these ligands was analyzed during the early onset of infection and on established lymphoblastoid cell lines (LCLs), which stably carry the virus. To identify the specific aspects modulated by infection with EBV, these findings were compared to those observed during normal B cell activation in the absence of EBV.

LMP2A is one of the few EBV antigens that can be expressed in three out of the four latency types that are part of the life cycle of EBV, and is expressed in most types of EBV-associated cancer. Given its frequent expression in EBV-infected B cells, LMP2A was identified as a good candidate for a possible mediator of immunoevasive functions during latency of EBV. LMP2A immunomodulatory functions were especially investigated under the light of CD8+ T cell recognition of infected cells. LMP2A was shown to interfere with the recognition of EBV-infected cells by CD8+ T cells of different EBV antigen specificities. LMP2A variably modulated the expression of other viral antigens and moderately affected the surface expression of MHC class I molecules. Moreover, LMP2A altered the surface expression of several immunomodulatory molecules. In particular, LMP2A downregulated the surface expression of the ligands for the co-activatory receptor NKG2D, which I showed to be involved in the recognition of infected cells by EBV-specific CD8+ T cells.

In addition, the immune response to EBV by γδ T cells was investigated. To this end, reactivity to EBV-infected cells of both short-term polyclonal γδ T cell lines and single γδ T cell clones was investigated. This led to the identification of a subpopulation of these cells, presenting a TCR with a δ1+ chain, showing an EBV-specific reactivity.

Summary

One of the EBV-specific δ1+ T cell clones showed an HLA-A2-restricted recognition of EBV-infected cells. Interestingly, while LMP2A hampered recognition of infected cells by EBV-specific CD8+ T cells, it increased recognition of infected cells by γδ T cells. These data suggest that the evolutionary pressure on EBV by the immune system specifically prompted the virus to develop immunoevasive strategies against detection by CD8+ T cells in the latent phase. Taken together, I have discovered the first example of an EBV latent protein that interferes with T cell recognition, have identified γδ T cells that are specifically active against EBV-infected cells, and have shown that complementary responses by CD8+ and γδ T cells shape EBV immunoevasion.

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1 Introduction

1.1 The immune system

Every day of our lives, we are in contact with microorganisms. With some of them, such as the bacterial flora in the gut or bacteria on the skin, we have established beneficial interactions. Others instead can be dangerous to humans and cause a variety of diseases. Four broad categories of pathogenic microorganisms are recognized: viruses, bacteria, fungi and parasites. In order to prevent the development of diseases, higher organisms are equipped with a complex network of tissues, organs, cells and molecules able to confront these pathogens. Tissues, such as the skin and the mucosae, protect the internal environment from the external environment. Organs, such as the lymphoid organs, generate cells responsible for the counteraction against pathogens, called immune response, and are the regions where this reaction is initiated. Immune cells, called lymphocytes, and molecules taking part in the immune response are the direct mediators of the immune response and divided into two groups, innate and adaptive immunity, according to their mechanism of action and the speed of reactivity to a pathogen.

Innate immunity serves as a first defense line. It occurs rapidly, but it is either unspecific or targets only a limited set of conserved microbial structures. Adaptive immunity takes more time for its formation and activation, but it is capable of eliminating infections more efficiently because of its high specificity and versatility. Clonal expansion ensures that immune responses with exactly the specificity required to combat the pathogens at hand are selected and amplified from a large pool of available specificities. Adaptive immunity can also lead to the development of a lifelong protective immunity, called immunological memory. This property of the adaptive immune response is of extreme importance because it allows a faster and stronger reactivity in case a pathogen is encountered again at later times. To mount a proper immune reaction against pathogens, both of these compartments can efficiently distinguish between self and nonself, but they differ in how they do it (Murphy 2011).

1.2 Innate immunity

Most of the microorganisms encountered daily are promptly detected and destroyed by innate immunity. This line of defense relies on preexisting mechanisms common to all individuals. The first barriers against infections are the epithelia and the mucosal epithelia. On top of conferring a physical barrier between the internal and the external environment, these tissues present numerous mechanical, chemical and microbiological properties to protect from infections. For example, epithelial cells are joined together by tight junctions and mucosal epithelia are often coated in mucus, preventing the adhesion of microorganisms to the surface. Epithelia produce antimicrobial peptides, which disrupt the cell membrane of microbes, and on some surfaces, enzymes attacking specific features of the bacterial cell wall are released. In addition, the commensal microbial flora inhabiting some epithelia competes with pathogens for nutrients and for attachment sites on epithelial cells, providing another layer of defense.

Once pathogens pass through these barriers, they encounter a molecular mechanism of defense, called the complement system, and the first cell defense line, the phagocytes. Complement is a system of plasma proteins and activation of these proteins leads to the generation of protein products binding covalently to the surface of the pathogen that can (i) directly induce the lysis of certain pathogens or (ii) be recognized by phagocytic cells that eliminate the pathogen. Phagocytic cells, such as granulocytes, macrophages and dendritic cells (DCs), lie beneath the epithelial barriers and monitor these tissues for invading microorganisms. Phagocytes recognize, engulf and destroy pathogens coated with complement proteins through the binding of the activated complement components to specific receptors present on the phagocytes' surface. Receptors for the complement proteins or for microbial molecular structures are called pattern recognition receptors (PRRs). Activation of phagocytes through these receptors induces the secretion of cytokines (chemokines, interleukins and TNF family molecules), which (i) recruit other immune cells to the site of infection, (ii) deliver modulatory signals to other innate and adaptive immune cells, and (iii) mobilize antigen-presenting cells (APCs) that induce the adaptive immune response. Other cells associated to innate immunity are natural killer (NK) cells. These cells are cytotoxic lymphocytes involved in non-specific viral and tumor immune surveillance. NK cells distinguish between healthy and infected or transformed cells by recognizing changes in the expression of cell-surface molecules on these cells (Murphy 2011).

1.3 Adaptive immunity

B and T lymphocytes are the major players in the adaptive immune response. These cells present highly specific antigen receptors generally referred to as B-cell receptor (BCR) and T-cell receptor (TCR). BCRs and TCRs recognize a small part (called epitope) of the molecular structure of a pathogen's complex macromolecule (usually referred to as antigen). An adaptive immune response is initiated when APCs take up antigens and present them to B and T cells. After this specific encounter, B cells differentiate to plasma cells, highly proliferating cells that produce a soluble version of the BCR, the antibodies. These molecules bind to the pathogens' surface, supporting the phagocytosis of the pathogens. After antigen encounter, T cells support clearance of the infection by coordinating the immune response of other cells or by directly killing infected cells. Characteristics of the adaptive immunity common to both B and T cells are: (i) production of highly specific lymphocyte antigen receptors by somatic DNA recombination, (ii) presentation on the cell surface of these highly specific receptors (iii) clonal expansion upon activation, (iv) establishment of immunological memory (Murphy 2011).

1.4 Antigen presentation to T cells

T cells can only recognize antigens when these are presented by highly polymorphic surface glycoproteins encoded by genes in the major histocompatibility complex (MHC). In the human system, the MHC proteins are also called human leukocyte antigens (HLAs). Surface MHC molecules associate to small peptides derived by degradation of pathogens' macromolecules. MHC molecules are highly unstable when peptides are not bound, and T cell recognition of antigenic peptides is possible only when these are loaded on a MHC molecule because the TCR binds and interacts directly with both the peptide and the MHC molecule. There are two types of MHC molecules.

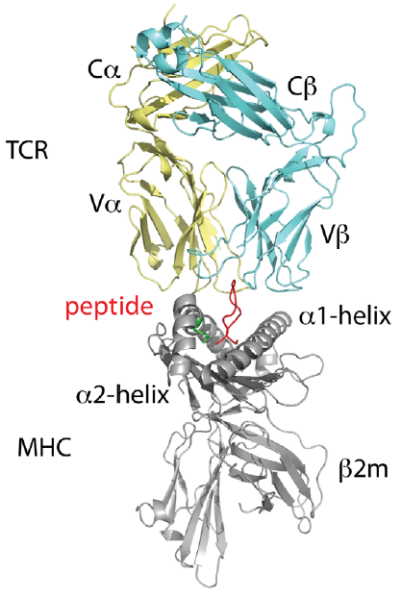


Figure 1.1. Cartoon showing the molecular structure of an αβ TCR and a peptide:MHC-I complex, and the interaction between them. The TCR molecule interacts with both the peptide and the MHC molecule. Thereby, TCR specificity is determined by both the antigenic peptide and the MHC molecule. Figure modified after (Reboul et al. 2012)

MHC class I (MHC-I) are expressed virtually by all cells and present peptides that originate from antigens located in the cytosol (both self and nonself antigens). MHC class II (MHC-II) expression is restricted to thymic epithelial cells and professional APCs and presented peptides originate in the vesicular endosomal system.

MHC-I molecules consist of two polypeptide chains: a polymorphic α chain (heavy chain, 44 kDa) encoded by the genes in the MHC, and β_2-microglobulin (β_2m, light chain, 12 kDa) a smaller nonpolymorphic chain not encoded by the MHC. The α chain consists of three extracellular α domains (α_1-α_3), a transmembrane domain and a cytosolic tail. The β_2m is noncovalently associated to the α_3 domain of the α chain. The MHC locus is located on chromosome 6 and contains more than 200 genes, many of them involved in immune response, while the β_2m gene locates to chromosome 15. The MHC-I α chains are encoded by HLA-A, -B and –C, highly polymorphic genes, referred to as "classical" MHC-class I molecules, and by HLA-E, -F and –G genes, which are less polymorphic and constitute the "non-classical" MHC-class I molecules.

The α_1 and the α_2 domains form the peptide-binding groove, where the peptide is presented for T cell recognition. MHC-I molecules usually bind 8-10 amino acids long peptides. Major differences between MHC-I molecules are found in the peptide-binding groove. This influences the peptides that the MHC-I molecule will bind, thus influencing the recognition by T cells.

MHC-II molecules consist of two chains, α and β, both anchoring to the plasma membrane. They are approximately equal in size (30 kDa) and each chain contains two extracellular domains (α_1/α_2 and β_1/β_2), a transmembrane domain and a cytosolic tail. The α_1 and β_1 domains of the two chains form the groove accommodating the peptide. Genes coding for MHC-class II molecules in humans are called HLA-DR, -DP, and –DQ. Each MHC-II molecule comprises of a α and β chain, which are encoded by separate genes in the HLA-II region that are designated as A and B, respectively (e.g. HLA-DQA1, HLA-DQB1). The overall structure of the MHC-II and MHC-I molecules is similar, but the binding groove is not capped at the sides in the MHC-II molecules, which permits them the binding of longer peptides, 13-25 amino acids long (Germain 1994).

Before MHC class I and II molecules reach the cell surface, they are loaded with peptides coming from the degradation of pathogens' antigens or from self antigens. Peptides loaded on MHC-I molecules derive from cytosolic degradation performed by

the proteasome, a multicatalytic protease, and cytosolic proteases. The peptides are transported into the endoplasmatic reticulum (ER) by the transporter associated with antigen processing (TAP) (Del Val et al. 2011). Here, they are further processed and trimmed by ER aminopeptidases (ERAPs) to produce peptides of the right length to fit into the binding groove of the MHC-I molecules (Serwold et al. 2002). MHC-I molecules are translocated during their synthesis into the ER, where they are joined with the β_2 subunit and loaded with the peptide. Peptide binding occurs before the MHC-I molecule completes its folding and is coordinated by the peptide-loading complex. Highly hydrophobic peptides or peptides generated in the ER or in the Golgi compartment reach the MHC-I molecules in a TAP-independent manner (Lautscham et al. 2003). Once the peptide:MHC-I complex is formed, it is transported to the cell surface, ready for interaction with CD8+ T cells.

MHC class II molecules bind peptides generated by acidic endosomal compartments. Upon synthesis in the ER and before acquiring their ligands, MHC-II molecules are associated with the invariant chain (Ii), which binds the peptide groove, preventing the binding of peptides. Once MHC-II reaches the endosomal compartment, proteases here located release Ii, allowing the loading and binding of other peptides, a reaction catalyzed by the chaperone-like molecule HLA-DM. Afterwards, the peptide:MHC-II complex is transported to the cell surface for interaction with CD4+ T cells (Murphy 2011).

Another class of antigen presenting molecules belongs to the CD1 gene group. CD1 molecules, called CD1a to CD1e, are specialized in binding glycolipids, and are expressed by DCs, monocytes and some thymocytes. These molecules are divided into two groups. Group 1, which is lacking in the mouse, contains the human molecules CD1a, CD1b, CD1c; Group 2 consists of CD1d, which is present in mice and humans. The molecule CD1e functions as a lipid chaperone. Since CD1 molecules can present cellular and microbial glycolipids, their probable task is to alert T cells to a pathogenically altered lipid repertoire, just as MHC molecules inform T cells about abnormal alterations in the protein repertoire of a cell. T cells recognizing Group 1 CD1 molecules do express neither CD4 nor CD8, and most of these T cells have a diverse repertoire of $\alpha\beta$ TCRs. The majority of CD1d-restricted T cells are less diverse, express a limited set of TCRα chains, and express NK receptors. For this reason they have been called invariant NKT (iNKT) cells. Interestingly, CD1 molecules have been

found to be ligands for populations from both αβ and γδ T cells (Murphy 2011; Adams 2014).

1.5 NK cells and NK coreceptors

Natural killer (NK) cells represent 5-15% of circulating lymphocytes. They are phenotypically characterized by lack of CD3, a component of the T cell receptor complex that is present in all T cells, and by the presence of CD56 (NCAM) (Cooper et al. 2001). They differ from T and B cells in that they do not present a receptor with somatic DNA rearrangement, but their function is coordinated by a combination of activating and inhibitory receptors specific for conserved molecules. These receptors

	Inhibitory Receptor	Ligand
KIR family	2DL1	HLA-C*02, 04, 05, 06
	2DL2/3	HLA-C*01, 03, 07, 08
	2DL5	Unknown
	3DL1	HLA-A/B with Bw4 motif
	3DL2	HLA-A*03, 11
KLR family	CD94:NKG2A	HLA-E
	NKR-P1A	LLT1
LIR/ILT family	LILRB1/ILT2/LIR1	HLA-A/B/C
	Activatory Receptor	**Ligand**
NCR family	NKp30	BAT-3
	NKp44	Viral haemagglutinin
	NKp46	Viral haemagglutinin
KLR family	CD94:NKG2C	HLA-E
	CD94:NKG2E	HLA-E
	NKG2D	MICA/B, ULBPs
KIR family	2DS1	HLA-C*02, 04, 05, 06
	2DS2	HLA-C*01, 03, 07, 08
	3DS1	HLA-A/B with Bw4 motif
	2DS3	Unknown
	2DS4	HLA-C*04
	2DS5	Unknown
	2DL4	HLA-G
Others	CD244 (2B4)	CD48
	CD16	IgG

Table 1.1. Inhibitory and activatory receptors on NK cells and their ligands. Modified after (Cheent & Khakoo 2009).

allow the recognition of “altered” or “missing” self. Upon activation, NK cells mediate direct cytotoxicity of the targeted cell. The balance between inhibitory and activatory receptors plays a critical role in governing the functions of this cell type.

Inhibitory NK receptors allow the cell to detect “missing self”. Killer cell immunoglobulin-like receptors (KIRs), killer C-type lectin-like receptors (KLRs) and the receptor of the leucocyte immunoglobulin-like receptor/immunoglobulin-like transcript family (LIR/ILT) form the inhibitory receptor repertoire of NK cells. The majority of these receptors detect changes in the surface levels of MHC-I molecules. Malignant or virally infected cells often downregulate the surface expression of MHC-I. The decreased expression of MHC-I releases the inhibitory signal induced by the inhibitory receptors, and permits activation of the NK cell.

Activating receptors mediate the activation of NK cells. Some of them recognize MHC-I molecules, a viral component (viral haemagglutinin), or the up-regulation of immunomodulatory molecules. Activating receptors include molecules from different families, KIRs, KLRs, and natural cytotoxicity receptors (NCRs), and unrelated receptors, such as the SLAM family member CD244 (2B4) or the antibody-binding Fc receptor CD16 (Cheent & Khakoo 2009; Murphy 2011).

Interestingly, NK cells in the same individual express variable numbers and different combinations of KIRs (Valiante et al. 1997). The distribution of KIRs in the NK population appears to be stochastically determined, and maintenance of an NK cell’s KIR repertoire appears to be guided by the methylation state of the KIR gene loci (Chan et al. 2003).

1.6 T lymphocytes

Immune cells originate in the bone marrow from hematopoietic stem cells. To complete their development, the precursors of T cells migrate from the bone marrow to the thymus, where they acquire their antigen specificity by recombining their T-cell receptor genes. This results in two broad T cell subsets, αβ T cells and γδ T cells, distinguished by the type of TCR chain expressed (αβ or γδ TCR). Each type of TCR is the result of somatic rearrangement of the genes encoding for the two constituent chains, occurring during T cell development in the thymic cortex (Murphy 2011).

1.6.1 αβ T cells

The majority of circulating T cells express an αβ TCR that has the potential to recognize a peptide:MHC complex. Both the α and the β chains of the TCR contain a variable amino-terminal region and a constant region, largely encoded by a V and a C gene segment, respectively. In between the larger V and C gene segments, smaller gene segments and non-templated nucleotides are inserted during TCR recombination. The additional segments are a J (joining) segment for the α chain, and a J and D (diversity) segment for the β chain. The TCR chain loci contain sets of several alternative gene segments, from which one is chosen during TCR recombination. The sequences of alternative V and J segments show large differences, and thus the choice of segments has a large impact on the specificity of the resulting TCR. The TCRα locus consists of 70-80 Vα gene segments, 61 Jα gene segments and a single Cα gene. The TCRβ locus consists of 52 Vβ gene segments and two separate clusters, each containing a single Dβ gene segment together with 6 or 7 Jβ gene segments and a single Cβ gene. The V gene segments encode for the complementary-determining region (CDR) 1 and 2 of each chain, while the junctions between the V(D)J form the CDR3. The highly variable CDR3 domains of the two chains form the center of the antigen-binding site of the T cell receptor (Figure 1.2).

Once the TCR is engaged in the recognition of the peptide-MHC complex, the complex

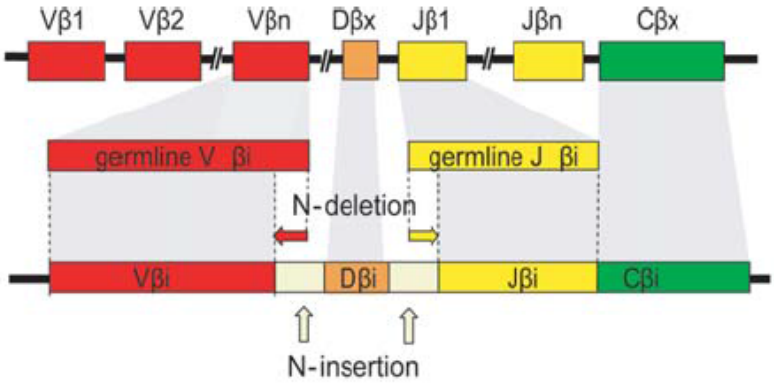


Figure 1.2. Cartoon showing V(D)J gene recombination. During T cell development, gene rearrangement leads to the combination of randomly selected V, (D), and J segments. The junctions between the different segments can be further modified by deletion and insertion of nucleotides, resulting in the creation of the hypervariable region CDR3, which determines the antigen-specific recognition of the T cells. Figure modified after (Bonarius et al. 2006)

of the cluster of differentiation 3 (CD3) mediates transmission of the signal. The CD3 complex is closely associated to the TCR and is formed of one CD3γ, one CD3δ and two CD3ε chains and the homodimeric ζ chain. These chains carry immune-receptor tyrosine-based activation motifs (ITAMs). Each ITAM contains two tyrosine residues that, once phosphorylated, function as docking sites for other signaling molecules leading as a final result to T cell activation. The CD3 complex does not participate in the recognition and in the binding of the antigen.

After maturation, naive T cells leave the thymus, enter the blood and lymphatic streams and regularly pass secondary lymphoid organs such as lymph nodes or the spleen. In lymphoid organs, they can make numerous contacts with DCs to screen the peptide:MHC complexes presented by these cells. By sampling the DCs, T cells have a high probability of encountering antigens derived from pathogens present in whatever location in the body. The activatory signals induced by the engagement of the TCR with the peptide:MHC complex is referred to as signal 1. Full activation of naive T cells needs additional stimulatory signals mediated by the interaction of coreceptors with their ligand counterparts on the APCs (signal 2) and by cytokines controlling the differentiation of the T cells (signal 3). Activation, proliferation (clonal expansion) and differentiation of a naive T cell after antigen recognition is called priming.

Mature αβ T cells can be further distinguished by the presence of either the CD8 or the CD4 coreceptors. These molecules recognize and bind the nonpolymorphic domains of the MHC-I (α_3 domain) or of the MHC-II (β_2 domain) molecules respectively, which increases the affinity of antigen recognition by T cell. Therefore, CD8+ T cells recognize antigens coming from the cytoplasm and presented on MHC-I molecules, while CD4+ T cells recognize antigens coming from the cellular vesicles and presented by MHC-II. Most CD8+ T cells differentiate to cytotoxic lymphocytes (CTLs), which kill their target cells. In contrast to CD8+ T cells, CD4+ T cells can differentiate into several functional classes: T_H1, T_H2, T_H17, regulatory T cells (T_{reg}) and T follicular helper cell (T_{FH}). Differentiation into the different subsets of CD4+ T cells is mediated by different cytokines produced by the priming APCs. T_H1 cells, for which the signature cytokine is IFN-γ, are important for activation of macrophages to destroy phagocyted bacteria, for induction of cytotoxic T cell proliferation, and for inducing B cells to produce IgG or IgA antibodies. Some T_H1 cells can directly kill virus-infected cells in the manner of CD8+ T cells, as shown in the EBV system (Adhikary et al.

2006). T_H2 cells, producing IL-4, IL-5, IL-10 and IL-13, promote B cells to produce antibodies with isotypes including IgE, and direct the immune response toward an anti-parasitic or allergic type of immunity. T_H17 release IL-17, IL-21 and IL-22 and recruit acute inflammatory cells to the site of infection. T_{reg} produce inhibitory molecules such as IL-10 and TGF-β, suppressing immune function. These cells are important to prevent excessive reactions and to maintain tolerance to self-antigens. T_{FH} cells produce IL-4 and IL-21 and are specialized in promoting T cell help to B cells. After priming, the naive T cells develop to effector and memory T cells of the same specificity. At a subsequent encounter with the same antigen, these cells can react and expand rapidly, without the need of undergoing the priming step (Palmer & Weaver 2010; Murphy 2011).

1.6.2 γδ T cells

As mentioned, T cells can be divided into two large groups, according to the type of T cell receptor (TCR) they express. Classical CD8+ killer and CD4+ helper T cells generally carry an αβ TCR. In contrast, γδ T cells usually do not express CD8 or CD4. They generally represent between 2 to 10% of lymphocytes in peripheral blood, but can be more strongly expanded in the blood in response to infection, and constitute a considerable lymphocyte population in epithelial tissues (Vantourout & Hayday 2013). These cells are often considered to be the link between innate and adaptive immunity, because they present features identified with either type of immune response. There are distinct subgroups among γδ T cells that are distinguished by the subclass of γδ TCR they express, and such subgroups specifically accumulate in different tissues (Itohara et al. 1990). Interestingly, in some tissues γδ T cells are almost monoclonal. This permits to have a large number of cells ready to promptly counteract pathogens, without the dependence on clonal expansion for an efficient immune response. This feature recalls a principle of innate immunity. On the other hand, like αβ T cells, the TCR of γδ T cells derives from somatic V(D)J recombination. Compared to the α and β loci, γ and δ chains derive from a more limited repertoire of V and J segments (Chien & Konigshofer 2007). The TCRδ locus is embedded in the TCRα locus and it contains three Dδ gene segments, four Jδ gene segments, and a single Cδ gene segment. The δ locus codes for three δ-specific V gene segments but shares five more V segments

that can also be part of a TCRα chain. The γ TCR locus has twelve Vγ gene segments, and resemble the TCRβ locus in having two C genes, each with its own set of J genes (three in one set, two in the second one). Even though the number of available gene segments is smaller, the CDR3 region of both γ and δ chains can have a higher degree of variation compared to the α and β chains because (i) during rearrangement it can incorporate multiple D gene segments, (ii) all D gene segments can be read in all three open reading frames, and (iii) N-nucleotides can be inserted into junctions of the J gene segments (Chien & Konigshofer 2007). This leads to a contrast between the limited diversity of the CDR1 and CDR2 loops of the γδ TCR, determined only by the small choice of possible V gene segments, and the CDR3 that has high potential for diversity (Rock et al. 1994). Another interesting feature of the γδ TCRs is that CDR3 regions of both γ and δ chains are more variable in length than αβ TCRs. The high variability of the CDR3, both in length and in sequence, might reflect the target variability or different affinities to the target of γδ T cells.

1.6.2.1 γδ T cell subsets

In humans, γδ T cells expressing a δ1 chain are enriched in epithelial tissues, while T cells with a δ2 chain are mostly found in peripheral blood and the δ2 chain is usually paired to a γ9 chain (γ9δ2 T cells). Cells with the γ9δ2 TCR are known to recognize phosphoantigens (Gober et al. 2003), while several antigens have been proposed as targets for cells expressing the δ1 chain (Vantourout & Hayday 2013).

γ9δ2 cells represent the most abundant subpopulation of γδ T cells in peripheral blood. These cells were shown to recognize phosphoantigens such as isopentenyl pyrophosphate (IPP), an ubiquitous intermediate of isoprenoid and steroid biosynthesis, and hydromethyl-but-2-enyl-pyrophosphate (HMBPP), a bacterial metabolite (Morita et al. 2007). In mammalian cells, phosphoantigens such as IPP are produced as intermediates of the mevalonate pathway, which is essential for the synthesis of cholesterol, steroid hormones and other molecules. Most bacteria make use of an alternative pathway, the methylerythritol 4-phosphate (MEP) pathway, of which HMBPP is an intermediate. Interestingly, γ9δ2 cells can be activated by both IPP and HMBPP, but the bacterial counterpart is much more potent (Hintz et al. 2001). In human cells, phosphoantigens are quickly turned over. However, during tumor

transformation, these molecules can accumulate, allowing the recognition of malignant cells by the γ9δ2 T cells (Morita et al. 2007). Recognition of phosphoantigens by γ9δ2 T cells is dependent on cell-cell contact (Morita et al. 1995). Phosphoantigens need to be presented by particular cell types in order to be recognized by γ9δ2 T cells (Allison et al. 2001). These data suggest that phosphoantigens need to be presented by an antigen-presenting molecule. In particular, γ9δ2 T cell activation was shown to be greatly enhanced by monocytes (Eberl & Moser 2009). To date, several proteins have been linked to recognition by γ9δ2 T cells: the chaperone GroEL (Fisch et al. 1990), the F1-ATPase (Mookerjee-Basu et al. 2010), an aminoacyl-tRNA synthetase (Monkkonen et al. 2006) and, most recently and convincingly, butyrophilin 3A1 (Vavassori et al. 2013).

T cells with a δ1 cells are less frequent in peripheral blood (between 0.1 and 3% of lymphocytes). They are often associated with mucosal surfaces. Various evidence suggests that circulating δ1 T cells are different from the ones in epithelia, suggesting a correlation between localization, function and phenotype. There are various suggestions as to which antigens are recognized by these T cells. The NKG2D ligands MICA and MICB have been proposed to be recognized directly by the δ1 TCR (Groh et al. 1998; Das et al. 2001). In addition, subpopulations of δ1 T cells were shown to recognize the CD1c (Spada et al. 2000) and sulfatide-loaded CD1d molecule (Bai et al. 2012). Interestingly, CD1d was also found to be recognized by peripheral δ3+ T cells (Mangan et al. 2013). Most likely, the δ1 T cell population is highly heterogeneous, which would explain the identification of such various molecules as targets. Possibly, different populations might have different targets, different properties and different roles. In contrast to γ9δ2 T cells, δ1 T cells appears to be more closely involved in control of viral infections, as they were shown to strongly expand after HIV, HCMV and HSV infections (Hinz et al. 1994; Boullier et al. 1995; Maccario et al. 1995; Lafarge et al. 2001; Pitard et al. 2008).

1.6.3 T cell coreceptors

Specific engagement of the TCR with the peptide:MHC complex activates the T cells. However, intensity and quality of antigen recognition may depend on their interaction

with costimulatory or coinhibitory receptors. To date, various classes of coreceptors have been identified. Here, I will discuss some of those that were relevant for my work.

The B7 family consists of structurally related cell-surface proteins, and comprises stimulatory and inhibitory members. B7 molecules include CD86 (B7.1), ICOS-L (B7-H2), and PD-L1 (B7-H1). CD86 is a molecule constitutively expressed on APCs at low levels, but activation of APCs leads to its upregulation. CD86 can bind to the co-stimulatory receptor CD28 or to the inhibitory receptor CTLA-4 on T cells, but it was shown to be bound by CD28 more effectively (Collins et al. 2002). CD28 is constitutively expressed on naive CD8+ and CD4+ T cells, serving as positive stimulator of cell growth and cell survival. CTLA-4 is induced on the surface of T cells upon T cell activation and it suppresses T cell responses (Chen & Flies 2013). ICOS-L is constitutively expressed on DCs and monocytes, but also on non-lymphoid cells (Collins et al. 2005). It interacts with the receptor ICOS (Inducible COStimulator), which is upregulated on activated T cells. ICOS is particularly important for CD4+ T cell helper functions to B cells (Greaves & Gribben 2013). PD-L1 is constitutively expressed and upregulated upon activation on APCs, but it can also be expressed on non-lymphoid cells. Its receptor, PD-1, is expressed during the effector phase of T cells and limit the responses of both CD4+ and CD8+ T cells. In the context of viral infection, PD-1 may play a role in contraction of the response after acute infection (Brown et al. 2010), but it has also been associated with T cell dysfunction, especially in the context of tumor immunity (Greaves & Gribben 2013).

The activatory receptor NKG2D was first identified as an NK receptor. However, it is now well established that NKG2D is expressed also on CD8+ T cells, γδ T cells and subpopulations of CD4+ T cells (Zafirova et al. 2011). Ligands for this receptor are MICA, MICB and the ULBP molecules. The NKG2D ligands function as “stress”-alerting molecules and are expressed on the cell surface after heat shock, injury, viral infection and tumor transformation (Raulet et al. 2013).

The intracellular adhesion molecule ICAM-1 and the integrin LFA-1, which consists of the CD11a and the CD18 subunits, play an important role in the formation of the immunological synapse between T cells and APCs. Interaction of LFA-1 on the T cell with ICAM-1 on DCs highly increases antigen sensitivity of T cells (Bachmann et al. 1997) and is necessary for the development of memory (Scholer et al. 2008). On the other side, T cell MHC-II triggering on naive B cells enhances LFA-1 “stickiness” on

these cells, resulting in a very stable cell-cell contact that disfavours functional T cell activation and leads to the development of regulatory T cells (Gunzer et al. 2004).

The SLAM family member CD48 is solely expressed on hematopoietic cells. Its receptor, 2B4, is expressed on NK and T lymphocytes and, according to the presence or the absence of the cytoplasmic adaptor SAP, can induce activatory or inhibitory signals (Parolini et al. 2000).

Costimulatory receptors OX40 and 4-1BB of the TNF receptor superfamily play a substantial role in regulating effector T cell responses. Expression of both receptors is induced on T cells after TCR engagement (Croft 2009). OX40 promotes proliferation and survival of both CD4+ and CD8+ T cells, whereas 4-1BB preferentially promotes the expansion and survival of CD8+ T cells (Chen & Flies 2013). Ligands for these receptors are inducible on APCs (Croft 2009). Another member of the TNF receptor superfamily is the receptor CD40, an activatory receptor expressed by APCs including B cells. Its ligand, CD40-L, is expressed on CD4+ T helper cells reacting to TCR ligation. The interaction between CD40 and its ligand presented by helper T cells is essential for B cell activation and consecutive expansion and differentiation (see below).

Family	Ligand	Receptor	Functionality
B7 family	CD86	CD28	+
		CTLA-4	-
	PD-L1	PD-1	-
	ICOS-L	ICOS	+
NK receptors	MICA/B	NKG2D	+
	ULBPs	NKG2D	+
Integrin	CD11a (LFA-1)	ICAM-1	+
Adhesion molecule	ICAM-1	LFA-1	+
SLAM family	CD48	2B4	+
TNF family	4-1BB-L	4-1BB	+
	OX40-L	OX40	+
	CD40-L	CD40	+

Table 1.2. List of activatory and inhibitory receptors playing a role in T cell activation and reactivity analyzed during this study.

1.7 B lymphocytes and the humoral immune response

B lymphocytes recognize microbial antigens through the B-cell receptor (BCR). The BCR is composed by the pairing of two polypeptide chains, one of approximately 50 kDa, called heavy or H chain, and a second one, the light or L chain, of 25 kDa. Two types of light chains can be produced, λ and κ. Each heavy chain is linked to a light chain by a disulfide bond, and the two heavy chains are likewise linked to each other by disulfide bonds. Like the TCR, the functional BCR genes are formed by somatic gene recombination. The light chain derives from V-J rearrangement, while the heavy chain derives from a V-D-J rearrangement. The light λ chain locus encodes 29 to 33 functional Vλ gene segments and 4 or 5 Jλ genes. The number of Cλ genes presents variations between individuals. The κ locus has about 38 functional Vκ gene segments, 5 Jκ genes and a single Cκ gene. The heavy chain can be encoded by 40 different VH segments, recombining with 23 DH genes and 6 JH genes, and has a 9 CH gene segments (Murphy 2011).

Once a B cell encounters its antigen, the surface BCR-antigen complex is internalized, the antigen is degraded, and the derived peptides are loaded on MHC-II molecules. The peptides are presented on the B cell surface by MHC-II to specific CD4+ helper T cells that react to the TCR ligation by upregulating the CD40 ligand (CD40L). CD40L is bound by the receptor CD40 on the B cell. The recognition of the antigen (signal 1) and the engagement of the receptor CD40 (signal 2) synergize in driving B cell activation and inducing B cell proliferation, with formation of the germinal center, and differentiation. Activation of a B cell depends on the receiving of both these stimulatory signals (Graham et al. 2010). Nevertheless, maximal proliferation of B cells is achieved when the B cell receives an additional stimulation through pattern-recognition receptors (PPR) such as Toll-like receptors (TLRs), recognizing microbial components like unmethylated CpG DNA or their mimics (signal 3) (Ruprecht & Lanzavecchia 2006). Upon activation, B cells differentiate to memory cells and plasma cells, which start producing high amounts of antibodies (immunoglobulins, Ig), the soluble version of their BCR.

Igs are highly diverse and have diversified effector functions thanks to somatic hypermutation and class switching, modifications occurring in activated B cells. Somatic hypermutation further diversifies the rearranged V regions of the Ig genes by introducing mutational changes that may lead to a higher affinity to the antigen. Class

switching combines the same assembled V(D)J region – i. e. the same antigen specificity - with different C-region isotypes. Different classes of antibodies perform different functions. IgD and IgM are the first antibodies produced and are coexpressed on the surface of a naive B cell. Upon primary activation, B cells become IgM+IgD–. Once class-switching takes place, IgM is not produced anymore, but one of several other classes (IgG, IgA and IgE) will be produced. IgG are found in blood and in extracellular fluid where they bind to pathogens, neutralizing viruses, activating the complement and facilitating phagocytosis. IgA are the antibodies present in extracellular fluids within the body and in epithelial secretions. IgE resides below the body surfaces, mostly bound to IgE receptors on mast cells, and is accountable for allergic reactions (Murphy 2011).

1.8 Epstein-Barr Virus

Epstein-Barr virus (EBV) is a human gamma-1 herpesvirus with a preferred B cell tropism. EBV infects more than 95% of the adult population and, in healthy carriers, it persists in the form of a lifelong asymptomatic infection. The virus is mainly orally transmitted; in most cases primary infection occurs during childhood and is often asymptomatic or it can resemble a common cold or flu-like symptoms. If the first encounter with the virus occurs during adolescence or adulthood, acute infection with EBV can lead to infectious mononucleosis (IM), a self-limiting lymphoproliferative disease characterized by high fever, swollen lymph nodes, and general malaise lasting for several weeks (Rickinson et al. 2014).

Persistent viruses are never completely cleared by the immune system. It is therefore of extreme importance for the host to develop an effective immune response able to continuously repress the reactivation of these viruses. Indeed, in healthy carriers, EBV infection is kept under control by a diverse repertoire of antigen-specific T cells (Hislop et al. 2007b). The consequences of the lack of proper control of EBV infection are seen in the reactivation of infection in immunosuppressed patient, such as patients after transplantation, who lack EBV-specific T cell responses and are prone to develop EBV-associated lymphoproliferative disease, which is life-threatening and can be difficult to treat (Bollard et al. 2012). In these patients, transfer of EBV-specific T cells can be successfully used to treat EBV-associated diseases (Moosmann et al. 2010, 2012). As an oncovirus, EBV can also contribute to the development of several types of cancers

in immunocompetent patients, such as Burkitt lymphoma, nasopharyngeal carcinoma, EBV-associated gastric carcinoma and Hodgkin lymphoma (Rickinson 2014).

1.8.1 The replication cycle of EBV

After transmission, the virus establishes a lytic infection of epithelial cells and infiltrating B cells in the oropharynx, during which many viral proteins needed for the assembly and the release of new viral particles are expressed. From here, the virus colonizes the B cell compartment, establishing its growth programme ("latency III") in B cells in lymphoid tissues. In "latency III", several immunogenic EBV antigens, the latent membrane proteins (LMP1, -2A, -2B) and the Epstein-Barr nuclear antigens (EBNA1, -2, -3A, -3B, -3C, -LP), are expressed in EBV-infected B cells. This type of latency leads to intense cell proliferation and activation, and is found *in vitro* in EBV-induced lymphoblastoid cell lines (LCLs) and in post-transplant lymphoproliferative disease (Brink et al. 1997). EBV-infected B cells in lymphoid organs during primary and persistent EBV infection (Tierney et al. 1994) lead to an amplification of EBV load through proliferation of infected cells (Hochberg et al. 2004; Young & Rickinson 2004). Following the normal channels of B cell differentiation, the EBV-induced activated lymphoblastoid B cells enter the germinal center, where they gain access to the memory compartment, the site of long-term persistence (see below).

Many of these proliferating B cells are detected and recognized by virus-specific T cells. However, some of them manage to escape from recognition and elimination by suppressing expression of most EBV antigens, resulting in "true latency" or the EBV "default program". "True latency" or "Latency 0" characterizes quiescent infected memory B cells, without the expression of any viral protein (Babcock et al. 2000). "Latency 0" infected cells represent the virus reservoir in the immunocompetent host who has established an EBV-specific T cell and antibody response. During the "default program" ("latency I" and "latency II"), viral antigen expression is limited either to EBNA1, or to EBNA1 together with one or more LMPs (Young & Rickinson 2004). These latency types occur in Burkitt lymphoma (type I), nasopharyngeal carcinoma, gastric carcinoma (type I/II) and Hodgkin lymphoma (type II) (Rickinson 2014) and in centroblast and centrocytes, the proliferating and growing B cells forming the germinal centers (Babcock et al. 2000). Cells in "true" or "default" latency program express little

or no viral antigens and are, therefore, recognized by the immune response only with difficulty.

Occasionally, EBV-infected memory B cells will re-enter the germinal center, where different stimuli will lead to activation of different latency programs and reinitiation of the EBV infection cycle.

1.8.2 B cell activation by EBV: the role of LMP2A

As mentioned, during type III latency, EBV induces activation and proliferation of the infected B cells. Two main mediators of these effects are the latent membrane proteins LMP2A and LMP1. These two viral proteins mimic the activatory signals induced by the BCR (signal 1) and by the receptor CD40 (signal 2), respectively. Both are transmembrane proteins, and each of them has a cytoplasmic domain containing various motifs functioning as docking sites for cellular kinases that lead to activation of several signaling pathways (Pang et al. 2009; Dawson et al. 2012).

In B cells, LMP2A mimics the activatory signals of the B cell receptor (BCR) both *in vitro* and in a transgenic mouse model (Caldwell et al. 1998; Mancao & Hammerschmidt 2007), activating PI3K/Akt and ERK-MAPK signaling (Panousis & Rowe 1997; Fukuda & Longnecker 2004; Portis & Longnecker 2004; Anderson & Longnecker 2008). These signals promote B cell survival and inhibition of apoptosis. LMP2A was also shown to interfere with activation of EBV lytic cycle upon BCR engagement by preventing normal BCR activation (Miller et al. 1994a, b, 1995; Konishi et al. 2001; Merchant et al. 2001). In the absence of exogenous triggers of the lytic cycle, however, LMP2A supported basal levels of lytic cycle induction, suggesting a dual role of LMP2A in controlling EBV lytic cycle (Schaadt et al. 2005).

However, the influence of LMP2A on human B cell proliferation and transformation has been controversial: while some studies did not identify a role of LMP2A in these processes (Longnecker et al. 1992, 1993a, b; Speck et al. 1999; Konishi et al. 2001) other reports indicated that LMP2A increases the efficiency of B cell proliferation and transformation (Brielmeier et al. 1996; Mancao & Hammerschmidt 2007), implying that LMP2A-deleted EBV has reduced capacity to transform B cells.

LMP2A is one of few EBV latent antigens that are not only expressed in B cells in the transforming growth program (latency III), but also in tumors expressing the restricted antigen expression “default” programs such as Burkitt lymphoma, Hodgkin lymphoma,

nasopharyngeal carcinoma and gastric carcinoma (Rickinson 2014). Several studies in epithelial cells showed that LMP2A promotes survival, proliferation and transformation in these cells, implicating a role for LMP2A in the maintenance or in the progression of carcinomas. These reports showed that LMP2A enhances anchorage-independent growth (Fukuda & Longnecker 2007), inhibits differentiation (Scholle et al. 2000), interferes with the proapoptotic effects of TGFβ1 (Fukuda & Longnecker 2004) and induces the expression of the anti-apoptotic protein survivin (Hino et al. 2008). In addition, LMP2A enhances tumorigenesis *in vivo* (Scholle et al. 2000; Kong et al. 2010).

1.8.3 Cellular immunity in EBV infection

Cell mediated responses are crucial both in acute and in persistent infection. In virus-host interaction, both the adaptive and the innate immune response play fundamental role in the control of the infection.

1.8.3.1 Humoral response in EBV infection

The humoral immune response to active EBV infection shows an initial strong IgM production specific for the viral capsid antigen (VCA) and the development of IgG antibodies specific for the EBV early antigen (EA-D). While the antibody responses to these antigens rapidly decline after acute infection, the production of anti-VCA IgG antibodies rises slowly during acute infection and persists for life. In addition, IgG antibodies specific for the EBV nuclear antigen 1 (EBNA1) are produced and, as for the anti-VCA IgG antibodies, persist long-tern after the resolution of the infection. In seropositive hosts, viral reactivation can be indirectly detected by the presence of newly produced IgM antibodies (Klutts et al. 2004).

1.8.3.2 NK cell response in EBV infection

Several lines of evidence suggest an involvement of NK cells in the control of EBV infection, although a clear role for these cells in the control of EBV has not been established yet. First, male patients affected with X-liked lymphoproliferative disease (XLP), a fatal disease associated to mutations in the SAP gene, are prone to develop EBV-induced lymphomas (Nichols et al. 2005). Mutations in the adaptor protein SAP

lead to defective recognition of EBV-transformed B cells by NK cells (Benoit et al. 2000). The fact that these patients cannot control EBV infection, suggests an important role for NK cells in EBV immunity. Second, expansion of NK cells is observed during infectious mononucleosis (Williams et al. 2005; Balfour et al. 2013). Third, several *in vitro* studies showed that NK cells can inhibit EBV-induced B cell transformation (Kaplan & Shope 1985; Wilson & Morgan 2002; Strowig et al. 2008; Lunemann et al. 2013) or that NK cells can with some efficiency lyse lytically EBV-infected Burkitt lymphoma cells (Pappworth et al. 2007) and early EBV-infected B cells (Jochum et al. 2012a). Interestingly, in the case of lytically EBV infected Burkitt cell lines, NK lysis was dependent on the downregulation of MHC molecules, ligands for NK inhibitory KIR receptors, and on the upregulation of ligands for the agonistic receptor NKG2D on the surface of infected cells (Pappworth et al. 2007). A recent study showed that in patients with genetic deficiencies in the magnesium transporter MAGT1, who are, like XLP patients, particularly susceptible to EBV infection and EBV+ lymphomas, NKG2D plays an important role in the control of EBV infection by NK and CD8+ T cells (Chaigne-Delalande et al. 2013), further supporting a role of NK cells in EBV immunity.

1.8.3.3 T cell response in EBV infection

The development and maintenance of an efficient adaptive immunity is extremely important in control of life-lasting infections, such EBV. During IM, a marked expansion of CD8+ T cells is observed. This population proved to be oligoclonal in the TCR repertoire (Annels et al. 2000), with reactivities for lytic cycle proteins usually dominant (Pudney et al. 2005). While these highly expanded responses to lytic cycle antigens are strongly culled after IM, the response to latent cycle proteins is smaller during acute infection, but is stably maintained or even increased after the resolution of IM (Catalina et al. 2001; Woodberry et al. 2005). In long-term asymptomatic EBV carriers, a significant fraction of circulating CD8+ T cells is devoted to the control of EBV, with individual epitope-specific populations reaching 1-2% of the total CD8+ T cell pool (Bihl et al. 2006). Despite the fact that even during asymptomatic infection, it is likely for EBV to sustain recurrent activation, most of the memory cells have a resting phenotype. Nevertheless, *ex vivo* EBV-specific T cells can be rapidly activated

upon challenge with peptide-loaded targets, EBV-transformed B cells, or other forms of antigen-specific stimuli (Hislop et al. 2001).

A different role seems to be played by the response to EBV in the CD4+ T cell compartment. CD4+ T cells are not markedly expanded in IM, even though EBV-specific CD4+ T cells are detected at this time (Long et al. 2013). Interestingly, in contrast to the CD8+ T cell response, the latent-specific CD4 T cell pool is larger that the one directed to the lytic antigens (Long et al. 2013). While CD4+ T cells specific for latent antigens are often incapable of recognizing EBV-infected cells such as LCLs, CD4+ T cells specific for late lytic antigens can sensitively recognize lytically produced EBV antigen, and can directly and efficiently kill target cells that present such antigens (Adhikary et al. 2006, 2007). Thus, CD8+ and CD4+ T cells appear to play complementary roles in control of EBV infection.

1.8.3.4 γδ T cell response in EBV infection

In the context of EBV infection, less is known about the role of γδ T cells. γδ T cells were shown to expand during acute infectious mononucleosis (De Paoli et al. 1990), to proliferate after contact with LCLs (Hacker et al. 1992; Orsini et al. 1993) and after contact with EBV-positive Burkitt lymphoma lines (Hacker et al. 1992; Orsini et al. 1994; L'Faqihi et al. 1999). However, the literature gives controversial information regarding which population recognizes EBV-infected B cells (De Paoli et al. 1990; Lam et al. 1990; Qvigstad et al. 1990; Hacker et al. 1992; Orsini et al. 1993; Hyjek et al. 1997; Fujishima et al. 2007; Kong et al. 2009). In addition, after a number of studies were conducted early after the discovery of the γδ T cell population, less work has been done in recent years, and the knowledge about the role of γδ T cells in EBV infection has remained superficial.

1.8.4 Mechanisms of EBV immune escape

During lytic EBV replication, a majority of at least 70 viral proteins are co-expressed: in this situation, the virus would be extremely vulnerable to the immune system. Thus, a number of EBV proteins expressed in lytic cycle have been found to interfere with the display of viral antigens to CD8+ T cells. Among these, the BNLF2a protein diminishes the supply of peptide available in the ER for MHC class I antigen

presentation by inhibition of peptide translocation by the TAP transporter (Hislop et al. 2007a). BILF1 directly associates with MHC class I molecules rapidly after their biosynthesis and induces their internalization and degradation in lysosomes (Zuo et al. 2009). BGLF5 inhibits cellular protein biosynthesis thereby drastically reducing MHC-I surface levels (Zuo et al. 2008). EBV IL-10 homolog BCRF1 (or v-IL10) was shown to reduce MHC-I levels by decreasing mRNA and protein levels of the TAP1 and the LMP2 proteins, two of the subunits forming the immunoproteasome (Zeidler et al. 1997). Moreover, it has been described to block IFNγ release by CD8+ T cells (Swaminathan et al. 1993).

In contrast, it is less clear how B cells expressing the EBV latency III "growth program" manage to escape from recognition and elimination by virus-specific T cells. The sole known exception is EBNA1, which interferes in cis with its presentation to CD8+ T cells through its Gly-Ala repeat domain, which hampers the processing of EBNA1 by the proteasome, (Levitskaya et al. 1997) and prevents EBNA1 mRNA translation (Yin et al. 2003). The possibility of an involvement of LMP2A in the modulation of the immune response was suggested by the observation that LMP2A modulates signaling of type I/II interferon receptors in epithelial cells (Shah et al. 2009) and that the presence of LMP2A alters the expression of several immune-related genes (Portis et al. 2003). Beyond these hints, it is unclear whether the growth program uses other mechanisms to hamper T cell recognition of infected cells.

1.8.5 EBV in cancer

EBV is an oncovirus and is associated with numerous human malignancies, such as Burkitt lymphoma, nasopharyngeal carcinoma, EBV-associated gastric carcinoma and Hodgkin lymphoma (Rickinson 2014). Interestingly, although EBV has a preferential B cell tropism *in vitro* and, as far as is known, in healthy carriers *in vivo*, it is associated with tumors of both B and non-B cell origin. All EBV-positive B cell tumors present somatically mutated Ig genes, suggesting that at some point these cells have undergone a germinal center reaction. Non-B cell EBV-positive tumors arise from rare events in which the virus gains entry to a different host cell type. In both cases, EBV is present in these tumors expressing different sets of viral proteins. In Burkitt lymphoma, viral antigen expression is usually limited to EBNA1 (latency I) or to EBNA1 with low levels of one or more LMPs (latency I/II). This expression profile is common in non-B

cell tumors (nasopharyngeal carcinoma, gastric carcinoma). In Hodgkin lymphoma both EBNA1 and the LMPs are expressed at high levels (latency II), while in lymphoproliferative disease, usually developed in immunocompromised patients, EBV present a latency III expression profile (Rickinson 2014). This latter mode of latency is more immunogenic, which makes it plausible that such tumors usually arise only in conditions of immunodeficiency.

1.8.6 Immunotherapy

The balance between EBV reactivation and control of the infection by the immune system can be disrupted after transplantation of solid organs or hematopoietic stem cells (HSCT). After solid organs transplantation, the cellular immune response of the patient is kept at bay by immunosuppressive drug administration for the prevention of graft rejection. In allogeneic HSCT, the host immune system is ablated and reconstitution of cellular immunity is a slow process, which can again be impaired by the administration of immunosuppressive drugs during the first months after transplantation. In both cases, the patients will lack the EBV-specific T cell responses necessary to control EBV reactivation. To treat the development of post-transplant lymphoproliferative disease (PTLD), guidelines suggest an initial reduction of immune suppression (Bollard et al. 2012). However, this solution is not always possible or successful. Monoclonal antibodies specific for B cells (Rituximab, a specific antibody against CD20, an antigen expressed solely on B cells) are often effective, either alone or in combination with chemotherapy (Kuehnle et al. 2000; Savoldo et al. 2005). However, the depletion of the complete B cell population that is a regular consequence of anti-CD20 application further increases the risk of other infections in patients after HSCT. In addition, PTLD often recurs because the cellular immunity is not restored. Thus, strategies to restore T cell immunity by transfer of EBV-specific T cells have been developed and successfully applied (Heslop et al. 2010; Moosmann et al. 2012). The majority of PTLD express a type III latency, while a minority presents a type II latency expression profile (Bollard et al. 2012). Therefore, it is necessary to transfer T cells specific for the relevant expressed antigens. In earlier times, transfer of unmanipulated donor lymphocytes in patients treated with allogeneic HSCT has shown good efficacy. However, this strategy often causes severe GvHD, because alloreactive T cells are costransferred (Heslop et al. 1994). For this reason, various strategies to

expand *ex vivo* EBV-specific T cells have been tested. The use of LCLs as APCs to stimulate expansion of EBV-specific T cells was used with success; however, this strategy is laborious and time consuming (Heslop et al. 2010). Other strategies consist in the development of rapid *ex vivo* culture, for example by a single stimulation of T cells with DCs previously transfected with DNA plasmids encoding for EBV immunogenic proteins (Gerdemann et al. 2009). Another technique employs the use of tetrameric HLA-peptide constructs (tetramers) to select virus-specific T cell populations (Uhlin et al. 2010). Still another approach showed efficacy, in which virus-specific T cells were selected by EBV peptide stimulation of PBMCs and subsequent isolation of IFN-γ secreting T cells (Moosmann et al. 2010). The last three approaches yield low absolute numbers of EBV-specific T cells, but these cells have the potential to expand *in vivo*, providing potent antiviral activity. Whereas experience with therapeutic transfer of EBV-specific αβ T cells (CD8+ and, sometimes, CD4+) has been promising, the potential of EBV-specific immunotherapy employing different types of effector cells, such as γδ T cells, has not been explored.

1.9 Aim of the study

EBV efficiently infects B cells by taking advantage of the processes of normal B cell activation and differentiation. An important form of EBV infection *in vivo* is the "growth programme", also called latency III, which can be faithfully modeled *in vitro* by infection of primary B cells with EBV, resulting in generation of lymphoblastoid cell lines (LCLs). Latency III is also present in post-transplant lymphoproliferative disease. In the growth programme, EBV induces or mimics several cellular pathways of B cell activation, and thus drives B cell proliferation and its own amplification. However, it is unclear whether and how the virus modulates immunogenicity in this important form of infection. In principle, EBV-specific CD8+ T cells can recognize LCLs, but responses by some CD8+ and by CD4+ T cells appear to be blunted. Moreover, it has remained unclear whether γδ T cells can recognize this form of infection. The present study investigates different possibilities of how the virus controls and modifies the immunogenicity of B cells during latency III. One of the EBV latent viral proteins, LMP2A, triggers multiple signaling pathways, is expressed in latency III and also two other latency types (I and II), and is one of the few viral proteins expressed in most types of EBV-related malignancies. Therefore, LMP2A was a likely candidate to perform immunomodulatory functions during latent infection of EBV. The aim of the present study was to better understand what makes EBV one of the most successful persistent viruses, by addressing the following questions:

I. How does EBV infection affect the immunophenotype of the B cell, and how does this differ from the phenotype induced by physiological B cell activation? Is there evidence for immunoevasion specifically induced by EBV in latency III B cells?
II. Does LMP2A have immunomodulatory effect(s)? If yes, which immunological mechanisms are affected by the function of LMP2A?
III. Which effector cells are affected by the immunomodulatory effects mediated by LMP2A?
IV. Are γδ T cells involved in recognition of EBV-infected B cells? If yes, which γδ T cell subpopulation is active?

2 Materials

2.1 Eukaryotic cells

2.1.1 Primary cells

Peripheral blood mononuclear cells (PBMCs) were isolated from standard blood donations by anonymous healthy adult donors purchased in the form of buffy coats from the Institute for Transfusion Medicine, University of Ulm, Germany or from voluntary healthy adult blood donors providing written informed consent. All blood donors contributing to this work were anonymized. The institutional review board (Ethikkommission, Klinikum der Universität München, Grosshadern, Munich, Germany) approved this procedure. All work was conducted according to the principles expressed in the Helsinki Declaration. High- or low-resolution HLA types and virus carrier status of PBMC donors are listed in the following table:

	HLA-A	HLA-B	HLA-C	EBV status	HCMV status
Donor 1	*0101 *0201	*3906 *4402	*0702 *0704	? (EBNA-VCA+)	-
Donor 2	*0101 *2601	*3501 *5701	*0401 *0602	-	-
Donor 3	*0301 *6801	*0702 *3501	*0401 *0702	+	-
Donor 4	*0101 *2402	*0801 *1402	*0701 *0802	+	+
Donor 5	*2402 *2402	*1302 *1801	*0602 *1203	+	+
Donor 6	*110 *3101	*3503 *4001	*0304 *1203	+	-
Donor 7	*0201 *0301	*1529 *5101	*1203 *1402	+	-
Donor 8	*0201 *6801	*1501 *4402	*0102 *0501	+	+
Donor 9	*0201 *0201	*0702 *4002	*0202 *0702	+	+
Donor 10	*03 *24	*3501 *4002	*02 *04	+	-

Donor 11	*0101 *0201	*1801 *5101	*0501 *1502	+	-
Donor 12	*30 *31	*13 *27	*01 *06	+	-
Donor 13	*3201 *6601	*4002 *5101	*0202 *0202	+	+
Donor 14	*0201 *2902	*4402 *4501	*0602 *0602	+	+

2.1.2 Cell lines

B95.8	EBV B95.8 infected lymphoblastoid marmoset cell line	(Miller et al. 1972a; Miller & Lipman 1973)
293/TR-2	EBV-packaging cell line based on the human embryonic kidney (HEK) 293 cell line stably transfected with p2114, TR- producer line	(Delecluse et al. 1999)
293/2098	EBV-packaging cell line based on the human embryonic kidney (HEK) 293 cell line stably transfected with p2089, EBV WT 2089 producer line	(Delecluse et al. 1998)
293/2525	EBV-packaging cell line based on the human embryonic kidney (HEK) 293 cell line stably transfected with p2525, ΔLMP2A EBV producer cell line	(Mancao & Hammerschmidt 2007)
LL8	Mouse fibroblast cell line L929 stably transfected with human CD40L, for production of B-blasts	Leah Schirrmann, Andreas Moosmann
Daudi	Burkitt's lymphoma cell line, subcloned for EBV-loss, MHC class I deficient	(Klein et al. 1968)
Raji	Burkitt's lymphoma cell line, EBV positive	(PULVERTAFT 1964)
K562	Immortalized human chronic myelogenous leukemia cell line, MHC class I deficient	(Lozzio & Lozzio 1975)

2.2 Plasmids

p2670	Plasmid encoding the EBV glycoprotein BALF4/gp110	(Neuhierl et al. 2002)
p509	Plasmid encoding the EBV lytic transactivator BZLF1	(Delecluse et al. 1999)

2.3 Oligonucleotides

Oligonucleotides used in this work were ordered to and synthesized by Metabion (Munich, Germany). They are listed as follows in 5´→ 3´direction.

Primers for standard PCR:

PCR	Primer name	Sequence (5´→3´)	Product size (bp)	Annealing T (°C)
LMP2A bridging	L2BRC L2BRD	GCTTCCTCGTGCTTTACGGTATC AAGAACTTTGACCTGTTGTCCCTG	WT: - Δ: 481	52
LMP2A internal	L2INA L2BRD	CATTGCGGGTGGATAGCCTC AAGAACTTTGACCTGTTGTCCCTG	WT: 831 Δ: -	55
gp85	gp85c gp85d	TGGTCAGCAGCAGATAGTGAACG TGTGGATGGGTTTCTTGGGC	450	55
TCR seq Vδ1	Vδ1F2 CδR3	GAACAGAATGCAAAAAGTGGTC CACTTCAAAGTCAGTGGAGTGC	variable	56
TCR seq Vδ3	Vδ3F3 CδR3	GGCGAGTGGCAGTGAGG CACTTCAAAGTCAGTGGAGTGC	variable	56

Primers for quantitative real-time PCR:

Target	Forward primer	Reverse primer	Product size
Human IL-10	GCAGGTGAAGAATGCCTTTA	CCCTGATGTCTCAGTTTCGT	128
BZLF1 - unspliced	GCACATCTGCTTCAACAGGA	CCAAACATAAATGCCCCATC	104
EBNA1	CGCAAGGAATATCAGGGAT-G	TCTCTCCTAGGCCATTTCCA	150
gp350	TTGTGAAATTTCGCCATCCT	CAAAACCCCGTGTACCTG	222
BCRF1 (vIL-10)	ACCTTAGGTATGGAGCGAAG	GGGAAAATTGTCACATTGGT	110
EBNA3A	TCCGCAGGTTTCCACTAG AT	GGGATCCGAAAAACTGGT CT	99
LMP2	ATCGCTGGTGGCAGTATTTT	GAGTATGCCAGCGACAATCA	105
GUSB	CGCCCTGCCTATCTGTATTC	TCCCCACAGGGAGTGTGTAG	91

2.3.1 Peptides

All peptides used in this study were synthesized by JPT (Berlin, Germany) to >70% purity, resuspended in 100% dimethyl sulfoxide (DMSO) at a final concentration of 10

mg/mL and stored at -20°C. In T cell assays, peptides were diluted to a final concentration of 2 µg/mL.

Peptides used in this work are listed in the following table and are designated by their first three amino acids in one-letter code.

Abbreviation	Peptide sequence	Antigen	HLA-restriction	Source
CLG	CLGGLLTMV	EBV, LMP2	A*0201	(Lee et al. 1993)
CRV	CRVLCCYVL	HCMV, IE-1	C*0702	(Ameres et al. 2013)
FLY	FLYALALLL	EBV, LMP2	A*0201	(Meij et al. 2002; Lautscham et al. 2003)
HPV	HPVGEADYFEY	EBV, EBNA1	B*3501	(Rickinson & Moss 1997)
RAK	RAKFKQLL	EBV, BZLF1	B*0801	(Bogedain et al. 1995)
RPP	RPPIFIRRL	EBV, EBNA3A	B*0702	(Hill et al. 1995)
VLE	VLEETSVML	HCMV, IE-1	A*0201	(Khan et al. 2002)
YVL	YVLDHLIVV	EBV, BRLF1	A*0201	(Saulquin et al. 2000)

2.4 Antibodies

Antibodies used in this study are listed as follows. If not otherwise stated, all antibodies were made in mouse.

specificity	clone	isotype	labeling	quantity	company
MICA	159227	IgG2b	-	50 µg/mL	R&D Systems
MICB	236511	IgG2b	-	50 µg/mL	R&D Systems
ULBP1	3F1	IgM	-	50 µg/mL	Santa Cruz
ULBP1	170818	IgG2a	-	50 µg/mL	R&D Systems
ULBP2-5-6	165903	IgG2a	PE	12.5 µg/mL	R&D Systems
ULBP3	166510	IgG2a	PE	12.5 µg/mL	R&D Systems
ULBP4	6E6	IgG2b	-	50 µg/mL	Santa Cruz
CD48	BJ40	IgG1	PE	5 µg/mL	BioLegend
4-1BB-L (CD137L)	5F4	IgG1	PE	5 µg/mL	BioLegend

OX40L (CD252)	11C3.1	IgG1	PE	5 µg/mL	BioLegend
CD11a	G43-25B	IgG2a	PE	0.5 µL	BD Bioscience
ICOS-L (B7-H2)	2D3	IgG2b	PE	2.5 µg/mL	BioLegend
PD-L1 (B7-H1)	29E.2A3	IgG2b	APC	5 µg/mL	BioLegend
CD86 (B7-2)	IT2.2	IgG2b	APC	5 µg/mL	BioLegend
CD86 (B7-2)	37301	IgG1	PE	2.5 µg/mL	R&D Systems
CD54 (ICAM-1)	HCD54	IgG1	PE	2.5 µg/mL	BioLegend
CD54 (ICAM-1)	HCD54	IgG1	APC	2.5 µg/mL	BioLegend
TCR αβ	IP26	IgG1	APC	4 µL	BioLegend
TCR γδ	B1	IgG1	PE	1 µL	BioLegend
TCR Vδ2	B6	IgG1	PerCP	1 µL	BioLegend
TCR Vδ2	B6	IgG1	FITC	1 µL	BioLegend
TCR Vδ1	TS8.2	IgG1	FITC	2 µL	Thermo Scientific
TCR γ9	B3	IgG1	PE	1 µL	BioLegend
CD314 (NKG2D)	149810	IgG1	-	6.25 µg/mL	R&D Systems
CD314 (NKG2D)	1D11	IgG1	APC	7.5 µg/mL	BioLegend
CD314 (NKG2D)	1D11	IgG1	PE-Cy7	0.5 µL	BioLegend
CD159a (NKG2A)	Z199	IgG2b	APC	4 µL	Beckman Coulter
CD158b (KIR2DL2/3)	CHL	IgG2b	PE	2 µL	BD Pharmingen
CD158e1 (KIR3DL1)	DX9	IgG1	FITC	2 µL	BioLegend
CD158a (KIR2DL1)	HP-3E4	IgM	FITC	6 µL	BD Bioscience
CD56 (NCAM)	N901 (HLDA6)	IgG1	PE	1 µL	Beckman Coulter
CD56 (NCAM)	HCD55	IgG1	PE-Cy5	1 µL	BioLegend
CD56 (NCAM)	HCD56	IgG1	BV421	1 µL	BioLegend
CD19	HIB19	IgG1	FITC	1.5 µL	BioLegend
CD19	HIB19	IgG1	PE	1.5 µL	BioLegend
CD19	HIB19	IgG1	APC	1.5 µL	BioLegend
CD3	HIT3a	IgG2a	PE-Cy5	1 µL	BioLegend
CD4	RPA-T3	IgG1	FITC	1 µL	BioLegend
CD4	RPA-T4	IgG1	PE	1 µL	BioLegend
CD4	RPA-T4	IgG1	PE-Cy5	1 µL	BioLegend
CD8	RPA-T8	IgG1	FITC	1 µL	BD Pharmigen
CD8	RPA-T8	IgG1	APC	1 µL	BioLegend
CD8	RPA-T8	IgG1	PB	1 µL	BioLegend
HLA-ABC	W6/32	IgG2a	APC	1-0.5 µL	BioLegend
CD80	2D10	IgG1	FITC	5 µg/mL	BioLegend
HLA-A2	BB7.2	IgG2b	PE	3 µg/mL	BioLegend
HLA-B7	BB7.1	IgG1	PE	2 µL	Millipore
HLA-C/E	DT9	IgG2b	-	-	Veronique Braud, Nice, France
HLA-A3	4i87	IgM	-	-	US Biological

HLA-Bw6	REA143	human IgG1	PE	-	Miltenyi Biotec
isotype control	-	human IgG1	PE	-	SouthernBiotech
isotype control	133304	IgG2a	-	-	R&D Systems
isotype control	MOPC-173	IgG2a	PE	-	BioLegend
isotype control	MOPC-173	IgG2a	APC	-	BioLegend
isotype control	133303	IgG2b	-	-	R&D Systems
isotype control	133303	IgG2b	APC	-	R&D Systems
isotype control	MG2b-57	IgG2b	PE	-	BioLegend
isotype control	MG2b-57	IgG2b	APC	-	BioLegend
isotype control	11711	IgG1	-	-	R&D Systems
isotype control	MOPC-21	IgG1	PE	-	BioLegend
isotype control	MOPC-21	IgG1	APC	-	BD Bioscience
isotype control	MM-30	IgM	-	-	BioLegend
Anti-mouse IgG	Poly4053	Goat Ig	APC	5 µg/mL	BioLegend
Anti-mouse IgG/IgM	115-136-068	Goat Ig	APC	5 µg/mL	Jackson ImmunoResearch

MHC-I:peptide pentamers for staining of EBV- and HCMV-specific CD8+ T cells were purchased by ProImmune, Oxford, England.

CRV/HLA-C*0702 MHC-I:peptide monomers were generated and provided by Dr. Michael Neuenhahn and Prof. Dirck Busch (Institute for Medical Microbiology, Immunology and Hygiene, Technische Universität München).

2.5 Cell culture media and additives

The following media and additives were used for cultivation of eukaryotic cell lines.

Name	Use	Source
RPMI 1640	Standard cell culture medium	Gibco Invitrogen, Karlsruhe, Germany
Opti-MEM	Cell culture medium for transfection	Gibco Invitrogen, Karlsruhe, Germany
DMEM	Cell culture medium for adherent cells (HEK293)	Gibco Invitrogen, Karlsruhe, Germany
Fetal calf serum (FCS)	Nutritive substance	Gibco Invitrogen, Karlsruhe, Germany
Penicillin/streptomycin	Antibiotics for inhibition of bacterial growth	Gibco Invitrogen, Karlsruhe, Germany
Sodium selenite	Supplement of the essential element selenium	ICN Biochemicals, Aurora, USA
0.05% Trypsin-EDTA	Detachment of adherent cells	Gibco Invitrogen, Karlsruhe, Germany
Cyclosporin A	Immunosuppressant, inhibits T and NK cell growth during the establishment of EBV-infected B cell lines	Novartis, Nürnberg, Germany
Geneticine G-418 sulphate	Antibiotic for selection of eukaryotic cells	Gibco Invitrogen, Karlsruhe, Germany
Hygromycin B	Antibiotic for selection of eukaryotic cells	Gibco Invitrogen, Karlsruhe, Germany
Polyethyleneimine (PEI) MW: 25000	Mean for transfection of eukaryotic cells	Sigma-Aldrich, St. Louis, USA
αBCR $F(ab')_2$ fragments	$F(ab')_2$ fragments for BCR engagement for activation of B cells	Jackson Immuno Research, West Grove, USA
CpG DNA	Ligand for TLR9 for activation of B cells	(Iskra et al. 2010), Metabion, Germany

The following cytokines were used for cultivation of specific cell types.

Name	Use	Source
Recombinant human IL-2	Cultivation of T cells	Novartis, Nürnberg, Germany
Recombinant human IL-4	Cultivation of CD40-stimulated B cells	R&D Systems, Minneapolis, USA

2.6 Commercial kits

Human IFN-γ ELISA Kit (ALP)	Mabtech, Nacka Strand, Sweden
Human IL-10 ELISA Kit (ALP)	Mabtech, Nacka Strand, Sweden
MACS® IFN-γ secretion assay	Miltenyi Biotec, Bergisch Gladbach, Germany
MACS® Human B Cell Isolation Kit II	Miltenyi Biotec, Bergisch Gladbach, Germany
MACS® TCR γ/δ+ T cell Isolation kit, human	Miltenyi Biotec, Bergisch Gladbach, Germany
RNeasy® Mini Kit	Qiagen, Hilden, Germany
QIAamp® DNA Blood Mini Kit	Qiagen, Hilden, Germany
NucleoSpin® Gel and PCR Clean-up	Macherey-Nagel, Düren, Germany
QuantiTect® Reverse Transcription Kit	Qiagen, Hilden, Germany
SYBR Green LC480 Mix	Roche, Basel, Switzerland

2.7 Enzymes

Taq-DNA Polymerase	NewEngland BioLabs, Ipswich, USA
Pfu-DNA Polymerase	Thermo Fisher Scientific, Waltham, USA

2.8 Chemicals, reagents, buffers

10 mM dNTPs	Thermo Fisher Scientific, Waltham, USA
Biocoll Separating Solution	Biochrom, Berlin, Germany
Calcein acetoxymethylester (AM)	Invitrogen, Karlsruhe, Germany
Dimethyl sulfoxide (DMSO)	Carl Roth GmbH, Karlsruhe, Germany
DNA agarose	Biozym, Hessisch Oldendorf, Germany
DNA loading dye (6x)	Fermentas, St. Leon-Rot, Germany
Ethidium bromide	Carl Roth GmbH, Karlsruhe, Germany
FACS Flow/Clean/Rinse	BD Biosciences, Heidelberg, Germany
GeneRuler™ DNA Ladder Mix	Fermentas, St. Leon-Rot, Germany
Heparin-Natrium 25,000	Ratiopharm, Ulm, Germany
NaOH (50 mM)	Carl Roth GmbH, Karlsruhe, Germany
Para-nitrophenyl phosphate (pNPP)	Carl Roth GmbH, Karlsruhe, Germany
PBS Dulbecco (w/o Mg^{2+})	Biochrom, Berlin, Germany
TAE (Tris, acetate, EDTA)	Carl Roth GmbH, Karlsruhe, Germany
Tris/HCl (1M)	Carl Roth GmbH, Karlsruhe, Germany
Triton™ X-100	Carl Roth GmbH, Karlsruhe, Germany
Trypan blue	Merck, Darmstadt, Germany

2.9 Consumables

6-/12-/48-/96-well flat-bottom plates	BD Biosciences, Heidelberg, Germany
96-well round-bottom plates	Nunc A/S, Roskilde, Denmark
96-well V-bottom plates	Hartenstein, Würzburg, Germany
BD Falcon™ cell strainer 100 µm	BD Biosciences, Heidelberg, Germany
BD Falcon™ conical tubes 15mL, 50 mL	BD Biosciences, Heidelberg, Germany
BD Falcon™ Polystyrene round-bottom tubes 5 mL	BD Biosciences, Heidelberg, Germany
Cell culture flasks (25/80/175 cm^2)	Nunc A/S, Roskilde, Denmark
Cryo tube vials 1.8 mL	Nunc A/S, Roskilde, Denmark
Immunoplates MaxiSorp® 96-well flat bottom	Nunc A/S, Roskilde, Denmark
Infuject® 50 mL syringes and needles	Dispomed Witt oHG, Gelnhausen, Germany
PCR reaction tubes (0.2 µL single tubes, 0.2 µL stips)	Thermo Fisher Scientific, Waltham, USA
Pipette tips (Diamond® TowerPack™) (0.1-10 µL, 10-100 µL, 100-1000 µL)	Gilson Inc. Middleton, USA
Plastic pipettes (2 mL, 5 mL, 10 mL, 25 mL)	Greiner Bio-One, Kremsmünster, Austria
Reaction tubes (0.5 mL, 1.5 mL, 2 mL)	Eppendorf, Hamburg, Germany
Single use Pasteur pipettes 3.2 mL	Carl Roth GmbH, Karlsruhe, Germany
Syringe filter Minisart® 0.2 µm	Sartorius AG, Göttingen, Germany
Syringe filter Nalgene™ 0.8 µm	Nalge Company, Rochester, USA

2.10 Laboratory equipment and devices

Centrifuge “Centrifuge 5415 R”	Eppendorf, Hamburg, Germany
Centrifuge “Rotanta 460 R”	Hettic AG, Bäch, Switzerland
Centrifuge “Rotanta 46 RSC”	Hettic AG, Bäch, Switzerland
Centrifuge “Heraeus Pico 21”	Thermo Fisher Scientific, Waltham, USA
Cytofluorometer “FACS Calibur”	BD Biosciences, Heidelberg, Germany
Cytofluorometer “LS Fortessa”	BD Biosciences, Heidelberg, Germany
Freezer -20°C	Liebherr, Bilberach an der Riss, Germany
Freezer -80°C “Hera freeze”	Heraeus Holding, Hanau, Germany
Fridge	Bosch, Gerlingen-Schillerhöhe, Germany
Gel documentation system	Vilber Lourmat, Eberhardzell, Germany
Gel electrophoresis chambers	Peqlab Biotechnologie, Erlangen, Germany
Glassware	Schott AG, Mainz, Germany

Irradiation device Gammacell 40 (Cs-137)	Atomic Energy of Canada Limited, Ottawa, Canada
Ice machine AF 200	Scottsman, Milan, Italy
Incubator CO_2 O_2 control	Heraeus Holding, Hanau, Germany Binder, Tuttlingen, Germany
Inverted microscope Axiovert 25	Zeiss, Jena, Germany
Laminar flow hoods	BDK, Sonnenbühl-Genkingen, Germany
LightCycler 480	Roche, Basel, Swizerland
Magnetic stirrer "IKAMag REO"	IKA®-Werke GmbH, Staufen, Germany
Microwave "compact microwave oven"	Reston Lloyd, Sterling, USA
Nanodrop ND-1000 spectrophotometer	Peqlaq Biotechnologie, Erlangen, Germany
Neubauer counting chamber	Paul Marienfel, Lauda-Königshofen, Germany
PCR-machine "Biometra® Tgradient"	Biometra, Göttingen, Germany
pH Tester (HI98108, pHep®)	Hanna® Instruments, Smithfield, USA
Pipetboy acu	INTEGRA Biosciences, Fernwald, Germany
Plasticware	Brand, Wertheim, Germany
PowerPac 200 power supply	Bio-Rad, München, Germany
Precision balance "SPB 55"	Scaltec instruments, Göttingen, Germany
Pipettes (0.1-2 µL 2-20 µL, 20-200 µL, 100-1000 µL)	Gilson, Mettmenstetten, Switzerland
Thermomixer compact	Eppendorf, Hamburg, Germany
Transferpipette (2.5-25 µL, 20-200 µL)	Brand, Wertheim, Germany
Tumble roller mixer TRM-50	IDL GmbH, Nidderau, Germany
Ultra pure water device "Aquintus"	membraPure, Hennigsdorf, Germany
Universal Microplate reader EL-800	BIO-TEK Instruments, Winooski, USA
Vacuum pump	Fröbel, Labortechnik, Lindau, Germany
Vortex mixer, VM-300	NeoLab, Heidelberg, Germany
Wallac Victor^2 1420 Multilabel-Counter	PerkinElmer, Waltham, USA
Water baths	GFL, Burgwedel, Germany

2.11 Databases and software

The IMGT database was used for this study for the identification of the CDR3 region of the sequenced δ chain of the TCR of γδ T cell clones (http://www.imgt.org/)

Software used for this study for designing experiments, data analysis and data illustration:

Name	Application	Source
Adobe® Illustrator® CS5	Illustration and graphic design	Adobe System Inc., San José, USA
Bookends 11.3.7	Reference management	Sonny Software, Chevy Chase, USA
Cell Quest™ Pro	FACS analysis	BD Biosciences, Heidelberg, Germany
Diva Software	FACS analysis	BD Biosciences, Heidelberg, Germany
FlowJo 9.4.11	FACS analysis	TreeStar Inc., Ashland, USA
KC4	ELISA measurement, calculation of standard curves	TreeStar Inc., Ashland, USA
LC480 Software Service Pack 3	Relative quantification for quantitative PCR	Roche, Basel, Switzerland
MacVector 12.7.5	Nucleotide and protein sequence analysis and alignment	MacVector Inc., Cary, USA
Microsoft® Word 2011	Writing and work processing	Microsoft, Redmond, USA
Microsoft® Excel 2011	Data analysis	Microsoft, Redmond, USA

2.12 Services

The EBV and HCMV IgG serostatus of the blood donors was determined by the Max von Pettenkofer-Institute, München, Germany.

High or low resolution HLA typing of blood donors was performed on PBMCs using PCR-based methods by IGMG/Labor Dr. Klein und Dr. Rost, Martinsried, Germany.

3 Methods

3.1 Molecular biology methods

3.1.1 Agarose gel electrophoresis

PCR products were separated and visualized according to their size by agarose gel electrophoresis. 1-1.5% agarose gels were prepared in 1x TAE buffer. For visualization, ethidium bromide was added at the final concentration of 0.1 μg/mL. 1x TAE was used as running buffer. The nucleic acid was mixed with 6x loading dye (Fermentas) prior to loading onto the agarose gel. The molecular weight marker GeneRuler^{TM} DNA Ladder Mix (Fermentas) was used to verify the size of the DNA fragments. Gels were documented by ultraviolet (UV) light irradiation (gel documentation system, Vilber Lourmat)

3.1.2 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was used to detect the presence of endogenous EBV in CD40L-stimulated B-blasts and in mutant LCLs. DNA extraction from these cells was performed with the aid of the DNA extraction kit QIAamp® DNA Blood Mini Kit (Qiagen). PCR reactions were carried on in 0.5 μL tubes in a total volume of 25 μL, using the Taq Polymerase (NewEngland BioLabs) with the supplied buffer. A standard reaction mix contained 100-150 ng DNA template, 2.5 μL of the 10x Taq Buffer, 10 pmol of each primer, 0.5 μL dNTPs (10 mM, Fermentas) and 0.2 μL of the Taq Polymerase. Sterile water was added up to 25 μL of final the reaction volume. A standard PCR program included initial denaturation at 95°C for 45 seconds, followed by 40 cycles of (i) denaturation at 95°C for 45 seconds, (ii) annealing of the primers at the primer-specific temperature for 45 seconds, and (iii) product elongation at 68°C according to the expected product size (Taq polymerase elongates at 1000 bp/min). A final step of 5 minutes at 68°C was performed to enable the completion of abortive elongation reactions.

The amplification of cDNA from γδ T cell clones to be sent for sequencing was performed in a similar fashion, but with the use of the Pfu DNA Polymerase (Thermo

Scientific) with the supplied buffer. Pfu DNA Polymerase is proof-reading and shows a reduced error rate. For the reaction mix, 1 µL of cDNA product, 4 µL of the 10x Pfu Buffer, 10 pmol of each primer, 0.5 µL dNTPs (10 mM, Fermentas) and 0.5 µL of the Pfu Polymerase were used. Sterile water was added up to 40 µL of the final the reaction volume. A standard PCR program included initial denaturation at 94°C for 2 minutes, followed by 40 cycles of (i) denaturation at 94°C for 30 seconds, (ii) annealing of the primers at the primer-specific temperature for 30 seconds, and (iii) product elongation at 72°C according to the expected product size (Pfu polymerase elongates at 500 bp/min). A final step of 10 minutes at 72°C was performed to enable the completion of abortive elongation reactions. Out of this reaction, 10 µL were used to control the efficiency of the reaction by agarose gel electrophoresis and the remaining 30 µL were sent for DNA sequencing after purification with the kit NucleoSpin® Gel and PCR Clean-up (Qiagen).

3.1.3 Reverse transcriptase-PCR

Total RNA was extracted from LCLs or from γδ T cell clones with the aid of the RNeasy® Mini Kit (Quiagen) according to the manufacturer instructions. cDNA synthesis from RNA was performed by Quantiscript Reverse Transcriptase with the QuantiTect® Reverse Transcription Kit (Quiagen). Usually, 1 µg of RNA was used for reverse transcription.

3.1.4 Quantitative PCR

Quantitative PCR was performed on 96-well plates in a LightCycler 480 (Roche). PCR products were detected with the aid of SYBR Green LC480 Mix (Roche). SYBRGreen is an intercalating marker: intercalation into double-strand DNA results in fluorescence. Three technical replicates were performed for each sample, as well as for a negative control free of template to identify possible contaminations. Total reaction volume of 10 µL contained 5 pmol of each primer, 2 µL of diluted cDNA template and 5 µL of 2x SYBRGreen reaction mix. cDNA stocks was diluted at least 5-fold with sterile water because of PCR efficiency. Relative mRNA expression of other genes was expressed in proportion to the housekeeping gene β-Glucuronidase (GUSB) (de Brouwer et al. 2006; Iskra et al. 2010).

A standard qPCR program included initial denaturation at 95°C for 10 minutes, followed by 45 cycles of (i) denaturation at 95°C for 10 seconds, (ii) annealing of the primers at 60°C for 10 seconds and acquisition of fluorescence, and (iii) product elongation at 72°C for 10 seconds. The 45 cycles were followed by 5 seconds at 95°C and 10 seconds at 65°C for the dissociation and annealing of the DNA strands before the quality control step. Afterwards, continuous heating to 95°C was performed with the acquisition of fluorescence (melting curve) to verify the amplification of only one DNA product.

3.2 Cell culture methods

3.2.1 Cell culture conditions

All eukaryotic cells used in this study were cultivated in an incubator with humidified atmosphere at 37°C and 5% CO_2. Handling of cells was carried out in laminar flow hoods.

Standard cell culture medium was RPMI 1640 with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 100nM sodium selenite (ICN).

Stimulator cell line LL8 stably expressing the human CD40 ligand was generated by stable transfection of L929 mouse fibroblasts with an expression plasmid for human CD40 ligand carrying a G418-selectable marker, followed by two rounds of single-cell cloning under selection. LL8 was cultivated until confluence and splitted once a week by washing twice with PBS and detachment with 0.5% Tryspin/EDTA for 7-10 minutes at room temperature. Detached cells were pelleted by centrifugation and replated at a 25-50-fold lower cell number in fresh standard cell culture medium.

Suspension cells (LCLs, K562, Daudi, Raji) were cultivated in flasks and expanded once or twice a week as necessary.

293HEK-EBV derived lines were cultivated in standard cultivation medium with the addition of 100 µg/mL hygromycin B to preserve the stably transfected maxi-EBV plasmid. At confluence, cells were splitted once a week by washing twice with PBS.

Cells were detached with 0.5% Tryspin/EDTA for 7 minutes at 37°C. Detached cells were pelleted by centrifugation and expanded approximately 20-fold in fresh standard cell culture medium with the addition of hygromycin B.

In general, cells were pelleted by centrifugation for 8-10 minutes at 12000 rpm at room temperature.

Cell count was determined using a Neubauer counting chamber. Before counting, cell suspensions were diluted 2-10 fold with 0.5% trypan blue solution in order to visualize dead cells. Cells with intact cell membrane are not permeable to the negatively charged trypan blue. By contrast, dead cells and debris are stained by the dye and appear blue under the microscope.

3.2.2 Cyopreservation of cells

Long-term preservation of eukaryotic cells was achieved by storing the cells in the presence of dimethyl sulfoxide (DMSO) in the gas phase over liquid nitrogen. Cells were pelleted by centrifugation and resuspended in a cold solution containing 45% RPMI, 45% FCS and 10% DMSO. Cell suspensions were aliquoted in 1.8 mL cryovials, pre-cooled on ice and stored as soon as possible at -80°C. After a few days the aliquots were transferred to the liquid nitrogen tank.

Frozen cells were thawed as quickly as possible by adding pre-warmed standard cell culture medium to the cryovial. The DMSO-containing solution was discarded by washing the cells once with 20-25 mL of medium. After centrifugation, the cell pellet was resuspended in the required medium and cells were taken into culture.

3.2.3 Isolation of peripheral blood mononuclear cells

Peripheral blood from healthy donors was collected and directly heparinized. A maximum of 25-30 mL of blood was distributed into 50 mL Falcon tube and diluted with sterile PBS until reaching 40 mL of volume. Slowly, 10 mL of Pancoll were added to the diluted blood by layering it at the bottom of the tube. Samples were centrifuged at 2000 rpm for 25 minutes without acceleration and without brake. The Pancoll

solution allows the separation of mononuclear cells (PBMCs) from red blood cells and granulocytes. During centrifugation, PBMCs collect at the interface between the Pancoll and the plasma. After discarding part of the plasma/PBS phase, PBMCs were carefully recovered avoiding the uptake of the Pancoll and transferred to a new 50 mL Falcon. Recovered PBMCs were washed 3-5x with sterile PBS, counted and resuspended in medium for further uses.

3.2.4 Virus production

Virus-producing cell lines for recombinant EBV 2089 (EBV WT), derived from EBV strain B95.8 (Delecluse et al. 1998), for its ΔLMP2A-deleted derivative EBV 2525 (Mancao & Hammerschmidt 2007) and for the virus-like particle TR-EBV strain (Delecluse et al. 1999) were provided by Wolfgang Hammerschmidt . Virus-producing cell lines were cultured in 15-cm dishes and reached 70% density before replacing the standard cultivation medium with 13 mL Opti-MEM (Invitrogen). PEI diluted 1:1000 (w/v) in sterile water was used for transfection and 120 µL PEI was diluted in 1 mL Opti-MEM, stirred vigorously, incubated for maximum 5 minutes at RT. Per 15-cm dish of 293HEK-derived EBV producer cell lines, the viral lytic cycle was induced by transient transfection of 10 µg each of the expression plasmids coding for transcription factor BZLF1 (p509) and glycoprotein gp110/BALF4 (p2670), diluted in 1 mL Opti-MEM. The PEI/Opti-MEM solution was incubated with the DNA/Opti-MEM solution for 20 minutes at RT and applied dropwise to the cells. Incubation of the cells with the PEI/DNA solution was performed overnight or for at least 6 hours. On the following day, the cells were supplied with standard full RPMI media. EBV-containing supernatant was harvested three days later, centrifuged to reduce cellular debris, filtered (0.8 µM), and stored at 4°C. Titer of infectious virus was determined by infecting Raji cells for three days and quantifying GFP-positive cells by flow cytometry as described (Altmann & Hammerschmidt 2005). Infection of B cells was performed at 0.1 virus units per cell.

The marmoset-derived lymphoblastoid cell line B95.8 spontaneously produces EBV in significant amount (Miller & Lipman 1973). Thus, virus containing supernatant was harvested from B95.8 cultures, cell debris were eliminated by centrifugation and filtration (0.8 µM), and virus containing supernatant was stored at 4°C.

3.2.5 Establishment of permanent B cell lines

CD40-stimulated and lymphoblastoid cell lines (LCLs) were established from primary B cells purified from freshly isolated PBMCs. Untouched B cells were negatively isolated using Human B Cell Isolation Kit II (Miltenyi Biotec). Enrichment of B cells was verified by flow cytometry (anti-CD19 clone HIB19; anti-CD3 clone HIT3a) and was in the range of 95-98%. B cells were plated at 100.000 cells/well in 24-well plates on an adherent cell layer of irradiated (180 Gy) CD40 ligand-expressing LL8 cells in standard medium supplemented with 1 mg/mL cyclosporine A to inhibit possible residual T cell activity. Cyclosporin A was kept in the culture up to 4-5 weeks after infection/stimulation. In case of the establishment of CD40-stimulated B cell lines, IL-4 was added to the culture at a concentration of 2.5 ng/mL, and cells were replated weekly onto a fresh layer of LL8 cells with expansion as necessary. For the establishment of LCLs, B cells were infected with 0.1 virus units per cell. Half of the culture supernatant was exchanged at day 1 post infection to diminish possible cell death due to toxicity of viral supernatant. Outgrowing cultures were transferred after 1-2 weeks to a fresh plate, and further cultivated without LL8 cells. Presence of mutant EBV in the LCLs and absence of endogenous EBV wild type in the LCLs and CD40L-stimulated B blasts were confirmed by PCR every few weeks. Proliferating lines were analyzed and used in T cell assays between 1.5 and 4 moths after establishment.

3.3 T cell clones

3.3.1 Generation of EBV-specific and HCMV-specific CD8+ T cells clones

EBV-specific T cells were directly isolated from PBMCs of EBV-seropositive, HLA-typed donors after stimulation with a matched peptide using the IFN-γ secretion assay (Miltenyi Biotec), according to the manufacturer instructions. For single T cell cloning, isolated IFN-γ-secreting cells were seeded into round-bottom 96-well plates at 0.7 or 2.5 cells per well in 200 µL of medium supplemented with 1000 U/mL rIL-2, 10^5/mL irradiated (50 Gy) HLA-matched LCLs, and 1.5×10^6/mL of a mixture of irradiated (50 Gy) allogeneic PBMCs from at least three different donors. Outgrowing T cell clones were expanded in round-bottom 96-well plates by restimulating every 2 weeks under

the same conditions. When necessary, cells were splitted and medium was exchange. Monoclonality of the cultures was tested by flow cytometry.

3.3.2 Generation of monoclonal γδ T cell lines

Monoclonal γδ T cell lines were established using the limiting dilution method. Freshly isolated PBMCs were used to isolate γδ T cells with the aid of the TCRγ/δ+T cell Isolation Kit – human (Miltenyi Biotec) for purification of untouched γδ T cells. The instructions of the manufacturer were followed. Efficiency and purity of the separation was verified by flow cytometry and usually was above 95%. Subsequently, the purified γδ T cells were plated on a U-bottom 96-well plate at a concentration of 0.5 cells/well, together with irradiated (50 Gy) PBMCs from 3 different donors at a concentration of $1x10^6$ cells/mL, irradiated (50 Gy) LCLs from 3 different donors at a concentration of $0.2x10^6$ cells/mL and 500 U/mL IL-2, in order to stimulate γδ T cell growth. After two weeks, half of the medium was exchanged. Single cell clones started to outgrow after 3-4 weeks after the first stimulation. Every 3 weeks, γδ T cell clones were restimulated under the same conditions. When necessary, cells were splitted and medium was exchange. Monoclonality of the cultures was tested by flow cytometry.

3.4 Immunological methods

3.4.1 ELISA for quantification of cytokine release

For IFN-γ ELISA, effector cells (25.000 cells/well) and target cells (50.000 cells/well) were co-cultivated in 200 µl/well in a V-bottom 96-well plate at 37°C and 5% CO_2. For IL-10 ELISA, LCLs were plated at a concentration 500.000 cells/mL in a 12-well plate or in a V-bottom 96-well plate and incubated at 37°C and 5% CO_2. In both cases supernatants were harvested after 16-18 hours. ELISA was performed according to the manufacturer's instructions (Mabtech, Nacka, Sweden). Antibodies were diluted in sterile PBS w/o Mg2+ and added to the plates in 50 µL/well. Plates were rinsed 4-5 times with PBS + 0.05% Tween. Unless otherwise noted, all incubation steps were carried out at room temperature. ELISA plates (Nunc MaxiSorp Immunoplates) were coated with a monoclonal „capture“ antibody (2 µg/mL) specific for the appropriate cytokine by overnight incubation at 4°C. The following day, plates were rinsed to wash away unbound antibody and unspecific binding sites were blocked by incubation with

200 µL standard culture medium for 30 minutes. After washing, 50 µL of the co-culture supernatant to be analyzed was applied to the plates for 2 hours, to allow cytokines to bind to the specific antibody. For quantification purposes, a cytokine standard was prepared and incubated in parallel on each plate. Plates were washed and incubated with a second cytokine-specific biotinylated antibody (1 µg/mL) for 1 hour. The antibody „sandwich“ was labeled by incubation for 1 hour with Streptavidin-ALP (diluted 1:1000), which binds to the antibody sandwich via biotin. Detection was achieved by applying 50 µL/well ALP substrate solution containing *para*-nitrophenylphosphate (*p*-NPP). ALP catalyzes the hydrolysis of colorless *p*-NPP to the yellow *p*-nitrophenol. The ALP substrate solution was prepared immediately before use by 5-fold dilution in sterile water of the ALP stock solution (50% diethanolamine and 5 mg/ml *p*-NPP in sterile water at pH 9.5) with the addition of the ALP cofactor Mg2+ (MgSO4) in final concentration of 0.8 mM. The presence of *p*-nitrophenol was detected with a spectrophotometer at 405 nm. The standard curve for the calculation of the concentration in each sample was performed with the KC4 software (TreeStar Inc.).

3.4.2 Cytotoxicity assay

The cytotoxic activity of γδ T cells against target cells was measured by calcein-release assay. Target cells (2x10^6 cells) were labeled with 5 µg/mL calcein acetoxymethylester (Invitrogen) in 1 mL medium. After incubation for 30 minutes at 37°C, labeled cells were washed 3-4 times with sterile PBS in a 15 mL Falcon tube. Following, this 5000 target cells/well were plated in V-bottom 96-well plates (200 µL/well). For each target cell type, spontaneous release (no effector cells, 0% lysis) and maximum release (addition of 0.5% of Triton-X 100, 100% lysis) was determined. Effector cells were counted, washed once and plated at the appropriate target:effector ratio (usually 1:10, see figure legends) and co-incubated with target cells for 3 hours at 37°C and 5% CO2. Thereafter, 150 µl of supernatant were collected and transferred to a fresh flat bottom 96-well plate. Fluorescence intensity at 485/535 nm (excitation/emission) was measured in a Wallac Victor^2 counter (Perkin-Elmer).

3.4.3 Blocking studies

To evaluate the role of different receptors in CD8+ T cell and in γδ T cell effector function activities (cytokine release, cytotoxic lysis), the interaction between the given receptor and its ligand(s) was blocked by specific purified antibodies. Each antibody was added either to the target or to the effector cells at a pre-established concentration and incubated for 1 hour at 37 °C, 5% CO_2 prior to the addition of the effector or target cells. As a control, matched isotype controls were used.

Specificity	Clone	Isotype	Purchased from	Final conc
NKG2D LEAF	1D11	IgG1, mouse	Biolegend, San Diego, USA	50 μg/ml
isotype LEAF	MOPC-21	IgG1, mouse	Biolegend, San Diego, USA	50 μg/ml
HLA-I LEAF	W6/32	IgG2a, mouse	Biolegend, San Diego, USA	100 μg/ml
isotype	MPC-11	IgG2a, mouse	Biolegend, San Diego, USA	100 μg/ml
γδ-TCR LEAF	B1	IgG1, mouse	Biolegend, San Diego, USA	50 μg/ml
isotype LEAF	MOPC-21	IgG1, mouse	Biolegend, San Diego, USA	50 μg/ml
IL-10R	3F9	IgG2a, mouse	Biolegend, San Diego, USA	20 μg/ml
isotype	RTK2758	IgG2a, mouse	Biolegend, San Diego, USA	20 μg/ml
Il-10	JES3-9D7	IgG1, mouse	Biolegend, San Diego, USA	20 μg/ml
isotype	RTK2071	IgG1, mouse	Biolegend, San Diego, USA	20 μg/ml
HLA-A2	BB7.2	IgG2b, mouse	Biolegend, San Diego, USA	50 μg/ml
isotype	MPC-11	IgG2b, mouse	Biolegend, San Diego, USA	50 μg/ml

LEAF indicates low-endotoxin, azide-free

3.5 Phenotypical analysis of cells by flow cytometry

3.5.1 Staining of cell surface antigens

To measure the expression of different surface molecules, cells were stained with specific antibodies. Generally, between 100.000 and 200.000 cells were stained in a V-bottom 96-well plate or in a 1.5 mL reaction tube (Eppndorf). All centrifugation steps were carried out in a centrifuge suitable for 96-well plates at 1200 rpm for 5 minutes at 4°C or in a table centrifuge at 3200 rpm for 4 minutes at 4°C. Pre-cooled buffer (PBS + 2% FCS) was used as washing buffer. If not stated otherwise, incubation with the

antibody diluted in buffer was always carried out in a final volume of 20 µl. Each antibody was used at the appropriate concentration, according to manufacturer´s instructions or previous titration analysis. Then, cells were incubated for 20 minutes on ice, washed (twice with 200 µL buffer in case of the V-bottom 96-well plate, once with 1.5 mL buffer for the 1.5 mL tube), resuspended with the suitable volume of buffer, usually 100 µL, and directly analyzed. In case the sample was to be analyzed during the following days, cells were resuspended in fixing solution (PBS + 1% Paraformaldehyde). Fixed samples were stored in the refrigerator at 4-6°C for up to one week.

In the case of unlabeled antibodies, cells were first stained with the unlabeled antibody by incubation for 20 minutes on ice, followed by a washing step (three times with 200 µL buffer in case of the V-bottom 96-well plate, twice with 1 mL buffer for the 1.5 mL tube). The sample was then incubated again for 20 minutes on ice with a labeled secondary antibody specific for the Fc part of the primary antibody. If necessary, following the washing step (three times with 200 µL buffer in case of the 96-well plate, twice with 1 mL buffer for the 1.5 mL tube), the sample was incubated for a third step with a mix of labeled antibodies specific for other antigens. This last incubation step was followed by another washing step (twice with 200µL buffer in case of the 96-well plate, once with 1.5 mL buffer for the 1.5 mL tube) and the resuspension in a suitable volume of buffer. Cells were analyzed on a BD Bioscience FACS Calibur or on a LS Fortessa flow cytometer (BD Biosciences). Data analysis was performed using the FlowJo software (TreeStar Inc.).

3.5.2 Peptide/HLA class I multimer staining

Monoclonality of EBV- and HCMV-specific CD8+ T cells was confirmed *ex vivo* using the MHC class I multimer technology. This method uses the natural ligand for a given TCR, the MHC-I:peptide complex, to stain T cells and evaluate their epitope specificity. To increase the avidity of this interaction, multimerization of MHC-I:peptide complexes to tetramers or pentamers is used.

200.000-300.000 T cells were first stained with 1 µL unlabeled multimer for 10 minutes at room temperature in a final volume of 30 µl in a V-bottom 96-well plate.

After two rounds of washing with 200 µL staining buffer (PBS+2%FCS), cells were incubated with Fluorotag-PE for 20 minutes on ice in a volume of 30 µl, followed by two times washing with 200 µL buffer. In case cells were to be stained with labeled antibodies, the sample was then incubated with the mix of labeled antibodies in 20 µL volume for 20 minutes on ice. The sample was washed once with 200 µL buffer and resuspended in a suitable amount of buffer for analysis.

CRV-specific CD8+ T cells were stained using an MHC-StrepTactin multimer reagent (Streptamer) as described (Knabel et al. 2002). CRV/HLA-C*0702 MHC-I:peptide monomers were generated and provided by Dr. Michael Neuenhahn and Prof. Dirck Busch (Institute for Medical Microbiology, Immunology and Hygiene, Technische Universität München). Multimerization of the streptamer complexes for stabilization of the interaction between the streptamer and the TCRs was achieved by incubating the monomers with PE-labeled Streptactin for 45 minutes on ice at molar ratios of MHC molecule/StrepTactin molecule 1:1. Cells were incubated with an aliquot of the assembled multimer containing 2.5 µg CRV/HLA-C*0702 monomer in a total volume of 30 µL staining buffer for 20 minutes on ice. In case cells were to be stained with labeled antibodies, the sample was incubated at the same time with the multimer and with the mix of labeled antibodies for 20 minutes on ice. The sample was washed once with 200 µL buffer and resuspended in a suitable amount of buffer for analysis.

4 Results

4.1 Modulation of the immunophenotype of B cells in cellular activation and EBV infection

Infection with a virus induces numerous changes in a cell. Once cells are infected, they alert the immune system of the infection and support the recognition and the elimination of the infected cells in various fashions. One way to signal the threat by infection is the modification of the cell surface levels of immunomodulatory molecules. On the other hand, viruses strive to reduce infection-related alterations that could be detected by the immune system. EBV represents an extreme case, because it triggers B cell activation pathways that usually render a B cell highly immunogenic, and it is only incompletely known whether it interferes with some of the signals that trigger responses of immune effector cells. Therefore, I investigated the immunophenotypic changes induced in B cells early after EBV infection (Figure 4.1). Freshly isolated peripheral blood mononuclear cells (PBMCs) were infected with the recombinant EBV "wild type" strain 2089 (Delecluse et al. 1998) (WT, Figure 4.1A and B) or with its parental EBV wild type strain B95.8 (Miller et al. 1972b) (WT, Figure 4.1C), and surface expression of immune effector molecules was analyzed by flow cytometry after staining with specific antibodies. Already at day 3 after infection, EBV-infected B cells upregulated the B7 family member CD86, ligand for the costimulatory receptor CD28 on T cells, and the adhesion molecule ICAM-1, important in the formation of the immunological synapse (Figure 4.1A). A stable increase of the surface expression of these two molecules was observed during the whole duration of the experiment. Surface expression of MICB, one of the ligands for the coactivatory receptor NKG2D on T and NK cells, reached its peak already at day 3 after infection, and was constant for all later time points (Figure 4.1A). Figure 1B and 1C show the surface expression at day 7 of the complete panel of analyzed molecules. Day 7 was chosen as representative of EBV-induced modifications, because at this time a dominant population of EBV-transformed/activated cells was present in the culture, as evidenced by cell enlargement and increased scatter in flow cytometry (Iskra et al. 2010). Infection with EBV was compared to pseudo-infection with virus-like particles (TR-) and to non-infected cells (Figure 4.1B), or only to non-infected cells (Figure 4.1C). The EBV mutant "TR-"

lacks the packaging signals (Terminal Repeats) (Delecluse et al. 1999), and upon induction of the lytic cycle of the packaging cell line, virus-like particles devoid of the viral DNA genome are produced. Of notice, the virus-like particles still contain some viral and cellular mRNAs (Jochum et al. 2012b). At day 7, we detected upregulation of the coinhibitory B7 molecule PD-L1, and downregulation of the costimulatory molecules ICOS-L, another B7 family member, and of CD48, ligand of the SLAM family member 2B4. However, surface expression of CD48 was decreased not only on EBV-infected cells but also on uninfected cells on day 7. I did not detect modification of surface expression of CD11a, a component of the integrin LFA-1, its counterpart

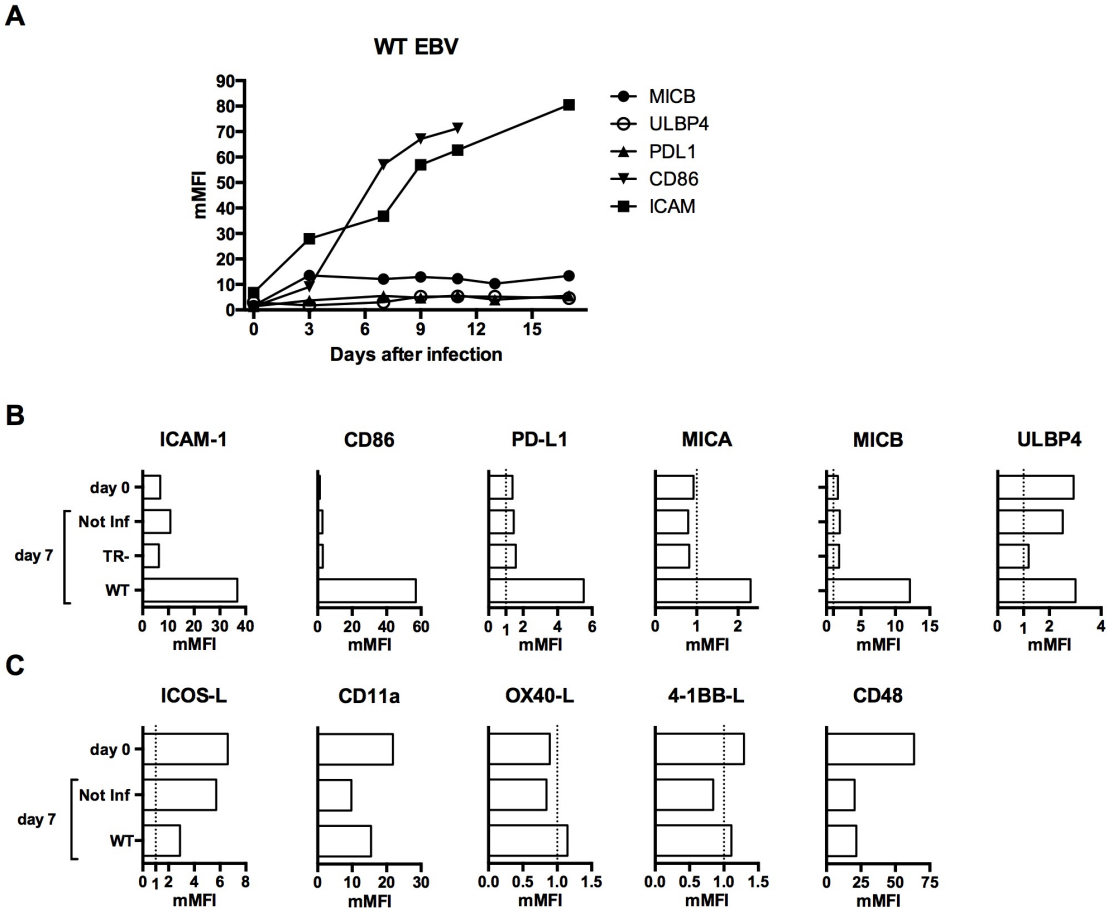


Figure 4.1. Induction of the surface expression of immunomodulatory molecules on B cells by EBV infection. (A) PBMCs (donor 2) were infected with recombinant EBV wild-type strain 2089 (WT), and expression of the indicated surface molecules on B cells was determined by flow cytometry after staining with specific monoclonal antibodies. Expression of the indicated surface molecules was determined at day 0, 3, 7, 9, 11, 13 and 17 after infection. (B, C) PBMCs (donor 2) were infected with EBV recombinant 2089 (WT in panel B) or native recombinant strain B95.8 (WT in panel C), EBV virus-like particles (TR-), or cultivated without EBV (Not Inf). Expression of the indicated surface molecules on B cells was determined at day 7 after infection by flow cytometry after staining with specific monoclonal antibodies and compared to expression at day 0 on untouched B cells. The multiplicity of mean fluorescence intensity (mMFI) was determined by dividing MFI of specific antibody staining by MFI of isotype control.

ICAM-1, or the coactivating members of the TNF superfamily OX40-L and 4-1BB-L. Interestingly, at later time points (day 9) I detected upregulation of the NKG2D ligand ULBP4 (Figure 4.1A). Expression of other NKG2D ligands, ULBP1, 2, 3, 5 and 6 was not detected, even at late time points (data not shown). With the exception of changes in CD48 expression, none of these modifications could be detected in non-infected cells or in cells infected with virus-like particles, suggesting that active EBV infection was responsible for the modification of the surface levels of these immunomodulatory molecules.

A number of recent reports suggested that EBV infection induces upregulation of ligands for the receptor NKG2D, which is important as a coactivator on T and NK cells (Pappworth et al. 2007; Kong et al. 2009; Wiesmayr et al. 2012; Chaigne-Delalande et al. 2013). Therefore, I analyzed the surface expression of all NKG2D ligands in a tighter time frame directly after infection, to determine the kinetics of regulation of these molecules (Figure 4.2). EBV infection of B cells induced upregulation of MICB already at day 1 after infection, and a moderate upregulation of ULBP1 at later time points. I could not observed upregulation of ULBP4, probably because the time points analyzed in this experiment were too early for its detection.

Subsequently, to determine whether the modifications observed in the surface expression of immunomodulatory molecules was specifically induced by EBV, I compared EBV infection to "classical" B cell activation. B cell activation through the BCR was achieved with $F(ab')_2$ fragments specific for human IgA, IgG and IgM

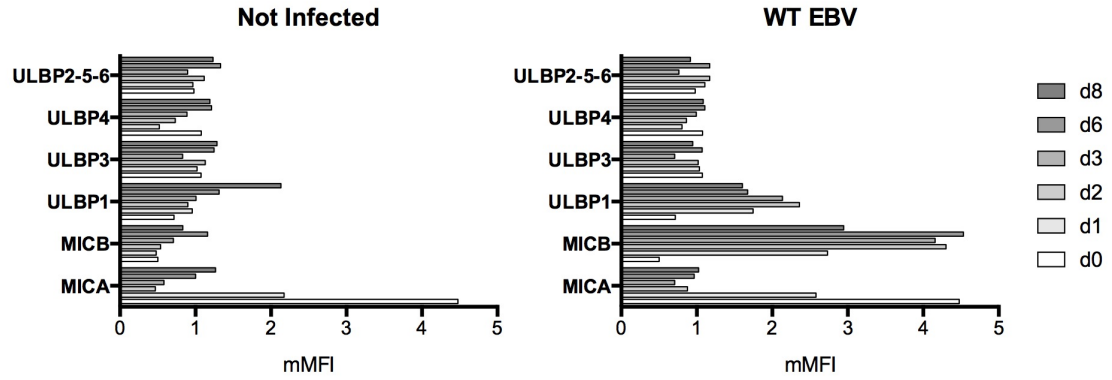


Figure 4.2. Surface expression of NKG2D ligands on B cells early after infection with EBV. PBMCs (donor 6) were infected with EBV wild type strain 2089 (WT EBV) or left untouched (Not Infected), and expression of the indicated NKG2D ligands on B cells was determined by flow cytometry after staining with specific monoclonal antibodies. Expression of the indicated molecules was determined at day 0, 1, 2, 3, 6 and 8 after infection. The multiplicity of mean fluorescence intensity (mMFI) was determined by dividing MFI of specific antibody staining by MFI of isotype control.

(αBCR, signal 1) (Ruprecht & Lanzavecchia 2006); coactivation by helper T cells was mimicked by plating B cells on a layer of murine fibroblasts overexpressing human CD40 ligand (CD40L) together with the addition of IL-4 (CD40L/IL-4, signal 2) (Wiesner et al. 2008); unmethylated CpG DNA oligonucleotide 2006 was added in

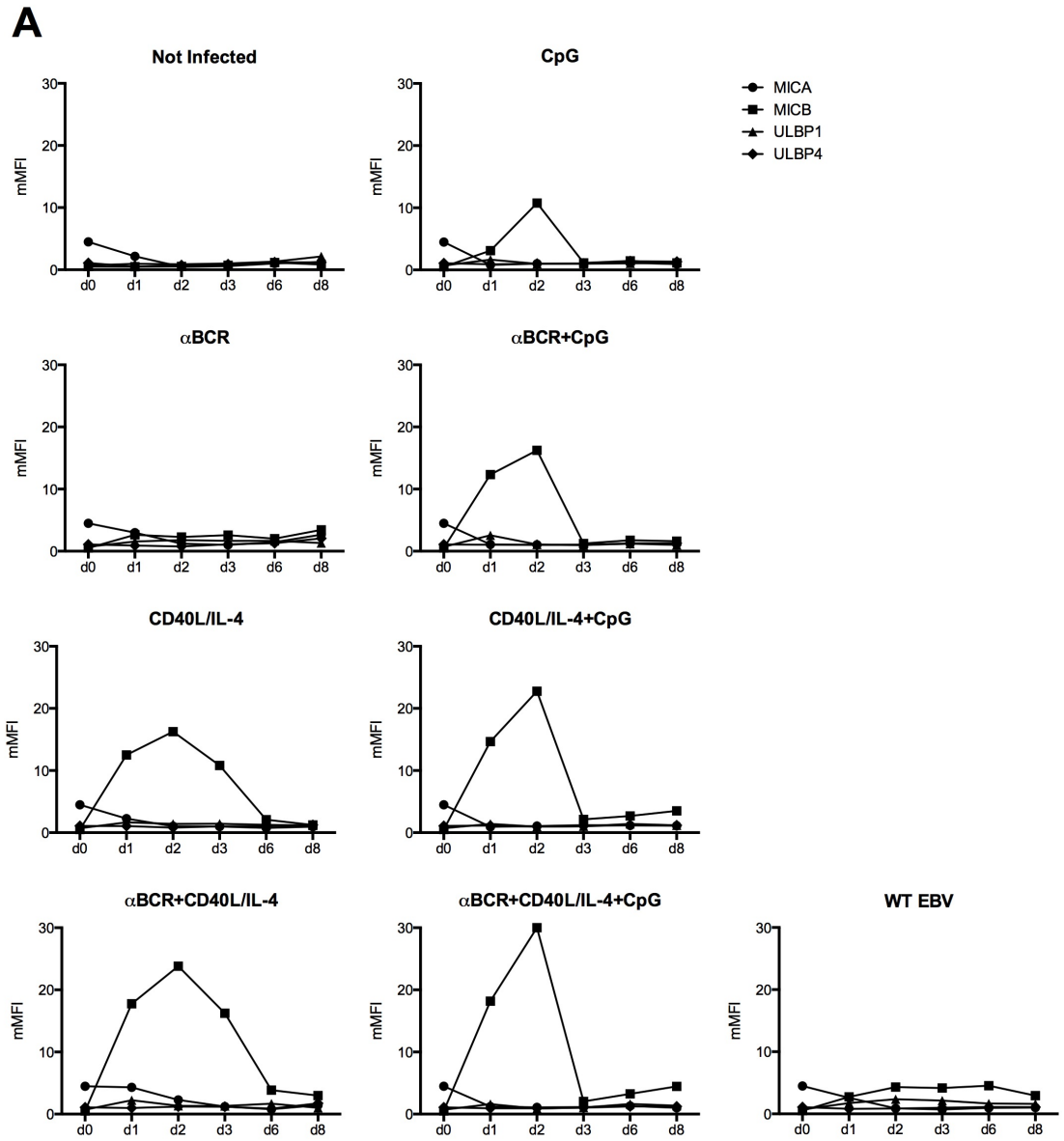
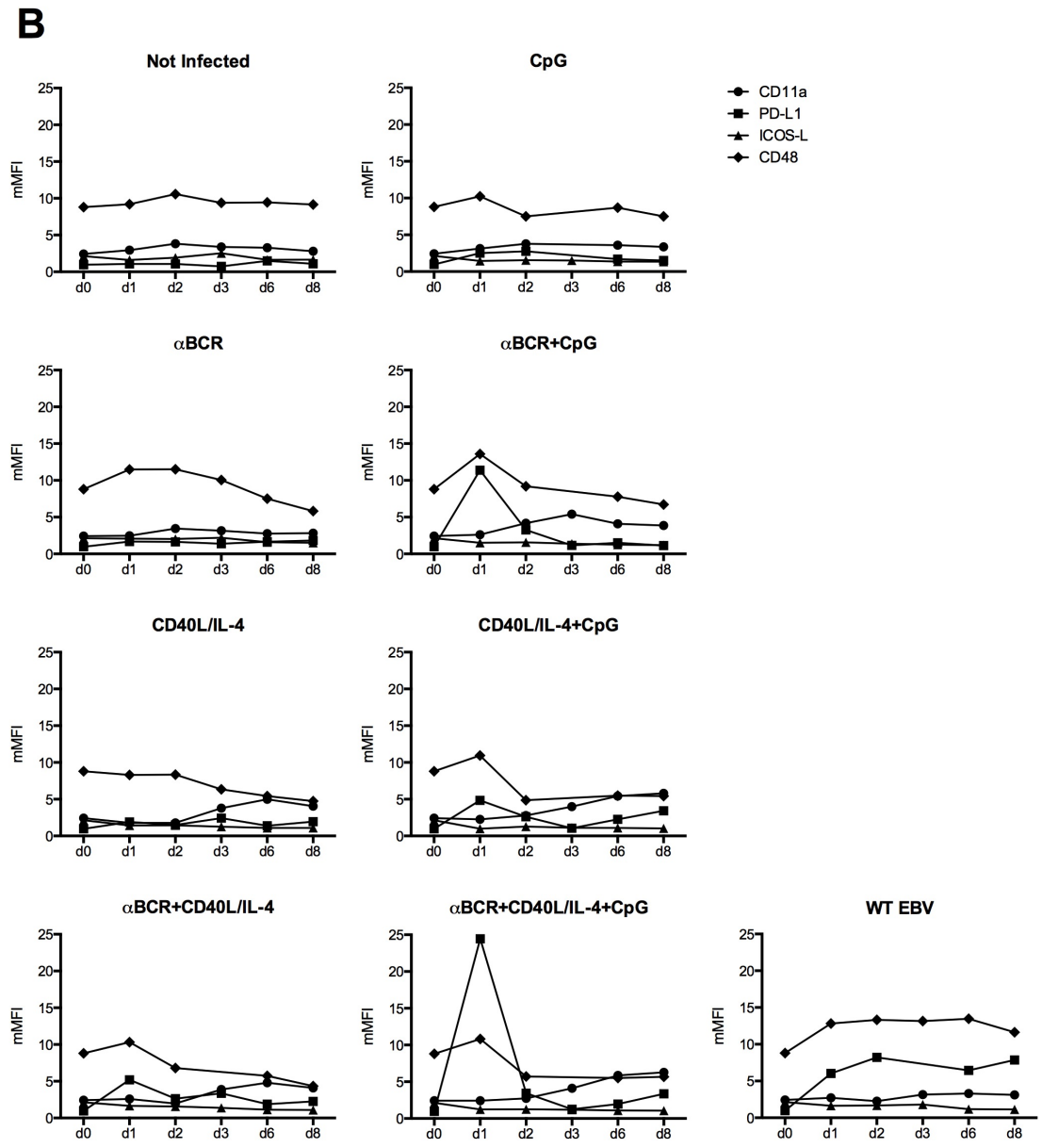
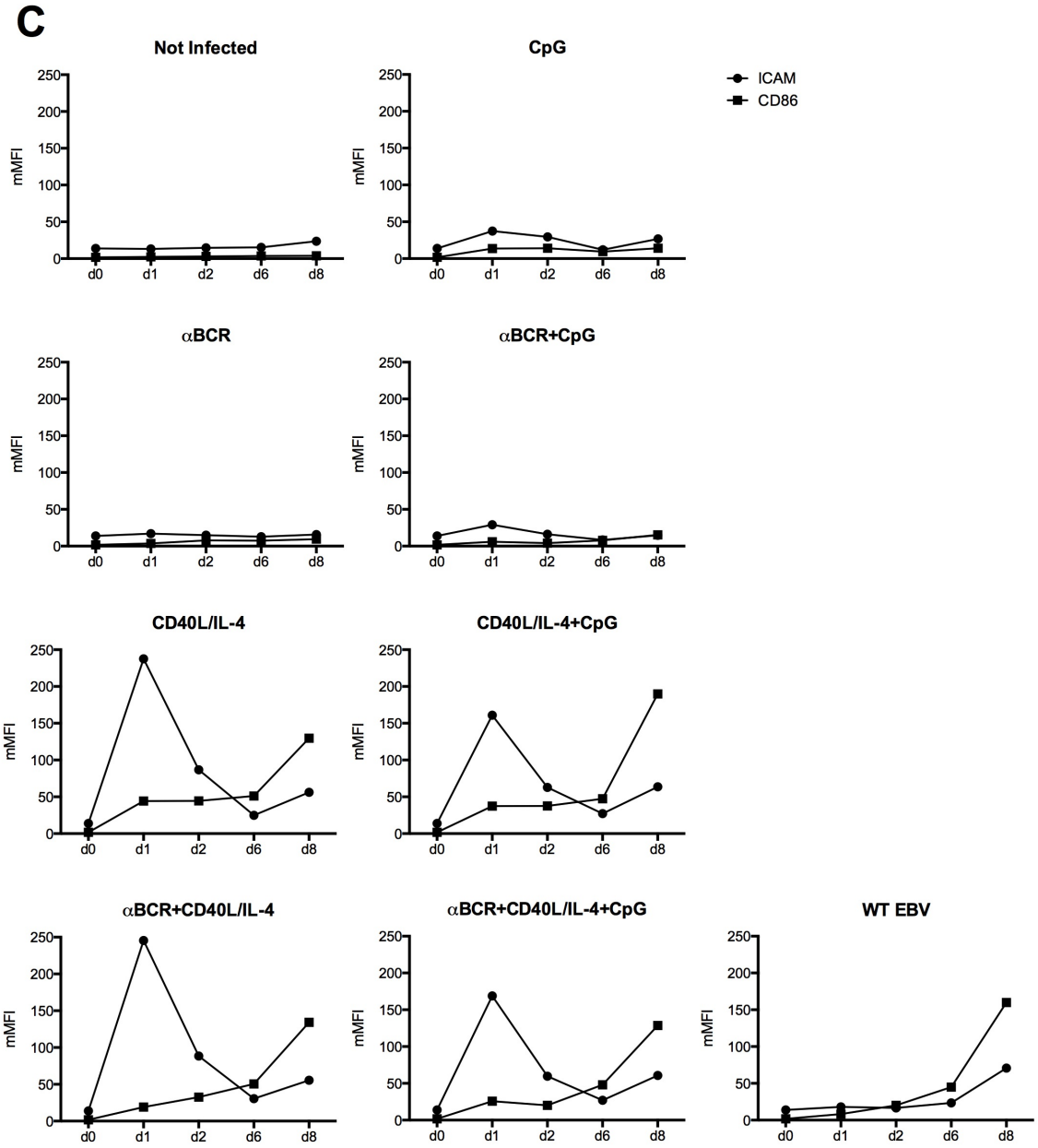


Figure 4.3. Induction of surface expression of agonistic or antagonistic molecules on B cells after B cell activation. PBMCs (donor 6) were activated with F(ab´)$_2$ fragments specific for human IgA, IgG and IgM to activate the BCR receptor (αBCR, signal 1 in B cell activation), with mouse L929 cells stably expressing human CD40L plus IL-4 (CD40L/IL-4, signal 2 in B cell activation), with CpG DNA to activate TLR9 (CpG, signal 3 in B cell activation), or with all possible combinations of these stimuli. These conditions were compared with infection with EBV WT strain 2089 or with cultivation of untouched, uninfected cells in standard medium alone (Not Infected). Expression of the indicated surface molecules on B cells was determined by flow cytometry after staining with monoclonal antibodies. Flow cytometric analyses were performed on day 0, 1, 2, 3, 6 and 8 after activation/infection. The multiplicity of mean fluorescence intensity (mMFI) was determined by dividing MFI of specific antibody staining by MFI of isotype control.

soluble form to activate toll-like receptor 9 (TLR9), a PRR receptor (CpG, signal 3) (Hartmann & Krieg 2000). These stimuli were used alone or in all possible combinations and were compared to infection with EBV WT 2089 (WT EBV) or to uninfected cells (Not Infected). Stimulation of B cells through the activation of different cellular mechanisms, induced upregulation of several immune effector molecules on the B cell surface. The NKG2D ligand MICB was most efficiently upregulated by CD40L/IL-4 alone, or by combinations of at least two stimuli (Figure 4.3A). The highest induction of this ligand was obtained on B cells stimulated with all three compounds. Induction was transient, reaching its maximum at day 2 after stimulation, and going back to near-basal levels later. I could not observe upregulation of any other NKG2D ligands (Figure 4.3A and data not shown). Activation of B cells



also induced a fast but transient expression of the inhibitory B7 family molecule PD-L1 (Figure 4.3B). Interestingly, to observe upregulation of this molecule, at least two stimuli were needed. Again, strongest expression was obtained when all three stimulatory signals were combined. It appeared that αBCR+CpG were more important for PD-L1 induction than CD40L/IL-4. In contrast, the stimulatory B7 molecule CD86 was induced by CD40L/IL-4 activation, while αBCR or CpG alone had no effect on the expression of this molecule. CD86 expression reached its maximum surface expression level at the last day of analysis, especially by combining CD40L/IL-4 with CpG stimulation (Figure 4.3C). In this case, combination of the three stimuli still resulted in upregulation of CD86, but the surface expression level reached for this molecule was



comparable to the induction by CD40L/IL-4 alone, suggesting that activation of the BCR partially counteracted the upregulation of this molecule induced by CD40L/IL-4+CpG. It is noteworthy that induction of CD86 by EBV is slightly lower during the first few days after infection, but reached levels similar to those during CD40L/IL-4+CpG stimulation at the last analyzed time point, day 8. For the other B7 molecule analyzed in this experiment, ICOS-L, I could not detect any relevant changes in its expression (Figure 4.3B).

In contrast to what was seen with EBV (Figure 4.1, Figure 4.3B), activated B cells showed upregulation of the integrin CD11a, which was delayed relative to most other induced markers. CD11a seemed to be induced in particular by CD40L/IL-4 or by the combinatory activation of two or more B-cell activatory pathways. The expression of its interaction partner, ICAM-1, followed a pattern of expression similar to PD-L1 (Figure 4.3C). Upregulation of ICAM-1 was strongly marked at day 1 after activation, but it was transient and quickly compressed already at day 2. In this case, strongest induction was observed by CD40L/IL-4 alone or in combination with αBCR, although the contribution of αBCR was small. EBV infection, on the other end, did not cause early induction of ICAM-1, but led to a slow progressive upregulation of this molecule. Expression of the SLAM family member CD48 showed only small changes after any kind of stimulus, with a small peak of expression on day 2 followed by a slow but stable decrease (Figure 4.3B). Modulation of OX40-L and 4-1BB-L expression levels was not observed (data not shown). Untouched B cells did not show any modulation of expression for any of the molecules analyzed in this experiment. It is interesting to notice that the stimulus that had the strongest effects on the largest number of immunomodulatory molecules was CD40L/IL-4. These results show that “classical” B cell activation induces the upregulation of a variety of immunomodulatory molecules often in a pattern different from EBV infection. Although there are several similarities in the expression patterns, EBV infection of B cells does not simply mimic cellular activation pathways, but induces in early-infected B cells a specific, virus-induced immunophenotype. In particular, suppression of MICB and ICAM-1 peak expression and induction of sustained PD-L1 expression are hallmarks of EBV infection compared to normal B cell activation. This observation is consistent with the hypothesis that EBV must modify the standard activation-induced gene expression program in infected cells in order to avoid being eliminated by immune effector cells.

4.2 LMP2A decreases recognition of infected cells by CD8+ T cells

To investigate the influence of LMP2A on the interaction between EBV-infected cells and cellular immunity, I established EBV-transformed B cell lines (LCLs) with a mutant EBV virus deleted for LMP2A (Mancao & Hammerschmidt 2007). This virus is deleted for the promoter and the first exon of LMP2A on the background of the EBV strain B95.8. Expression of LMP2B is still possible in this mutant. B cells were purified from freshly isolated PBMCs (Figure 4.4) and infected with the LMP2A-deficient virus (ΔLMP2A) and with the parental LMP2A-expressing virus, the wild type strain 2089 (WT). In line with previous findings (Brielmeier et al. 1996; Mancao & Hammerschmidt 2007), I found that the ΔLMP2A virus had reduced efficiency of B cell transformation (data not shown). From now on, all LCLs designated "WT" had been established with the recombinant 2089 strain that carries the complete B95.8 wild-type EBV genome. To facilitate the establishment of LMP2A-deficient LCLs, I infected primary B cells with both WT and mutant EBV in the presence of LL8 fibroblasts that overexpress human CD40L. Outgrowing B cell cultures were further expanded in the absence of these stimulator cells. Established LCLs were regularly

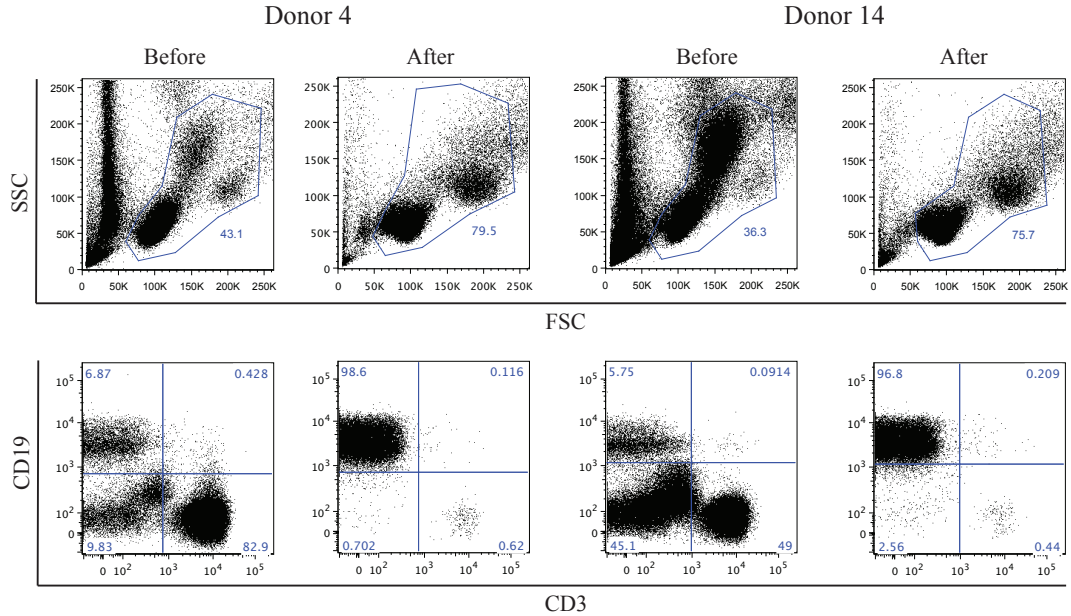


Figure 4.4. Purification of peripheral B cells. The flow cytometry plots show examples of the efficiency of B cell purification from PBMCs from two donors by immunomagnetic depletion of non-B cells (B Cell Isolation Kit II, Miltenyi Biotec). Mononuclear cells were identified in the SSC/FCS graph and gated to eliminate debris and dead cells. Within the lymphocyte population, B cells were identified as CD19+/CD3-. Purity of the purified B cell population reached always 95-99%. Before: PBMCs before purification; After: B cell fraction after purification. FSC, forward scatter; SSC, side scatter.

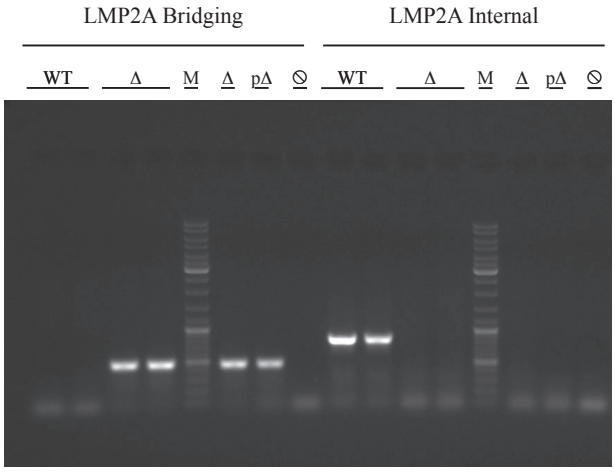


Figure 4.5. ΔLMP2A LCLs were tested by PCR for the presence of endogenous wild-type EBV. ΔLMP2A LCLs (Δ, in this example established from donor 9) were tested by PCR every few weeks to detect the presence/reactivation of endogenous EBV. LMP2A bridging PCR was performed using primers binding conserved sites flanking the LMP2A deletion; a 481 bp product was expected only in the presence of the mutant EBV genome. Efficient generation of a PCR product from WT genomes was not expected due to its large size. LMP2A internal PCR was performed using one primer binding in a region present in both the WT and ΔLMP2A genomes together with a primer binding inside the genome fragment deleted in the mutant. Therefore, a product of 831 bp would be expected only in the presence of wild-type EBV. DNA preparations from WT LCLs (WT) and from the packaging cell line (pΔ) for the ΔLMP2A strain (LMP2A$^-$ 2525 293HEK) were used as controls, together with a PCR reaction performed without added DNA (∅).

examined for the presence of the LMP2A deletion and for the absence of endogenous wild type EBV (Figure 4.5). Under these conditions, LCLs transformed with the WT or with the ΔLMP2A virus could be established with similar efficiency and did not exhibit differences in proliferation, cell density, survival, or longevity in culture (data not shown). This observation suggested that the presence of LMP2A was crucial for the proper establishment of EBV latent infection *in vitro*, but it was not essential for the growth of established LCLs.

I analyzed the reactivity of EBV-specific CD8+ T cells in response to WT and ΔLMP2A LCLs (Figure 4.6). Interestingly, CD8+ T cell clones specific for epitopes from all tested EBV latent antigens (EBNA1, EBNA3A, LMP2) showed a higher IFN-γ release in response to ΔLMP2A LCLs than to WT LCLs (Figure 4.6A). CD8+ T cells specific for the LMP2 epitope CLG recognized ΔLMP2A LCLs, as expected, because the CLG peptide is derived from a transmembrane region shared between LMP2A and LMP2B. In contrast, CD8+ T cell clones specific for lytic-cycle antigens (BRLF1, BZLF1) showed weak recognition of LCLs, and no difference in IFN-γ release could be detected after contact with WT or ΔLMP2A LCLs (Figure 4.6B). This is in accordance

with long-standing observations showing that LCLs express low to undetectable amounts of lytic-cycle genes (Pudney et al. 2005). Thus, LMP2A interfered with CD8+ T cell recognition of various EBV latent antigens, but was not able to detectably modulate the recognition of EBV lytic-cycle antigens by CD8+ T cells due to their low baseline level of recognition.

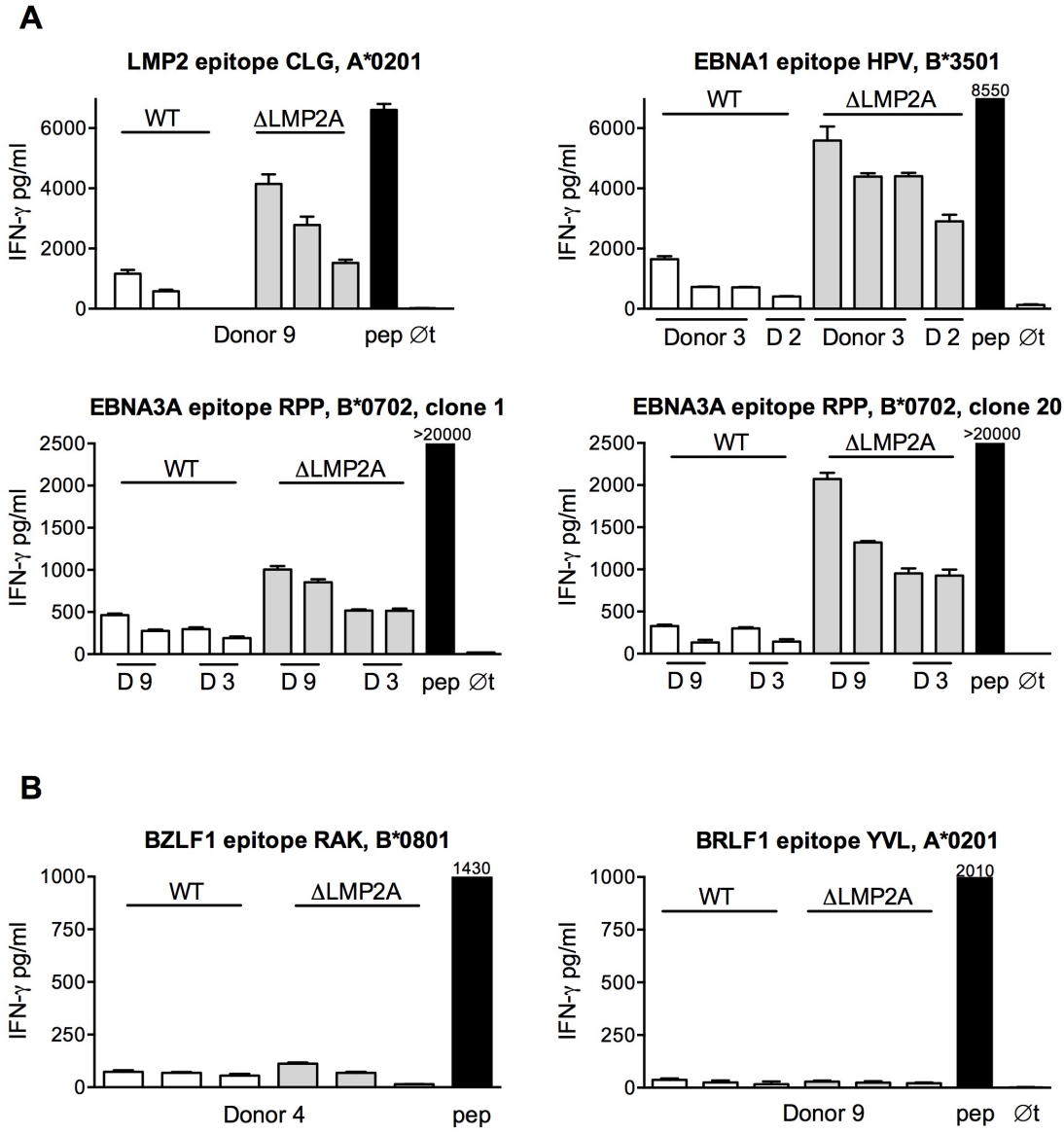


Figure 4.6. Recognition of EBV-transformed B cells by CD8+ T cells in the presence or absence of LMP2A. (A, B) CD8+ T cell clones specific for epitopes from EBV latent antigens (A) or EBV lytic-cycle antigens (B) were coincubated with HLA-matched LCLs that were infected with EBV expressing LMP2A (WT) or deleted for LMP2A (ΔLMP2A). After 16 hours, IFNγ release in the supernatant was quantified by ELISA. EBV epitope peptides are designated by their first three amino acids in one-letter code. "D", donor; "pep", WT LCLs exogenously loaded with corresponding peptide; "∅t", T cells alone. Data are represented as mean + standard error of the mean (SEM) for three replicates. Data are representative for two or more independent experiments performed for each T cell clone.

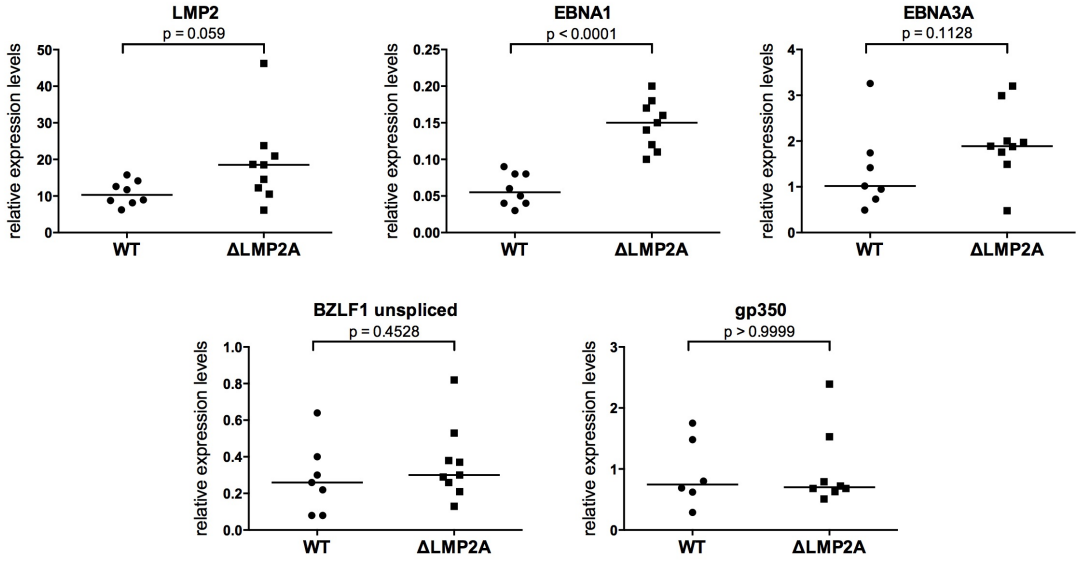


Figure 4.7. mRNA expression of EBV latent and lytic-cycle genes in LCLs in the presence or absence of LMP2A. Transcript levels of LMP2, EBNA1, EBNA3A (latent genes), BZLF1 and gp350 (lytic-cycle genes) were measured by quantitative RT-PCR in LCLs established with WT or ΔLMP2A EBV (donors 1, 3, 7, 9). Expression relative to transcript levels of the housekeeping gene β-glucuronidase (GUSB) is represented. Each dot represents a single measurement for a single cell line. Per condition, six to nine independently established LCLs from 4 different donors were analyzed. The horizontal line indicates the median. Statistical analysis was performed with the Mann-Whitney test.

Now I tested whether the observed difference in cell recognition could be related to mere differences in antigen expression between WT and ΔLMP2A LCLs. I quantitatively analyzed transcript levels of various latent and lytic EBV antigens in WT and ΔLMP2A LCLs from different donors (Figure 4.7). Independently generated LCLs showed some variation between each other in their expression of EBV transcripts, even though established with the same virus. On average, transcript levels of several EBV latent antigens (EBNA1, EBNA3A, LMP2) appeared to be increased in ΔLMP2A LCLs. However, this effect reached $p < 0.05$ only for EBNA1. No difference between WT and ΔLMP2A LCLs was seen for median expression of the lytic-cycle genes BZLF1 and gp350. These results suggest that LMP2A downmodulates the expression of latent antigens in EBV-infected B cells, in particular EBNA1. This effect may contribute to the reduced presentation of these antigens to CD8+ T cells. However, at least for latent antigens other than EBNA1, modulation of T cell recognition (Figure 4.6) appeared too intense to be likely explained by differences in antigen expression alone.

To investigate whether the differential reactivity of CD8+ T cells against B cells infected with WT or ΔLMP2A was mediated by mechanisms beyond availability of

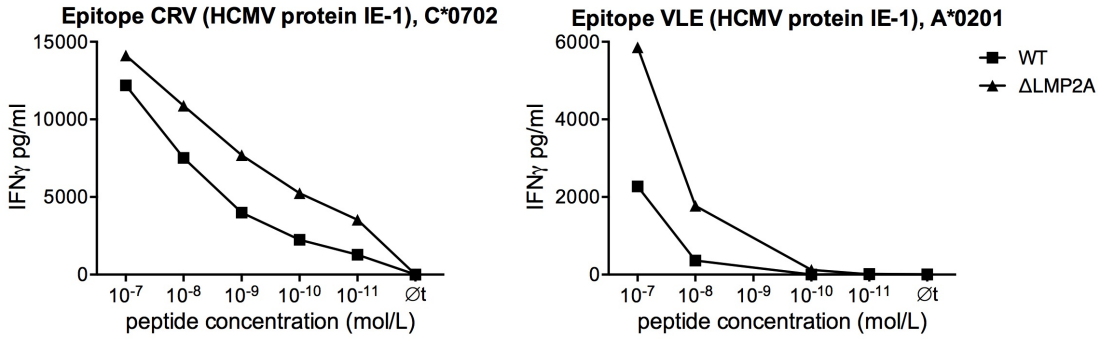


Figure 4.8. CD8+ T cell recognition of heterologous peptides on LCLs in the presence or absence of LMP2A. WT and ΔLMP2A LCLs (donor 9) were loaded with titrated peptides CRV and VLE from the HCMV antigen protein IE-1, and were coincubated for 16 hours with antigen-specific CD8+ T cell clones. IFNγ release by CD8+ T cells into the supernatant was quantified by ELISA. "∅t", T cells alone.

EBV antigens, I exogenously loaded WT and ΔLMP2A LCLs with peptides from the human cytomegalovirus (HCMV) protein IE-1 (CRV and VLE), and I analyzed the recognition of peptide-loaded LCLs by HCMV-specific CD8+ T cell clones (Figure 4.8). CD8+ T cells specific for the two analyzed HCMV peptides showed a better recognition of peptide-loaded ΔLMP2A LCLs than peptide-loaded WT LCLs. These data demonstrate that endogenous levels of antigen expression, or a potential interference of LMP2A with antigen processing, cannot be the only factors accountable for the difference in recognition of mutant LCLs to WT LCLs. This suggested that LCL-expressed surface or secreted molecules contributed to the differences in CD8+ T cell recognition.

To identify other factors playing a possible role in CD8+ T cell reactivity, I investigated soluble and cell surface molecules that may influence the interaction between EBV-infected cells and CD8+ T cells. EBV codes for a homologue of the human anti-inflammatory cytokine IL-10, and LMP2A was shown to increase IL-10 production in infected B cells (Incrocci et al. 2013). This possibility is intriguing because IL-10 is known to reduce antiviral activity of various immune effector cells (Zeidler et al. 1997; Bejarano & Masucci 1998; Jochum et al. 2012a). Therefore, I analyzed the release into the supernatant of IL-10 by WT and ΔLMP2A LCLs. Higher amounts of IL-10 were secreted by WT LCLs than LCLs lacking LMP2A (Figure 4.9A). In contrast, transcription levels for human and viral IL-10 did not mirror levels of secreted IL-10, and for human IL-10 there was even an opposite trend (Figure 4.9B). This suggests an effect of LMP2A on post-transcriptional regulation of IL-10. To determine whether differences in IL-10 release could influence the reactivity of CD8+

T cell clones, I used specific antibodies to neutralize IL-10 in the supernatant (Figure 4.9D), or to block IL-10 receptor on CD8+ T cells (Figure 4.9C). In both cases, I did not detect an increase of recognition for WT or ΔLMP2A LCLs. These data suggest that, even though ΔLMP2A LCLs produce lower amounts of IL-10, this does not affect the ability of CD8+ T cells to recognize EBV-infected B cells.

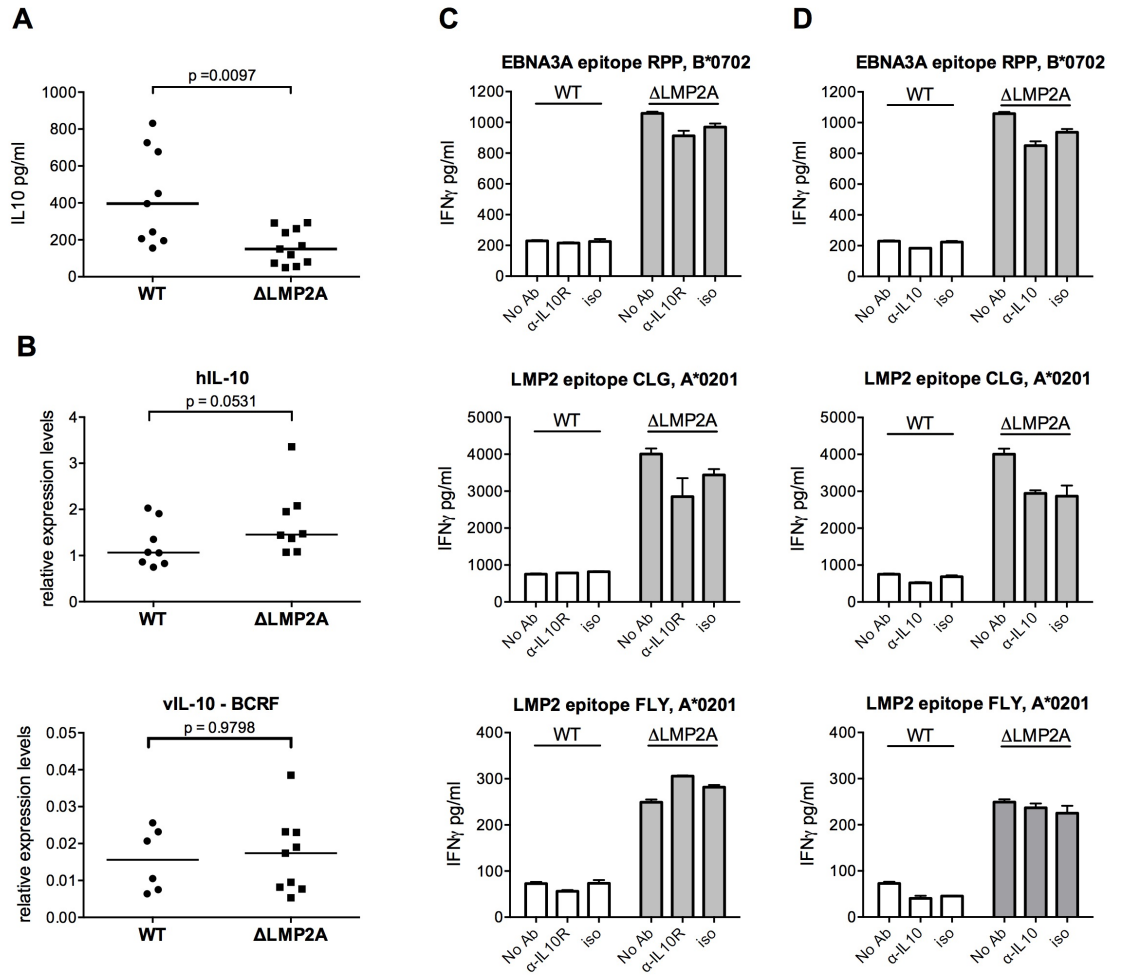


Figure 4.9. Role of LCL-secreted IL-10 in CD8+ T cell recognition in the presence or absence of LMP2A. (A) WT and ΔLMP2A LCLs (donors 1, 3, 7, 8, 9) were incubated at $0.5x10^6$ cells/ml for 18 hours, and released IL-10 was evaluated in ELISA. Each dot represents the mean of quadruplicates for a single independently established cell line. Shown data are representative for three independent experiments. LCLs established from 5 different donors were analyzed. The horizontal line indicates the median. Statistical analysis was performed with the Mann-Whitney test. (B) mRNA levels for human IL-10 and BCRF1 (viral IL-10) were measured by quantitative RT-PCR in WT and ΔLMP2A LCLs (donors 1, 3, 7, 9) and are displayed relative to transcript levels of the housekeeping gene β-glucuronidase (GUSB). Each dot represents a single measurement for a single cell line. LCLs established from 4 different donors were analyzed. Shown data are representative for two independent experiments. The horizontal line indicates the median. Statistical analysis was performed with the Mann-Whitney test. (C, D) Reactivity of EBV-specific CD8+ T cell clones against WT or ΔLMP2A LCLs (donor 9) was determined after blocking the IL-10 receptor on the T cells (C) or in the presence of neutralizing IL-10-specific antibody (D). For both conditions, matched isotype control antibodies were used as control where indicated ("iso"). T cells had been pre-treated with the antibodies for 1 hour before incubation with LCLs. After 16 hours, IFNγ release in the supernatant was quantified by ELISA. Mean and range of duplicates are shown.

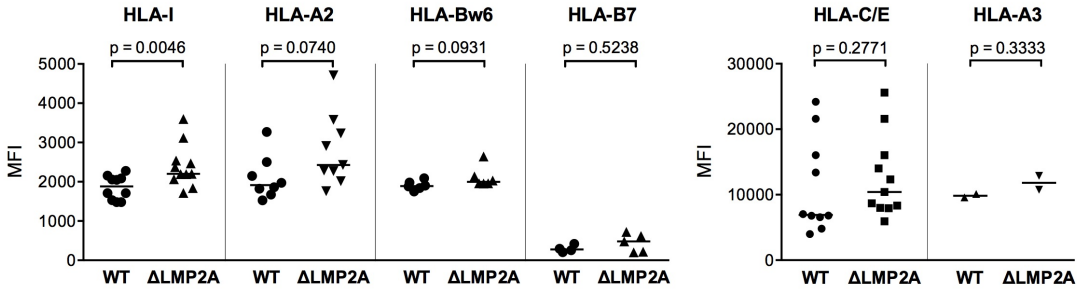


Figure 4.10. Expression of MHC class I on the surface of LCLs in the presence or absence of LMP2A. WT and ΔLMP2A LCLs were stained with antibodies specific for total HLA class I ("HLA-I") or individual allotypes as indicated. Stained cells were analyzed by flow cytometry. Each dot represents a single measurement for a single independently established cell line. Lines established from 5 different donors were analyzed. Shown data are representative for three independent experiments. MFI: mean fluorescent intensity. The horizontal line indicates the median. Statistical analysis was performed with the Mann-Whitney test.

Now I analyzed surface molecules involved in the interactions between CD8+ T cells and LCLs. First, I asked whether the absence of LMP2A could affect the surface levels of MHC class I molecules. To this end, I stained ΔLMP2A and WT LCLs with specific antibodies for total MHC class I and for individual MHC class I allotypes, and analyzed the cells by flow cytometry (Figure 4.10). Total MHC class I levels were moderately but significantly ($p=0.0046$) increased in LCLs deleted for LMP2A as compared with WT LCLs. A similar tendency was observed for some of the individual MHC allotypes, but this did not reach $p < 0.05$. Thus, LMP2A moderately reduces MHC-I levels in LCLs, which may contribute to the reduced recognition by CD8+ T cells.

Antigen-specific activation of CD8+ T cells can be modulated by a variety of surface molecules on the antigen-presenting cell. Thus, I examined the expression on the surface of LCLs of relevant immunomodulatory molecules, for which an upregulation was seen early after EBV infection (Figure 4.1), and tested whether their expression was altered in the absence of LMP2A (Figure 4.11). Indeed, I found strong differences in expression for some such molecules. Contrary to what might have been expected, the coinhibitory B7 family molecule PD-L1 (B7-H1) was strongly induced in ΔLMP2A LCLs, whereas there was no difference in the expression of the B7 molecule CD86. Also counter-intuitively, the integrin CD11a was strongly downregulated in the absence of LMP2A, while its interaction partner ICAM-1 showed no differences in ΔLMP2A versus WT LCLs. So far, these alterations could not explain the increased susceptibility of ΔLMP2A cells, but rather would suggest the contrary. When I examined surface expression of NKG2D ligands, interestingly I found that latent EBV infection induced

the expression of MICA, MICB and ULBP4. As shown above in Figures 4.1, 4.2 and 4.3, these molecules were not expressed on primary B cells and early EBV infection induced upregulation of MICB and, at later time points of ULBP4, but not of MICA (Figure 4.1, 4.2 and 4.3). This demonstrates that MICA induction is a slow process in EBV infection that becomes apparent only at later times than those tested above (days 0–8). While expression of MICB did not differ between mutant and WT LCLs, a marked increase for MICA and ULBP4 was detected on ΔLMP2A LCLs as compared to WT LCLs (Figure 4.11). Similarly to early EBV infection, WT or ΔLMP2A LCLs did not express the other five NKG2D ligands (ULBP1, 2, 3, 5, 6) on their surface (data not shown). This phenotypic analysis identified NKG2D ligands as effector molecules that potentially contributed to increased recognition of ΔLMP2A LCLs by CD8+ T cells.

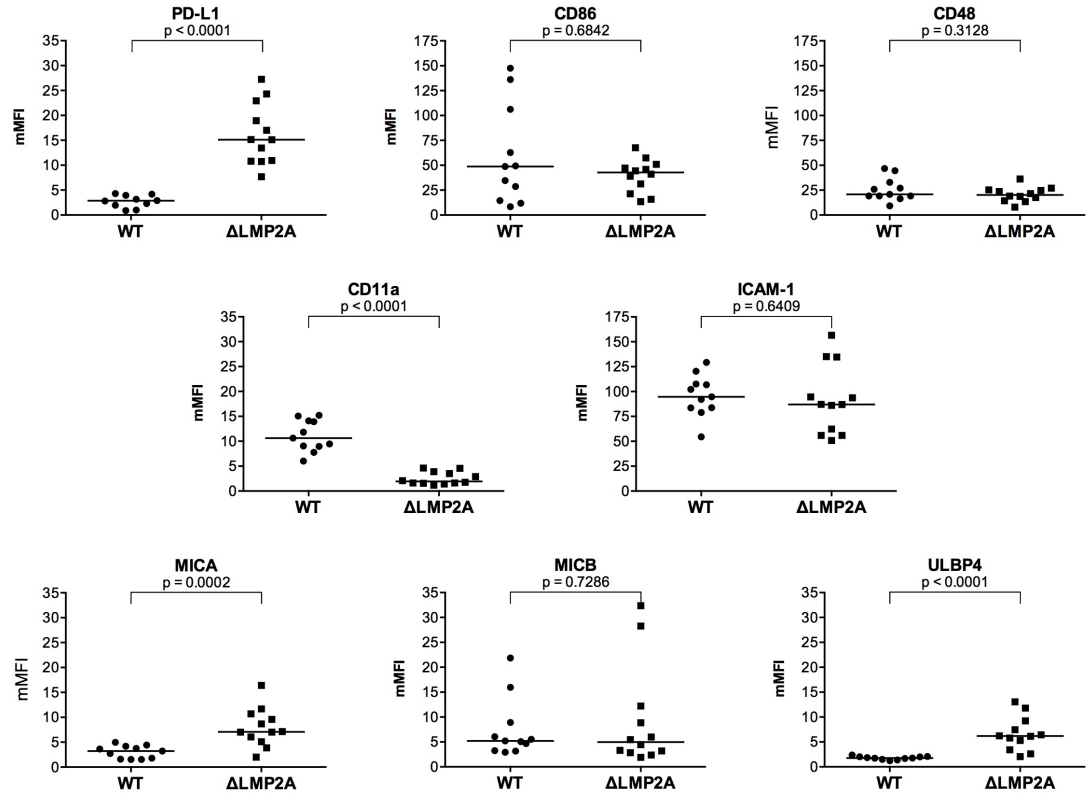


Figure 4.11. Immunophenotype of EBV-infected B cells in the presence or absence of LMP2A. Expression of the indicated antigens on the surface of WT and ΔLMP2A LCLs (donors 2, 3, 4, 9) was determined by flow cytometry after staining with monoclonal antibodies. The multiplicity of mean fluorescence intensity (mMFI) was determined by dividing MFI of specific antibody staining by MFI of isotype control. Each dot represents a single analysis of an independently generated LCLs. LCLs from 4 donors were analyzed. The horizontal line indicates the median. Statistical analysis was performed with the Mann-Whitney test.

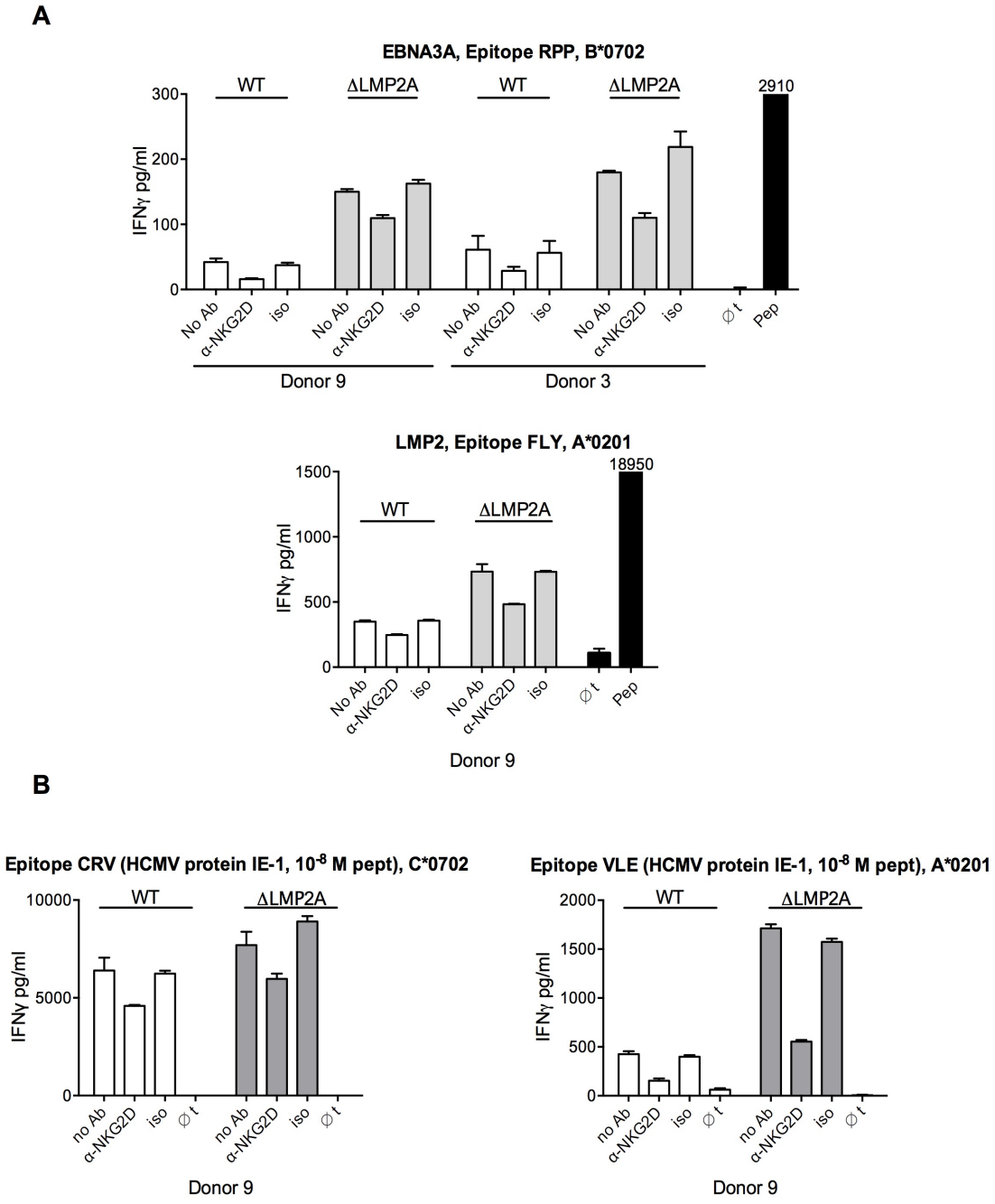


Figure 4.12. Effect of NKG2D blocking on CD8+ T cell recognition of LCLs in the presence or absence of LMP2A. (A) CD8+ T cell clones specific for epitopes from EBV latent antigens EBNA3A (RPP) and LMP2 (FLY) were coincubated with WT and ΔLMP2A LCLs in the presence of an anti-NKG2D antibody or matched isotype control where indicated. T cells had been pre-treated with the antibodies for 1 hour before incubation with LCLs. After 16 hours, release of IFNγ into the supernatant was evaluated by ELISA. "pep", WT LCLs exogenously loaded with corresponding peptide; "∅t", T cells alone. Mean and range of duplicates are shown. (B) WT and ΔLMP2A LCLs were loaded with peptides CRV and VLE from the HCMV antigen protein IE-1, and were coincubated for 16 hours with antigen-specific CD8+ T cell clones. Treatment with anti-NKG2D or isotype control antibodies and determination of IFNγ release were done as before. "∅t", T cells alone. Data are representative for two or more independent experiments performed for each T cell clone. Mean and range of duplicates are shown.

To assess the functional relevance of NKG2D ligands, I analyzed the reactivity of CD8+ T cell clones against WT or ΔLMP2A LCLs after blocking the receptor NKG2D on the effector cells with a specific antibody (Figure 4.12). NKG2D blocking consistently decreased the IFN-γ release by EBV-specific CD8+ T cells after contact with EBV-infected cells (Figure 4.12A). A reduction was found in the reactivity of CD8+ T cells to both WT and mutant LCLs, but in absolute terms the reduction was higher for ΔLMP2A LCLs as compared to WT LCLs. Likewise, NKG2D blocking on HCMV-specific CD8+ T cell clones recognizing peptide-loaded LCLs also led to a reduction of IFN-γ release (Figure 4.12B). Thus, NKG2D ligands on LCLs contribute to their recognition by CD8+ T cells, irrespective of the antigen specificity of the CD8+ T cells. Thus, LMP2A reduces CD8+ T cell recognition of EBV-infected B cells, among other mechanisms, by reducing the expression of NKG2D ligands.

4.3 Evidence for a role of γδ T cells in control of EBV infection

To elucidate the involvement of γδ T cells in the immunity against EBV, I incubated freshly isolated PBMCs from different donors with autologous or allogeneic LCLs, and analyzed the resulting expansion of γδ T cells (Figure 4.13). In the majority of the stimulation reactions (6 out of 8), contact with LCLs induced expansion of γδ T cells. Both the δ2+ and the δ2– subpopulations contributed to expansion (Figure 4.13B).

Higher average expansion was observed for δ2– T cells than for δ2+ cells (Figure 4.13A), and the proportion of cultures in which any expansion was observed was also

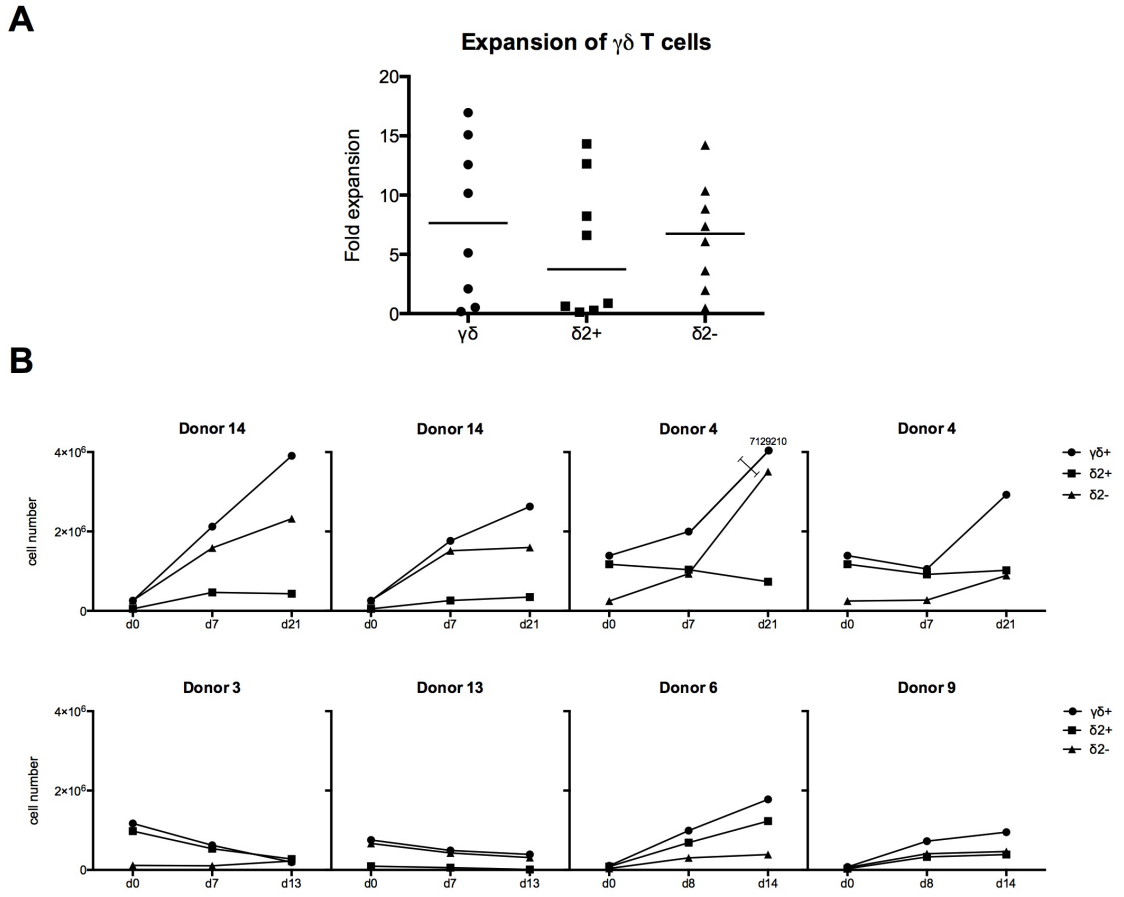


Figure 4.13. Expansion of γδ T cells after contact with LCLs. PBMCs from donors 3, 4, 6, 9, 13 and 14 were stimulated with matched (donor 3) or mismatched LCLs (all other stimulation reactions). Expansion of γδ T cells, δ2+ T cells or δ2- T cells (determined as γδ+/δ2-) was determined by flow cytometry after staining with specific monoclonal antibodies. (A) Fold expansion of γδ T cell, δ2+ T cells or δ2- T cells is calculated as the ratio between the percentage identified by flow cytometry of the indicated population present at the last day of analysis (day 13, 14 or 21) and the percentage present in the culture at day 0, multiplied by total expansion of the culture. The horizontal lines indicate the median. (B) Kinetics of expansion for each stimulation. Cell numbers were calculated from percentages of each cell type and the total expansion rate reached at each time point of analysis (day 0, 7 or 8 and day 13, 14 or 21) in the culture are shown.

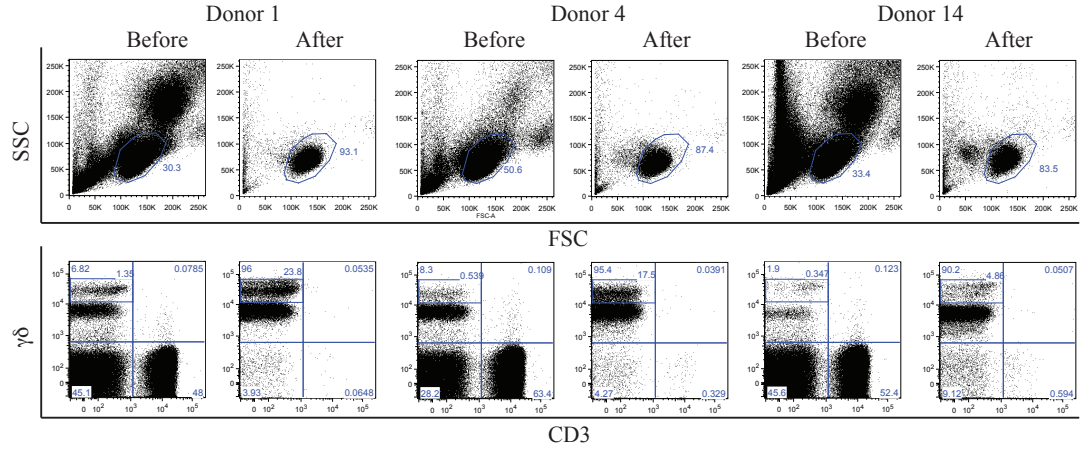


Figure 4.14. Purification of peripheral γδ T cells for the establishment of single cell clones. Flow cytometry graphs show examples of the efficiency of γδ T cells purification from PBMCs from three donors (Before: before purification; After: after purification). Lymphocytes were identified in the SSC/FSC graph and gated to eliminate debris and dead cells. In the lymphocyte population, γδ T cells were identified as γδ+/CD3+. Purity of the purified γδ cell population reached 90-95% in each case.

higher for δ2– than for δ2+ cells. These results showed that both of the major γδ T cell subpopulations can react against EBV infected cells, but suggested that the role of δ2– T cells may be more important.

I decided to establish single γδ T cell clones, in order to address which population was actively involved in the immunity against EBV. γδ T cells were purified from freshly isolated PBMCs (Figure 4.14), plated at a density of 0.7 cells/well, and stimulated with a mixture of irradiated PBMCs and LCLs coming from at least three allogeneic donors. Interestingly, different types of γδ T cells could be established as single cell clones (Table 4.1). Figure 4.15 shows examples of the single cell clones established. In total, I established 317 clones from five different donors. A majority of these clones were

	N° γδ clones	δ1+	δ2+	γ9+ δ1+	γ9+ δ2+	γ9+ δ1– δ2–	δ1– δ2–
Donor 1	107	14	55	nd	nd	nd	3
Donor 4	81	45	18	nd	nd	nd	6
Donor 8	28	2	17	2	17	1	1
Donor 9	22	14	2	3	2	0	0
Donor 14	79	29	36	nd	nd	nd	2

Table 4.1. Summary of the T-cell receptor phenotype of established γδ T cell clones from 5 donors. γδ T cells purified from freshly isolated PBMCs were plated at a density of 0.7 cells/well and stimulated with a mixture of irradiated PBMCs and LCLs coming from at least three allogeneic donors. Individual outgrowing T cell cultures were analyzed by flow cytometry for expression of different TCR chains. The table summarizes the results of these analyses. Not all clones were analyzed with the whole set of antibodies; therefore, the total number of clones will not always matched the identified γδ T cell subpopulations. Nd: not determined.

Table 4.2. Phenotypic analysis of the established γδ T cell clones from 5 donors.
γδ T cells purified from freshly isolated PBMCs were plated at a density of 0.7 cells/well and stimulated with a mixture of irradiated PBMCs and LCLs coming from at least three allogeneic donors. The tables show the results of these stimulations. γδ T cell subpopulations were determined by flow cytometry after staining with specific monoclonal antibodies. Clones that showed a combined γδ+/αβ+ low-expression phenotype were marked as αβ+? The tables also indicate for which of the T cell clones it was possible to perform at least one cellular cytotoxicity (lysis) assay (#). For γδ-specific staining, which showed a wider range of staining intensities, +/++/+++ indicates positive staining, with low/intermediate/bright intensity. For the other antibodies, a + sign indicates positive staining. -: negative staining, nd: not determined, smear: variable degree of positivity within one clone, αβ+?: γδ+/αβ+ low phenotype, #: lysis assay was performed.

Donor 1	γδ	δ1	δ2	CD56	lysis
1	++	-	+	+	nd
2	++	-	+	+	nd
3	++	-	-	+	#
5	++	-	+	+	nd
7	++	-	+	+	nd
8	+++	+	-	+	#
9	+	-	+	+	nd
10	++	-	+	+	nd
11	+++	-	-	+	nd
12	++	-	+	smear	nd
13	+	-	+	+	nd
14	++	-	+	+	nd
15	++	-	+	+	nd
16	++	-	+	smear	nd
17	++	-	+	smear	#
18	++	-	+	+	nd
19	++	-	+	+	nd
20	+	-	+	smear	nd
21	+++	+	-	+	#
22	+	-	+	+	nd
23	++	+	-	smear	#
24	+	-	+	+	nd
25	++	-	+	+	nd
26	++	-	+	+	nd
27	++	-	+	smear	nd
28	++	-	+	+	nd
29	++	-	+	smear	nd
30	++	-	+	smear	nd
31	++	-	+	+	nd
32	+++	+	-	+	#
33	++	-	+	+	nd
34	++	+	-	smear	#
35	++	-	+	+	nd
36	++	-	+	+	nd
37	++	-	+	+	nd
38	++	-	+	smear	nd
39	+	-	+	+	nd
40	++	-	+	smear	#
41	++	-	+	smear	nd
42	++	-	+	+	nd
43	++	-	+	+	nd
44	++	-	+	+	nd
45	++	-	+	+	nd
46	++	-	-	smear	#
47	++	+	-	smear	#
48	++	-	+	+	nd
49	+	-	+	+	nd
50	++	-	+	+	nd
51	+	-	+	+	nd
52	++	-	+	+	#
53	++	-	+	+	nd
54	++	-	+	+	nd
55	++	-	+	smear	#
56	+++	+	-	smear	#
57	+	-	+	nd	nd
59	++	-	+	+	nd
60	++	-	+	+	nd
61	++	-	+	+	nd
63	++	-	nd	smear	nd
64	+++	-	nd	smear	nd
65	++	-	+	+	nd
66	++	-	+	+	nd
67	++	-	+	+	nd
68	++	-	+	+	nd
69	++	-	+	+	nd
70	+	-	+	+	nd

71	++	-	+	smear	nd
72	++	-	nd	+	nd
73	++	-	nd	+	nd
75	++	-	nd	+	nd
76	++	-	nd	+	nd
80	++	-	nd	+	nd
81	++	-	nd	+	nd
82	++	-	nd	+	nd
83	+++	+	nd	smear	nd
84	++	-	nd	+	nd
85	++	-	nd	+	nd
86	++	-	nd	+	nd
91	+	-	nd	+	nd
92	++	-	nd	smear	nd
93	++	-	nd	+	nd
95	+++	+	nd	+	#
96	++	-	nd	+	nd
97	++	-	nd	+	nd
99	++	-	nd	+	nd
100	++	-	nd	+	nd
102	++	-	nd	+	nd
104	++	-	nd	+	nd
105	+++	+	nd	+	nd
106	++	-	nd	+	nd
107	++	-	nd	+	nd
108	++	-	nd	+	nd
109	++	-	nd	+	nd
110	++	-	nd	+	nd
112	++	-	nd	+	nd
113	++	-	nd	+	nd
115	++	-	nd	+	nd
116	++	-	nd	+	nd
117	++	-	nd	+	nd
118	++	-	nd	smear	nd
120	++	-	nd	+	nd
122	++	-	nd	+	nd
123	++	-	nd	+	nd
119 /αβ+?	+++	+	nd	smear	#
58 /αβ+?	+++	+	-	smear	nd
62 /αβ+?	+++	+	-	smear	#
94 /αβ+?	+++	+	nd	smear	#

Donor 4	γδ	δ1	δ2	CD56	lysis
1	+++	-	-	+	#
2	++	-	+	+	#
3	+++	+	-	smear	#
4	+++	+	-	smear	#
5	+++	+	-	smear	#
6	++	+	-	+	#
7	+++	+	-	smear	nd
8	++	-	+	+	nd
9	+++	+	-	smear	#
10	+++	+	-	smear	#
11	++	-	+	+	nd
12	++	+	-	smear	#
13	+++	+	-	smear	#
14	+++	+	-	+	nd
15	++	-	+	+	nd
16	+++	+	-	smear	#
17	++	-	+	smear	nd
18	++	-	+	+	nd
19	++	-	+	smear	nd
20	+++	+	-	smear	#
21	+++	+	-	smear	#
22	+++	+	-	smear	nd
23	+++	+	-	smear	nd
25	++	-	+	smear	nd
26	++	+	-	+	#
27	+++	+	-	smear	#
28	++	-	-	smear	nd
29	++	+	-	smear	#
30	++	-	+	smear	nd
31	+++	+	-	smear	#
32	+++	+	-	smear	#
33	++	-	-	smear	#
34	+++	+	-	smear	#
35	++	-	+	+	nd
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37	++	-	-	smear	#
38	+++	+	-	smear	#

39	++	-	+	+	nd
40	+++	+	-	smear	#
43	+	-	+	smear	nd
44	+++	+	-	smear	#
46	+++	+	-	smear	#
47	+++	+	-	smear	#
48	+++	+	-	smear	#
49	+++	+	-	smear	#
50	+++	+	-	smear	#
51	+++	+	-	smear	#
52	+	-	+	+	nd
53	++	-	+	smear	nd
54	+++	+	nd	smear	#
55	++	-	nd	+	nd
56	+++	+	-	smear	#
57	++	-	nd	+	nd
58	++	-	nd	smear	nd
59	++	-	nd	+	nd
60	++	-	nd	smear	nd
61	++	-	nd	+	nd
62	+++	+	nd	smear	#
63	+++	+	nd	smear	#
64	++	-	nd	+	nd
65	++	-	nd	+	nd
66	++	-	+	+	#
67	+++	-	nd	+	nd
68	++	-	nd	+	nd
69	++	-	nd	+	nd
70	++	-	+	smear	nd
71	++	+	-	smear	#
73	+++	+	nd	smear	#
75	+++	+	nd	smear	#
76	+++	+	-	smear	#
77	+++	+	nd	smear	#
78	+++	-	nd	+	nd
79	+++	+	nd	smear	#
80	+++	+	nd	smear	nd
89	+++	+	-	smear	#
92	++	-	+	smear	nd
94	++	-	+	smear	#
95	++	-	-	smear	#
96	+++	-	-	smear	#
42/αβ+?	++	+	-	+	#
45/αβ+?	++	+	-	smear	nd

Donor 8	γ9	δ2	δ1	Lysis
A4	+	nd	-	nd
B1	+	+	-	nd
B5	+	+	-	nd
B6	+	+	-	nd
B8	+ bright	+	-	#
C1	+ bright	nd	-	nd
C2	+ bright	+	-	nd
C11	+ bright	-	-	#
C3	+	+	-	nd
C4	+	+	-	nd
C9	+	+	-	nd
D1	+ dim	nd	-	#
D3	+ dim	nd	-	nd
D6	+	+	-	nd
E5	+	+	-	nd
F2	+	nd	-	nd
F7	+	nd	+	#
G11	+	+	-	nd
G5	+	+	-	nd
G9	+	+	-	nd
H1	+ dim	nd	-	nd
H2	+	+	-	nd
H6	+ dim	+	-	nd
H7	+	nd	+	#
I1	+	nd	-	nd
I4	+	+	-	#
I6	+ dim	nd	-	nd
I7	+ bright	+	-	#

Donor 14	γδ	δ1	δ2	CD56	Lysis
1	++	+	-	+	#
2	+	-	+	+	#
3	++	-	+	+	nd
4	+	-	+	+	nd
6	+++	-	-	+	#
7	++	+	-	+	#
8	++	-	+	+	nd
9	+++	-	-	+	#
11	++	-	+	+	nd
12	++	+	-	+	#
13	++	-	+	+	nd
14	++	+	-	+	#
15	++	-	+	+	nd
16	++	-	+	+	nd
17	++	-	+	+	nd
18	++	+	-	smear	#
19	++	-	+	+	nd
20	+	-	+	+	nd
21	++	-	+	+	nd
22	++	+	-	smear	#
23	++	-	+	+	nd
24	++	-	+	+	nd
25	+	-	+	+	nd
26	++	-	+	+	nd
27	+	-	+	smear	nd
28	++	-	+	+	nd
29	+	-	+	+	nd
30	++	+	-	+	#
32	+	-	+	+	nd
33	++	+	-	+	#
34	++	-	+	+	nd
35	++	-	+	+	nd
37	++	+	-	+	#
38	+	-	+	+	nd
39	++	+	-	+	#
40	++	+	-	+	nd
41	++	-	+	+	nd
42	++	-	+	+	nd
43	++	-	+	+	nd
44	++	-	+	+	nd
45	++	-	+	+	nd
46	++	+	-	+	#
47	+++	+	-	+	#
48	++	-	+	+	nd
49	++	-	+	+	nd
51	+	-	+	+	nd
52	++	+	-	smear	#
54	++	-	nd	+	#
55	+	+	nd	+	nd
56	++	-	nd	+	nd
57	++	-	nd	+	nd
58	+	+	nd	+	#
59	++	-	nd	+	nd
61	+	+	nd	+	#
62	++	-	nd	+	nd
66	+	+	nd	+	nd
69	+++	-	nd	+	nd
70	++	+	nd	+	nd
71	+	+	nd	+	nd
76	++	+	nd	+	nd
78	++	+	nd	+	#
79	++	+	nd	+	#
81	++	-	+	+	nd
82	+	-	+	+	nd
83	++	+	nd	+	nd
84	++	-	nd	+	nd
85	++	-	nd	+	nd
86	++	+	nd	+	#
87	++	+	-	+	nd
88	+	-	+	+	nd
89	++	+	-	+	#
91	+++	-	nd	+	nd
92	+++	+	-	+	#
93	++	-	+	+	nd
94	++	-	+	+	nd
95	+++	-	nd	+	nd
96	++	-	nd	+	nd
36/αβ+?	++	+	-	smear	nd

Donor 9	γ9	δ2	δ1	KIR	NKG2A	Lysis
A3	-	-	+	-	-	#
A4	-	nd	-	nd	nd	nd
A5	-	nd	+	nd	nd	nd
A6	-	nd	+	nd	nd	nd
A7	+	nd	+	nd	nd	#
B1	-	-	+	-	+	#
B2	-	nd	-	nd	nd	nd
B4	-	-	+	-	smear	#
B6	-	-	+	2DL2/3+ 3DL1+	-	#
B7	+	nd	+	nd	nd	#
B8	-	nd	+	nd	nd	#
C3	-	nd	+	nd	nd	nd
C5	-	nd	-	nd	nd	nd
C6	-	nd	+	nd	nd	nd
C8	+	nd	-	nd	nd	#
D2	-	-	+	-	-	#
D3	-	-	+	-	-	#
D5	+	+	-	nd	nd	#
D8	+	+	-	nd	nd	#
E1	+	-	+	nd	nd	#
E2	-	-	+	-	-	#
E6	-	-	+	-	+	#

analyzed for their TCR phenotype by flow cytometry (Table 4.2). From each of the donors, I obtained both δ2+ and δ2– clones, although with different efficiencies for each TCR expression phenotype. From donor 8, nearly all of the outgrowing clones were δ2+ T cell clones, except for two clones expressing a γ9δ1+ TCR and another with a γ9+/δ1–/δ2– TCR (Table 4.2, donor 8). From donor 1, only 13% of the established clones possessed a δ1 chain, while for the other three donors (4, 9, 14), about half of the clones were δ1+ and the other half were δ2+ (also identified as δ1–). It is of notice that I could establish clones presenting a rare phenotype of γ9δ1+ TCR, and clones that were δ1–/2–, and therefore expressing another of the available but less frequently expressed delta chains. Some rare clones showed a peculiar and unexpected γδ+/αβ+low phenotype (Table 4.2). All γδ T cell clones expressed the receptor NKG2D with similar intensity (examples in Figure 4.16) and the marker CD56, although there was sometimes a broad CD56 intensity distribution within a clone (Table 4.2). Table

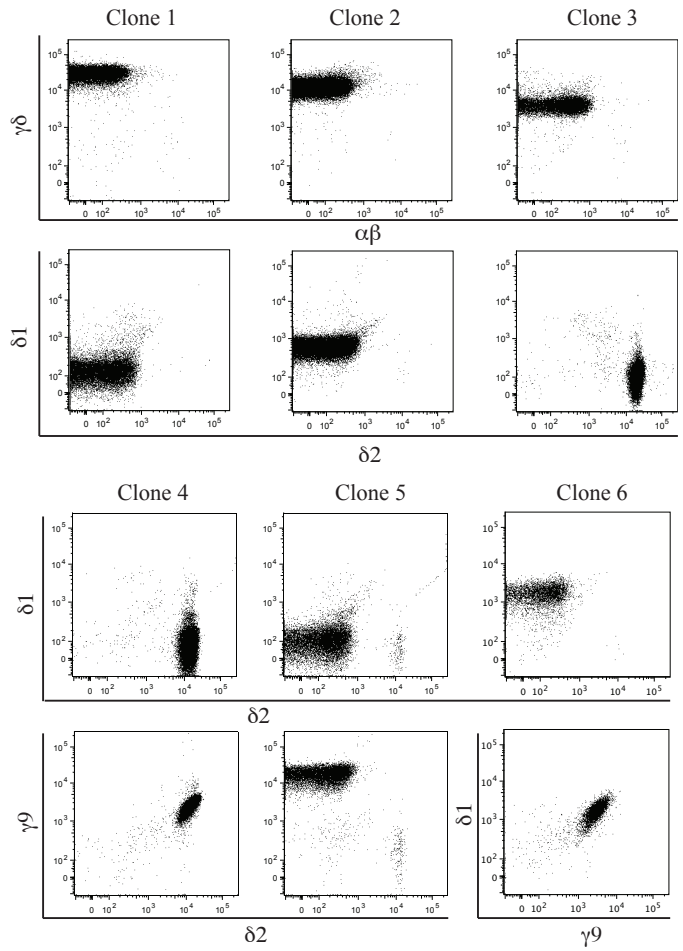

Figure 4.15. Flow cytometry staining of γδ T cell clones. TCR expression and clonal phenotype of cloned γδ T cells was analyzed by flow cytometry with the aid of specific monoclonal antibodies recognizing the αβ TCR, the γδ TCR, δ1, δ2 or γ9 chains. In the established cultures, I identified clones with a δ1– / δ2– TCR (clone 1), with δ1+ TCR (clone 2), and with a δ2+ TCR (clone 3). Taking into account the γ chain as well, clones were identified with a γ9δ2+ TCR (clone 4), with a γ9+/ δ1– / δ2– TCR (clone 5) and with a γ9δ1+ TCR (clone 6). Examples of 317 clones established from five different donors are shown (see Table 4.1).

4.2 also indicates for which T cell clones it was possible to perform at least one lysis assay against EBV-infected cells. This series of experiments showed that stimulation of γδ+ T cells with irradiated PBMCs and LCLs is an efficient method to obtain γδ+ single cell clones, even from infrequent γδ subpopulations.

To identify whether γδ T cells could specifically recognize EBV-infected cells, I performed lysis assays, in which γδ T cell clones were challenged with autologous LCLs or autologous B-blasts (Figure 4.17, and data not shown). Indeed, some of the clones analyzed lysed EBV-infected B cells with different degrees of efficiency, but not activated B-blasts. Thus, some γδ T cell clones may recognize a molecular marker related to EBV infection but not to endogenous B cell activation. Interestingly, the opposite case was found for some of the γδ T cell clones (Figure 4.17), which indicates that there are some antigenic determinants that are induced by standard B cell activation pathways, but suppressed in EBV infection.

The γδ T cell clones were further analyzed by performing lysis assays with autologous or allogeneic LCLs, with other EBV-positive or EBV-negative B cell lines (Raji and Daudi, respectively), and with a known, broadly recognized target for NK cells (K562, a CML cell line) (Figure 4.18). Interestingly, γ9δ2+ T cell clones had no or very low

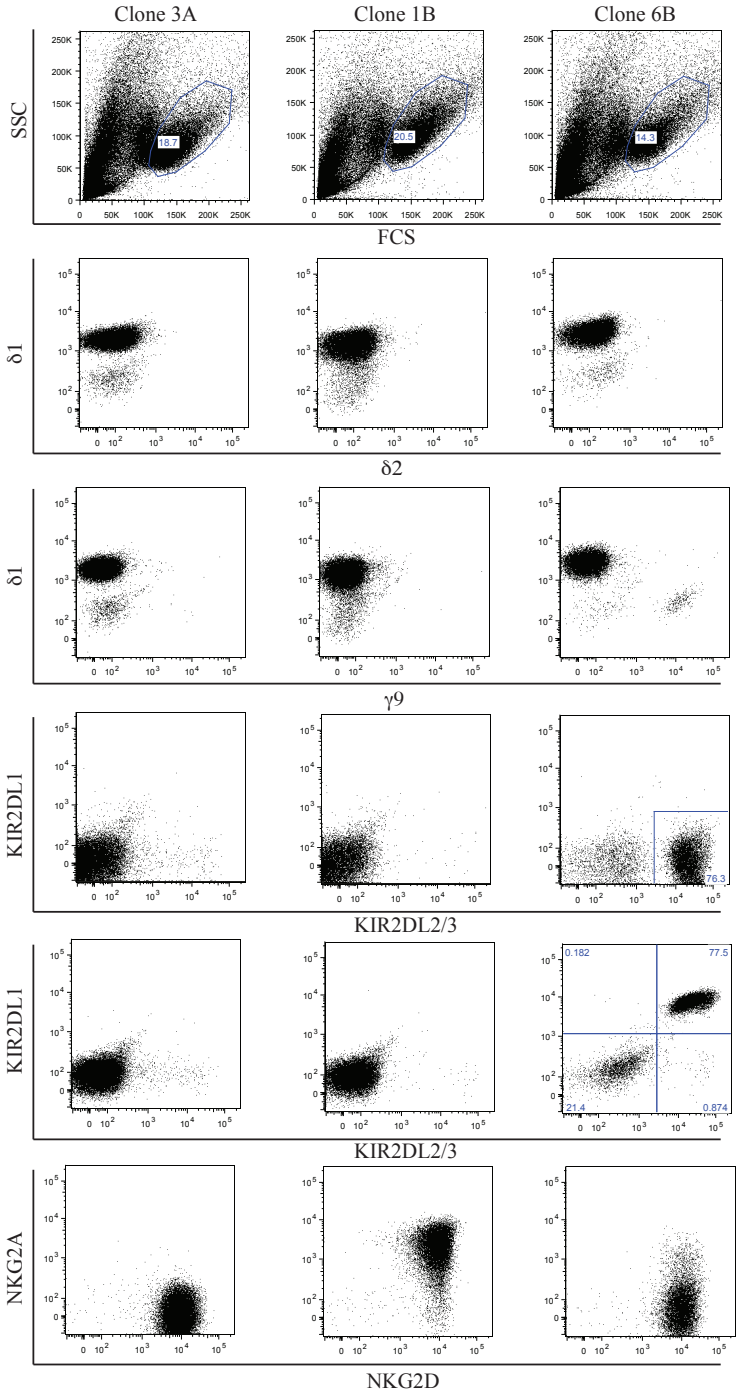

Figure 4.16. Expression of NK receptors by γδ T cell clones. The expression of several NK receptors (KIR and NKG2 group receptors) was analyzed on γδ T cell clones by flow cytometry after staining with specific monoclonal antibodies. The clones analyzed were from donor 9.

reactivity against EBV-infected B cells, but did recognize the other cell lines with different degrees of intensity (Figure 4.18B and data not shown). Meanwhile, δ1+ T cells generally recognized and induced lysis of at least one LCL line and showed low recognition of the B cell lines Raji and Daudi, but different intensities of lysis of the CML line K562. These data suggest that the major population playing a role in the control of EBV infection is the δ1+ T cell population. It is of interest that some δ1+ T cell clones recognized autologous LCLs, while others recognized only allogeneic targets. This observation suggests that δ1+ T cells recognize LCLs in different ways, possibly by recognizing different molecular targets on these cells.

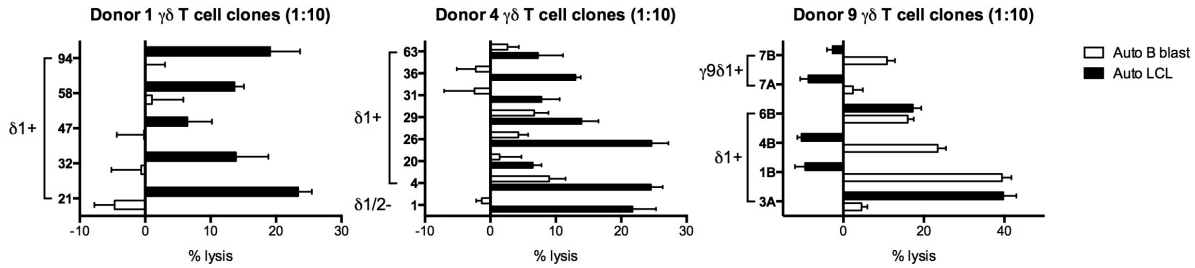

Figure 4.17. Recognition of autologous B-blasts or EBV-infected cells by γδ T cell clones. γδ T cell clones from donors 1, 4 and 9 were challenged in a lysis assay with autologous B-blasts or autologous LCLs at a target:effector ratio of 1:10. Mean and standard error of the mean (SEM) of four replicates are shown.

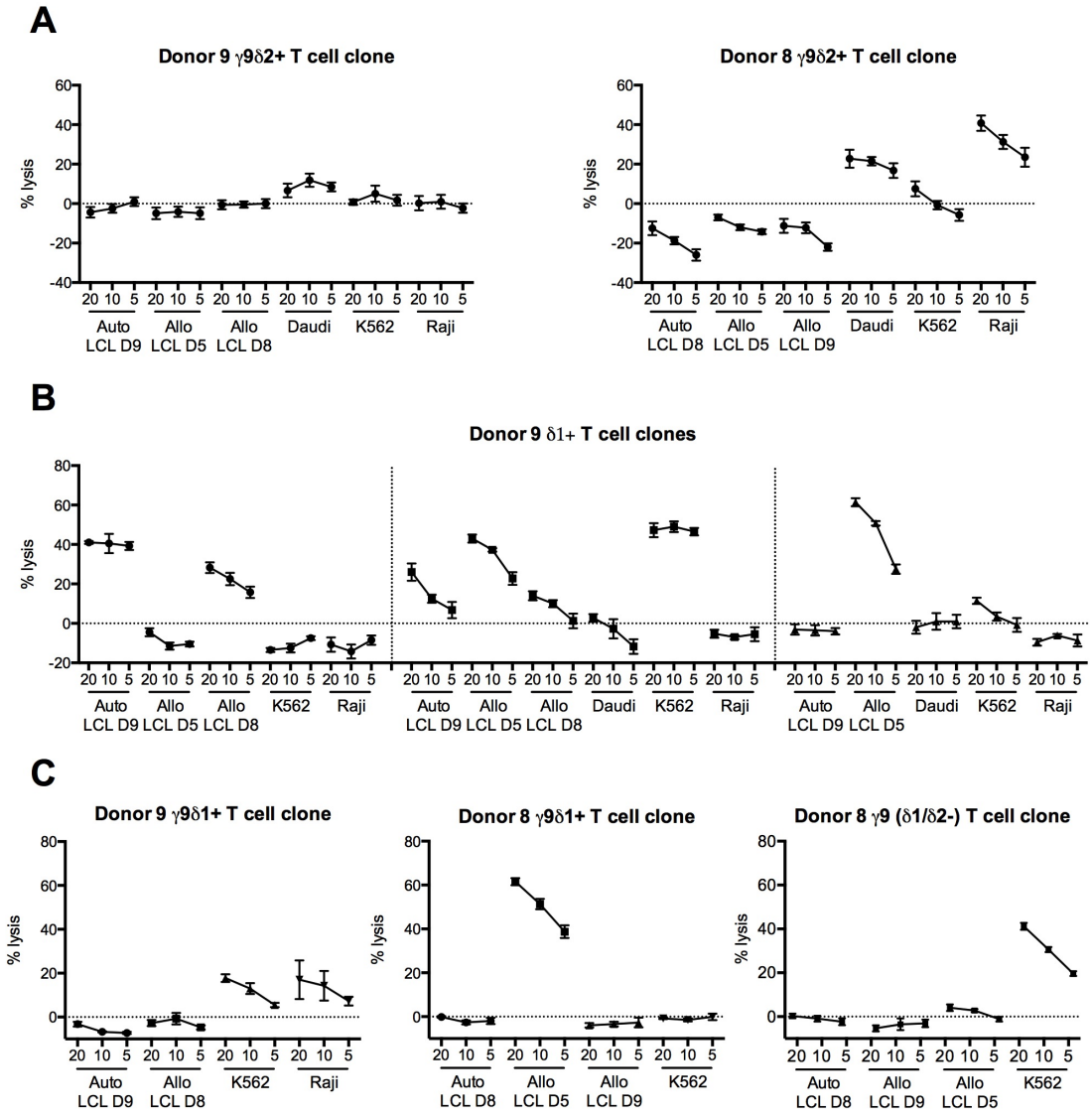

Figure 4.18. Recognition of autologous or allogeneic EBV-infected cells by γδ T cell clones. γδ T cell clones from donors 8 and 9 were challenged in a lysis assay with autologous or allogeneic LCLs and, where indicated, with the cell lines Daudi, K562 and Raji at a target:effector ratio of 1:5/10/20. Mean and standard error of the mean (SEM) of four replicates are shown.

Clone	V-gene segment	V-region AA	NDN	J-region AA	J-gene segment
D1 #8	TRDV1*01	CALGE	RTSLIYWGFNV	TAQLFF	TRDJ2*01
D1 #21	TRDV1*01	CALGE	RTSLIYWGFNV	TAQLFF	TRDJ2*01
D1 #94	TRDV1*01	CALGE	FRAYLGGYVRLK	YTDKLIF	TRDJ1*01
D1 #95	TRDV1*01	CALG	HSVLLPWGIRD	TDKLIF	TRDJ1*01
D4 #04	TRDV1*01	CALGE	AFARLRRGDTRL	YTDKLIF	TRDJ1*01
D4 #20	TRDV1*01	CALGEL	LSTGG	YTDKLIF	TRDJ1*01
D4 #29	TRDV1*01	CALGE	PVGSWIPDNAD	TDKLIF	TRDJ1*01
D4 #36	TRDV1*01	CALGEL	NSPTGNTGGYFVGSN	TDKLIF	TRDJ1*01
D9 #3A	TRDV1*01	CALGE	QLPILPEVWGIKGD	DKLIF	TRDJ1*01
D14 #14	TRDV1*01	CALGE	SAFPTTGGST	YTDKLIF	TRDJ1*01
D14 #22	TRDV1*01	CALGE	SAFPTTGGST	YTDKLIF	TRDJ1*01
D4 #1	TRDV3*01	CAF	REIHVLG	TDKLIF	TRDJ1*01 F
D14 #6	TRDV3*01	CAF	SALQ	YTDKLIF	TRDJ1*01 F
D14 #9	TRDV3*01	CAF	SALQ	YTDKLIF	TRDJ1*01 F

Table 4.3 Sequences of the δ TCR chain for some γδ T cell clones. mRNA was extracted from γδ T cell clones, cDNA synthesized, the δ TCR cDNA was amplified by PCR and sent for sequencing. Primers for the δ TCR were positioned in the V-gene segment and in the C-gene segment to amplify the CDR3 region. The sequences are aligned according to their usage of V and J regions. The table indicates the amino acid composition of the CDR3 region for each clone, specifying the amino acids encoded by the V-segment and the J-segment. Underlined amino acids are homologues to the D-gene segments. The IMGT online tool was used to facilitate allocation of amino acid sequences to the individual gene segments (www.imgt.cines.fr) {Lefranc et al., 1999, #30971}. TCR nomenclature according to {Giudicelli and Lefranc, 1999, #871} was used.

To assess whether there was a correlation of TCR primary structure with reactivity of the γδ T cells, I analyzed the TCR sequences of the δ TCR chain of some δ2-negative T cell clones (Table 4.3). The mRNA was extracted from γδ T cell clones, cDNA was synthesized, the δ TCR cDNA was amplified by PCR and commercially sequenced. Primers for the δ TCR were positioned in the V-gene segment and in the C-gene segment, bridging the hypervariable region (CDR3 region). In Table 4.3, the CDR3 sequences are arranged according to their expression of different V and J gene segments. Interestingly, sequences obtained from clones from 4 different donors showed a large heterogeneity in their CDR3 sequences. Only in the case of three pairs of clones that came from the same donor, identical δ-CDR3 sequences were found. Thus, δ2– γδ T cell clones with different specificities carry different γδ TCRs. Thus, the sequences identified here could be used in the future as molecular markers for identification of EBV-specific γδ T cells across donors.

To further clarify how γδ T cells mediate the lysis of EBV-infected B cells, I performed a lysis assay with an antibody specific for the receptor NKG2D, blocking the

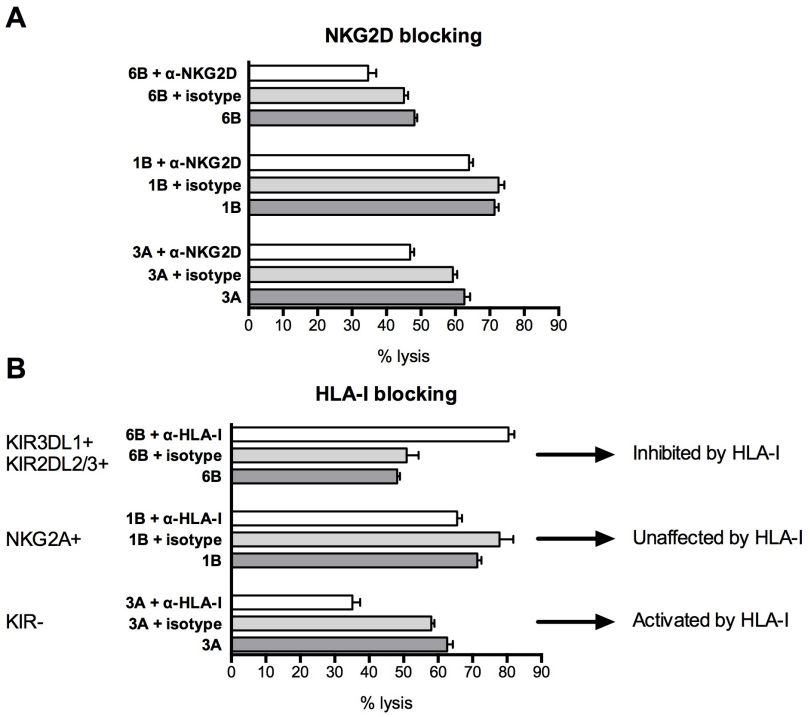

Figure 4.19. Effect of NKG2D and HLA class I blocking on lysis efficiency of LCLs by γδ T cell clones. γδ T cell clones from donor 9 were challenged in a lysis assay with autologous (clone 3A) or allogeneic (clones 6B and 1B) LCLs at a target:effector ratio of 1:10 in the presence of an anti-NKG2D (A) or anti-HLA-I antibody (B) or matched isotype control where indicated. Means and range of duplicates are shown.

interaction with its ligands on the target cells. As shown in figure 4.19A, blocking of NKG2D partially reduced the efficiency of LCL lysis by the analyzed γδ T cell clones. Interestingly, clone 6B from donor 9 showed a reactivity pattern that appeared to be related to its expression of inhibitory NK receptors (Figure 4.16). It is interesting to notice that a dominant cell population within this clone – if it is in fact a single-cell clone – co-expressed at least two inhibitory KIR receptors that are specific for HLA class I molecules, KIR2DL1 and KIR2DL2/3 (Figure 4.16). When I blocked the interaction between KIRs present on the T cell clone and their ligands with a specific antibody targeting HLA class I molecules, lysis was increased (Figure 4.19B), which is in accordance with the idea that the reactivity of clone 6B is at least partially regulated by inhibitory KIRs. For another clone expressing the inhibitory molecule NKG2A but no other inhibitory KIR receptors (Figure 4.16), I could not see any differences in its functions (Figure 4.19B). NKG2A is inhibited by the nonpolymorphic MHC molecule HLA-E, which is also bound by the anti-HLA antibody. However, it has been observed before that LCLs express comparatively small amounts of HLA-E (Braud et al. 1997; Corrah et al. 2011), so a lack of regulation of this T cell clone through the NKG2A/HLA-E axis in this experiment can be easily explained. For the third clone

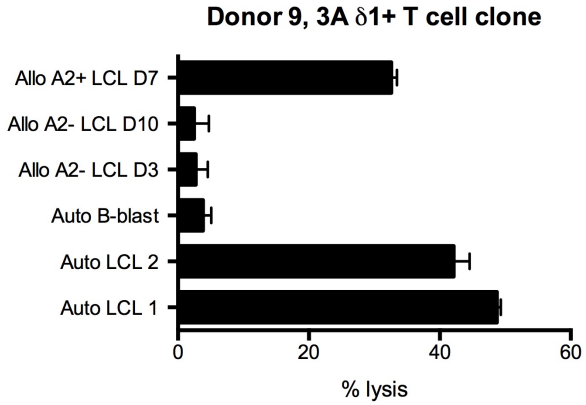

Figure 4.20. Recognition of matched, partially matched or mismatched LCLs by the δ1+ T cell clone 3A from donor 9. This δ1+ T cell clone was challenged in a lysis assay with autologous CD40-stimulated B blasts, autologous LCLs, and partially HLA-matched (HLA-A*0201+) or fully mismatched (A2–) LCLs, at a target:effector ratio of 1:10. Mean and standard error of the mean (SEM) of four replicates is shown.

analyzed, in contrast to expectation, I observed a reduction of recognition when its interaction with HLA-I molecules was blocked (figure 4.19B). I investigated this clone in more detail, and analyzed its recognition of autologous, partially matched or mismatched LCLs (Figure 4.20). Patterns of lysis of several LCLs from different donors confirmed that the reactivity of this clone was associated with the presence of the HLA-A2 molecule on the target cell. Indeed, when I performed blocking experiments with specific antibodies targeting only the HLA-A2 molecule, I observed a clear reduction of the reactivity of this clone to autologous LCLs (Figure 4.21). Recognition of LCLs by this clone was also dependent on the γδ TCR, as shown by blocking (Figure 4.21). In addition, this experiment confirmed the involvement of the receptor NKG2D in the recognition of EBV-infected cells. Taken together, these results show that the δ1+ γδ T cell population contains different types of effector cells that specifically kill EBV-infected cells through recognition of different determinants, and some of these effector cells have HLA-I-restricted reactivity.

To investigate the effect of LMP2A on the effector activity of δ1+ T cells, we performed a lysis assay challenging δ1+ T cell clones with WT or ΔLMP2A LCLs

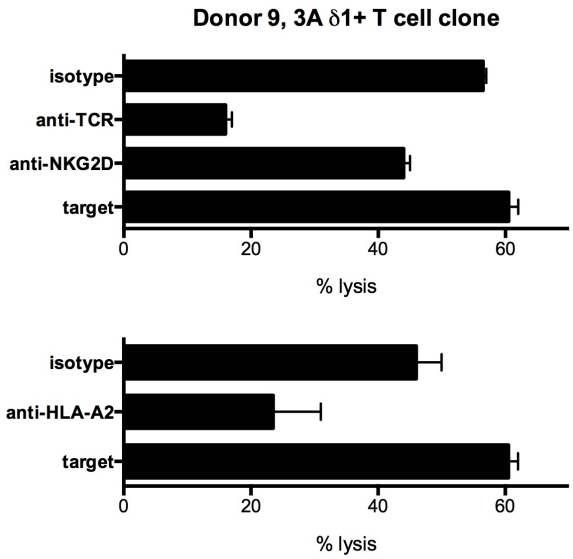

Figure 4.21. Effect of γδ TCR, NKG2D and HLA-A2 blocking on the lysis efficiency of the δ1+ T cell clone 3A from donor 9. The δ1+ T cell clone 3A from donor 1 was challenged in a lysis assay with autologous LCLs at a target:effector ratio of 1:10, in the presence of an anti-γδ TCR, anti-NKG2D or anti-HLA-A2 antibody or matched isotype control where indicated. Mean and range of duplicates are shown.

(Figure 4.22). Interestingly, recognition of cells infected with the mutant virus showed a decreased recognition by δ1+ T cell clones. Thus, the presence of LMP2A favors recognition of EBV-infected cells by γδ T cells, but hampers recognition of infected cells by CD8+ T cells as examined above. This suggests that CD8+ T cells and γδ T cells are predominantly triggered by different determinants or coactivators on LCLs. It appears that the immunomodulatory properties of LMP2A have evolved to strike a balance between prevention of control by CD8+ T cells and by γδ T cells.

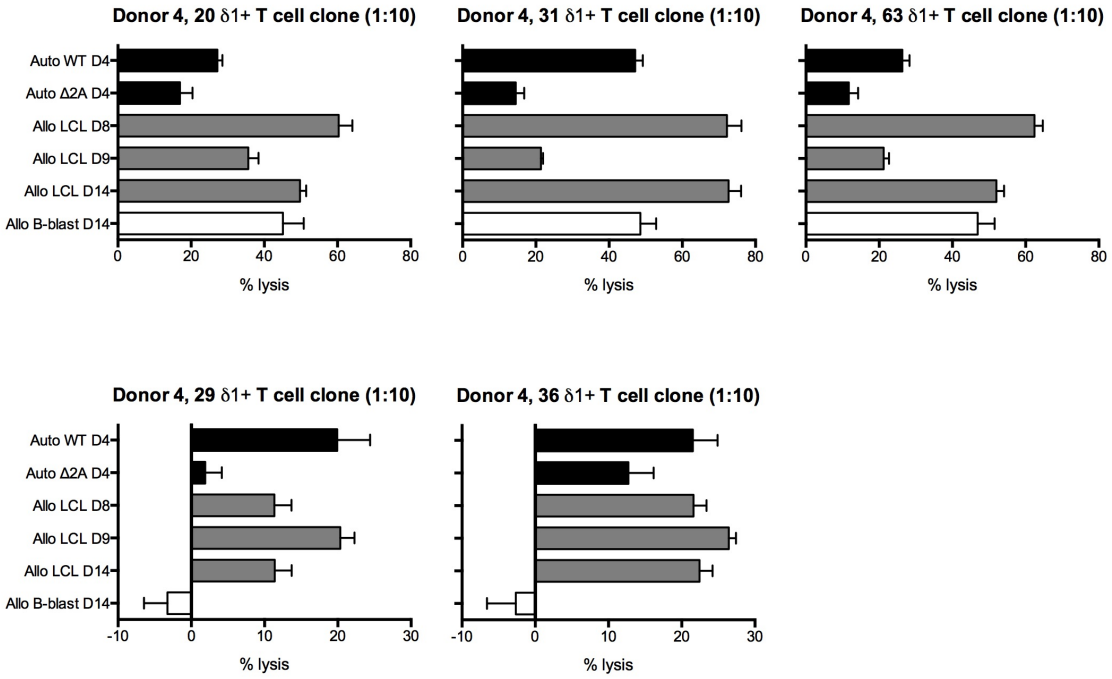

Figure 4.22. Recognition of WT and ΔLMP2A LCLs by δ1+ T cell clones. Five different δ1+ T cell clones from donor 4 were challenged in a lysis assay with autologous WT and ΔLMP2A LCLs, and with allogeneic LCLs or B-blasts at a target:effector ratio of 1:10. Means and standard error of the mean (SEM) of four replicates are shown.

5 Discussion

Epstein-Barr virus has a deep and intricate impact on the human immune system. Two processes come to mind that vividly illustrate this effect: the remarkable efficiency of transformation of normal human B cells by EBV *in vitro* (Pope et al. 1968), and the capability of EBV to induce strong virus-specific T cell responses in virus carriers for a lifetime (Hislop et al. 2007b). The discovery of Epstein-Barr virus (EPSTEIN et al. 1964), the first identified human tumor virus, even preceded the insight that lymphocytes can be divided into subsets with different functions, B and T cells (Miller & Mitchell 1967; Mitchell & Miller 1968). Thus, it is not surprising that the concepts of how EBV interacts with the immune system were only gradually developed, in parallel to the elucidation of the principles of immune function.

When EBV transforms normal human B cells, B-"lymphoblastoid" cells are formed that closely resemble B lymphoblasts. The latter are B cells that have been activated by executing their capacity to recognize antigen (signal 1), receiving T cell help (signal 2), or detecting invariant microbial components (signal 3). All of these signals are important elements for the mobilization of a B cell response against pathogens, and exert the strongest effect if present in combination (Ruprecht & Lanzavecchia 2006). Many of the viral functions that mediate B cell transformation have been characterized in close detail (Young & Rickinson 2004), including the functions of LMP2A, which mimics signal 1 (Merchant et al. 2001), and of LMP1, which mimics signal 2 (Graham et al. 2010). However, the apparent close similarity of lymphoblasts and lymphoblastoid cells, and the compelling analogy of viral transforming molecules with cellular activation pathways, have lead to a view of EBV infection of B cells that has neglected an important distinguishing aspect: while physiological mechanisms of B cell activation are designed to elicit and sustain immune responses, EBV has necessarily evolved to evade immune responses that are mobilized against the virus. Thus, it can be predicted that B cell activation by EBV infection on one hand, and by physiological triggers of signals 1, 2, and 3 on the other hand, produce B cells with important functional differences regarding their capability to interact with other cells of the immune system. Strikingly, not much is known so far on mechanisms employed by EBV that limit the immunogenicity of EBV-transformed B cells. To the contrary, the

focus of research has mainly been on the immune-stimulatory capacity of EBV-transformed lymphoblastoid cells. This is understandable because EBV LCLs have proven their suitability as a tool to expand EBV-specific T cells for scientific and also for immunotherapeutic use (Wallace et al. 1982; Rickinson & Moss 1997; Adhikary et al. 2007; Bollard et al. 2012). Moreover, a number of immunomodulatory EBV genes has been identified that reduce T cell recognition of infected cells in lytic cycle, but these immunomodulators are not active in latently infected cells (Hislop et al. 2007a; Zuo et al. 2008, 2009; Jochum et al. 2012a).

Therefore, the goal of my work presented in this thesis was to identify factors that modulate the interaction of EBV-infected B cells with antiviral immune cells. In the first part of my experiments, I comparatively analyzed changes in the immunophenotype that are triggered by EBV infection versus non-viral triggers of signals 1, 2, and 3, and identify immune effector molecules that are differentially regulated in lymphoblastoid cells and lymphoblasts. In the second part, I describe that latent membrane protein LMP2A counteracts CD8+ T cell recognition of latently infected cells. In the third part, I show that γδ T cells can specifically recognize EBV-infected cells, and describe that their recognition of LCLs appears to follow different rules than recognition by CD8+ T cells.

5.1 Effect of EBV infection and non-viral B cell activation on immunophenotype

In the first part of the present work, I present a detailed analysis of the modulation of the expression of relevant immunomodulatory molecules in the context of EBV-mediated and non-viral B cell activation. It is now appreciated that costimulatory and coinhibitory molecules have a crucial role in regulating activation, effector function, subset differentiation and survival of T cells (Welten et al. 2013). Therefore, the modulation of the surface expression of these molecules is relevant to understand how a virus such as EBV can influence the cellular immune response of the host.

EBV is known to induce activation of B cells through its viral proteins LMP1 and LMP2A (Dawson et al. 2012). In B cells, LMP2A mimics signal 1, the activatory signal of the B cell receptor (BCR) both *in vitro* and in a transgenic mouse model (Caldwell et al. 1998; Mancao & Hammerschmidt 2007). LMP2A promotes B cell survival and inhibition of apoptosis by activating the PI3K/Akt and ERK-MAPK signaling pathways (Panousis & Rowe 1997; Fukuda & Longnecker 2004; Portis & Longnecker 2004; Anderson & Longnecker 2008).

LMP1 acts as a functional mimic of signal 2, T helper cell–mediated CD40 ligation on B cells, but in comparison to the receptor CD40, LMP1 does so in a constitutive, dysregulated manner (Graham et al. 2010). Studies regarding the activatory functions of LMP1 have been mainly performed in epithelial cells, fibroblasts or HEK293 cells and showed that LMP1 activates the canonical and non-canonical NF-κB and PI3K/Akt pathways (Dawson et al. 2003; Eliopoulos et al. 2003; Luftig et al. 2004; Thornburg & Raab-Traub 2007; Kung & Raab-Traub 2010) and leads to the activation of the ERK and JNK kinases (Kieser et al. 1997; Roberts & Cooper 1998). The activation by LMP1 of these cellular pathways positively affects cell proliferation, survival and transformation. Since EBV mimics B cell activation and takes advantage of the same cellular pathways as physiological B cell activation, EBV-induced B cell activation is often compared to non-viral B cell activation: identifying similarities and differences is useful to gain information on how EBV influences the molecular changes induced by these pathways. Indeed, my data show that early EBV infection induces the upregulation of the B7 molecules CD86 and PD-L1, of the integrin ICAM-1 and of the NKG2D ligands MICB and ULBP4.

The surface molecule CD86 is upregulated on APCs upon activation. It can bind to the co-stimulatory receptor CD28 or to the inhibitory receptor CTLA-4 on T cells, but it was shown that CD28 binds CD86 more effectively in comparison to CTLA-4 (Collins et al. 2002). This observation suggests a predominantly stimulatory rather than inhibitory function for CD86. CD28 is constitutively expressed on naive CD8+ and CD4+ T cells, for which it serves as positive stimulator of cell growth and cell survival (Acuto & Michel 2003). CD28 is also expressed on γδ T cells; however, for these cells its role is still controversial (Ribot et al. 2011). CD28 ligation itself does not trigger activation of its own cellular pathways, except in the special case of "CD28 superagonists" (Mikami & Sakaguchi 2014), but it rather amplifies TCR signaling, which makes the difference between T cell activation or T cell death especially in the event of weak TCR signals (Acuto & Michel 2003). Priming of virus-specific CD4+ T cells is strongly reduced in CD28 knock-out mice, but requirement of CD28 co-stimulation for CD8+ T cell activation was shown to be variable in several viral infection models (Salek-Ardakani et al. 2009). Our data show that B cell activation and EBV infection induced a similar extent of CD86 upregulation, suggesting that EBV-induced activation and B cell activation through BCR/CD40/TLR engagement were comparable in regard to this parameter. Expression of CD86 was variable in latently EBV infected cells, but showed a downward trend as compared to early infection. In the context of B cell activation, upregulation of CD86 may help the activation and priming of T cells. In return, this signal leads to induction of CD40L on the T cell, supporting B-cell-mediated humoral responses (Acuto & Michel 2003). It is difficult, however, to predict the role of the upregulation of this molecule especially in the context of EBV infection, since there is no knowledge on whether CD86 is involved in the priming of EBV-specific T cells or in the support of recognition of EBV-infected cells.

Another B7 family member whose surface expression is influenced by B cell activation is PD-L1. The ligand PD-L1 is expressed on both lymphoid and non-lymphoid cells. Increasing evidence suggests that PD-L1 expression on non-lymphoid cells prevents tissue destruction by suppressing autoreactive lymphocytes (Okazaki & Honjo 2006). Its receptor PD-1 is found on activated T cells, and its engagement induces T cell inactivation or anergy (Okazaki & Honjo 2006). PD-1 interferes with activatory TCR signaling by recruiting negative regulators to the site of the immunological synapse

(Kulpa et al. 2013). Interestingly, blocking of the interaction between PD-1 and PD-L1 was demonstrated to restore T cell function in various contexts (Okazaki & Honjo 2006), for example in a model of chronic viral infection (Barber et al. 2006). Several reports showed the presence of PD-1 on EBV-specific CD8+ T cells, especially during and after IM (Sauce et al. 2009; Greenough et al. 2010), and blocking of PD-1/PD-L1 interactions between NK or T cells and EBV-infected B cells was recently shown to increase NK and T cell reactivity (Durand-Panteix et al. 2012). In accordance with these findings, I found that EBV stably induced PD-L1 expression, both in early and in established latent infection. The induction of expression of PD-L1 by EBV, especially early after infection, may support the escape of EBV-infected cells and help establish infection. The induction or maintenance of PD-L1 expression to promote immune escape or T cell exhaustion has been already demonstrated in the context of other viral infections (Kirchberger et al. 2005; Benedict et al. 2008; Kulpa et al. 2013). Inhibition of T cell activity during the early phase of infection establishment may provide enough time for EBV to build up an EBV-carrying B cell population and establish sustained latent infection in its host. Thus, it will be interesting to investigate to what extent the blocking of the interaction between PD-1 and PD-L1 influences recognition by T cells of early EBV-infected B cells.

The expression of PD-L1 on B cells after non-viral activation followed completely different kinetics. On these cells, PD-L1 expression was first induced and then downregulated within 48 hours after stimulation, while EBV-infection induced a slower increase to a level that remained stable already after 48 hours. While upregulation of PD-L1 by EBV could be a mechanism designed to avoid detection of infection, the rapid induction of PD-L1 upon B cell activation might be of importance in T cell activation control. In this regard, it is interesting to notice that PD-L1 surface expression is maximal when all three B cell activatory stimuli are combined but, in contrast to the expression of other immune effector molecules studied here, upregulation of PD-L1 appears to be mainly driven by the combination of BCR and TLR9 signaling. CD40L alone or in combination with only one other stimulus was not a good inducer of PD-L1. However, CD40L further increased PD-L1 expression by anti-BCR combined with CpG. It would be interesting to investigate whether and how the differences in PD-L1 expression after different types of stimulation could affect T cell functions.

Upregulation of the adhesion molecule ICAM-1 on non-virally activated B cells followed a kinetic similar to PD-L1 expression. Detection of ICAM-1 was highly increased 24 hours after stimulation, but showed a drastic decrease of expression already at 48 hours post-stimulation. As components of the immunological synapse, the integrin LFA-1 on the T cell side and its ligand ICAM-1 on the target cell side play an important role in stabilizing interaction between T cell and target cell, and in promoting T cell activation (Wulfing et al. 1998). Additionally, LFA-1 can be expressed by antigen-presenting cells, and ICAM-1 by T cells. Anti-LFA-1 antibodies inhibit CD4+ T cell proliferation, cytokine production, and induction of activation antigens. These observations support a model in which blocking of LFA-1/ICAM-1 interaction prevents TCR-mediated signaling events, such as upregulation of the activation antigen IL-2Rα (Hathcock et al. 1994). It is however interesting to note that the highest surface expression level detected for ICAM-1 upon B cell activation was observed with CD40L/IL-4 activation alone or in combination with BCR activation, whereas the addition of CpG had no major effects on ICAM-1 expression. This provides an example of a partial division of labor among different physiological B cell stimuli: whereas TLR signaling triggered by CpG DNA preferentially influences the expression of molecules such as PD-L1 as described above, the induction of ICAM-1 was largely driven by CD40 and BCR stimulation, but not supported by CpG stimulation. It is possible that rapid downregulation of ICAM-1 after B cell activation may positively influence the functional outcome of B cell:T cell contacts, because it was shown that transient contacts of a T cell with an APC are typical for the interaction of T cells with dendritic cells, which leads to the differentiation of effector T cells, whereas overly prolonged contacts of a T cell with an APC may render T cells anergic, or favor their differentiation to regulatory T cells (Reichardt et al. 2007). In contrast to B cell activation, EBV infected B cells showed a slow induction of ICAM-1 expression, and expression of this molecule was maintained in latently infected B cells.

On the other hand, the expression of CD11a, the α chain of the integrin LFA-1, the interacting counterpart of ICAM-1, was only influenced by non-viral B cell activation. In non-viral B cell activation, CD11a showed a slow upregulation during the late stages of B cell activation (day 6, day 8 after stimulation). LFA-1 is thought to help in formation and stability of the immunological synapse. Indeed, supporting this hypothesis, it was shown that T cell MHC-II triggering on naive B cells enhances LFA-

1 “stickiness” on these cells, resulting in a very stable cell-cell contact (Gunzer et al. 2004). Moreover, activation of B cells mediated by follicular DCs is dependent on the presence of integrins such as LFA-1 (Koopman et al. 1991). LFA-1 was shown to form a ring around clusters of BCR molecules, stabilizing the cell:cell contact and reducing the threshold of antigen required by B cell for activation (Koopman et al. 1991). In addition, interaction between LFA-1 and ICAM-1, expressed on the B cell and on the APC respectively, prevents apoptosis of the B cell in the germinal center (Arana et al. 2008). This mechanism becomes of particular importance in the case of a limited amount of antigen for B cell activation. The differential expression of the two intergins ICAM-1 and CD11a during B cell activation may reflect the different roles of these molecules in providing an anchor for B cell–T cell or B cell–APC interactions. If we compare early and latent EBV infection of B cells in regard to CD11a surface expression levels, it appears that EBV does not modulate the expression of this molecule after early infection, but its levels are stably maintained throughout EBV infection. A more comprehensive series of measurements over a longer time will provide an even better insight into the effect of EBV on CD11a surface expression.

We also analyzed upregulation of NKG2D ligands after non-viral and EBV infection-mediated B cell activation. NKG2D is an agonistic receptor present on T and NK cells, which recognizes a variety of “stress-induced” ligands that are upregulated on the cell surface in conditions such as malignant transformation, viral infection or heat shock. Increased expression of NKG2D ligands after EBV infection was described earlier (Pappworth et al. 2007; Kong et al. 2009; Zhang et al. 2012; Chaigne-Delalande et al. 2013), but a comprehensive analysis of NKG2D ligand expression on EBV-infected B cells was still missing. Our analysis of the eight known NKG2D ligands showed that early EBV infection induced the expression of two of them (MICB, ULBP4) and the transient but moderate upregulation of a third NKG2D ligand, ULBP1. MICB and ULBP4 were also expressed on the surface of latently EBV infected B cells, MICB higher than ULBP4. Interestingly, in latently infected B cells a third NKG2D ligand, MICA, was also upregulated. After non-viral B cell activation, I observed only a transient expression of MICB, but no expression of any other NKG2D ligand. If we compare the expression of NKG2D ligands between EBV infection and B cell activation, a few differences become apparent. First, EBV induces not only MICB but also transient ULBP1 expression and ULBP4 upregulation, even though at later time

points. Second, EBV slowly induced and maintained the expression of MICB on the cell surface, while activated B cells presented a transient expression of this molecule. Could the expression of MICB on activated B cells play a role in attracting help from NKG2D+ T helper cells? While the role of NKG2D on cytotoxic T and NK cells is well established (Zafirova et al. 2011), not so much is known about the role of this activatory receptor on CD4+ T cells. What is known is that NKG2D+ CD4+ T cells are a rare subpopulation in healthy humans (Groh et al. 2003; Alonso-Arias et al. 2011). In contrast, NKG2D+ CD4+ T cells have been isolated in different malignancies (Garcia-Chagollan et al. 2013; Romero et al. 2014; Yu et al. 2014) and in chronic inflammatory disease (Camus et al. 2014). Interestingly, a population of virus-specific NKG2D+ CD4+ T cells has been identified in carriers of the herpesvirus HCMV (Saez-Borderias et al. 2006). Therefore, it cannot be ruled out that EBV-specific CD4+ T cells with this phenotype may exist in EBV carriers, and that MICB may play a role in priming or activation of these cells.

B cell activation, but not EBV-induced activation, also influenced the expression of the SLAM family member CD48. CD48 surface expression was slightly upregulated 24 hours after B cell stimulation. Induction of the expression of this molecule was rapidly reversed and it was followed by a slow reduction of its expression. CD48 is a molecule solely expressed on hematopoietic cells. Its receptor, 2B4, is expressed on NK and T lymphocytes and it can induce both activatory and inhibitory signals, accordingly to the presence or the absence of the cellular mediator SAP (Parolini et al. 2000). Activation of T and NK cells by 2B4 and other SAP-interacting receptors is fundamental for efficient control of EBV-infection. In fact, male patients affected with X-linked lymphoproliferative disease (XLP), a fatal disease caused by mutations in the SAP gene, are prone to develop life-threatening EBV-induced lymphomas (Nichols et al. 2005). This suggests a relevant role for this receptor-ligand pair in the recognition and clearance of EBV-transformed B cells by NK cells (Benoit et al. 2000). It has been recently appreciated that SAP also play a role in the generation and maintenance of humoral immunity. Mutations in SAP impair the ability of CD4+ T cells to interact with B cells, leading to a shortage of adequate T cell contact-dependent help for B cells (Qi et al. 2008). Like for CD11a, early or latent EBV infection of B cells did not show strong effects on the surface expression of CD48. Comparing CD48 expression levels between early EBV infection of B cells and LCLs, it appears that CD48 might be

slowly upregulated upon EBV infection. Further experiments with a longer kinetic would be necessary to reach a conclusive answer.

When we directly compare these results on non-virally activated B cells and EBV-infected B cells, we need to keep in mind that EBV infection is expected to act more slowly on B cells on a population level *in vitro* due to the experimental conditions that are available. Stimulation with CD40L-expressing cells (Wiesner et al. 2008) or with CpG DNA (Hartmann & Krieg 2000; Ruprecht & Lanzavecchia 2006) is rapid and efficient *in vitro*, because these stimuli are applied in a form that quickly targets most or all B cells that are present in the culture. In contrast, EBV infection of primary B cells is quantitatively less efficient, because only a fraction of the B cells can be functionally infected with EBV *in vitro* (Iskra et al. 2010), which has several reasons. Although different B cell subsets (naive and memory) can be infected by EBV *in vitro* with similar efficiency (Heath et al. 2012), only a fraction of EBV bound to the B cell surface can reach the cell nucleus and initiate viral gene expression (Shannon-Lowe et al. 2005). After EBV reaches the nucleus, intracellular expression of viral B-cell–activatory molecules first needs to be turned on before they can initiate changes in expression of cellular immune effector molecules on the cell surface, a process that can take hours to days (Iskra et al. 2010; Jochum et al. 2012b). Within this time window, a proportion of B cells undergo apoptosis, because they apparently do not receive sufficient survival signals under *in vitro* culture conditions. EBV counteracts this tendency by early expression of two antiapoptotic molecules that are functionally homologous to Bcl-2 (Altmann & Hammerschmidt 2005), but their efficiency is not absolute, and therefore apoptosis is prominent in EBV-infected B cell cultures around 2–6 days after initiation of culture (Altmann & Hammerschmidt 2005; Iskra et al. 2010). It follows that B cell immunophenotypes after EBV infection and non-viral stimulation should be compared qualitatively rather than quantitatively.

5.2 Role of LMP2A in evading recognition of latently EBV-infected cells by CD8+ T cells

In the second part of my experiments for this thesis, I analyzed the role of the EBV latent membrane protein LMP2A in modulating the immunophenotype of infected B cells and in altering their recognition by immune effector cells. These studies were made possible by my identification of a method to generate LMP2A-deleted LCLs with high efficiency. In this method, primary B cells are infected with LMP2A-deleted EBV in the presence of CD40L-expressing stimulator cells, but further expanded without such an exogenous stimulus. My analyses showed for the first time that LMP2A interferes with CD8+ T cell recognition of latently infected B cells (LCLs), and I could identify several mechanisms that contribute to this immunoevasive function of LMP2A. First, LMP2A lowered the amount of antigen available for CD8+ T cell recognition by decreasing mRNA expression levels of EBV latent antigens targeted by CD8+ T cells, in particular EBNA1. Second, LMP2A downregulated MHC class I molecules on the cell surface to a certain degree. Third and perhaps most important, two ligands of the coactivatory receptor NKG2D were strongly upregulated in LMP2A-deficient LCLs. I could demonstrate that blocking of NKG2D reduced T cell recognition of infected cells, and this reduction was particularly strong in LMP2A-deleted LCLs. These results show that LMP2A hampers CD8+ T cell recognition of infected cells by combining a variety of mechanisms, and establish the importance of NKG2D ligands in CD8+ T cell recognition of EBV-infected B cells.

LMP2A is part of the latent protein repertoire of EBV. Much of the protein is embedded in cell membranes, because it contains twelve transmembrane regions that follow each other closely. Its N-terminal cytoplasmic tail contains various motifs functioning as docking sites for cellular kinases that lead to activation of intracellular signaling pathways (Pang et al. 2009; Dawson et al. 2012). As mentioned above, LMP2A promotes B cell survival and inhibition of apoptosis by mimicking the activatory signals of the BCR (Caldwell et al. 1998; Mancao & Hammerschmidt 2007), and by activating the PI3K/Akt and ERK-MAPK pathways (Konishi et al. 2001; Portis & Longnecker 2004; Anderson & Longnecker 2008). In addition, by preventing normal BCR activation, LMP2A was shown to interfere with BCR-induced activation of the EBV lytic cycle (Miller et al. 1994a, b, 1995; Konishi et al. 2001). However, in the

absence of exogenous triggers of lytic cycle, LMP2A supported the induction of basal levels of lytic cycle gene expression. This suggests a dual role of LMP2A in controlling EBV lytic cycle (Schaadt et al. 2005).

The influence of LMP2A on human B cell proliferation and transformation has been controversial: while some studies did not identify a role of LMP2A in these processes (Longnecker et al. 1992, 1993a, b; Speck et al. 1999; Konishi et al. 2001), other reports indicated that LMP2A increases the efficiency of B cell proliferation and transformation (Brielmeier et al. 1996; Mancao & Hammerschmidt 2007). My experience with establishing LCLs with an EBV mutant deleted for LMP2A confirmed that LMP2A is indeed important for establishment of EBV latent infection *in vitro*, because the outgrowth of LCLs from EBV-infected primary B cell cultures was strongly impaired when the virus was deleted for LMP2A. However, if an additional stimulus such as CD40 activation was provided, LCLs deleted for LMP2A could be efficiently generated, and were then maintained autonomously. This showed that LMP2A is not essential for maintenance and proliferation of established LCLs, as long as the B cell receptor is expressed in the transformed B cells (Mancao & Hammerschmidt 2007).

In hindsight, the immunomodulatory effects of LMP2A may also have contributed to the differential outgrowth of WT and ΔLMP2A EBV-infected B cells in culture that I observed. Although I performed EBV infection with B cells purified from PBMCs, the purity of the B cells was obviously not absolute, but in the range of 95–98%, and some T and NK cells were necessarily still present. Some of these T cells, with potential support by NK cells, may have selectively prevented outgrowth of ΔLMP2A LCLs, due to their higher potential to activate T cells. It is known that immune effector cells in PBMCs can abolish outgrowth of B cells infected with EBV *in vitro*. In fact, this phenomenon of outgrowth regression first demonstrated the existence of EBV-specific cellular immunity (Moss et al. 1978), and forms the basis of a simple assay to quantitate EBV-specific T cell immunity in bulk (Frisan et al. 2001). Later, it was shown that EBV antigen-specific cytotoxic CD8+ T cells, and cytotoxic CD4+ T cell populations that target unknown antigens expressed on LCLs, both contribute to regression of EBV-infected B cells (Nikiforow et al. 2001; Gudgeon et al. 2005). In accordance with standard methods (Roskrow et al. 1998; Moosmann et al. 2002), I used cyclosporin A in our EBV infection cultures to prevent activation of residual effector T

cells against outgrowing EBV-infected B cells. However, the experience of our and other laboratories shows that the effect of cyclosporin A in culture is not an absolute one, and occasionally the outgrowth of EBV-transformed B cells from infected cultures is prevented by T cells in spite of the presence of cyclosporin A, a problem that can be circumvented by establishing multiple parallel EBV-infected microcultures from each donor (Roskrow et al. 1998; Moosmann et al. 2002). Thus, it can be easily imagined that ΔLMP2A LCLs are more susceptible to attack by T cells than WT LCLs, and this difference may also have contributed to reduce outgrowth of LMP2A-deleted LCLs observed earlier (Brielmeier et al. 1996). Whether this hypothesis is true remains to be verified in more detailed regression assays with precisely defined numbers of admixed primary T cells.

LMP2A is not only expressed in B cells expressing the transforming "growth" program or latency III program, but also in EBV-positive tumors expressing an even more restricted panel of viral antigens, such as Burkitt lymphoma, Hodgkin lymphoma, nasopharyngeal carcinoma, and gastric carcinoma (Rickinson 2014). By enhancing anchorage-independent growth (Fukuda & Longnecker 2007), inhibiting differentiation (Scholle et al. 2000), interfering with the proapoptotic effects of TGFβ1 (Fukuda & Longnecker 2004) and inducing the anti-apoptotic protein survivin (Hino et al. 2008), LMP2A promotes survival, proliferation and transformation in epithelial cells. In addition, LMP2A was shown to enhance tumorigenesis *in vivo* (Scholle et al. 2000; Kong et al. 2010)), strongly suggesting a role for LMP2A in the maintenance or in the progression of LMP2A-expressing malignancies.

While a number of EBV-encoded immunoevasins that operate in lytic cycle have been identified as discussed above (Hislop et al. 2007a; Rowe et al. 2007; Zuo et al. 2009), only one EBV antigen expressed in latently infected cells, EBNA1, was shown to interfere with antigen presentation to CD8+ T cells. Importantly, EBNA1 does not generally interfere with antigen presentation, but specifically inhibits its own processing in cis by blocking proteasomal processing (Levitskaya et al. 1997). It has been unknown whether other latent EBV antigens can affect recognition by T cells. An immunomodulatory role of LMP2A was previously suggested by reports showing that the presence of LMP2A alters the expression of several genes related to immunity (Portis et al. 2003), and demonstrated an interference of LMP2A with signaling of type I/II interferon receptors (Shah et al. 2009). My data demonstrated that the presence of

LMP2A markedly reduced the reactivity by EBV-specific CD8+ T cells against LCLs. Notably, this was true for all the latent viral antigens investigated (LMP2, EBNA1, EBNA3A). In contrast, for the tested lytic antigens, BZLF1 and BRLF1, I could not identify differences in recognition by CD8+ T cells. This absence of a detectable effect of LMP2A on recognition of lytic antigens is due to the negative or very low baseline expression of these antigens in LCLs (Pudney et al. 2005; Hislop et al. 2007b), which means that latently WT EBV-infected B cells are unreliably or not at all recognized by CD8+ T cells specific for lytic-cycle antigens, as my data confirm. Thus, LMP2A is the first EBV latency antigen for which an in-trans interference with recognition of infected cells by EBV-specific CD8+ T cells has been demonstrated, independent of the identity of the antigen recognized.

Interestingly, the latent antigens targeted by the CD8+ T cells analyzed in our experiments are processed in the infected cells by different pathways for their presentation: the CLG and FLY epitopes are TAP-independent epitopes, with FLY being immunoproteasome-dependent (Lautscham et al. 2001, 2003), while the RPP and HPV epitopes are TAP-dependent (Tellam et al. 2004). In addition, a reduction in CD8+ T cell reactivity was mediated by LMP2A in the context of different HLA allotypes (HLA A*0201, B*0702, B*3501 and HLA A*0201, C*0702 for the peptide-loading experiments). By demonstrating effects of LMP2A on specific CD8+ T cell recognition of LCLs that were exogenously loaded with peptides derived from CMV, I further showed that LMP2A has immunomodulatory effects that are independent of a specific antigen or MHC-I allotype, and are mediated by secreted molecules and cell surface molecules including NKG2D ligands.

However, LMP2A influenced antigen-specific T cell recognition at different levels. Besides the antigen-processing machinery, other factors can influence the successful presentation of an antigen, such as antigen availability and levels of MHC class I molecules. An effect of LMP2A on these processes was observed in our experiments, showing that LMP2A had a tendency to reduce mRNA expression of several EBV latent genes as well as MHC-I surface levels. Both aspects could be causally related, because an increased supply of peptides available for loading on MHC class I may increase the overall levels of MHC-I present at the cell surface.

Nontheless, some of the effects of LMP2A on the EBV-infected B cell appear to preferentially affect particular antigens. This was suggested by my observation that

LMP2A differentially affected the mRNA levels of other latent-cycle antigens, and mRNA coding for the nuclear antigen EBNA1 was downregulated more strongly than other viral latency mRNAs. This is particularly interesting, because in several EBV-associated malignancies of EBV latency II type, such as nasopharyngeal carcinoma and Hodgkin lymphoma, LMP2A and EBNA1 are often the only two EBV protein-coding genes expressed in the tumor cells (Herbst et al. 1991; Deacon et al. 1993; Bell et al. 2006). Thus, LMP2A may interfere with presentation of EBNA1-derived peptides not only in the LCL model studied here, but also in latency II type tumors, which could have important effects on the overall visibility of these tumors for the immune system.

I also addressed the question whether cytokines could mediate part of the effects of LMP2A on LCLs by CD8+ T cells. In this regard, IL-10 was an interesting candidate, because it is an immunomodulatory cytokine (Ng et al. 2013) that is constitutively produced at high levels by EBV-transformed B cells (Finke et al. 1993), and a recent report showed that LMP2A increased IL-10 production in Burkitt lymphoma lines (Incrocci et al. 2013). EBV encodes a viral homologue of human IL-10 also known as viral IL10 (vIL-10), which is encoded by the early lytic gene BCRF1 (Hsu et al. 1990). Both human and viral IL-10 have been shown to interfere with EBV immunity at various levels. vIL-10 was shown to reduce IFN-γ release by PBMCs after contact with LCLs in which the lytic cycle was artificially induced (Swaminathan et al. 1993) and to inhibit, when added exogenously, the control of EBV-infected B cell outgrowth by T cells (Bejarano & Masucci 1998). vIL-10 contributes to downregulation of the transporter of antigen processing 1 (TAP1) and MHC-I in the early phase of B cell infection (Zeidler et al. 1997), although recognition of early-infected B cells by EBV-specific CD8+ T cells was not increased in the absence of vIL-10 (Jochum et al. 2012a). However, the presence of vIL-10 impaired NK cell reactivity (Jochum et al. 2012a).

Our data showed that LCLs lacking LMP2A released lower amounts of IL-10 compared to WT LCLs, which was not associated with reduced IL-10 mRNA levels. IL-10 mRNA present multiple copies of potential mRNA destabilizing motifs, and previous reports showed that IL-10 transcription and secretion can be widely divergent (Powell et al. 2000; Nemeth et al. 2005). Thus, IL-10 secretion is strongly modulated by post-transcriptional processes. Importantly, in my experiments I found that the difference in IL-10 production did not affect the reactivity of CD8+ T cell clones

against infected B cells, as shown by blocking and neutralizing experiments. This is compatible with the observation of Jochum et al. that WT EBV and vIL-10-deleted EBV are equally recognized by CD8+ T cells in the early phase of infection (Jochum et al. 2012a). Nonetheless, my finding does not exclude an indirect influence of altered IL-10 secretion on T cell recognition of LCLs. Indeed, human and viral IL-10 have been shown to downregulate MHC-I surface expression on freshly infected B cells and monocytes, and to interfere with the expression of other surface molecules on monocytes (Zeidler et al. 1997; Salek-Ardakani et al. 2002). Therefore, LCL-secreted IL-10 may act back on the LCLs, reducing in a downregulation of MHC-I molecules observed for WT LCLs as compared to ΔLMP2A LCLs that translates later into a differential recognition by CD8+ T cells, although there is no direct impact of IL-10 levels on the T cells themselves.

CD8+ T cells recognize their targets through the specific interaction of the TCR with the peptide:MHC-I complex. As explained above, the intensity and quality of antigen recognition by CD8+ T cells depend on their interaction with costimulatory receptors (Greaves & Gribben 2013) and adhesion molecules (Springer & Dustin 2012) on the surface of the antigen-presenting cell. Thus, I analyzed the influence of LMP2A on surface expression of the B7 molecules PD-L1 and CD86, of the integrin CD11a and the adhesion molecule ICAM-1, of ligands for the receptor NKG2D, and of the SLAM family member CD48.

As mentioned, the inhibitory receptor PD-1 is found on activated T cells or on virus-specific T cells in chronic infection (Barber et al. 2006) and on EBV-specific CD8+ T cells during and after IM (Sauce et al. 2009; Greenough et al. 2010). Interestingly, surface expression of PD-L1, PD-1 ligand, is increased on LCLs and on EBV-positive PTLD (Kim et al. 2008; Durand-Panteix et al. 2012; Green et al. 2012). Notably, it was recently shown that blocking of PD-1/PD-L1 interactions could increase cytotoxic activity of T and NK cells against EBV-infected B cells (Durand-Panteix et al. 2012). PD-L1 expression may additionally mediate toxic effects to EBV-infected cells because it was shown that cross-linking of PD-L1 by a specific antibody induced their apoptosis (Kim et al. 2008). Counter-intuitively, we found PD-L1 to be downregulated in LCLs in the presence of LMP2A. Thus, it is very unlikely that differential PD-L1 expression contributed to increased T cell reactivity to ΔLMP2A LCLs. However, it would be interesting to investigate in the future whether PD-L1 can decrease recognition of WT

and ΔLMP2A LCLs. Hypothetically, because of higher PD-L1 expression in ΔLMP2A LCLs, blocking of PD-L1, or of PD-1 on the side of the T cell, may even increase the difference in reactivity of T cells against WT versus ΔLMP2A LCLs.

As mentioned before, the receptor-ligand pair ICAM-1 and LFA-1 plays an important role in promoting T and B cell activation and interaction between T cell and antigen-presenting cell (Wulfing et al. 1998; Arana et al. 2008). Our analyses showed that the levels of ICAM-1 were equally high on the surface of WT and ΔLMP2A LCLs, suggesting a similar capability of these LCL variants to interact with LFA-1 on T cells and thereby stabilize T cell recognition. Of interest, the expression of CD11a, the alpha chain of LFA-1, on LCLs itself was strongly decreased in the absence of LMP2A. Although LFA-1 on B cells is important for their BCR-mediated activation upon contact with antigen (Carrasco et al. 2004), it seems unclear whether an "inverse" interaction of LFA-1 on B cells and ICAM-1 on T cells could influence T cell-mediated recognition of EBV-infected B cells. However, in a very speculative model B-cell-expressed LFA-1 could compete in cis or in trans for B-cell expressed ICAM-1 with LFA-1 expressed by effector T cells. Thus, we cannot rule out that the decreased levels of LFA-1 on ΔLMP2A LCLs contribute to their increased recognition by EBV-specific T cells.

EBV infection was described to increased expression of NKG2D ligands (Pappworth et al. 2007; Kong et al. 2009; Wiesmayr et al. 2012; Chaigne-Delalande et al. 2013). Our analysis of the eight NKG2D ligands showed that latent EBV infection indeed induced the expression of three of them (MICA, MICB, ULBP4) and that induction of MICA and ULBP4 was further increased in the absence of LMP2A. In addition, we demonstrated that blocking of NKG2D on CD8+ T cells distinctly affected the recognition of LCLs by these effector cells. A recent study has shown that in patients with genetic deficiencies in the magnesium transporter MAGT1, who are particularly susceptible to EBV infection and EBV+ lymphomas, NKG2D plays an important role in the control of EBV infection by NK and CD8+ T cells (Chaigne-Delalande et al. 2013).

A role for NKG2D in control of EBV-associated cancer has been further illustrated in a mouse model of LMP1-induced cancer, which could be therapeutically targeted through NKG2D (Zhang et al. 2012). Targeting of the NKG2D ligand MICB by an EBV-encoded miRNA may decrease susceptibility of EBV-infected B cells to lysis by

NK cells (Nachmani et al. 2009). Thus, NKG2D ligands represent important coagonists for EBV-specific adaptive or innate immunity, and it appears an efficient strategy for the virus to decrease surface expression of NKG2D ligands through the action of LMP2A.

Taken together, I describe here for the first time an immunomodulatory function of the EBV protein LMP2A, and show that LMP2A affects escape of infected B cells from recognition by CD8+ T cells. A broad range of immunoevasive mechanisms is mediated by LMP2A in EBV-infected B cells. Thus, it will be urgent to determine the role played by LMP2A in evasion from T and NK cell recognition in different types of EBV-associated lymphoproliferative and malignant disease. Because LMP2A decreases T cell recognition independent from the identity of the antigen and the MHC allotype, an important function of LMP2A could be to shield the spectrum of mutated cellular antigens in malignantly transformed cells from recognition by T cells.

5.3 γδ T cells in the control of EBV infection

In the third part of this thesis, I studied the reactivity of γδ T cells against EBV-infected B cells. I demonstrated that γδ T cells efficiently recognize and exert cytotoxicity against EBV infected cells. I found that the γδ cells mediating this effect carry a T cell receptor containing the Vδ1 chain. Some cells within this subpopulation could recognize EBV-infected B cells in an apparently EBV-specific fashion. Moreover, I identified one δ1+ T cell clone that specifically recognized autologous EBV-infected B cells in an HLA-A2-restricted manner.

In the context of EBV infection, not much is known about the role of γδ T cells. γδ T cells were shown to expand during acute infectious mononucleosis (De Paoli et al. 1990), to proliferate after contact with LCLs (Hacker et al. 1992; Orsini et al. 1993) and after contact with EBV-positive Burkitt lymphoma cells (Hacker et al. 1992; Orsini et al. 1994; L'Faqihi et al. 1999). However, the literature gives controversial information regarding which population recognizes EBV-infected B cells. In some studies, the population showing major expansion was the δ1+ T cell population (Hacker et al. 1992; Orsini et al. 1993), while others reported expansion of other γδ T cell populations (De Paoli et al. 1990; Qvigstad et al. 1990). Various groups also reported lysis of LCLs by the δ1+ T cell population (Mami-Chouaib et al. 1990; Qvigstad et al. 1990; Hyjek et al. 1997; Fujishima et al. 2007), by δ2+ T cells (Kong et al. 2009) and by polyclonal lines (Lam et al. 1990), or production of IFNγ after contact with LCLs in bulk short-term cultures (Bhaduri-McIntosh et al. 2008). It is interesting to notice that expansion of γδ T cells was also observed in cytotoxic lymphocyte cultures (CTL) stimulated with LCLs prior infusion to treat EBV-positive post-transplant lymphoproliferative diseases, reaching even 30% of the total culture mix (Comoli et al. 2001; Savoldo et al. 2006). In one of the treated patients, the CTL status was analyzed again 15 weeks after CTL infusion and it was observed that the γδ T cell population remained present in frequencies that were very close to those present in the pre-infusion CTL mix (Comoli et al. 2001). This evidence suggests an involvement of γδ T cells in the control of EBV infection.

By stimulating freshly isolated PBMCs with LCLs, I confirmed that γδ T cells regularly expand in the presence of EBV-infected cells, and that both δ2+ and δ2– subpopulations were expanded after contact with LCLs. The δ2– subpopulation showed

a higher average expansion than the δ2+ subpopulation. Interestingly, there were differences in the expansion efficiency between different donors. For two donors the expansion of γδ T cells was minimal (Donor 3 and 13). In these cases, I suggest that the overall stimulatory reaction may be composed of a compression of irrelevant γδ T cells and an expansion of a subpopulation of "EBV-specific" γδ T cells. In these two cases, it would have been interesting to continue the analysis for a longer time frame.

All donor analyzed were EBV-seropositive, and four out of six donors were also CMV-seropositive. The CMV status is relevant, because CMV-seropositive donors present higher numbers of δ2– T cells in peripheral blood than CMV-negative donors (Dechanet et al. 1999; Pitard et al. 2008). However, CMV serostatus did not appear to influence the expansion of γδ T cells after contact with LCLs. We also compared stimulation efficiencies by different LCLs. PBMCs from two donors (donors 4 and 14) were stimulated in two separate reactions with two different allogeneic LCLs. For one donor, the kinetic of the expansion was similar in the two stimulatory reactions, even though one LCL was slightly more efficient in inducing γδ T cell expansion. For the second donor, instead, the expansion of γδ T cell, and especially of δ2– T cell population, was strongly increased in one of the two reactions. This suggests that heterogeneity between LCLs used for γδ T cell stimulation can strongly affect expansion efficiency of these effector cells, and that specific interactions between γδ T cells and donor-specific alloantigens on LCLs might affect the selective expansion of a desired γδ T cell subpopulation.

To further investigate which γδ T cell population mediates an effector function in the control of EBV infection, I established γδ single T cell clones. From all donors examined, I could obtain δ2+ and δ2– clones, although with different efficiencies in different donors. From one donor almost all clones established presented a δ2+ TCR. From a second donor, only 13% of the established clones were δ1+ T cells, while for the other three donors the δ2+ and δ2– clones were present in similar proportions. It is noteworthy that I could also establish clones that are rarely found in peripheral blood, presenting a γ9δ1+ TCR or, in another case, a δ1–/δ2– TCR, which therefore expressed another of the available but less frequently used delta chains (Casorati et al. 1989). I also identified some rare clones that presented a peculiar and unexpected low affinity interaction with a αβTCR-specific antibody, in addition to staining with γδ-specific

antibodies. αβ+ T cell clones presenting a Vδ1 chain have been already identified (Ueno et al. 2003). However, in that case the TCR composition showed an occurred recombination between the Vδ1 gene segment with a Jα gene segment. In my case, TCR sequencing for one of these peculiar clones (clone 94, donor 1) showed the presence of a classic Vδ1-Jδ1 chain. All the clones analyzed, irrespective for their TCR composition, expressed high levels of the agonistic receptor NKG2D and expressed variable levels of CD56, a marker for NK cells that is also found in subpopulations of CD8+ T cells. In contrast, only a few clones between those analyzed showed expression of other NK receptors. These data confirm that stimulation of γδ+ T cells with LCLs is an efficient method to obtain γδ+ single cell clones, even from infrequent γδ subpopulations.

Subsequently, I investigated whether these clones could mediate cytotoxicity of EBV-infected B cells. My results demonstrated that the population performing higher and broader LCL cytotoxicity was the δ1+ T cell population. In contrast, δ2+ T cell clones showed no or low levels of cytotoxicity when challenged with LCLs. γ9δ1+ and δ1/2– T cell clones had variable effector activity against LCLs. Different δ1+ T cell clones had different specificities, as shown by their different degree of recognition of allogeneic or autologous LCLs. Importantly, several clones appeared to specifically recognize autologous LCLs, but not activated autologous CD40-activated B lymphoblasts, suggesting recognition of an EBV antigen or a specifically EBV-induced antigen. The different patterns of recognition of EBV-infected B cells by different clones confirm the idea that γδ+ T cells are highly heterogeneous, further supporting the hypothesis that this T cell population can recognize a variety of molecular targets. In order to obtain more precise data regarding the specificity mediated by different types of γδ T cell receptors, I analyzed the TCR sequences of some of the clones that had an effector activity against EBV-infected B cells. These data showed that, even though the recognition of EBV-infected cells appears to be mostly related to the presence of the δ1 TCR chain, the CDR3 regions of the analyzed δ1 chains are highly variable both in length and in their amino acid sequence. In three cases, I identified two clones from the same donor that had identical CDR3 regions. Generally, however, the variability of TCR sequences of clones from the same donor and between donors was high, supporting the idea that they recognize different molecular targets. High variability in the length, and in the amino acid and nucleotide sequence was also

observed in the TCRs from γδ T cells after infection with HCMV, another herpesvirus (Dechanet et al. 1999). The only feature that appeared more frequent in γδ T cells after infection with HCMV than in the absence of HCMV infection was the presence of phenylalanine and tryptophan, two apolar aromatic bulky residues, in positions P3 and P6 of the δ1 chain of the TCR (Dechanet et al. 1999). In the sequences obtained from EBV-specific δ1+ T cells in my work, no conserved motifs were identified, further supporting the idea of the recognition of different molecular targets by these cells. Generally, information regarding the TCR sequences for δ1+ T cells, but also for other γδ subpopulation, is limited, and the correlation between TCR sequence and specificity of the γδ T cells has rarely been investigated, most likely because only a few γδ T cell-specific ligands have been identified so far (Chien & Konigshofer 2007). Therefore, these data are a good starting point for further analysis focusing on the correlation between TCR sequences and reactivity of γδ T cells.

Interestingly, when further investigating the mechanism of recognition for three δ1+ T cell clones, I found that for all of them, the costimulatory receptor NKG2D was a mediator of cytotoxicity against LCLs, as shown by anti-NKG2D blocking studies. This is of particular interest, because in the case of the herpesvirus HCMV the receptor NKG2D was not involved in the recognition of HCMV-infected cells by γδ+ T cells (Halary et al. 2005). A possible reason for this could be that HCMV uses several immunoevasive strategies to downregulate the surface expression of NKG2D ligands during infection (Muller et al. 2010). My evidence further supports the hypothesis that NKG2D ligand expression on LCLs is an important factor for recognition of EBV-infected cells by T cells of different types.

One γδ T cell clone, which expressed the inhibitory KIR receptors KIR2DL2/3 and KIR3DL1, presented a high increase in its cytotoxic activity against EBV-infected B cells upon antibody blocking of MHC-I, the ligand of these inhibitory KIRs. These data support the hypothesis that γδ+ T cells expressing NK receptors can have an NK-like behavior (Rothenfusser et al. 2002). Moreover, these observations suggest that the co-receptor repertoire of γδ+ T cells can strongly affect the effector functions of these cells, possibly much more than for αβ+ T cells. Therefore, this characteristic of γδ+ T cells needs to be considered when analyzing the functionality of these cells.

Another γδ T cell clone showed the opposite behaviour, resulting in a surprising decrease of the lysis of LCLs after blocking with anti-MHC-I antibody, suggesting an MHC-dependent mechanism of recognition of EBV-infected cells. The hypothesis of HLA-restricted γδ T cells is not new. Several groups investigated this hypothesis, especially early after the discovery of γδ T cells, but the evidence gathered to support or discard this hypothesis appeared to be contradictory at that time. A number of different reactivity patterns of γδ T cells were described. For some γδ T cells, alloreactive MHC-dependent activity was found (Matis et al. 1987). Some authors did not find classical MHC-restricted reactivity (Borst et al. 1987; Lam et al. 1990; Maccario et al. 1993), whereas others identified γδ T cells that recognized polymorphic determinants of MHC molecules (Ciccone et al. 1989; Matis et al. 1989; Flament et al. 1994)}. Later, when it became accepted that γδ T cells followed different rules of antigen recognition than αβ T cells, the hypothesis of HLA-restricted reactivity of γδ T cells was abandoned by most researchers, and research efforts focused on the identification of alternative molecules serving as molecular targets (Pardoll et al. 1987; Janeway et al. 1988; Constant et al. 1994). Nevertheless, the idea that some γδ T cells may be HLA-restricted has not been abandoned (Knutson & Disis 2002). My data show a possible resolution of these apparent contradictions, showing that δ1+ T cells are a heterogeneous group of cells with different specificities and modes of regulation.

By testing the MHC-dependent γδ T cell clone against a panel of HLA-matched and mismatched LCLs, I found that recognition correlated with expression of HLA-A2 on the LCLs. Blocking experiments with HLA-A2-specific antibody confirmed this finding. Recognition of LCLs by this clone was γδ TCR-dependent, and receptor NKG2D was also involved in the recognition of EBV-infected B cells.

Interestingly, when the reactivity of δ1+ T cell clones against WT LCLs was compared to the reactivity against LCLs lacking LMP2A, δ1+ T cells showed a decreased recognition of ΔLMP2A LCLs. Therefore, the presence of LMP2A in EBV-infected cells favors the recognition of LCLs by δ1+ T cells but hampers the recognition of the same cells by CD8+ T cells. This observation implies that these two subsets of effector cells interact differently with their target and suggests that CD8+ T cell and γδ T cell reactivity is triggered by different determinants and, possibly, by different coactivators. In addition, it underlines the balance that is established between the need by EBV to

evolve escape mechanisms to hide from immune recognition and the need by the host to develop efficient strategies for the control of EBV infection.

Taken together, these results show that δ1+ T cells represent the γδ T cell subpopulation mediating an immune response against EBV and that different γδ T cells have different anti-viral properties. Moreover, we identified a clone that specifically recognizes EBV-infected B cells in an HLA-restricted manner.

There have been many hints in the literature that γδ T cells are involved in recognition of EBV-infected cells. However, it has remained unclear and controversial which types of γδ T cells are involved in EBV-specific recognition, and which receptors and mechanisms are responsible for it. The results of my analyses do not comprehensively answer these questions, but they clearly show that specific interaction of γδ T cells and EBV-infected B cells takes place, and leads to killing of EBV-infected B cells. Thus, it is likely that γδ T cells have a role in control of EBV *in vivo*. My findings provide a solid starting point for more intense investigations of this question in the future, and indicate which lines of research will be most promising.

My use of a large panel of single-cell clones has allowed me to clearly distinguish the reactivity of different types of γδ T cells. First, I showed that certain δ1+ T cells specifically recognize EBV-infected lymphoblastoid cells, whereas such recognition was never found for δ2+ T cells, the major γδ T cell population in peripheral blood. Thus, if δ2+ T cells are sometimes found to be expanded in the presence of EBV *in vitro* or *in vivo* (De Paoli et al. 1990; Kong et al. 2009), my results suggest that this is unlikely to be driven by direct recognition of EBV+ lymphoblastoid cells. Second, I found that only specific subsets of δ1+ T cells recognize EBV-infected B cells from the autologous host, while sparing EBV-negative or allogeneic B cells, and I identified examples of specific δ1+ TCR sequences that are involved in this recognition. Thus, γδ T cells with these or closely related TCRs should be specifically studied in a larger panel of healthy donors and patients. Third, I confirmed the earlier finding that certain γδ T cells specifically react with certain allogeneic B cells. It will be very important for future immunotherapeutic applications of γδ T cells, for example after stem cell transplantation (Scheper et al. 2013), that potentially protective and potentially pathogenic γδ T cells are precisely distinguished and separately analyzed. Fourth, I identified an example of an EBV-specific δ1+ T cell that only recognized HLA-A2-positive EBV-infected targets, and is most likely an HLA-restricted γδ T cell. This

refutes the notion that γδ T cells can never be HLA-restricted (Lam et al. 1990; Maccario et al. 1993). HLA-restricted γδ T cells may be a very important antiviral effector population, and should be more intensely investigated in the future. Although γδ T cells are sometimes considered to be "innate-like" immune effectors due to the supposedly small diversity of their T cell receptor repertoire, my data clearly argue that γδ TCRs are highly diverse, and that apparently subtle differences in their TCR primary structure lead to distinctly different reactivities. Although some γδ T cells show a pattern of reactivity reminiscent of natural killer cells, there are good arguments to consider γδ T cells part of the adaptive immune system, at least the δ1+ subset. The exact principles that guide their antiviral activity, and their potential in immunotherapeutic application, remain to be fully explored in the future.

6 Abbreviations

α-	anti-, antibody specific for
Akt	protein kinase B
Allo	allogeneic
ALP	alkaline phosphatase
AM	acetoxymethylester
APC	Antigen-presenting cell
APC	allophycocyanin
ATP	adenosine triphosphate
Auto	autologous
β_2m	β_2-microglobulin
B7-H1	B7 homolog 1
BCR	B-cell receptor
BV421	brillant violet 421
CD	cluster of differentiation
cDNA	complementary DNA
CDR	complementarity-determining region
CML	chronic myeloid leukemia
conc	concentration
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte antigen 4
D	donor
DNA	deoxyribonucleic acid
DC	dendritic cell
ΔLMP2A	LMP2A deleted
DMEM	Dulbecco's modified Eagle Medium (cell culture medium)
DMSO	dimethyl sulfoxide
dNTP	deoxynucleotide triphosphate
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERAPs	ER aminopeptidases
ERK	extracellular-signal-regulated kinase
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FSC	forward scatter
gp	glycoprotein
GUSB	β-glucuronidase

GvHD	graft versus host disease
HCMV	human cytomegalovirus
HEK	human embryonic kidney
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HMBPP	hydromethyl-but-2-enyl-pyrophosphate
HSCT	hematopoietic stem cell transplantation
HSV	herpes simplex virus
ICAM-1	intracellular adhesion molecule 1
ICOS	inducible costimulator
ICOS-L	inducible costimulator ligand
IE-1	immediate early protein 1
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IM	infecious mononucleosis
IPP	isopentenyl pyrophosphate
iso	isotype control
ITAM	immunpreceptor tyrosine-based activation motif
kDa	kilodalton
KIR	killer cell immunoglobulin-like receptor
KLR	killer C-type lectin-like receptors
L	ligand
LCL	lymphoblastoid cell line
LEAF	low-endotoxin, azide-free
LFA-1	lymphocyte function-associated antigen 1
LIR/ILT	receptor of the leucocytes immunoglobulin-like receptor/immunoglobulin-like transcript family
LMP	latent membrane protein
MAGT	magnesium transporter
MAPK	mitogen-activated protein kinase
MEP	methylerythritol 4-phosphate
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MHC-I	MHC class I
MHC-II	MHC class II
MIC	MHC class-I polypeptide-related sequence
mMFI	multiplicity of mean fluorescence intensity
mRNA	messenger RNA
NCAM	neural cell adhesion molecule
NCR	natural cytotoxicity receptor
nd	not determined
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer

NKG2A	natural killer group 2 A
NKG2D	natural killer group 2 D
No Ab	no antibody
Not Inf	not infected
Opti-MEM	reduced serum media Eagle's Minimum Essential Medim (cell culture medium)
PB	pacific blue
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pΔ	packaging cell line
PD-1	programmed death-1
PD-L1	programmed death-ligand 1
PE	phycoerythrin
PE-Cy5	phycoerythrin-cyanine 5
PEI	polyethyleneimine
pep	peptide
PerCP	peridinin chlorophyll
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
pNPP	para-nitrophenyl phosphate
PRRs	pattern recognition receptors
PTLD	post-transplant lymphoproliferative disease
R	receptor
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute (cell culture medium)
RT-PCR	real time PCR
SAP	Signaling lympocytic activation molecule-associated protein
SEM	standard error of the mean
SLAM	signaling lymphocyte activation molecule
SSC	side scatter
TAE	Tris, acetate, EDTA
TAP	transporter associated with antigen processing
TCR	T-cell receptor
TGF	trasforming growth factor
TLR	toll-like receptor
TNF	tumor necrosis factor
TR	terminal repeats
ULBP	UL-16-binding protein
UV	ultraviolet
v	viral
VCA	virus capsid antigen
WT	wild type
XLP	X-linked lymphoproliferative disease
∅t	no target

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