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The T cell repertoire specific for the IE-1 protein of human cytomegalovirus: diversity, function and evasion

von
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Erklärung

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Summary

The present study investigates the diversity and function of the T cell repertoire specific for the immediate-early 1 (IE-1) antigen of HCMV and analyzes the viral countermeasures to recognition of this antigen, with an aim to improve antiviral immunotherapy. Among HCMV antigens, IE-1 is a dominant target of CD8+ T cells. Superior protective function of IE-1-specific CD8+ T cells is expected because T cells recognizing immediate-early infection can destroy infected cells before these produce new virus. CD8+ and CD4+ T cells specific for IE-1 could be expanded *in vitro* from all healthy HCMV carriers by stimulation of peripheral blood cells with autologous IE-1-expressing activated B cell lines (mini-LCLs). Specific T cell clones were established from expanded T cells by limiting dilution and used to define epitope specificities and the impact of viral immunoevasion on T cells with different specificities.

CD4+ T cells against IE-1 were a quantitatively small but customary component of the HCMV-specific T cell repertoire. In each donor, 3–7 IE-1 epitopes were identified (27 in total) and presented by HLA-DR, DQ or DP allotypes, and some epitopes were shared between donors. CD4+ epitopes were distributed over the entire IE-1 sequence. All IE-1-specific CD4+ T cell clones produced Th1 cytokines (IL-2, IFN-γ, TNF-α), some of them also Th2 cytokines (IL-4), and only few were cytotoxic. CD4+ T cell clones had distinct reactivity against HCMV-infected dendritic cells (DCs), which was especially strong for HLA-DP-restricted clones, indicating that IE-1 presentation by different class II allotypes may be differentially regulated.

The CD8+ T cell response to IE-1 was found to be extremely large and focused on 1 or 2 epitopes per donor, often shared between donors. Unexpectedly, newly identified CD8+ T cells restricted through a very frequent HLA-C allotype, C*0702, dominated the T cell response to IE-1 in carriers of this allele, and recognized HCMV-infected cells much more efficiently than HLA-A and B-restricted T cells. Detailed analyses using HCMV strains deleted for all four or three of the HLA class I-specific immunoevasins US2/3/6/11 revealed allotype-specific patterns of interference with T cell recognition by each of US2, US3 and US11. A concerted action of several immunoevasins already at the immediate-early phase of infection was necessary to block antigen presentation by most HLA-A and B allotypes. HLA-C*0702 was selectively excluded from viral immunoevasion, and CD8+ T cell recognition through HLA-C*0702 but not HLA-A or -B allotypes was specifically preserved. Conversely, killing of infected cells by NK cells carrying an HLA-C-specific inhibitory receptor was specifically inhibited. Thus, HCMV immunoevasion strikes a balance between excessive recognition by CD8+ T cells and by NK cells. As a consequence, HLA-C-restricted antigen presentation is selectively preserved and HLA-C-restricted T cells may be superior effectors of HCMV-specific vaccines and cellular immunotherapies.

Zusammenfassung

Diese Arbeit untersucht die Diversität und Funktion des spezifischen T-Zellrepertoires gegen das Antigen *immediate-early 1* (IE-1) von HCMV und die viralen Maßnahmen gegen seine Erkennung. Ziel ist die Verbesserung der antiviralen Immuntherapie. IE-1 ist ein dominantes Zielantigen HCMV-spezifischer CD8+-T-Zellen. Eine protektive Wirkung gerade IE-1-spezifischer T-Zellen wird erwartet, da T-Zellen gegen Antigene der IE-Phase infizierte Zellen zerstören können, bevor neues Virus gebildet wird. Spezifische CD8+- und CD4+-T-Zellen gegen IE-1 konnten *in vitro* aus Blut jedes gesunden HCMV-Trägers durch Stimulation mit autologen IE-1-exprimierenden B-Zelllinien (mini-LCLs) expandiert werden. Spezifische T-Zellklone wurden hergestellt, um die Zielepitope der T-Zellen zu ermitteln und die virale Immunevasion gegenüber T-Zellen verschiedener Spezifitäten zu untersuchen.

CD4+ T-Zellen gegen IE-1 waren in HCMV-Trägern zwar in geringer Zahl, jedoch regelmäßig vorhanden. Je Spender wurden 3–7 IE-1-Epitope identifiziert (27 insgesamt), die durch HLA-DR, DQ oder DP präsentiert wurden, manche dieser Epitope in mehreren Spendern. Die CD4+-T-Zellepitope waren über den Großteil der IE-1-Sequenz verteilt. IE-1-spezifische T-Zellklone sezernierten Th1-Zytokine (IL-2, IFN-γ, TNF-α), manche auch Th2-Zytokine (IL-4), nur wenige waren zytotoxisch. Alle CD4+-T-Zellklone waren ausgeprägt reaktiv gegen HCMV-infizierte dendritische Zellen (DCs), am stärksten HLA-DP-restringierte Klone, ein Hinweis auf eine differentielle Antigenpräsentation durch verschiedene Klasse-II-Allotypen.

Die CD8+-T-Zellantwort gegen IE-1 erwies sich als sehr stark. Sie richtete sich gegen 1 bis 2 Epitope pro Spender, bei verschiedenen Spendern oft dieselben. Unerwartet dominierten bisher unbekannte HLA-C-restringierte CD8+-T-Zellen die Immunantwort gegen IE-1, restringiert durch den häufigen Allotyp HLA-C*0702. Solche T-Zellen erkannten die HCMV-Infektion weit effizienter als HLA-A- und HLA-B-restringierte T-Zellen. Untersuchungen mit HCMV-Rekombinanten, in denen alle vier oder drei der vier HLA-Klasse-I-spezifischen Immunevasine US2/3/6/11 deletiert waren, ergaben distinkte Allotyp-spezifische Muster der Inhibition der T-Zellerkennung durch US2, US3 und US11. Bei den meisten HLA-A- und HLA-B-Allotypen war die Kooperation mehrerer Moleküle der US2/3/6/11-Gruppe in der IE-Phase der Infektion notwendig, um die Antigenpräsentation zu unterbinden. HLA-C*0702 war von der viralen Immunevasion ausgenommen, und die Erkennung durch HLA-C*0702-restringierte T-Zellen, nicht aber HLA-A/B-restringierte T-Zellen, war selektiv bewahrt. Umgekehrt war die Zytolyse von infizierten Zellen durch NK-Zellen, die einen inhibitorischen Rezeptor für HLA-C trugen, spezifisch inhibiert. Die Immunevasion durch HCMV scheint so ausbalanciert zu sein, dass eine exzessive Erkennung einerseits durch CD8+ T-Zellen, andererseits durch NK-Zellen vermieden wird. Infolgedessen wird die HLA-C-restringierte Antigenpräsentation selektiv bewahrt. Daher könnten HLA-C-restringierte T-Zellen besonders geeignete Effektoren einer HCMV-spezifischen Impfung oder zellulären Immuntherapie sein.

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1 Introduction

The human body provides an ideal living environment for infectious organisms such as parasites, fungi, bacteria and viruses. However, the microorganisms encountered daily in the life of a healthy individual cause disease only sporadically. This is due to the evolvement of a complex network of cells, tissues, and organs, positioned throughout the body - the immune system -, which provides a broad variety of host defense mechanisms. To selectively attack "foreign" invaders and not cells of the host organism, the immune system has the ability to distinguish between "self" and "non-self". Also malignant cells with "altered self" can be recognized and eliminated which plays a major role in tumor surveillance.

If a pathogen penetrates the physical barriers of the body, it is immediately confronted with the unspecific defense strategies of the innate immune system, which is constitutively present and ready to be mobilized upon infection. In addition, vertebrates have evolved a more specific second line of defense, the adaptive immune system, which is effective with a delay of 4–7 days and is initiated by components of the innate immune response. During initial exposure to a pathogen the adaptive immunity develops an immunological memory, which leads to an enhanced response to subsequent encounters with the same pathogen and is the basis for vaccination. The defense strategies of both the innate and the adaptive immune response comprise humoral (secretory molecules) as well as cellular components. (Janeway et al., 2006)

1.1 Cellular immune response mechanisms

All immune cells originate from hematopoietic stem cells in the bone marrow which give rise to myeloid cells (including neutrophils, monocytes, macrophages, dendritic cells (DCs)) and lymphoid cells (B cells, T cells, natural killer (NK) cells). During an immune response, these cells exhibit complex interrelationships that allow them to fine-tune reactions to almost any pathogen. Neutrophils, monocytes, macrophages, DCs and NK cells are important mediators of the innate immune response. They possess pattern recognition receptors (PRRs) like Toll-like receptors (TLRs), which recognize broad molecular patterns (PAMPs = pathogen associated molecular patterns) present in a number of different pathogens but not in host cells. In this way, innate immune mechanisms can act efficiently to a variety of different pathogens. Neutrophils and tissue macrophages are the first to arrive in large numbers at a site of infection. Monocytes, which differentiate into macrophages, and immature DCs are recruited later. These cells are primarily phagocytic cells that engulf microorganisms and digest them intracellularly. They possess a variety of cell surface receptors through which they can bind infectious agents. For example, as a result of the activation of a catalytic cascade of serum proteins, bacteria are coated with complement factors and can be targeted by phagocytes via complement receptors. Recognition of pathogens by PRRs also activates phagocytes to secrete a range of cytokines (chemokines,

interleukins, TNF-α) that have important local and systemic effects contributing to both innate and adaptive immunity. For example, IL-8, MCP-1 and RANTES are chemoattractants that function in recruiting leukocytes, monocytes, and other effector cells from the bloodstream to sites of infection. NK cells are cytotoxic lymphocytes involved in non-specific immune responses to intracellular infections with bacteria and viruses, and further function in tumor surveillance. Circulating NK cells can be activated in response to cytokines and recognize changes in the expression of cell-surface proteins induced by infection or growth transformation.

B and T lymphocytes are the major players of the adaptive immune system. The specificity of the adaptive immune response resides in the receptors of T and B cells, the TCR and BCR, respectively, which recognize discrete antigens of a particular pathogen. The actual portions or fragments of an antigen that react with lymphocyte receptors are termed epitopes. To induce specific immune responses, mature DCs and macrophages stimulated during the initial immune response act as antigen-presenting cells (APCs) to T and B lymphocytes. After exposure to an antigen, B cells differentiate into plasma cells that produce and release specific antibodies (a soluble form of their BCRs) into the bloodstream. By binding to the surface of extracellular pathogens, antibodies enable phagocytosis of the pathogens via Fc receptors. T cells contribute to immune responses in two different ways: helper T cells coordinate the entire immune response by secretion of cytokines (including interleukins, growth factors, interferons), while cytotoxic T cells directly contact infected cells and destroy them. (Janeway et al., 2006)

1.1.1 Antigen presentation in the human system

As key players of the adaptive immune response, T lymphocytes continuously examine the intracellular and extracellular milieu for signs of infection, which become manifested as antigenic peptides presented on the surface of infected cells, phagocytes, or other cells that have taken up microbial material. The scaffolds for the presentation of these peptides are highly polymorphic glycoproteins, which are encoded in the major histocompatibility complex (MHC). In the human system, the MHC proteins are also called human leukocyte antigens (HLAs) as they were first discovered through antigenic differences between white blood cells from different individuals. There are two classes of MHC molecules, MHC class I and MHC class II, which show distinct protein structures (Fig. 1.1), expression patterns on host cells, and peptide loading mechanisms, and are targeted by different T cell subsets.

MHC class I molecules consist of two polypeptide chains, an α chain (heavy chain, 44 kDa) and a smaller β chain (light chain, 12 kDa), the β_2-microglobulin (β_2m). The heavy chain comprises three extracellular α domains (α_1–α_3), a transmembrane domain and a cytosolic tail. The two polypeptide chains are noncovalently associated by interaction of β_2m and α_3 domain, which both have an immunoglobulin-like structure. The α_1 and α_2 domains form a groove on the sur-

face of the MHC molecule, which is the site of peptide binding. The groove is bounded by an eight-stranded beta-pleated sheet on the bottom and two alpha helices on the sides and can accommodate peptides of approximately 8–10 amino acids in length. Whether a particular peptide can bind to a particular MHC molecule depends on the amino acid residues that line the groove and make contact with the peptide. These are the residues that are the most polymorphic in the MHC molecule. MHC class II molecules are heterodimers of an α and a β chain, which show approximately equal size (30 kDa) and are both anchored in the cell membrane. Each polypeptide chain, α and β, consists of two domains, α_1/α_2 and β_1/β_2, respectively, a transmembrane domain and a cytosolic tail. The peptide-binding groove is formed by the α_1 and β_1 domains and shows similar structure than for MHC class I molecules. However, the peptide binding groove is more open in MHC class II molecules compared to MHC class I molecules, so that the ends of peptides bound to MHC class II molecules are not buried within the groove. Consequently, MHC class II molecules can accommodate longer peptides of approximately 13–25 amino acids. (Germain, 1994)

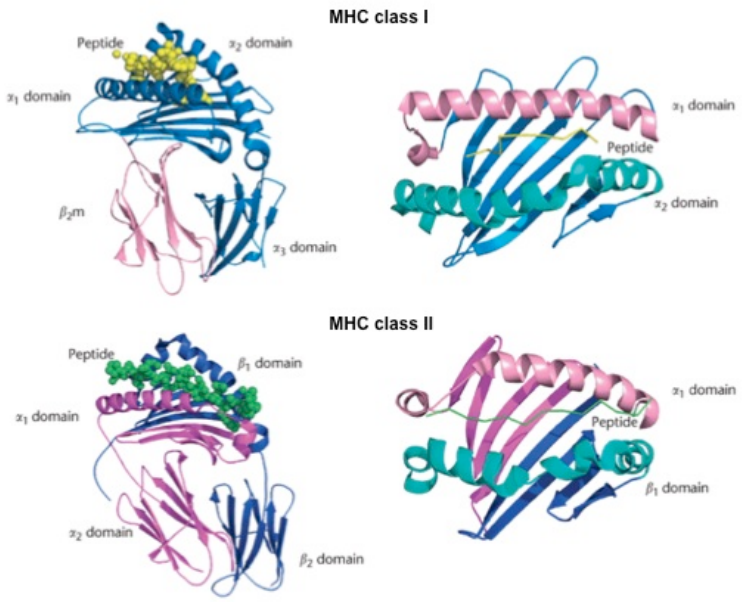

Fig. 1.1: Structure of MHC class I and II molecules. MHC class I molecules consist of an α chain subdivided into α_1–α_3 domains**,** transmembrane region and cytosolic tail, and a non-covalently associated β_2-microglobulin. The peptide binding groove is formed by the α_1 and α_2 domains and can accommodate peptides of approximately 8–10 amino acids. MHC class II molecules are heterodimers of an α and a β chain. Each chain consists of two extracellular domains, α_1/α_2 and β_1/β_2, a transmembrane and a cytosolic region. The peptide-binding groove is formed by the α_1 and β_1 domains and can bind peptides of 13–25 amino acids. The transmembrane and cytosolic regions have not been part of the structural analysis and are not shown. Modified according to (Liu and Gao, 2011).

The genes encoding the MHC class I α chain and the MHC class II α and β chains are located in the MHC. In humans this gene cluster is also termed HLA complex and is located on the short arm of chromosome 6 (Fig. 1.2). The gene for β_2-microglobulin is located on chromosome 15. The MHC is polygenic, i.e. it contains several different MHC class I and MHC class II genes, and it is highly polymorphic, i.e. there exist multiple allelic variants of each gene within the population. Alleles of an MHC gene are codominantly expressed, and both gene products are able to present antigens to T cells. The HLA protein encoded by an allele is termed the allomorph. The particular combination of HLA alleles found on a single chromosome is termed

the HLA haplotype. The combination of HLA class I and II allotypes expressed by a person is termed the HLA type.

The HLA complex spans approximately 4 Mb and comprises class I, II and III regions. The class III region encodes a variety of different proteins including complement factors (e.g. C2, C4), the heat shock protein HSP70, and TNF-α and -β. In the class I region are six genes which encode HLA class I heavy chains and give rise to six class I isoforms, the highly polymorphic HLA-A, -B and -C molecules (classical MHC class I molecules), and the less polymorphic HLA-E, -F and -G molecules (non-classical MHC class I molecules). The products of class II genes also form three major isoforms of MHC class II molecules termed HLA-DR, -DQ and -DP. However, each of DR, DQ and DP is assembled by an α and a β chain, which are encoded by separate genes in the HLA class II region designated as A and B, respectively (e.g. HLA-DQA1 and HLA-DQB1). The part of the HLA class II region that encodes HLA-DR molecules is more complex than those encoding the other class II isotypes, because there are several functional β-chain genes (DRB1, DRB3, DRB4, DRB5) and their number varies between different haplotypes with only DRB1 being always present. The β-chain products of all the expressed DRB genes associate with the α chain encoded by HLA-DRA, which is non-polymorphic. In contrast to DRA, DQA and DPA are polymorphic. Therefore, the number of different MHC molecules is further increased by the combinations of DQ and DP α and β allotypes encoded by different chromosomes. The exact number of different MHC class II molecules expressed in one individuum depends on how many genes and which alleles are present on each chromosome. The MHC class II region also encodes proteins with other important immunological functions, for example tapasin, the TAP1:TAP2 peptide transporter, and immunoproteasome subunits (LMP2, LMP7). (Marsh, 2000)

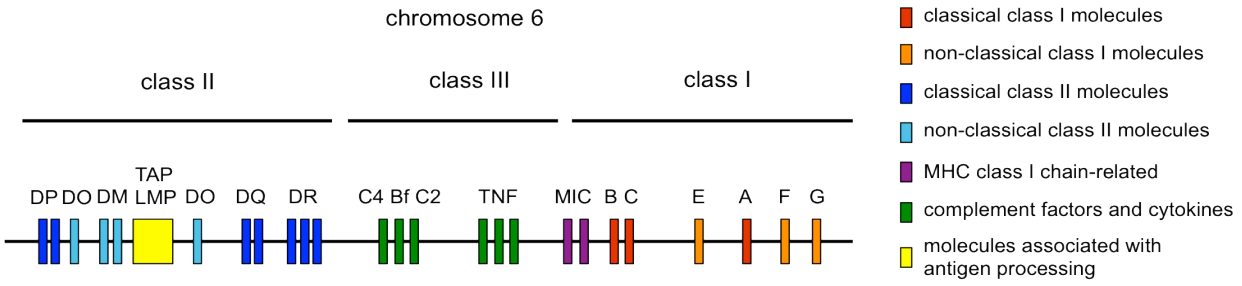

Fig. 1.2: Organization of the human major histocompatibility complex (HLA complex). HLA class I heavy chain genes are located in the class I region of the HLA complex and encode classical HLA class I molecules HLA-A, -B and -C, as well as non-classical HLA molecules HLA-E, -F, -G. HLA class II molecules involved in antigen presentation are HLA-DR, -DQ and -DP. These molecules are heterodimers of an α and a β chain, both which are encoded in the HLA class II region of the HLA complex. According to (Trowsdale, 2002).

As individuals carry different sets of HLA molecules, these surface glycoproteins themselves can become foreign antigens when organs or cells are transplanted. The T lymphocytes of an immunocompetent transplant recipient trigger an alloreactive immune response to the "foreign" HLA molecules on grafted cells. This situation is termed host versus graft (HVG) reaction, can result in transplant rejection and is repressed by immunosuppressive drug administration. However, after transplantation into an immunocompromised host, the immunocompetent T lymphocytes within the graft recognize the HLA molecules on the recipient's cells as alloantigens and respond with proliferation and host tissue damage. This situation is known as graft versus host disease (GVHD) and includes clinical manifestations like diarrhea, erythema, fever, pain and ultimately death. Matching of donor's and recipient's HLA type increases the success rate of grafts. However, genetic differences at other loci encoding minor histocompatibility antigens may still trigger GVHD or graft failure.

Loading of MHC class I and II molecules with peptides requires the processing of antigens into smaller fragments. Cytosolic proteins from a variety of sources including viruses are degraded by the proteasome and cytosolic proteases. The resulting peptides are then transported through the transporter associated with antigen processing (TAP) complex into the endoplasmic reticulum (ER) where they are further trimmed by ER aminopeptidases (ERAPs) to produce peptides of usually 8–10 aa for presentation on MHC class I molecules (Serwold et al., 2002). Very hydrophobic peptides and peptides generated in the ER or Golgi compartment can also reach the MHC class I pathway in a TAP-independent manner (Lautscham et al., 2003). MHC class I heavy chains are translocated during their synthesis into the lumen of the ER and assembled with β_2m. The loading of MHC class I heterodimers with the short peptides is coordinated by the peptide-loading complex, which consists of the TAP components TAP1 and TAP2, the ER-resident chaperone-like proteins ERp57, calreticulin, tapasin, and protein disulfide isomerase (PDI) (Park et al., 2006). This complex also has a function in peptide editing to select for the presentation of stable peptide-MHC class I complexes (Wright et al., 2004). MHC class I/peptide-complexes leave the endoplasmic reticulum and are transported through the Golgi compartment to the cell surface where they present the peptides to CD8+ T cells. MHC class I molecules typically present peptides derived from proteins synthesized in the same cell. However, exogenous proteins internalized by DCs and macrophages can be fed into the MHC class I pathway by retrotranslocation into the cytosol or by processing with endosomal proteases and loading onto MHC class I in the endocytic compartment itself. This phenomenon is referred to as cross-presentation (Cresswell et al., 2005).

MHC class II molecules are loaded with antigenic peptides in endosomal or lysosomal vesicles termed the MHC class II compartment (MIIC). Exogenous proteins access the MIIC through phagocytosis and endocytosis, endogenous cellular or viral proteins by a process involving autophagy (Crotzer and Blum, 2010). Antigenic peptides of an appropriate length for binding to

MHC class II molecules are generated by lysosomal proteases. The biosynthetic pathway for MHC class II-dependent peptide presentation starts with the ER assembly of MHC class II dimers in complex with the invariant chain (Ii). Ii blocks the peptide-binding groove of MHC II molecules, thus preventing peptide occupation in pre-lysosomal compartments. This complex traffics to the acidic MIIC, where Ii is subjected to sequential proteolysis. The final cleavage product, class II-associated Ii peptide (CLIP), can be replaced with high-affinity antigenic peptides, an exchange reaction that is catalyzed by the chaperone-like molecule HLA-DM. After transport to the cell surface, MHC class II/peptide-complexes are presented to CD4+ T cells. (Neefjes et al., 2011)

MHC class I molecules are expressed on almost all nucleated body cells, thus allowing the direct recognition and elimination of virus-infected or malignant cells by cytotoxic T cells. By contrast, constitutive expression of MHC class II molecules is restricted to specialized cells including thymic epithelial cells and professional APCs (DCs, macrophages, B cells) (Jensen, 2007). However, during an immune response MHC class II gene expression can be induced in other cells by IFN-γ, which is predominantly made by activated T cells and NK cells. IFN-γ also upregulates MHC class I surface levels by increasing expression of MHC class I α chain, β_2-microglobulin, tapasin, and TAP components (Schroder et al., 2004). Furthermore, IFN-γ induces the expression of the immunoproteasome, which is normally also restricted to professional APCs. In the immunoproteasome, the three catalytically active β subunits of the constitutive 26S-proteasome are replaced by the IFN-γ inducible subunits LMP2 (β1i), LMP7 (β5i) and MECL-1 (β2i) (Groettrup et al., 2001). These exchanges together with the IFN-γ-induced proteasome-associated activator PA28 alter the cleavage specificity of the proteasome, thus extending the range of antigenic peptides for MHC presentation (Schroder et al., 2004).

Several APCs are also used *in vitro* for antigen processing and presentation on MHC class I and II molecules. DCs are considered to be the most potent APCs due to their unique ability to prime antigen-inexperienced (naive) T cells and their enhanced capacity to reactivate memory T cells. Monocyte-derived DCs are already used in the clinic as vaccines to induce therapeutic anti-tumor T cell responses (Chiang et al., 2011). Unfortunately, the application of DCs is limited due to their rareness in the peripheral blood and difficulty to expand them *ex vivo* (von Bergwelt-Baildon et al., 2002). Another important group of APCs are B cells. However, resting B cells have found to be inactive or tolerogenic as APCs to T cells due to a lack of costimulatory molecule expression (Matzinger, 1994). Infection of primary B cells with the B-tropic Epstein-Barr virus transforms the B cells into long-term proliferating lymphoblastoid cell lines (LCLs), which display an activated phenotype characterized by high-level expression of MHC class I and II molecules, as well as costimulatory molecules (B7.1, B7.2, ICAM-1 and LFA-3) (Kilger et al., 1999). Deletion of EBV genes essential for lytic replication gives rise to so-called mini-EBV vectors that do not support release of progeny virus from infected cells (Kempkes et al., 1995).

Mini-EBVs can accommodate additional genes, which are then expressed in the mini-(m)LCLs and presented to CD8+ and CD4+ T cells (Kilger et al., 1999; Moosmann et al., 2002). A frequently used EBV-free alternative to activate B cells *in vitro* is the cocultivation of B cells with CD40-ligand (Wiesner et al., 2008). Also this method has shown to efficiently reactivate antigen-specific T cells in vitro (Schultze et al., 1997).

1.1.2 T lymphocytes

During T cell development, the precursor T cells migrate from the bone marrow into the thymus where they undergo differentiation into several T cell subsets, which have been defined according to their expressed surface markers, functional properties and secreted effector molecules. Two distinct T cell lineages, α:β and γ:δ T cells, can be further distinguished based on the polypeptide chains (α, β vs. γ, δ) that make up the main constituents of their antigen-specific receptors (TCRs). The majority of T cells in the blood and secondary lymphoid organs expresses an α:β TCR that recognizes antigenic peptides in the context of HLA molecules. The smaller population of γ:δ T cells predominate in the mucosal epithelium and recognizes a heterogenous class of TCR ligands (peptidic and non-peptidic, e.g. phospholipids) usually in an HLA-independent manner, for example in context of the non-polymorphic MHC-Ib protein CD1 (Champagne, 2011). Expression of either an α:β or γ:δ TCR requires the successive rearrangement of the germline gene segments V, (D), J, which encode the two TCR chains. Due to allelic exclusion, each T cell bears a TCR of only one specificity at its surface. As a result of random V(D)J rearrangements, TCRs of many specificities are generated, including some that do not recognize self MHC or are reactive to self antigens. It is the role of the thymus to ensure that T cells that go to the periphery are self-MHC-restricted, but are unable to react with self antigens. During a process known as positive selection, only T cells survive that are able to bind to self MHC molecules expressed by cortical thymic epithelial cells. T cells bearing a TCR that recognizes self MHC molecules associated with self peptides expressed by thymic epithelial cells, DCs and macrophages are deleted in a process called negative selection. (Starr et al., 2003)

The peptide binding specificity of the TCR is determined by the variable (V) regions of the two receptor chains, which have immunoglobulin-like structure. Accessory molecules also called coreceptors expressed on T cells stabilize the interaction between TCR and MHC/peptide complex. Mature α:β T cells generally express one of the coreceptors CD8 or CD4. CD8 molecules interact with the α_3 domain of MHC class I proteins, whereas CD4 molecules target the β_2 domain of MHC class II proteins. Following antigen interaction, activation signals are transduced to the cell by the CD3 complex, which is closely associated with the two TCR chains. The CD3 complex is composed of one γ, one δ, two ε and 2 ξ chains, which interact with second messengers (e.g. the ζ chain-associated protein kinase ZAP70) that forward the activation signal to the

nucleus. The molecules of the CD3 complex are all invariant and do not contribute to the TCR binding specificity. (Rudolph et al., 2006)

Activation of naive CD8+ and CD4+ T cells, a process which is termed T cell priming and takes place in the lymph nodes, requires the simultaneous delivery of a costimulatory signal in addition to the interaction of the TCR with the antigenic peptide and self MHC. This second signal comes from the engagement of costimulatory cell surface molecules (e.g. CD28 on T cells) with their ligands (e.g. CD80), which are only expressed by professional APCs, usually DCs. Therefore, only these professional APCs are able to initiate the clonal expansion of naive T cells and their differentiation into armed effector cells. Once a naive T cell has been primed, subsequent recognition of its specific antigen leads to an immune attack without the need for costimulation.

CD8+ and CD4+ T cells make up the majority of effector T lymphocytes. CD8+ T cells are primarily cytotoxic and can directly kill infected target cells. Depending on the cytokine milieu, antigen concentration, type of APCs and costimulatory molecules, naive CD4+ T cells can differentiate into three main effector subsets termed T helper (Th) 1, Th2 and Th17, which are characterized by the production of distinct cytokines and effector functions. To combat intracellular pathogens, Th1 cells (producing IL-2, IFN-γ, TNF-α and TNF-β) can mobilize the cellular arm of the immune system by activating macrophages and stimulating cytotoxic CD8+ T cell proliferation. For elimination of extracellular pathogens, Th2 cells (producing IL-4, IL-5, IL-6, IL-10, IL-13 and IL-25) promote humoral immune responses by stimulating B cells to secrete antibodies of particular isotypes. Th17 cells (IL-17, IL-21, IL-22) are highly pro-inflammatory and play a role in protection against certain extracellular pathogens, but also in inflammation and autoimmunity (Bettelli et al., 2007). A subpopulation of CD4+ T cells referred to as regulatory T cells (T_{reg}, CD25+, Foxp3+) suppresses immune functions to prevent excessive reactions and to maintain tolerance to self antigens. T_{reg} are released from the thymus as a distinct lineage called natural T_{reg}. However, T_{reg} can also be induced in the peripheral lymphoid organs after antigen priming by expression of transcription factors including Foxp3. (Luckheeram et al., 2012)

After elimination of the invading pathogen, a fraction of effector T lymphocytes persists as circulating memory cells in the absence of antigenic stimulation and has the capacity to expand rapidly upon secondary antigen challenge. Memory T cells can be distinguished from naive T cells according to the surface expression of CD45RA and the lymph node homing receptors CCR7 and CD62L (L-selectin) (Willinger et al., 2005). Naive T cells express all of these proteins. Among the memory T cells, three subsets are defined: effector memory T cells (T_{EM}: CCR7-, CD62L-, CD45RA-) express chemokine receptors (e.g., CXCR3) for migration to inflamed tissues and display immediate effector function; central memory T cells (T_{CM}: CCR7+, CD62L+, CD45RA-) express lymph node homing receptors and lack immediate effector func-

tion, but efficiently stimulate dendritic cells and differentiate into CCR7- effector cells upon secondary stimulation (Sallusto et al., 1999); effector memory T cells reexpressing CD45RA (T_{EMRA}: CCR7-, CD62L-, CD45RA+) are considered to be late differentiated cells, with limited proliferative but high functional capacity (D'Asaro et al., 2006). Recent data even suggest the existence of a stem cell-like memory T cell population (CCR7+, CD62L+, CD45RA+) with enhanced self-renewal capacity and the ability to differentiate to central memory, effector memory and effector T cells (Gattinoni et al., 2011). Antigen-specific memory T cells can be reactivated and expanded *in vitro* from peripheral blood mononuclear cells (PBMCs) with autologous (from the same donor) APCs like monocyte-derived DCs or activated B cells (CD40-stimulated or EBV-transformed) that are transduced or loaded with the antigenic peptide (described in 1.1.1).

1.1.3 Natural killer cells

NK cells are an early component of the innate immune defense and continuously survey the host cells for altered expression of surface molecules caused by intracellular pathogens or tumor transformation. Following cell-to-cell contact, they can induce apoptosis in target cells by either of two mechanisms: release of cytoplasmic granules containing membrane-disrupting perforin and serin proteases granzymes or engagement of target-cell death receptors (e.g.Fas/CD95) with their cognate ligands (e.g. FasL) expressed on the NK cell membrane (Trapani and Smyth, 2002). NK cells can further shape adaptive immune responses through release of immunoregulatory cytokines or direct interaction with DCs (Vivier et al., 2011).

NK cells comprise 5-10% of peripheral blood lymphocytes and are phenotypically characterized by lack of CD3 (T cell marker) and presence of CD56 (NCAM). Two subsets with distinct roles during the immune response can be distinguished according to their CD56 surface density (Cooper et al., 2001). $CD56^{dim}$ cells (low density), the majority of circulating NK cells in the blood, are primarily cytolytic and have only little ability to produce cytokines in response to activation. By contrast, $CD56^{bright}$ cells (high density) constitute only 10% of total NK cells in the blood but are the dominant NK cell population in secondary lymphoid tissues. They have only low lytic activity but instead are potent producers of immunoregulatory cytokines (IFN-γ, TNF-α, GM-CSF). IFN-γ is considered the prototypic NK cell cytokine, which enhances MHC expression, promotes Th1 differentiation and has broad antiviral and growth-inhibitory effects (Caligiuri, 2008).

To distinguish healthy cells from infected or transformed cells, NK cells are equipped with an array of germline-encoded activating and inhibitory surface receptors. The net income of positive and negative signaling events triggers cytolytic programs, as well as cytokine or chemokine secretion (Vivier et al., 2004). NK cell activating receptors selectively detect molecules that are only present upon infection or cellular transformation. Important members of the broad panel of

structurally distinct activating receptors are the natural cytotoxicity receptors (NCR), CD16 (FcγRIIIA) and the C-type lectin-like receptor NKG2D. NCRs (e.g. NKp46) play a role in NK cell-mediated cytotoxicity against tumor cells (Moretta et al., 2001), but can also bind viral components like hemagglutinins from influenza virus (Mandelboim et al., 2001). CD16 (FcγRIIIA), the low-affinity receptor for immunogloblin G (IgG), is abundantly expressed on $CD56^{dim}$ cells and can bind to and destroy IgG-coated cells in a process termed antibody-dependent cellular cytotoxicity (ADCC) (Cooper et al., 2001). NKG2D, which is among the best characterized NK cell receptors**,** is expressed on all NK cells as a homodimer. It can induce both a cytotoxic and a cytokine-mediated response upon engagement with stress-inducible ligands including the MHC class I chain-related (MIC) A and B molecules and the UL16-binding proteins 1, 2, 3 and 4 (ULBP1–4) (Sutherland et al., 2001).

NK cells use inhibitory receptors to monitor the absence of constitutively expressed self molecules ("missing self") on target cells. Upon engagement with its self ligand, the inhibitory receptor sends a signal to the cell that antagonizes activating signaling pathways, thus preventing cytolysis of normal cells and limiting the production of inflammatory cytokines (Vivier et al., 2004). The panel of inhibitory receptors is divided into two main groups according to their specificity for MHC class I molecules or non-MHC ligands. An example for the less diverse group of receptors targeting non-MHC molecules is the killer cell lectin-like receptor G1 (KLRG1), which is expressed on a subset of NK cells and binds to classical cadherins (E, N, R). For example, E-cadherin is expressed in healthy tissues but is downregulated in metastasizing epithelial tumors (Ito et al., 2006). The more diverse group of HLA class I-specific inhibitory receptors comprises three main classes which all display variegated expression patterns in peripheral blood NK cells: killer-cell immunoglobulin-like receptors (KIRs), killer-cell lectin-like receptors (KLRs, e.g. CD94-NKG2A) and the leukocyte immunoglobulin-like receptor 1 (LILRB1/ILT-2/CD85j) (Fig. 1.3). LILRB1 appears to play only a minor role compared to KIRs and NKG2A and binds with low affinity to a conserved amino acid stretch in the α_3 domain of MHC class I molecules (Chapman et al., 1999a). The ligand for CD94-NKG2A is the non-classical MHC class I molecule HLA-E, which presents peptides derived from leader sequences of other HLA class I molecules, thus allowing NK cells to indirectly monitor the total MHC class I expression on target cells (Borrego et al., 1998). The KIR receptors specifically bind HLA-A, -B and -C in a peptide-dependent manner and can thereby discriminate between different peptides bound to the same HLA allotype (Hansasuta et al., 2004). KIRs are type I transmembrane glycoproteins with two (KIR2) or three (KIR3) Ig-like domains in the extracellular region and possess either a short (S) or long (L) cytoplasmic tail (Pegram et al., 2011). KIRs with short cytoplasmic tails probably arose by gene duplication and mediate activation instead of inhibition. However, under normal circumstances, activating KIRs have lower affinity for their respective ligands than their inhibitory counterparts, which allows the inhibitory signal to dominate and to

minimize the risk of autoimmunity (Lanier, 2005). Conservation of activating KIRs through evolution may result from selective pressure imposed by pathogens (Arase et al., 2002).

KIRs are encoded by a diverse and polymorphic gene family consisting of 15 genes and 2 pseudogenes that are closely linked on chromosome 19. Two groups of KIR haplotypes, designated A and B (Fig. 1.3), with different gene content and allelic polymorphism have been identified within the population (Uhrberg, 2005). KIR subfamilies recognize polymorphisms of HLA class I molecules. A dimorphism at position 80 of the α_1 domain divides HLA-C molecules into two groups of KIR2DL ligands, with the C1 group (Asn^{80}) being recognized by KIR2DL2 and 3, and the C2 group (Lys^{80}) being recognized by KIR2DL1. Similarly, there is a dimorphism in the C-terminal region of the α_1 domain (aa 77–83) of HLA-B molecules that correlates well with the serological classification of HLA-B (and certain HLA-A) molecules into Bw4 and Bw6 groups. Only members of the Bw4 group have been identified as KIR ligands, namely for KIR3DL1 (Parham, 2005). Finally, a few HLA-A allotypes (A03, A11) have been shown to engage KIR3DL2 (Lanier, 2005).

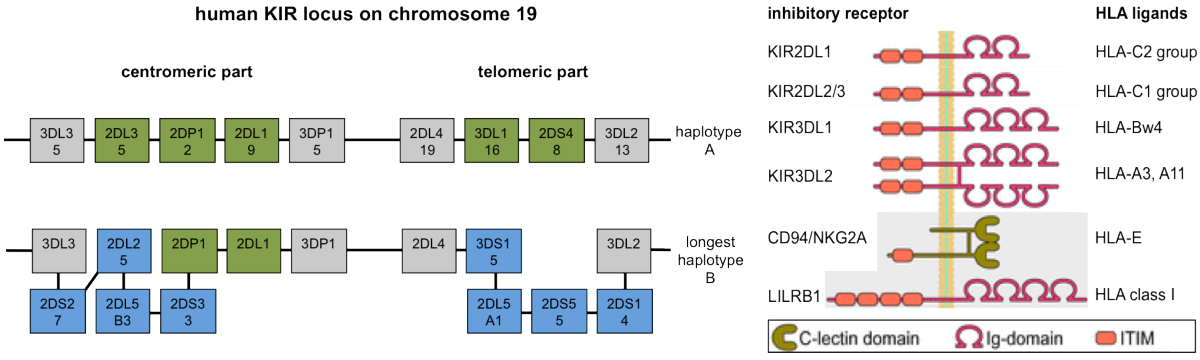

Fig. 1.3 Organization of the human KIR locus and HLA class I-specific receptors. Left: Numerous KIR haplotypes with different gene content and allelic variants are found in the human population. The shortest (haplotype A) and the longest (from the group of haplotypes B) KIR haplotypes are aligned. Grey=conserved genes; green=genes that can be present in haplotype A and B; blue=genes/alleles specific for haplotype B. The number of confirmed allelic variants is indicated below the name of each gene. According to (Parham, 2005) **Right:** NK cell inhibitory receptors specific for HLA class I molecules. Modified according to (Lanier, 2005).

The NK cells of an individual express variable numbers and combinations of the genome-encoded KIRs, giving rise to many NK cell subsets regulated by different self HLA ligands (Valiante et al., 1997). The decision of which KIRs are to be expressed on each NK cell seems to be primarily random, but is fixed by the methylation of KIR gene loci (Chan et al., 2003). However, there is evidence for modulation of the KIR repertoire by the host HLA haplotype (Schonberg et al., 2011). Interestingly, NK cells that do not express a self HLA-specific inhibitory KIR nontheless remain non-reactive to self-tissue. There are some mechanisms postulated which might ensure this self-tolerance (Orr and Lanier, 2010). The "licensing" hypothesis proposes that NK cells are initially unresponsive and that engagement of their inhibitory receptors

with host MHC class I molecules during development licenses or "arms" them to become potent effectors against MHC class I-deficient target cells (Kim et al., 2005). In accordance with this hypothesis, NK cells that do not express KIRs for self-MHC molecules are not armed and therefore not responsive.

1.2 Human cytomegalovirus

Human cytomegalovirus (HCMV), also called human herpesvirus 5, belongs to the β-subgroup of the herpesvirus family (Davison, 2007b). Like other herpesviruses, including herpes simplex virus type 1 and 2 (HSV-1, -2), varicella-zoster virus, HHV-6, HHV-7, Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV), HCMV is a widespread pathogen that commonly infects humans. In human populations, HCMV has a seroprevalence between 50 and 90%, depending on the geographic and socioeconomic conditions as well as ethnic backgrounds (Cannon et al., 2010). A remarkable biological property of HCMV with high clinical importance, and a feature common to all members of the herpesvirus family, is its ability to establish lifelong persistence within the host following initial infection.

1.2.1 HCMV infection and diseases

HCMV is commonly acquired by direct contact with an infected person who excretes actively replicating virus with body fluids like urine, saliva and breast milk. Furthermore, HCMV can be transmitted by blood transfusion or organ and bone marrow grafts (Boppana and Fowler, 2007). The majority of primary infections of healthy individuals are asymptomatic. In some cases, primary infection can result in a mononucleosis-like syndrome, which is clinically indistinguishable from primary EBV infection, with symptoms including sore throat, fever, fatigue, myalgia, lymphadenopathy and mild hepatitis (Sissons and Carmichael, 2002). After primary infection, HCMV persists lifelong in a disguised state called "viral latency", but undergoes periodic asymptomatic reactivation with shedding of infectious virus. Primary infection as well as reactivation from latency is hold in check by effective defense mechanisms of the host's immune system. As a consequence, HCMV disease is restricted to the immunocompromised or immunologically immature host. HCMV disease can affect various tissue types (Vancikova and Dvorak, 2001). Disease symptoms like organ dysfunction results from both direct cytopathic effects of the virus and indirect damage caused by antiviral immune defense mechanisms (Britt, 2007b).

Actively replicating HCMV present during pregnancy, whether from primary maternal infection or from reactivation, is congenitally transmitted to the fetus in 40% of cases and can confer harm to the immunologically immature host (Revello and Gerna, 2002). The earlier the mother ac-

quires the infection, the more severe are the damages, even including intrauterine death (Vancikova and Dvorak, 2001). Approximately 10–15% of the transplacentally infected babies have severe symptoms at birth including liver disease, encephalitis or pneumonitis. Their perinatal mortality rate is approximately 10%. Among the surviving babies, 70–80% suffer permanent neurological sequelae like mental retardation, visual impairment or hearing loss (Boppana et al., 1992). HCMV can further be transmitted from the mother to the newborn through the genital tract at delivery or postnatally through breast feeding and can thereby cause CMV-associated symptoms, especially pneumonitis and hepatitis (Hamprecht et al., 2005).

Serious HCMV disease is further caused by opportunistic infection of immunocompromised patients whose cellular immunity, the principal host defense against HCMV, is impaired. High risk for HCMV disease is consistently associated with transplantation and acquired immunodeficiency syndrome (AIDS) through HIV infection. In HIV patients, CMV disease occurs with advanced immunosuppression (CD4+ T cell count <100 cells/µL, high viral load) and is usually caused by reactivation of latent virus rather than primary infection. The most common clinical manifestation is retinitis, followed by gastrointestinal disease and encephalitis. With introduction of highly active anti-retroviral therapy (HAART) in 1995–1996 to enable persistent immune reconstitution, the incidence of CMV disease has significantly declined but still remains an important issue in persons who are not receiving HAART or who failed to respond (Boeckh and Geballe, 2011). HCMV infection or reactivation after transplantation is a consequence of immunosuppressive medication applied to avoid graft rejection. The risk of CMV disease clearly depends on the type and intensity of the immunosuppressive regimen used. For example, the administration of anti-lymphocyte antibodies such as OKT3 results in the release of large amounts of proinflammatory cytokines, which may promote HCMV reactivation from latency (Fietze et al., 1994). In solid organ transplantation (SOT), the combination of a seronegative patient and a seropositive donor bears the highest risk for HCMV infection by virus reactivation from the graft. Reactivation of endogenous HCMV in seropositive recipients is less common. In the SOT recipient, HCMV can cause a febrile illness with fever and leukopenia, as well as tissue-invasive disease, which manifests as hepatitis, pneumonitis, pancreatitis, colitis or myocarditis (Crough and Khanna, 2009). Furthermore, HCMV has been implicated in rejection of the transplanted organ (Evans et al., 2000). In contrast to the SOT setting, HCMV infections following allogeneic hematopoietic stem cell transplantation (HSCT) are more frequently caused by reactivation of latent virus present in the seropositive recipient. In HSCT, pneumonitis is the most serious CMV-associated complication, causing high mortality rates even with antiviral treatment (Sissons and Carmichael, 2002).

Today, antiviral prophylaxis and preemptive therapy strategies are widely used in SOT/HSCT and have led to a reduction of CMV disease during the time they are applied (typically the first 3 months). However, late CMV disease (>100 days post transplantation) continues to be a clinical

problem. The currently approved drugs are ganciclovir (GCV), foscarnet (FOS) and cidofovir (CDV). Several side effects including nephrotoxicity and myelotoxicity limit their application. Furthermore, resistance to all three drugs resulting from mutations in the drug target, the viral DNA polymerase, has been described (Lurain and Chou, 2010). To improve safety and to overcome resistance, several new anti-HCMV compounds with other modes of action are continuously investigated in clinical trials (Boeckh and Geballe, 2011).

1.2.2 Biology of HCMV

Among the human herpesviruses, HCMV has the largest genome, with a size of ~235 kb comprising up to 250 protein-coding open reading frames (ORFs) (Murphy et al., 2003). The double-stranded linear DNA genome is divided into two segments that contain unique long (UL) or unique short (US) sequences and are bounded by internal and terminal repeats (Davison, 2007a). Viral genes are named with the prefix of the segment in which they are located, followed by a sequential number. Genomes of commonly used laboratory strains like AD169 and Towne have multiple mutations, deletions, and rearrangements compared to fresh clinical isolates (Bradley et al., 2009). This is due to extensive laboratory passage in fibroblasts which selects for mutations adapted to the cell culture growth and can cause differences in virulence and tissue tropism, for example loss of the ability to replicate in endothelial cells (Cha et al., 1996; Prichard et al., 2001).

The viral genome is enclosed in an icosahedral nucleocapsid, which is surrounded by a dense protein-containing matrix termed the tegument and a lipid-bilayered envelope extensively spiked with glycoproteins (e.g. gB, gH, gL, gM, gN, gO) (Liu and Hong Zhou, 2007). The tegument contains the majority of the virion proteins, with the most abundant tegument protein being the lower matrix phosphoprotein 65 (pp65, UL83 gene product). Other major tegument proteins are the core virion maturation protein pp150 (large matrix phosphoprotein; UL32), the virion transactivator pp71 (upper matrix protein, UL82), the membrane-associated myristylated phosphoprotein pp28 (UL99), and the high molecular weight tegument protein (UL48). Some tegument proteins play a structural role for the assembly of virions, but also for disassembly upon cell entry. Other tegument proteins fulfill diverse functions at different stages of the virus life cycle including viral entry, modulation of host cell response and DNA replication (Kalejta, 2008). Virions also contain cellular components including lipids and structural proteins, which may play an important role in stabilizing infectious particles, as well as cellular and viral RNAs (Liu and Hong Zhou, 2007). Viral mRNAs appear to be packaged selectively into virions and have been proposed to facilitate initiation of viral infection upon cell entry (Bresnahan and Shenk, 2000). HCMV-infected cells in culture produce two other types of particles in addition to infectious virions: noninfectious enveloped particles (NIEPs) and dense bodies. NIEPs contain

an identical assortment of envelope, tegument, and capsid proteins than infectious virions but lack viral genomes. Dense bodies are enveloped tegument proteins that lack nucleocapsids and are primarily composed of the viral pp65 protein (Varnum et al., 2004).

HCMV enters human fibroblast cells through direct fusion with the cell membrane (Compton et al., 1992). Envelope glycoproteins mediate both the attachment to cell surface receptors (e.g. heparan sulfate proteoglycans, integrins, platelet-derived growth factor receptor) and the membrane fusion event leading to the release of nucleocapsids and tegument into the cytoplasm (Compton and Feire, 2007). In epithelial and endothelial cells, HCMV enters through low-pH-dependent endocytosis mediated by additional viral envelope glycoproteins (Ryckman et al., 2006). After translocation of the nucleocapsids into the cell nucleus, viral DNA is released and viral genes are expressed in a temporal cascade comprising immediate-early (IE), early (E) and late (L) phase (Stinski, 1978). IE proteins are mainly transcriptional regulators required for initiation of the lytic replication program. IE-1/ IE-2 transcription factors induce the expression of E proteins, which are mainly involved in viral DNA replication. Finally, late genes encoding structural components of the virion or assembly proteins are expressed from the replicated genomes and permit the formation of new virions. After encapsidation of viral DNA, mature nucleocapsids traverse the nuclear membrane undergoing a process of envelopment and de-envelopment and acquire their tegument in perinuclear compartments. Virions further gain their lipid membrane and envelope glycoproteins by budding into Golgi-derived cytoplasmic compartments (Britt, 2007a). Finally mature virus particles exit the cell using the exocytotic pathway, thus completing one cycle of lytic replication approximately 72–96 hours post infection (Loewendorf and Benedict, 2010).

As demonstrated by the variety of tissue-specific diseases *in vivo*, HCMV shows a broad cellular tropism for lytic replication, including differentiated cells of the endoderm, mesoderm, and ectoderm, for example fibroblasts, endothelial cells, epithelial cells, smooth muscle cells, stromal cells, macrophages, dendritic cells, neuronal cells, glial cells, hepatocytes, and trophoblasts (Prosch et al., 1995; Compton and Feire, 2007). By contrast, the virus fails to replicate in poorly differentiated cells such as progenitor cells of the myeloid lineage, which are considered to be the main reservoir for latent HCMV (Sinclair and Sissons, 2006). In these cells, HCMV is maintained in a nearly silent state with only very low levels of gene expression (Sinclair and Sissons, 2006). The critical event for transition to productive infection is the expression of IE transcription factors IE-1/IE-2. IE-1/IE-2 expression is controlled by the major IE promoter/enhancer (MIEP), which is regulated by changes in chromatinization but also viral (pp71) and cellular factors like NF-κB and cyclic AMP (cAMP) (Stinski and Isomura, 2008). Differentiation of myeloid progenitors to macrophages and DCs results in specific chromatin remodelling of the MIEP to an open, transcriptionally active chromatin structure allowing IE gene expression (Reeves et al., 2005). HCMV reactivation further occurs in response to inflammation or stress via induction of cellular

factors (Crough and Khanna, 2009). Proinflammatory cytokines are released during allogeneic transplantation, AIDS or sepsis. Among them, TNF-α is considered to be a key mediator of HCMV reactivation via NF-κB induction in latently infected cells (Prosch et al., 1995). Similarly, stress-induced catecholamines and proinflammatory prostaglandins promote HCMV reactivation through the cAMP pathway (Prosch et al., 1995; Kline et al., 1998).

1.2.3 Effectors of the antiviral immune response

The host defense against HCMV begins very early after infection with non-specific innate immune mechanisms that mediate direct elimination of HCMV-infected cells but also priming of the subsequent adaptive immune response. HCMV is detected by Toll-like receptors (TLRs) at the very earliest stages of viral life cycle, upon initial virus-cell contact. The interaction of viral structural proteins including gB/gH with TLR2 triggers the secretion of inflammatory cytokines that activate and recrute cells of the innate immune system, for example DCs, macrophages and NK cells (Compton et al., 2003). HCMV particles are also robust inducers of interferons and interferon-stimulated genes (ISGs) encoding, for example, various cytokines, thus establishing an antiviral state in the infected host cell (Boehme et al., 2004).

As integral part of the innate immunity, NK cells have an important role in the early control of viral infections. However, direct evidence for the role of NK cells in immunity to HCMV is limited. Patients with rare NK cell defects or deficiency are susceptible to life-threatening HCMV infections (Biron et al., 1989; Gazit et al., 2004). Furthermore, recovery of NK cell activity following HSCT has also been associated with survival of patients with CMV-related disease (Quinnan et al., 1982a). In addition, the murine (M)CMV model system provides evidence for a protective role of NK cells, as NK cell depletion in mice resulted in higher MCMV tissue titers and increased mortality (Scalzo and Yokoyama, 2008). However, a number of *in vitro* systems have shown that HCMV-infected cells are relatively resistant to NK cell-mediated lysis, suggesting that HCMV has evolved mechanisms to evade NK cell recognition (Wills et al., 2007). Indeed, a great variety of HCMV-encoded NK cell evasion proteins were identified the past years, thus providing indirect evidence for the importance of these cells in the innate response to HCMV.

HCMV is also a potent immunogen that triggers strong responses from both arms, humoral and cellular, of the adaptive immune system. However, the humoral, antibody-mediated immune response is not essential for the resolution of primary CMV infection and rather plays a role in restriction of viral dissemination and limiting the severity of the disease (Jonjic et al., 1994; Boppana and Britt, 1995). HCMV primary infection elicits antibodies specific for numerous HCMV proteins including structural proteins (e.g. pp65 and pp150), envelope glycoproteins (predominantly gB and gH) as well as non-structural proteins such as IE1 (Jackson et al., 2011). Virus-

neutralizing activity is predominantly mediated by gB-specific antibodies, which comprise at least 50% of the neutralizing antibodies in HCMV-infected individuals (Britt et al., 1990).

The restriction of HCMV disease almost exclusively to individuals with cellular immunodeficiency implicates that adaptive T cell responses are the predominant mechanism of HCMV control. This is supported by the observation that reconstitution of HCMV-specific T cell immunity and adoptive T cell transfer in immunocompromised patients correlates with protection from HCMV disease (Jackson et al., 2011). In seropositive humans, HCMV is targeted by an enormously high fraction of circulating T cells in the peripheral blood, comprising on average 10% of both the CD4+ and CD8+ T cell memory compartments (Sylwester et al., 2005). This fraction tends to increase with age ("memory inflation"), which may support the hypothesis that HCMV contributes to immune system exhaustion and dysfunction in the elderly (Moss, 2010). A protective role against HCMV is also described for a subset of gamma delta (γδ) T cells (Vδ2-) which expands following HCMV reactivation in kidney and stem cell transplant recipients and has the ability to kill HCMV-infected target cells *in vitro* (Halary et al., 2005; Knight et al., 2010). However, the target structures of the γδ T cells on the surface of HCMV-infected cells are not known so far.

A loss of HCMV control was primarily associated with the lack of HCMV-specific cytotoxic CD8+ T cells (CTLs) (Quinnan et al., 1982b; Reusser et al., 1991), which can directly kill HCMV-infected cells. HCMV-specific CD8+ T cell responses can target various viral antigens expressed at different stages during viral replication cycle and associated with diverse functions including structural proteins (pp150, pp50, pp28, gB, gH), transcription factors (IE-1, IE-2) and even immunomodulatory proteins (gpUS2–11) (Elkington et al., 2003; Sylwester et al., 2005). However, only a small number of these antigens, including the 72-kDa IE-1 protein and the tegument protein pp65, is prevalently recognized in a majority of healthy carriers, thus being considered as immunodominant (Khan et al., 2005; Sylwester et al., 2005). The reasons underlying this immunodominance are largely unresolved. As the most abundant structural protein, pp65 is introduced into host cells upon virus entry and is therefore able to sensitize HCMV-infected cells for pp65-specific CTLs already before *de novo* expression of viral proteins including immunoevasive proteins which interfere with MHC class I antigen presentation (Riddell et al., 1991; McLaughlin-Taylor et al., 1994). Over the past years, intensive studies on pp65-specific T cell responses led to the identification of various CD8+ T cell epitopes (Wills et al., 1996; Kondo et al., 2004). However, an even more important role as a T cell target has to be assumed for IE-1, as IE-1-specific CD8+ T cells, but not pp65-specific T cells, are associated with protection from viral disease in solid organ transplant patients (Bunde et al., 2005). Furthermore, a delay in IE-1-specific CD8+ T cell reconstitution correlated with HCMV disease after stem cell transplantation (Sacre et al., 2008). The IE-1 transcription factor is among the first proteins to be expressed in HCMV-infected cells during lytic infection (Stinski et al., 1983), thereby enabling

CTL interference at a very early stage of the viral life cycle. More importantly, virus reactivation from latency is initiated with the expression of IE proteins (Sinclair and Sissons, 2006). Therefore, IE-1-specific T cells and not T cells directed against structural or late expressed proteins are expected to efficiently control viral reactivation from latency before release of progeny virus. Indeed, there is direct evidence for an involvement of IE-1 epitope-specific CTLs in the immune surveillance of MCMV pulmonary latency by sensing of reactivated IE-1 gene expression (Simon et al., 2006). IE-1 therefore appears to be a preferable target of HCMV-specific immunotherapy, the more so since a variety of IE-1 epitopes restricted through different MHC class I allotypes have been identified (Kern et al., 1999; Khan et al., 2002; Elkington et al., 2003).

The role of CD4+ T cells in the control of HCMV is not as obvious as for CD8+ T cells. However, there is increasing evidence that CD4+ T cells are also an essential part of the immunity to HCMV. Analysis of the CD4+ T cell response to the entire HCMV proteome has also shown a very broad response, with an individual recognizing a median of 12 ORFs and with five ORFs (encoding gB, pp65, major capsid protein, pp28, IE-2) being recognized by more than half of the donors tested (Sylwester et al., 2005). So far, individual peptide epitopes have been identified in pp65, IE-1, gB and gH (Davignon et al., 1996; Kern et al., 2002; Elkington et al., 2004). HCMV-specific CD4+ T cells have been associated with helper function as maintenance of adoptively transferred HCMV-specific CD8+ T cells in HSCT patients was clearly dependent on the presence of the CD4+ T cells (Walter et al., 1995). This is further supported by the finding, that HCMV-specific CD4+ T cells primarily secrete the Th1 cytokines IFN-γ and TNF-α but rarely the Th2 cytokine IL-4 (Rentenaar et al., 2000; van Leeuwen et al., 2006). However, there is accumulating evidence that HCMV-specific CD4+ T cell subsets can also directly act upon infected cells, a feature that was thought to be restricted to cytotoxic CD8+ T cells (van Leeuwen et al., 2004). Cytotoxic CD4+ T cells have been identified against a number of HCMV antigens, including pp65, gB and gH (Hopkins et al., 1996; Elkington et al., 2004; Weekes et al., 2004).

1.2.4 Viral immune evasion strategies

During millions of years of coevolution with hominoids, HCMV has evolved a multitude of immune evasion proteins designed to silence the host's immune response, thus allowing the virus to survive in a hostile environment. The simplest way to avoid recognition by the immune system is to reduce viral antigen expression, which HCMV successfully employs during latency (Sinclair and Sissons, 2006). Latent HCMV might also avoid elimination by the immune system by expression of the viral IL-10 homolog cmvIL-10 (Slobedman et al., 2009), which is a potent immunosuppressor that downregulates inflammatory cytokine synthesis and interferes with MHC class II expression on antigen-presenting cells (Moore et al., 2001). During productive

infection, HCMV also modulates both arms of the immune response, innate and adaptive, by the expression of several homologues of cytokine receptors (UL144=TNFR), chemokines (pUL146/vCXC-1 and pUL147/vCXC-2) and chemokine receptors (e.g. pUS28/CCR1), as well as chemokine binding proteins (p21.5). HCMV further encodes two Fcγ receptor homologues, gp34 and gp68, which might bind HCMV-specific antibodies, thus protecting infected cells from phagocytosis, NK cell-mediated ADCC and complement activation. In addition, the three HCMV proteins IE-1, IE-2 and pp65 interfere with components of the type I IFN signaling pathway as well as ISG activation. (reviewed in (Miller-Kittrell and Sparer, 2009))

One central immune escape mechanism is the inhibition of NK cells, which are the key players of the antiviral innate immunity. Some HCMV-encoded proteins alter the surface expression of activating NK cell receptor ligands. The HCMV UL16-encoded glycoprotein binds the natural stress-induced ligands ULBP1, ULBP2 and MICB, thus disrupting their interaction with the activating NK cell receptor NKG2D (Dunn et al., 2003). Similarly, the HCMV-encoded micro-RNA miR-UL112 selectively targets MICB for downregulation in infected cells (Stern-Ginossar et al., 2007). Furthermore, the glycoprotein UL142 was found to downregulate the cell surface expression of another NKG2D ligand, MICA (Chalupny et al., 2006). The protein encoded by UL141 specifically targets and prevents the expression of the cell surface glycoproteins CD155 and CD112, which both function as ligands for the activating NK cell receptor DNAM-1 (Tomasec et al., 2005; Prod'homme et al., 2010). Finally, the structural protein pp65 was described to interfere with NK cell-mediated killing of HCMV-infected cells by interacting with the activating NK receptor NKp30 (Arnon et al., 2005). HCMV has also developed mechanisms to compensate for virus-mediated downregulation of MHC class I molecules, which, according to the "missing self"-hypothesis, renders infected cells sensitive for NK cell lysis. Viral glycoprotein UL18 binds the inhibitory receptor ILT-2 as a surrogate of endogenous MHC class I molecules (Chapman et al., 1999b). However, the function of gpUL18 has not been clearly defined as it was found to inhibit but also to activate NK cells *in vitro* (Prod'homme et al., 2007). Furthermore, HCMV-encoded UL40 protein can promote surface expression of HLA-E, the ligand for the inhibitory C-type lectin CD94/NKG2A, by providing UL40-derived TAP-independent peptides that bind to HLA-E (Tomasec et al., 2000). However, conflicting reports have found that UL40 either inhibited or had no effect on NK cell lysis of HCMV-infected cells (Falk et al., 2002; Wang et al., 2002).

A major focus of HCMV immune evasion lies on the inhibition of antigen processing and presentation in order to evade T cell recognition, which is the main HCMV defense mechanism. A pp65-associated kinase activity, probably from interaction with Polo-like kinase 1 (Gallina et al., 1999), was found to selectively block presentation of IE-1 protein-derived peptides (Gilbert et al., 1996). Early work observed that HCMV downregulated MHC class I molecules from the surface of infected cells (Barnes and Grundy, 1992; del Val et al., 1992). The genomic region

implicated in this phenomenon was US2–US11 (Jones et al., 1995) encoding the viral glycoproteins US2, US3, US6, US10, and US11, which have subsequently been found to modulate MHC class I (and II) antigen presentation (Fig. 1.4). GpUS3 is expressed during the IE period of the viral replication cycle and retains fully assembled MHC class I complexes in the ER by direct binding to the ER-resident chaperone tapasin (Zhao and Biegalke, 2003). Tapasin is essential for optimal peptide loading and surface expression of some MHC class I allotypes. Hence, susceptibility of MHC class I allotypes to US3 retention correlates with their tapasin dependence (Park et al., 2004). Additionally, gpUS3 reduces MHC class II antigen presentation by binding of newly synthesized MHC class II α/β heterodimers, thereby preventing their association with the invariant chain (Hegde et al., 2002). The early phase proteins gpUS2 and gpUS11 mediate the dislocation of MHC class I heavy chains from the ER to the cytosol for subsequent proteasomal degradation. Despite the similar outcome of their action, gpUS2 and gpUS11 use different molecular mechanisms involving diverse cellular factors of the ER-associated protein degradation (ERAD) pathway to mediate MHC class I destruction (Noriega et al., 2012). For example, gpUS2-mediated MHC degradation depends on the chaperones calnexin, BiP, and calreticulin, whereas gpUS11 action requires Derlin-1, a protein described to be involved in the removal of misfolded proteins (Miller-Kittrell and Sparer, 2009). Like gpUS3, gpUS2 and gpUS11 target MHC class I heavy chains in an allele-specific manner. Amino acid residues near the border between α_2 and α_3 domains are involved in the interaction with gpUS2 (Gewurz et al., 2001; Barel et al., 2006). By contrast, gpUS11 discriminates between HLA allelic variants based on their cytosolic tail residues. Furthermore, residues in the α_1 and α_2 domains may modulate gpUS11 targeting (Barel et al., 2003). In addition to its interference with MHC class I presentation, gpUS2 also inhibits MHC class II presentation by inducing the degradation of HLA-DR and -DM α chains (Tomazin et al., 1999). The HCMV early/late protein gpUS6 inhibits the translocation of antigenic peptides by the TAP complex, by preventing the binding of ATP to TAP1 (Ahn et al., 1997). Finally, gpUS10 binds free MHC class I heavy chains and delays their egress from the ER. GpUS10 is specific for HLA-G molecules (Park et al., 2010), whereas it does not block classical MHC class I maturation or surface expression (Jones et al., 1995). In addition to the immunoevasins encoded within the US2–11 region, the HCMV-encoded miRNA US4-1 plays a role in inhibiting antigen presentation to CTLs (Kim et al., 2011). US4-1 specifically targets the mRNA of ER aminopeptidase ERAP1, thus preventing ERAP1 translation and, consequently, the trimming of TAP-translocated precursors of MHC class I peptides (Saric et al., 2002).

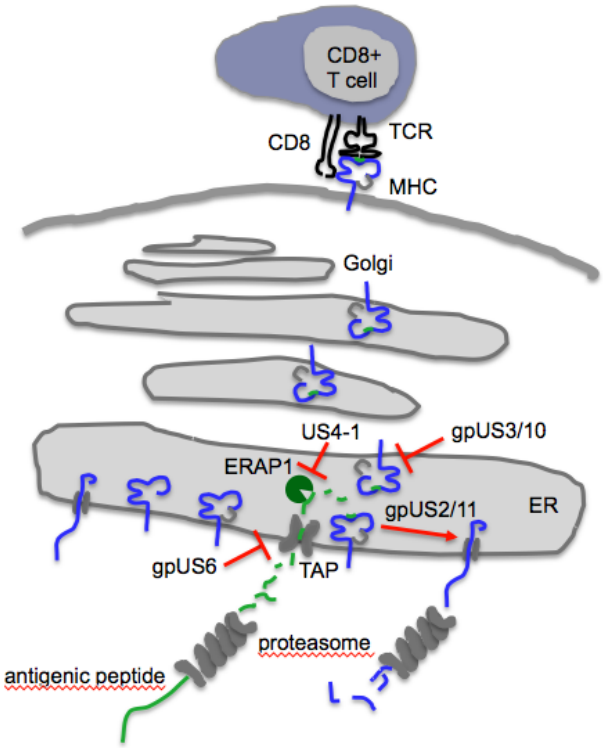

Fig. 1.4: HCMV-endoded glycoproteins and miRNA interfere with MHC class I antigen processing and presentation. Glycoprotein US3 retains fully-assembled MHC/peptide complexes in the ER. GpUS10 delays MHC egress from the ER. GpUS2 and gpUS11 individually mediate the retrograde translocation of MHC class I heavy chains from the ER to the cytosol and subsequent degradation at the proteasome. GpUS6 interacts with the TAP complex, thereby interfering with peptide transport from the cytosol into the ER and subsequent MHC loading. Micro-RNA US4-1 specifically targets the mRNA of ER-aminopeptidase ERAP1 and prevents its translation and, consequently, the trimming of TAP-translocated precursors of MHC class I peptides.

1.2.5 Immunotherapy

After transplantation, the reconstitution of B and T cell immunity is delayed due to immunosuppressive drug administration for prevention of GVHD, and this delay puts the patient at high risk of infectious complications, especially HCMV disease. Current antiviral drugs are limited by toxicities and lack of efficacy in established HCMV disease. Therefore, the design of adoptive immunotherapeutic strategies to correct antibody and T cell immunodeficiencies may be an attractive alternative. Passive administration of immunoglobulin preparations containing HCMV-specific antibodies has only provided limited success in the transplant setting. Despite some reports about improved disease outcome and survival (Falagas et al., 1997), there was no clear evidence that prophylactic immunoglobulin treatment lowered the incidence of HCMV disease (Ringden et al., 1987; Munoz et al., 2001). By contrast, in pregnant women, HCMV-specific immunoglobulin transfer significantly reduced the risk of congenital HCMV disease (Nigro et al., 2005).

Due to a strong correlation between recovery of HCMV-specific T cell immunity and protection from HCMV disease (Quinnan et al., 1982a; Reusser et al., 1991; Li et al., 1994), adoptive T cell transfer appears to be a more promising strategy for immunosuppressed patients. Indeed, patients who have received *ex vivo* expanded HCMV-specific T cells were protected from both primary and reactivating HCMV infection (Riddell et al., 1992; Peggs et al., 2003). Protective immunity could be successfully transferred to patients by the infusion of either HCMV-specific

CD8+ cytotoxic T cell clones or polyclonal T cell lines (Einsele et al., 2008). However, the maintenance of a protective CD8+ T cell immunity was depending on the presence of CD4+ helper T cell function (Walter et al., 1995; Einsele et al., 2002), suggesting that immunotherapy should not focus on CD8+ T cells alone. Over the last three decades, a number of strategies to isolate and expand HCMV-specific T cells for adoptive T cell therapy have been explored. Pioneer work on adoptive T cell transfer was done using HCMV-infected autologous fibroblasts for *in vitro* expansion of CD8+ T cell clones (Riddell et al., 1992; Walter et al., 1995). Other T cell expansion techniques use EBV-infected B cells or APCs retrovirally transduced with genes of interest as stimulator cells. Although being very effective approaches for T cell stimulation, these methods rise biosafety concerns due to the presence of live viruses and may therefore not be applicable to current good manufacturing practice (GMP) standards (Rauser et al., 2004). Other effective strategies for the generation of HCMV-specific T cells use DCs pulsed with specific antigens (Peggs et al., 2001) or synthetic peptides (Kleihauer et al., 2001). However, these methods are very labor and cost intensive and require several weeks for the generation of a clinical product. In an alternative approach, HCMV-specific T cells can be directly isolated from the peripheral blood of seropositive donors using HLA/peptide multimeric complexes (Cobbold et al., 2005). A new kind of multimeric HLA complexes called streptamers bind reversibly to the T cell receptor, thus offering the opportunity to transfer nearly "untouched" antigen-specific T cells (Knabel et al., 2002). A clinical study employing this principle in HCMV therapy after HSCT is ongoing (Schmitt et al., 2011). Furthermore, virus-specific T cells can be isolated within 36 hours by stimulation of PBMCs with synthetic peptides or antigen followed by IFN-γ capture-based isolation (Moosmann et al., 2010). The transfer of HCMV-specific T cells prepared with this procedure significantly reduced HCMV reactivation and disease in HSCT patients (Feuchtinger et al., 2010). These two methods allow rapid isolation of specific T cells without time-consuming *in vitro* expansion, thus avoiding T cell senescence, reducing the potential risk for contamination of the T cell preparation, and reducing the cost and complexity of production under clinical conditions.

Congenital HCMV infection is the leading infectious cause of mental retardation in children. In addition, HCMV seroprevalence is linked to an immune-risk phenotype in the very elderly that may predispose to early death (Pawelec and Derhovanessian, 2011). It appears that this cannot be resolved by other means than active immune prophylaxis. Therefore, HCMV is considered to be a prime candidate for eradication from the human population through vaccination. A successful vaccine strategy should stimulate both protective humoral and cell-mediated immunity. Therefore, more than 30 years ago, live-attenuated HCMV strains like AD169 and Towne were studied as vaccine candidates. Especially strain Towne was intensively investigated in seronegative volunteers and transplant recipients. Although most candidates developed humoral and T cell responses following vaccination, these were not protective against infection

with natural HCMV (Plotkin et al., 1991; Adler et al., 1995). Meanwhile, several other approaches for HCMV vaccine development have been attempted, for example recombinant virus vaccines (e.g. based on VSV, MVA or AdV), subunit vaccines, peptide vaccines and DNA vaccines (Schleiss, 2008). However, there is no licensed HCMV vaccine currently available, and the reasons for this are complex. For example, most efforts of HCMV vaccine development have focused on the antibody target gB and the T cell targets pp65 and IE1. However, during evaluation of the T cell responses in HCMV-seropositive individuals several HCMV proteins in addition to pp65 and IE-1 were identified as T cell targets (Sylwester et al., 2005) and their significance for mediation of protective immunity is not yet clear. Furthermore, it became obvious that neutralizing antibodies against gB are not able to prevent HCMV entry in epithelial and endothelial cells as this process does not involve gB but instead a complex of the glycoproteins gH/gL/UL128/130/131 (Revello and Gerna, 2010). Nevertheless, there are promising HCMV vaccine candidates, whose implementation could impact significantly on HCMV disease. The principle barrier to licensure of an HCMV vaccine is probably the lack of public awareness of the threat posed by congenital HCMV infection and the severe disabilities it produces in children (Schleiss, 2008).

1.3 Aims of the study

The IE-1 antigen of HCMV is a preferred target for antiviral immunotherapy. Its kinetics of expression, and studies in the mouse model, make it likely that IE-1-specific T cells protect from infection. However, many questions on IE-1-specific T cells were unanswered at the outset of the present study. IE-1-specific CD4+ T cells had been described, although some studies could not detect them in healthy donors. Furthermore, it was known that IE-1-specific CD8+ T cells are often dominant in healthy carriers, but most studies described that they do not recognize infected cells in the presence of viral immunoevasive proteins. Therefore, the aim of the present study was to address the following questions:

I. Can CD8+ and CD4+ T cells specific for the IE-1 protein be reliably expanded *in vitro* from the peripheral blood cells of healthy HCMV carriers by stimulation with mini-lymphoblastoid cell lines? Can specific CD8+ and CD4+ T cell clones be established?

II. Can new target epitopes of IE-1-specific CD8+ and CD4+ T cells be identified that will be of use in antiviral immunotherapy? How diverse are responses in healthy virus carriers, how many different epitopes are targeted, and which ones are recognized by dominant T cell populations?

III. What are the effector functions of IE-1-specific CD8+ and CD4+ T cells?

IV. Do IE-1-specific CD8+ and CD4+ T cells recognize infected cells, and which cell types? Is there a specialization for particular cell types, is there a preferential recognition of tissue cells (e.g. fibroblasts) or hematopoietic cells (e.g. dendritic cells)? What are the conditions for recognition?

V. Can it be confirmed that recognition of infected cells by IE-1-specific CD8+ T cells is inhibited by viral immunoevasins? Is this true for all IE-1 epitope specificities and HLA restrictions, or are there systematic differences? What is the role of individual viral immunoevasins in such a suppression? Can CD8+ T cell specificities be identified that control infection particularly well?

VI. Can IE-1 be used as a model antigen to obtain a better understanding of MHC class I immunoevasion by HCMV?

2 Materials

2.1 Bacteria

For preparation of plasmid DNA and cloning purposes, the chemically competent *E.coli* strains DH5α and DH10B (Invitrogen, Karlsruhe, Germany) were used:

genotype DH5α: F-, ϕ80dlacZΔM15, Δ(lacZYA-argF), U169, deoR, recA1, endA1, hsdR17(r_k^-, m_k^-), supE44, thi-I, gyrA96, relA1, λ^-.

genotype DH10B: F-, mcrA, Δ(mrr-hsdRMS-mcrBC), ϕ80dlacZΔM15, ΔlacX74, deoR, recA1, endA1, araD139, Δ(ara, leu)7697, galU, galK, λ^-, rpsL, nupG.

2.2 Eukaryotic cells

2.2.1 Primary cells

Peripheral blood mononuclear cells (PBMCs) were obtained from standard blood donations of healthy adult donors or from buffy coats received from the Institute for Transfusion Medicine, University of Ulm, Germany. All blood donors contributing to this work were anonymized. The institutional review board (Ethikkommission, Klinikum der Universität München, Marchioninistr. 25, 81377 Munich, Germany) approved the study. All work was conducted according to the principles expressed in the Helsinki Declaration. High- or low-resolution HLA types and virus carrier status of the PBMC donors are listed in Table 2.1.

Table 2.1: HLA types of donors and virus carrier status (n.d. = not determined)

Donor	HLA-A	HLA-B	HLA-C	HLA-DRB1	HLA-DR (B3/4/5)	HLA-DQ (A1/B1)	HLA-DP (A1/B1)	HCMV status	EBV status
AAJ	*0201 *0301	*3801 *4403	*0402 *1203	*0701 *1301	B3*0101 -- B4*0101 --	n.d.	n.d.	neg	neg
ABF	*02 *03	*35 *4001	*03 *15	n.d.	n.d.	n.d.	n.d.	pos	pos
ACR	*0301 *6801	*0702 *3501	*0401 *0702	*0101 *1501	n.d.	n.d.	n.d.	neg	pos
AEA	*0101 *2402	*0801 *1402	*0701 *0802	*0301 *1502	n.d.	n.d.	n.d.	pos	pos
AES	*2602 --	*3501 *4801	*0401 *0803	*1101 *1501	B3*0202 -- B5*0101 --	B1*0301 B1*0602	B1*0201 B1*0402	pos	neg
AJJ	*0201 --	*0702 *3503	*0401 *0702	*03 *14	n.d.	n.d.	n.d.	pos	pos
AJG	*0201 --	*3503 *5701	*04 *06	*07 *11	n.d.	B1*03 B1*03	n.d.	pos	pos
AJU	*0201 *24	*07 *40	*0304 *0702	*07 *13	B3*0202 -- B4*0101 --	A1*0103 A1*0201 B1*0202 B1*0603	A1*0103 -- B1*0401 B1*2001	pos	pos

Donor	HLA-A	HLA-B	HLA-C	HLA-DRB1	HLA-DR (B3/4/5)	HLA-DQ (A1/B1)	HLA-DP (A1/B1)	HCMV status	EBV status
ALT	*0201 --	*0702 *4002	*0202 *0702	*0401 *1501	B4*0103 B5*0101	B1*0301 B1*0602	B1*0401 B1*1101	pos	pos
AMD	*01 *11	*08 *1501	*0303 *0701	*03 *11	B3*0101 B3*0202	B1*02 B1*03	n.d.	pos	pos
ARZ	*0201 *2902	*4402 *4501	*0602 --	*04 *15	B4*0103 B5*0101	B1*03 B1*06	A1*0103 -- B1*0201 B1*0301	pos	pos
ASG	*0101 *2902	*0801 *4403	*0701 *1601	*13 *15	B3*0101 B5*0101	B1*0602 B1*0603	n.d.	pos	pos
ASI	*0201 --	*44 *51	*02 *05	*08 *11	n.d.	B1*03 B1*04	n.d.	neg	pos
ASM	*03 *33	*07 *14	*0702 *0802	*01 *15	n.d.	n.d.	n.d.	neg	n.d.
ATH	*02 *24	*44 *51	*02 *05	n.d.	n.d.	n.d.	n.d.	pos	pos
AYH	*0207 *0301	*2704 *4402	*0501 *1202	n.d.	n.d.	n.d.	n.d.	pos	pos
F43	*01 *24	*07 *57	*0602 *0702	*07 *15	B4 B5	B1*03 B1*06	n.d.	pos	n.d.
F45	*01 *68	*08 *14	*07 *08	*13 *15	B3 B5	B1*03 B1*06	n.d.	pos	pos
F46	*03 *11	*07 *3501	*0401 *0702	*13 *15	B3 B5	B1*06 --	B1*0401 B1*0402	pos	pos
F59	*02 *68	*18 *4001	*0701 --	*11 *13	B3	B1*03 B1*06	n.d.	pos	n.d.
F60	*02 *26	*07 *38	*0702 *1203	*0201 *0401	B3	B1*03 B1*06	n.d.	pos	pos
F61	*02 *34	*14 *44	*0401 *0802	*01 *07	B4	A1*0101 A1*0201 B1*02 B1*05	A1*0103 A1*0201 B1*0402 B1*1401	pos	pos
F62	*03 --	*3501 *5201	*0401 *1202	*0101 *1502	B5*0102	B1*05 B1*06	n.d.	pos	pos
F63	*03 *24	*18 *4001	*0401 *1504	*01 *08	--	B1*03 B1*05	B1*0401 --	pos	n.d.
F64	*02 *29	*1501 *44	*0304 *0701	*07 *13	B3 B4	A1*0103 A1*0201 B1*0202 B1*0603	A1*0103 A1*0201 B1*0201 B1*1101	pos	pos
F65	*24 *68	*35 *44	*0303 *1601	*14 *15	B3*0202 B5*0101	B1*05 B1*06	B1*0201 B1*0301	pos	n.d.
LM02	*03 *24	*07 *18	*0701 *0702	n.d.	n.d.	n.d.	n.d.	pos	pos
LM16	*0301 *3101	*0702 *0801	*0701 *0702	n.d.	n.d.	n.d.	n.d.	pos	n.d.
LM20	*0201 *2402	*0702 *3901	*0702 *1203	n.d.	n.d.	n.d.	n.d.	pos	n.d.
LT12	*11 *11	*07 *55	*0102 *0702	n.d.	n.d.	n.d.	n.d.	pos	n.d.
SA01	*0301 *6801	*0702 *5101	*0702 *1502	*0701 *1501	n.d.	n.d.	n.d.	pos	pos
SA03	*0101 *0301	*0702 *5701	*0602 *0702	*0701 *1501	n.d.	n.d.	n.d.	pos	pos
SA04	*0102 *0201	*0702 *4901	*0701 *0702	*1401 *1501	n.d.	n.d.	n.d.	pos	pos
SA05	*2301 *3001	*0705 *4901	*0701 *1505	*1301 *1501	n.d.	n.d.	n.d.	pos	pos
SA12	*01 *03	*07 *35	*0401 *0702	n.d.	n.d.	n.d.	n.d.	pos	pos
SA13	*03 --	*07 *52	*0702 *1202	n.d.	n.d.	n.d.	n.d.	pos	pos

Primary NK cell lines used in this study were all established from PBMCs of donor AJU with the following KIR genotype:

positive for genes encoding KIRs:	2DL1, 2DL3, 2DL4, 2DS4, 3DL1, 3DL2, 3DL3
negative for genes encoding KIRs:	2DL2, 2DL5, 2DS1, 2DS2, 2DS3, 2DS5, 3DS1

This genotype suggests AJU to be homozygous for KIR haplotype A (Uhrberg et al., 1997; Parham, 2005).

2.2.2 Cell lines

TR-2/293 EBV-packaging cell line based on the human embryonic kidney 293 cell line (Delecluse et al., 1999) (available in the Department of Gene Vectors, Helmholtz Zentrum München)

BHK-21 baby hamster kidney cells, derived from kidney of Syrian golden hamster (MACPHERSON and STOKER, 1962) (CCL-10™, purchased from ATCC)

Daudi B lymphoblastoid cell line, MHC class I-deficient (Klein et al., 1968) (purchased from ATCC)

K562 immortalized human chronic myelogenous leukemia cell line, MHC class I-deficient (Lozzio and Lozzio, 1975) (purchased from ATCC)

L721.221 B lymphoblastoid cell line, MHC class I-deficient (Shimizu and DeMars, 1989) (kindly provided by Elfriede Nößner, Helmholtz Zentrum München)

L721.221 C*0602 L721.221 stably transfected with the HLA-C*0602 gene (Falk et al., 1995) (kindly provided by Elfriede Nößner, Helmholtz Zentrum München)

L721.221 C*0702 L721.221 stably transfected with the HLA-C*0702 gene (Falk et al., 1995) (kindly provided by Elfriede Nößner, Helmholtz Zentrum München)

LL8 murine fibroblast L929 cell line stably transfected with the human CD40 ligand-encoding gene (L. Schirrmann and A. Moosmann, unpublished)

Mini-lymphoblastoid cell lines (mLCLs) and CD40-stimulated B cell lines were established within this work from PBMCs of the blood donors listed in Table 2.1.

T cell clones specific for pp65- and IE-1 were established within this work from PBMCs of the blood donors listed in Table 2.1. Systematic nomenclature includes a three-letter abbreviation for the anonymized donor (XYZ), P or M to indicate the target antigen (pp65 or IE-1, respectively), the number of the T cell clone, and a three-letter abbreviation for the recognized peptide.

XYZP#1 NLV	T cell clone number 1 of donor XYZ that recognizes NLV peptide of pp65
XYZM#1 CRV	T cell clone number 1 of donor XYZ that recognizes CRV peptide of IE-1

Human fibroblast cell lines used in this work:

cell line	description	source	HLA-A	HLA-B	HLA-C
BFF2	primary human foreskin fibroblast cell line from an adult donor	department of gene vectors	*02 *03	*35 *4001	*03 *15
CZF	primary human foreskin fibroblast cell line from an adult donor	department of gene vectors	*02 *02	*18 *60	*03 *05
MRC-5	human fetal lung fibroblast cell line	European collection of cell cultures	*0201 *2902	*0702 *4402	*0501 *0702
WI-38	human fetal lung fibroblast cell line	European collection of cell cultures	*0205 *6801	*0801 *5801	*0701 *0701

2.3 Viruses

Modified vaccinia virus Ankara (MVA) wild-type strain and recombinants were kindly provided by Naem Khan, Birmingham, UK (Khan et al., 2005).

MVA-wt	MVA wild-type strain
MVA-pp65	MVA recombinant with expression cassette for HCMV pp65 antigen
MVA-IE-1	MVA recombinant with expression cassette for HCMV IE-1 antigen

The following recombinant, modified mini-Epstein-Barr virus (mini-EBV) strains and corresponding viral plasmids were used in this study:

mini-EBV	plasmid 1495.A, no foreign antigen encoded
mini-EBV-pp65	plasmid 4014, pp65-expression cassette
mini-EBV-IE-1	plasmid 3457, IE-1-expression cassette

The mini-EBV plasmid 1495.A (Kempkes et al., 1995) contains all viral genes and elements necessary for transformation of primary B cells. Mini-EBVs coding for pp65 or IE-1 were constructed from 1495.A by Andreas Moosmann, Helmholtz Zentrum München, Germany (Moosmann et al., 2002) or Martina Wiesner, Helmholtz Zentrum München, Germany (Wiesner, 2005), respectively. Thereby, an expression cassette carrying the transgene's cDNA sequence under the SV40 promoter derived from the expression plasmid pSG-5 (Stratagene) was introduced into 1495.A by homologous recombination.

HCMV strain AD169 was kindly provided by Martin Messerle (Hannover, Germany).

Recombinant HCMV strains lacking three or all four HCMV-encoded immunoevasive proteins gpUS2, 3, 6 and 11 were kindly provided by Bodo Plachter, Mainz, Germany, and are listed below. These HCMV mutants were generated via BAC mutagenesis, by successive deletion of the individual sequences coding for US2, 3, 6 and 11 from the US gene locus as described in Besold et al., 2009.

virus strain	original name	immunoevasin encoded	reference
CMV-∆all	RV-KB6	-	Besold et al., 2009
CMV-US2	RV-KB13	gpUS2	Besold et al., 2009
CMV-US3	RV-KB7	gpUS3	Noriega et al., 2012
CMV-US6	RV-KB5	gpUS6	unpublished
CMV-US11	RV-KB9	gpUS11	Besold et al., 2009

2.4 Plasmids

p2670 — expression plasmid encoding the EBV glycoprotein BALF4/gp110 (Neuhierl et al., 2002)

p509 — expression plasmid encoding the EBV lytic transactivator BZLF1 (Delecluse et al., 1999)

pCMV-HLA-A*0201 — expression plasmid encoding recombinant HLA-A*0201 (kindly provided by Josef Mautner, Helmholtz Zentrum München)

pCMV-HLA-B*0702 — expression plasmid encoding recombinant HLA-B*0702 (kindly provided by Josef Mautner, Helmholtz Zentrum München)

pCMV-HLA-C*0702 — expression plasmid encoding recombinant HLA-C*0702 (kindly provided by Josef Mautner, Helmholtz Zentrum München)

pEGFP-C1 — expression plasmid for enhanced green fluorescent protein (EGFP) (purchased from Clontech, Mountain View, USA)

Plasmids coding for chimeric HLA molecules were constructed within this study:

pCMV-HLA-A2/C7 — expression plasmid encoding HLA-chimera HLA-A2/C7 (aa -24 to 175 of HLA-A*0201 and 176 to 342 of HLA-C*0702)

pCMV-HLA-C7/A2 — expression plasmid encoding HLA-chimera HLA-C7/A2 (aa -24 to 175 of HLA-C*0702 and 176 to 341 of HLA-A*0201)

2.5 Oligonucleotides

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

Cell lines were screened for the presence of a mini-EBV genome with primers recognizing mini-EBV-encoded chloramphenicol-acetyltransferase gene:

cam-up: TTC TGC CGA CAT GGA AGC CAT C

cam-down: GGA GTG AAT ACC ACG ACG ATT TCC

Cell lines were screened for the presence of a wild-type EBV genome with primers recognizing BXLF1 gene encoding gp85:

gp85c: TGG TCA GCA GCA GAT AGT GAA CG

gp85d: TGT GGA TGG GTT TCT TGG GC

Primers for detection of the gene encoding GAPDH (used as PCR amplification control; kindly provided by Stephanie Medele, Helmholtz Zentrum München, Germany):

GAPDH-F3: GAC ATC AAG AAG GTG GTG AAG CAG

GAPDH-B4: AAG TGG TCG TTG AGG GCA ATG

2.6 Peptides

All peptides used in this study were synthesized to >70% purity by JPT (Berlin, Germany), resuspended in 100% dimethyl sulfoxide (DMSO) and stored at -20°C. In the T cell effector assays, the DMSO concentration was kept below 0.1% (vol/vol).

Peptides derived from HCMV pp65 and IE-1 proteins used in this work are listed in Table 2.2. For published epitopes and epitopes identified within this work, the three letter abbreviation and HLA restriction are denoted. All peptides were stored at 5 mg/mL and diluted to a final concentration of 5 µg/mL in the assay mixtures.

Table 2.2: **Peptides derived from HCMV pp65 and IE-1 proteins.**

source/ description	antigen	peptide sequence	abbreviation for epitopes	aa position	HLA-restriction
AD169	**IE-1**	ATTFLQTMLR	ATT	32–41	A*6801
AD169	**IE-1**	CRVLCCYV		309-316	
AD169	**IE-1**	CRVLCCYVL	CRV	309–317	C*0702
CRV variant (Toledo, TB40E, Davis)	**IE-1**	CRVLCCY**I**L		309–317	C*0702
AD169	**IE-1**	DEEEAIVAY		379–387	B*1801
AD169	**IE-1**	EFCRVLCCY		307–315	

source/ description	antigen	peptide sequence	abbreviation for epitopes	aa position	HLA-restriction
AD169	**IE-1**	EFFTKNSAFPK		213–223	
AD169	**IE-1**	EFFTKNSAFPKT		213–224	
AD169	**IE-1**	EFFTKNSAFPKTT	EFF	213–225	DRB1*1501/ DRB5*0101
AD169	**IE-1**	EFFTKNSAFPKTTN		213–226	
AD169	**IE-1**	ELRRKMMYM	ELR	199–207	B*0801 B*1801
AD169	**IE-1**	FCRVLCCYV		308–316	
AD169	**IE-1**	FTKNSAFPKT		215–224	
AD169	**IE-1**	FTKNSAFPKTTN		215–225	
AD169	**IE-1**	FTKNSAFPKTTNG		215–226	
AD169	**IE-1**	FFTKNSAFPKT		214–224	
AD169	**IE-1**	FFTKNSAFPKTT		214–225	
AD169	**IE-1**	FFTKNSAFPKTTN		214–226	
AD169	**IE-1**	FFTKNSAFPKTTNG		214–227	
AD169	**IE-1**	IEFFTKNSAFPK		212–223	
AD169	**IE-1**	IEFFTKNSAFPKT		212–224	
AD169	**IE-1**	IEFFTKNSAFPKTT		212–225	
AD169	**IE-1**	KEVNSQLSL	KEV	42–50	B*4001
AD169	**IE-1**	NIEFFTKNSAF		211–221	
AD169	**IE-1**	QIKVRVDMV	QIK	88–96	B*0801
AD169	**IE-1**	RHRIKEHMLK	RHR	97–106	A*0301
AD169	**IE-1**	RVLCCYVL		310–317	
AD169	**IE-1**	RVLCCYVLE		310–318	
AD169	**IE-1**	TKNSAFPKTTN		216–226	
AD169	**IE-1**	TKNSAFPKTTNG		216–227	
AD169	**IE-1**	VLCCYVLEE		311–319	
AD169	**IE-1**	VLEETSVML	VLE	316–324	A*0201
phospho-VLE	**IE-1**	VLEE-**pT**-SVML		316–324	A*0201
phospho-VLE	**IE-1**	VLEET-**pS**-VML		316–324	A*0201
AD169	pp65	IPSINVHHY	IPS	123–131	B*3501
AD169	pp65	NLVPMVATV	NLV	495–503	A*0201
AD169	pp65	RPHERNGFTV	RPH	265–274	B*0702
AD169	pp65	TPRVTGGGAM	TPR	417–426	B*0702

Frequency and specificity of IE-1-specific T cells were analyzed by using a library of 120 peptides, each 15 amino acids in length, spanning the entire IE-1 sequence of HCMV strain AD169, with subsequent peptides overlapping in 11 amino acids (Table 2.3). Nine additional peptides containing sequence variants present in the HCMV strains Toledo and TB40E were included in the study (peptides #121-129).

IE-1 peptides were ordered from JPT as

IE-1 mix	"PepMix HCMVA IE-1"; mix of peptides #1-120; final concentration of 0.5 µg/ml/peptide in the assay mixtures
individual peptides	peptides #1-129; final concentration of 5 µg/mL in the assay mixtures

For mapping of IE-1 epitopes, the 129 peptides were subpooled in a cross-matrix fashion (Kern et al., 1999), such that every single peptide was contained in one "vertical" subpool (#1–12) and one "horizontal" subpool (#13–23) (Fig. 2.1).

Table 2.3: **IE-1 peptide library**

1	MESSAKRKMDPDNPD	34	LDKVHEPFEEMKCIG	67	LTHIDHIFMDILTTC	100	SDSLVSPPESPVPAT
2	AKRKMDPDNPDEGPS	35	HEPFEEMKCIGLTMQ	68	DHIFMDILTTCVETM	101	VSPPESPVPATIPLS
3	MDPDNPDEGPSSKVP	36	EEMKCIGLTMQSMYE	69	MDILTTCVETMCNEY	102	ESPVPATIPLSSVIV
4	NPDEGPSSKVPRPET	37	CIGLTMQSMYENYIV	70	TTCVETMCNEYKVTS	103	PATIPLSSVIVAENS
5	GPSSKVPRPETPVTK	38	TMQSMYENYIVPEDK	71	ETMCNEYKVTSDACM	104	PLSSVIVAENSDQEE
6	KVPRPETPVTKATTF	39	MYENYIVPEDKREMW	72	NEYKVTSDACMMTMY	105	VIVAENSDQEESEQS
7	PETPVTKATTFLQTM	40	YIVPEDKREMWMACI	73	VTSDACMMTMYGGIS	106	ENSDQEESEQSDEEE
8	VTKATTFLQTMLRKE	41	EDKREMWMACIKELH	74	ACMMTMYGGISLLSE	107	QEESEQSDEEEEEGA
9	TTFLQTMLRKEVNSQ	42	EMWMACIKELHDVSK	75	TMYGGISLLSEFCRV	108	EQSDEEEEEGAQEER
10	QTMLRKEVNSQLSLG	43	ACIKELHDVSKGAAN	76	GISLLSEFCRVLCCY	109	EEEEEGAQEEREDTV
11	RKEVNSQLSLGDPLF	44	ELHDVSKGAANKLGG	77	LSEFCRVLCCYVLEE	110	EGAQEEREDTVSVKS
12	NSQLSLGDPLFPELA	45	VSKGAANKLGGALQA	78	CRVLCCYVLEETSVM	111	EEREDTVSVKSEPVS
13	SLGDPLFPELAEESL	46	AANKLGGALQAKARA	79	CCYVLEETSVMLAKR	112	DTVSVKSEPVSEIEE
14	PLFPELAEESLKTFE	47	LGGALQAKARAKKDE	80	LEETSVMLAKRPLIT	113	VKSEPVSEIEEVAPE
15	ELAEESLKTFEQVTE	48	LQAKARAKKDELRRK	81	SVMLAKRPLITKPEV	114	PVSEIEEVAPEEEED
16	ESLKTFEQVTEDCNE	49	ARAKKDELRRKMMYM	82	AKRPLITKPEVISVM	115	IEEVAPEEEEDGAEE
17	TFEQVTEDCNENPEK	50	KDELRRKMMYMCYRN	83	LITKPEVISVMKRRI	116	APEEEEDGAEEPTAS
18	VTEDCNENPEKDVLA	51	RRKMMYMCYRNIEFF	84	PEVISVMKRRIEEIC	117	EEDGAEEPTASGGKS
19	CNENPEKDVLAELVK	52	MYMCYRNIEFFTKNS	85	SVMKRRIEEICMKVF	118	AEEPTASGGKSTHPM
20	PEKDVLAELVKQIKV	53	YRNIEFFTKNSAFPK	86	RRIEEICMKVFAQYI	119	TASGGKSTHPMVTRS
21	VLAELVKQIKVRVDM	54	EFFTKNSAFPKTTNG	87	EICMKVFAQYILGAD	120	GKSTHPMVTRSKADQ
22	LVKQIKVRVDMVRHR	55	KNSAFPKTTNGCSQA	88	KVFAQYILGADPLRV	121	LKAKARAKKDELKRK
23	IKVRVDMVRHRIKEH	56	FPKTTNGCSQAMAAL	89	QYILGADPLRVCSPS	122	ARAKKDELKRKMIYM
24	VDMVRHRIKEHMLKK	57	TNGCSQAMAALQNLP	90	GADPLRVCSPSVDDL	123	KDELKRKMIYMCYRN
25	RHRIKEHMLKKYTQT	58	SQAMAALQNLPQCSP	91	LRVCSPSVDDLRAIA	124	KRKMIYMCYRNVEFF
26	KEHMLKKYTQTEEKF	59	AALQNLPQCSPDEIM	92	SPSVDDLRAIAEESD	125	LTEFCRVLCCYILEE
27	LKKYTQTEEKFTGAF	60	NLPQCSPDEIMAYAQ	93	DDLRAIAEESDEEEA	126	CRVLCCYILEETSVL
28	TQTEEKFTGAFNMMG	61	CSPDEIMAYAQKIFK	94	AIAEESDEEEAIVAY	127	CCYILEETSVLLAKR
29	EKFTGAFNMMGGCLQ	62	EIMAYAQKIFKILDE	95	ESDEEEAIVAYTLAT	128	LEETSVLLAKRPLIT
30	GAFNMMGGCLQNALD	63	YAQKIFKILDEERDK	96	EEAIVAYTLATAGVS	129	ESDEEDAIAAYTLAT
31	MMGGCLQNALDILDK	64	IFKILDEERDKVLTH	97	VAYTLATAGVSSSDS		
32	CLQNALDILDKVHEP	65	LDEERDKVLTHIDHI	98	LATAGVSSSDSLVSP		
33	ALDILDKVHEPFEEM	66	RDKVLTHIDHIFMDI	99	GVSSSDSLVSPPESP		

	1	2	3	4	5	6	7	8	9	10	11	12
13	1	2	3	4	5	6	7	8	9	10	11	12
14	13	14	15	16	17	18	19	20	21	22	23	24
15	25	26	27	28	29	30	31	32	33	34	35	36
16	37	38	39	40	41	42	43	44	45	46	47	48
17	49	50	51	52	53	54	55	56	57	58	59	60
18	61	62	63	64	65	66	67	68	69	70	71	72
19	73	74	75	76	77	78	79	80	81	82	83	84
20	85	86	87	88	89	90	91	92	93	94	95	96
21	97	98	99	100	101	102	103	104	105	106	107	108
22	109	110	111	112	113	114	115	116	117	118	119	120
23	121	122	123	124	125	126	127	128	129			

Fig. 2.1: Cross-matrix orientation of 23 IE-1 peptide subpools containing 120 peptides of AD169 (#1–120) and 9 sequence variants present in the HCMV strains Toledo and TB40E (#121–129).

2.7 Antibodies

All antibodies used in this study were made in mouse and are listed as follows.

specificity	clone	isotype	conjugate	company
CD3	HIT3a	IgG2a	PE-Cy5	BioLegend, San Diego, USA
CD4	RPA-T4	IgG1	FITC	BD Biosciences, Heidelberg, Germany
CD8α	RPA-T8	IgG1	APC	BioLegend, San Diego, USA
CD56	HCD56	IgG1	PE-Cy5	BioLegend, San Diego, USA
HLA-A2	BB7.2	IgG2b	PE	BioLegend, San Diego, USA
HLA-A2	BB7.2	IgG2b	unlabeled	BioLegend, San Diego, USA
HLA-ABC	W6/32	IgG2a	PE	BioLegend, San Diego, USA
HLA-ABC	W6/32	IgG2a	unlabeled	BioLegend, San Diego, USA
HLA-B7	BB7.1	IgG1	PE	Millipore, Billerica, USA
HLA-DR	L234	IgG2a	unlabeled	BioLegend, San Diego, USA
HLA-DQ	SPV-L3	IgG2a	unlabeled	AbD Serotec, Kidlington, UK
HLA-DP	B7/21	IgG3	unlabeled	Abcam, Cambridge, UK
KIR2DL1 (CD158a)	HP-3E4	IgM	FITC	BD Biosciences, Heidelberg, Germany
KIR2DL2/3 (CD158b)	CH-L	IgG2b	PE	BD Biosciences, Heidelberg, Germany
KIR2DL2/3 (CD158b)	DX27	IgG2a	unlabeled	BioLegend, San Diego, USA
KIR3DL1	DX9	IgG1	FITC	BioLegend, San Diego, USA
NKG2A (CD159a)	Z199.1	IgG2b	APC	Beckman Coulter, Krefeld, Germany
isotype control	MOPC-21	IgG1	PE	BioLegend, San Diego, USA
isotype control	MOPC-173	IgG2a	PE	BioLegend, San Diego, USA
isotype control	MPC-11	IgG2b	PE	BioLegend, San Diego, USA

The following antibody-coupled beads were used for immunomagnetic separation:

MACS® CD3 MicroBeads, human	Miltenyi Biotec, Bergisch Gladbach, Germany
MACS® CD4 MicroBeads, human	Miltenyi Biotec, Bergisch Gladbach, Germany
MACS® PE MicroBeads	Miltenyi Biotec, Bergisch Gladbach, Germany

2.8 Peptide/HLA multimers

A PE-conjugated CRV/HLA-C*0702 streptamer was generated within a collaboration by Fabian Schlott (group of Michael Neuenhahn/Dirk Busch; Institute for Medical Microbiology, Immunology and Hygiene, Technische Universität München) and kindly provided for staining of CRV-specific CD8+ T cells. An unlabeled TPR/HLA-B*0702-Pentamer was ordered from (Proimmune, Oxford, England). Pentamer-positive cells were counterstained with Pro5 Fluorotag R-PE (Proimmune, Oxford, England).

2.9 Cell culture media and additives

E.coli were cultivated in LB (Luria Bertani) medium or on solid LB agar plates.

Luria Broth Base	Gibco Invitrogen, Karlsruhe, Germany

Select Agar	Gibco Invitrogen, Karlsruhe, Germany

The following antibiotics were used for selection purposes:

ampicillin sodium salt	Carl Roth GmbH, Karlsruhe, Germany
chloramphenicol	Sigma-Aldrich, St. Louis, USA
kanamycin sulfate salt	Sigma-Aldrich, St. Louis, USA

Eukaryotic cell culture

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

name	application/function	source
RPMI 1640	standard cell culture medium	Gibco Invitrogen, Karlsruhe, Germany
DMEM	cell culture medium for adherent cells (fibroblasts, BHK-21, TR-2/293)	Gibco Invitrogen, Karlsruhe, Germany
Opti-MEM	cell culture medium for transfection	Gibco Invitrogen, Karlsruhe, Germany
fetal calf serum (FCS)	nutritive substance	Biochrom, Berlin, Germany
penicillin/streptomycin	antibiotics, inhibition of bacteria growth	Gibco Invitrogen, Karlsruhe, Germany
sodium selenite	supplies an essential element (additive for RPMI 1640 and DMEM)	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 establishment of 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	Invitrogen, Karlsruhe, Germany

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

name	application	source
recombinant human IL-2 ("Proleukin S")	cultivation of T cells, NK cells	Novartis, Nürnberg, Germany
recombinant human IL-1β	establishment of DCs	Biochrom, Berlin, Germany
recombinant human IL-4	cultivation of CD40-stimulated B cells, establishment of DCs	R&D Systems, Minneapolis, USA
recombinant human IL-6	establishment of DCs	Biochrom, Berlin, Germany
recombinant human IFN-γ	cultivation of fibroblasts (enhancement of MHC expression)	PAN Biotech, Aidenbach, Germany
recombinant human GM-CSF	establishment of DCs	Genzyme, Neu-Isenburg, Germany
Prostaglandin E_2 (PGE_2)	establishment of DCs	Sigma, St. Louis, USA
recombinant human TNF-α	establishment of DCs	Biochrom, Berlin, Germany

2.10 Commercial kits

AP Conjugate Substrate Kit	Bio-Rad, München, Germany
Endofree® Plasmid Maxi Kit	Qiagen, Hilden, Germany
QIAprep® Spin Miniprep Kit	Qiagen, Hilden, Germany
Human IFN-γ ELISA Kit (ALP)	Mabtech, Nacka Strand, Sweden
Human GM-CSF ELISA Kit (ALP)	Mabtech, Nacka Strand, Sweden
Human TNF-α ELISA Kit (ALP)	Mabtech, Nacka Strand, Sweden
Human IL-2 ELISA Kit (ALP)	Mabtech, Nacka Strand, Sweden
Human IL-4 ELISA Kit (ALP)	Mabtech, Nacka Strand, Sweden
Human IL-10 ELISA Kit (ALP)	Mabtech, Nacka Strand, Sweden
Human IFN-γ ELISpot Kit (ALP)	Mabtech, Nacka Strand, Sweden
MACS® IFN-γ secretion assay	Miltenyi Biotec, Bergisch Gladbach, Germany
Nucleofector® Kit R	Lonza, Basel, Switzerland

2.11 Enzymes

restriction enzymes EcoRI, Esp3I, PvuII	Fermentas, St. Leon-Rot, Germany
Shrimp Alkaline Phosphatase (SAP)	Fermentas, St. Leon-Rot, Germany
T4 DNA ligase	Fermentas, St. Leon-Rot, Germany
Taq-DNA polymerase	Fermentas, St. Leon-Rot, Germany

2.12 Chemicals, reagents and buffers

actinomycin D (ActD)	Sigma-Aldrich, St. Louis, USA
Biocoll Separating Solution	Biochrom, Berlin, Germany
calcein acetoxymethylester (AM)	Invitrogen, Karlsruhe, Germany
cover oil	Biotest AG, Dreieich, Germany
cycloheximide (CHX)	Sigma-Aldrich, St. Louis, USA
dimethyl sulfoxide (DMSO)	Carl Roth GmbH, Karlsruhe, Germany
diethanolamine (DEA)	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
GeneRuler™ 100 bp DNA Ladder	Fermentas, St. Leon-Rot, Germany
Heparin-Natrium 25,000	Braun, Melsungen, Germany
human serum type AB	Cambrex, East Rutherford, USA
ionomycin calcium salt	Tocris Bioscience, Bristol, UK
magnesium sulfate heptahydrate	Carl Roth GmbH, Karlsruhe, 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
PCR reagents ($MgCl_2$, 2 mM dNTPs)	Fermentas, St. Leon-Rot, Germany
polyethyleneimine (PEI) M_W≈25 000 g/mol	Aldrich, St. Louis, USA
sodium dodecyl sulphate (SDS)	Carl Roth, GmbH, Karlsruhe, Germany
TAE (Tris, acetate, EDTA)	Carl Roth, GmbH, Karlsruhe, Germany

tetradecanoylphorbol acetate (TPA)	Sigma-Aldrich, St. Louis, USA
thiazolyl blue tetrazolium bromide (MTT)	Sigma-Aldrich, St. Louis, USA
Tris/HCl (1 M)	Carl Roth, GmbH, Karlsruhe, Germany
Triton™ X-100	Carl Roth GmbH, Karlsruhe, Germany
trypan blue	Merck, Darmstadt, Germany
TÜRK's solution	Merck, Darmstadt, Germany
Tween® 20	Carl Roth GmbH, Karlsruhe, Germany

2.13 Consumables

6-/12-/24-/48-/96-well flat-bottom plates	BD Biosciences, Heidelberg, Germany
96-well MultiScreen HTC Filter plates	Millipore, Billerica, USA
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 15 mL, 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
cell scrapers	Nunc A/S, Roskilde, Denmark
cryo tube vials 1.8 mL	Nunc A/S, Roskilde, Denmark
Immunoplates MaxiSorp® 96-w flat-bottom	Nunc A/S, Roskilde, Denmark
Infuject® 50 mL syringes and needles	Dispomed Witt oHG, Gelnhausen, Germany
PCR reaction tubes	Abgene, Hamburg, Germany
plastic pipettes (2 mL; 5 mL; 10 mL; 25 mL)	Greiner Bio-One, Kremsmünster, Österreich
polypropylene tubes 1.3 mL	Greiner Bio-One, Kremsmünster, Österreich
pipette tips (Diamond® TowerPack™) (0.110µL; 10100µL; 1001000µL)	Gilson Inc., Middleton, USA
reaction tubes 0.5 mL; 1.5 mL; 2 mL	Eppendorf, Hamburg, Germany
Reagent Reservoir Costar® 50 mL	Corning Inc., Corning, USA
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

2.14 Laboratory equipment and devices

bacteria incubator/shaker innova™ 4400	New Brunswick Scientific, Enfield, USA
"Centrifuge 5415 R"	Eppendorf, Hamburg, Germany
centrifuge "Avanti® J-26 XP"	Beckman Coulter, Krefeld, Germany
centrifuge "Multifuge 3 L-R"	Heraeus Holding, Hanau, Germany
centrifuge "Rotanta 46 RSC"	Hettich AG, Bäch, Switzerland
"FACS Calibur" cytofluorometer	Becton Dickinson, Heidelberg, Germany
Fluorescence microscope Axiovert 200M	Zeiss, Jena, Germany
forceps	Hartenstein, Würzburg, 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	Peqlab Biotechnologie, Erlangen, Germany
gel electrophoresis chambers	Peqlab Biotechnologie, Erlangen, Germany
glass ware	SCHOTT AG, Mainz, Germany
HP Scanjet G4050	Hewlett Packard, Böblingen, Germany
irradiation device Gammacell 40 (Cs-137)	Atomic Energy of Canada Limited, Ottawa, Kanada
HERAEUS PICO 21 centrifuge	Thermo Fisher Scientific, Waltham, USA
ice machine AF 200	Scotsman, Milan, Italy
incubator CO_2-AUTO-ZERO	Heraeus Holding, Hanau, Germany
incubator CO_2-Unitherm 170	UniEquip, Planegg, Germany
inverted microscope Axiovert 25	Zeiss, Jena, Germany
laminar flow hoods	BDK, Sonnenbühl-Genkingen, Germany
magnetic stirrer "IKAMag REO"	IKA®-Werke GmbH, Staufen, Germany
microwave "compact microwave oven"	Reston Lloyd, Sterling, USA
Nanodrop ND-1000 spectrophotometer	Peqlab Biotechnologie, Erlangen, Germany
Neubauer counting chamber	Paul Marienfeld, Lauda-Königshofen, Germany
Nucleofector I Device	Lonza, Basel, Switzerland
PCR-machine "Mastercycler Personal"	Eppendorf, Hamburg, 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
Reference® pipettes (0.510 µL; 10100 µL; 1001000 µL)	Eppendorf, Hamburg, Germany
Thermomixer compact	Eppendorf, Hamburg, Germany
Transferpette® (20200 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

2.15 Databases and software

The following **databases and bioinformatic tools** were used within this study:

IMGT/HLA database	supply of HLA sequences (www.ebi.ac.uk/imgt/hla/)
SYFPEITHI	epitope prediction (www.syfpeithi.de/)
BIMAS	epitope prediction (www-bimas.cit.nih.gov)

Software used within this study for design of experiments, data analysis and data illustration is listed as follows.

name	application	source
Adobe® Illustrator® CS5	illustration and graphic design	Adobe Systems Inc., San José, USA
Bookends 11.2.7.	reference management	Sonny Software, Chevy Chase, USA
CellQuest™ Pro	FACS analysis	BD Biosciences, Heidelberg, Germany
FlowJo 9.4.11	FACS analysis	TreeStar Inc., Ashland, USA
GraphPad Prism5	data illustration	GraphPad Software, La Jolla, USA
KC4	ELISA measurement, calculation of standard curves	TreeStar Inc., Ashland, USA
MacVector 12.0.6.	nucleotide and protein sequence analysis and alignment	MacVector Inc., Cary, USA
Microsoft® Word 2011	word processing	Microsoft, Redmond, USA
Microsoft® Excel 2011	data analysis and illustration	Microsoft, Redmond, USA

2.16 Services

The **HCMV and EBV 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 or cell lines from the donors using PCR-based methods by IMGM, Martinsried, Germany.

KIR typing of donor AJU was performed on PBMCs of this donor using PCR-based methods by IMGM, Martinsried, Germany.

3 Methods

3.1 Microbiological methods

3.1.1 Cultivation and storage of bacteria

Escherichia coli (*E. coli*) strain DH5α (Invitrogen) was used for propagation of the low size, high copy plasmids listed in 2.4. and for cloning purposes. *E. coli* strain DH10B (Invitrogen) was used for preparation of mini-EBV vector DNA listed in 2.3.

Bacteria from cryo-cultures were directly plated on LB agar plates and incubated overnight at 37°C. Resulting single cell colonies were used for inoculation of suspension cultures grown in LB medium at 37°C under constant agitation at 200 rpm. Plasmid-carrying bacteria were cultivated in presence of antibiotics (100 µg/mL ampicillin, 30 µg/mL chloramphenicol, 30 µg/mL kanamycin) to select for resistance genes.

For long-term storage, dense overnight cultures of *E. coli* were supplemented with 7% DMSO, transferred to cryovials and frozen at -80°C.

3.1.2 Transformation of bacteria

Transformation of bacteria with purified plasmid DNA or ligated DNA fragments was performed with chemically competent *E. coli* DH5α cells (Invitrogen) following the manufacturer's instructions. Up to 1 µg of plasmid DNA or 2–5 µL of ligation mixture was added to 50 µL of *E. coli* cells. After transformation, bacteria were plated on LB agar plates with the respective antibiotics and incubated overnight at 37°C.

3.2 Molecular biology methods

3.2.1 General DNA techniques

3.2.1.1 Plasmid DNA purification from *E.coli*

Small scale purification of low and high copy plasmid DNA for restriction enzyme analyses was performed by using the QIAprep® Spin Miniprep Kit (Qiagen). *E. coli* colonies were picked with pipette tips from the LB agar plates and incubated in 4 mL LB medium overnight under constant agitation (200 rpm). Approximately 2 mL of dense bacteria culture were used for plasmid preparation according to the manufacturer's protocol.

Large scale purification of low and high copy plasmid DNA for transfection of eukaryotic cells was performed by using the Endofree® Plasmid Maxi Kit (Qiagen) according to the manufacturer's protocol. For high copy plasmids (all plasmids listed in 2.4), 400 mL of saturated overnight

bacteria culture was used for purification (yield ≤ 1 mg DNA). For low copy plasmids (mini-EBV plasmids listed in 2.3), 2x 400 mL of dense overnight bacteria culture were necessary to isolate sufficient vector DNA for mini-EBV production (yield ≤ 100 µg).

The nucleic acid concentration of plasmid preparations was determined by absorbency readings at 260 nm with the Nanodrop ND-1000 spectrophotometer (Peqlab Biotechnologie). Samples with an absorbance ratio 260 nm/280 nm between 1.8 and 2.0 were considered as appropriately pure.

3.2.1.2 Restriction enzyme digest, dephosphorylation, ligation

Restriction endonuclease digests of DNA were performed with enzymes from the manufacturer Fermentas. Using 10 units of the chosen enzyme for each µg of DNA, reaction mixtures were incubated in the recommended reaction buffer for 1 h at 37°C (recommended temperature for the selected enzymes Esp3I, EcoRI and PvuII).

After restriction enzyme digest, Shrimp Alkaline Phosphatase (SAP, Fermentas) was used according to the manufacturer's instructions to remove 5' phosphates from free DNA ends in order to avoid spontaneous religation of vector DNA.

Ligations of digested vector DNA and insert DNA were performed using the T4 DNA Ligase (Fermentas) according to the manufacturer's protocol. In general, 50 ng of vector DNA were mixed with the appropriate amount of insert DNA at a vector/insert ratio between 1:2 and 1:10.

3.2.1.3 Agarose gel electrophoresis

DNA fragments from restriction enzyme digests and PCR products were separated according to their size by agarose gel electrophoresis. 1.0–2.0% agarose gels were prepared in 1x TAE buffer. For visualization, ethidium bromide was added in a final concentration of 0.1 µg/mL. The nucleic acid was mixed with 6x loading dye (Fermentas) prior to loading onto the agarose gel. The molecular weight markers GeneRuler^{TM} DNA Ladder Mix and GeneRuler^{TM} 100 bp DNA Ladder (Fermentas) were used to determine DNA fragment size. 1x TAE served as running buffer. Gels were documented by ultraviolet (UV) light irradiation (gel documentation system, Peqlab Biotechnologie).

3.2.2 Analysis of B cell lines for the presence of EBV-specific sequences

Each mini-EBV-transformed lymphoblastoid cell line (mLCL) established within this study was checked by polymerase chain reaction (PCR) for the presence of a mini-EBV-specific sequence (chloramphenicol acetyltransferase, cam) using primers cam-up and cam-down (product size: 294 bp).

EBV-free CD40-stimulated B cell lines and mLCLs were checked for contamination with lymphoblastoid cells transformed by endogenous wild-type EBV of seropositive donors. Primers gp85c and gp85d (product size: 461 bp) were specific for the gene BXLF1, encoding viral glycoprotein gp85, which is present in wild-type EBV strains and laboratory EBV strain B95.8, but not in recombinant mini-EBV vectors. B95.8-transformed LCLs were used as a PCR-control for detection of wild-type EBV-specific sequence. In PCR reactions of CD40-stimulated B cells, primers specific for GAPDH (GAPDH-F3 and -B4) were used as a control for genomic DNA amplification for the case that wild-type EBV sequence could not be detected.

For PCR, crude DNA was isolated from mLCLs, LCLs and CD40-stimulated B cells by alkaline lysis. 1 mL of dense cell culture containing approximately 1x 10^6 cells was harvested by centrifugation in a 1.5 mL reaction tube. After complete removal of the supernatant, the cell pellet was resuspended in 25 µL 50 mM NaOH, covered with oil and incubated at 95°C for 10 minutes. The lysate was cooled on ice, neutralized with 4 µL 1 M Tris/HCl (pH 7) and centrifuged at maximum speed for 1 minute. 1 µL of supernatant (below the cover oil) containing the genomic DNA was used in the PCR reaction mixture.

For mLCLs, the primer pairs cam-up/cam-down and gp85c/gp85d, and for CD40-stimulated B cells, the primer pairs gp85c/gp85d and GAPDH-F3/GAPDH-B4 were added into the same reaction mixture at a ratio of 1:1. The PCR reaction mixture is listed as follows:

	[µL]
DNA	1.00
Taq-buffer	5.00
Taq DNA polymerase	1.25
primers each	2.50
dNTPs (2 mM)	5.00
$MgCl_2$ (25 mM)	5.00
H_2O	22.75
total	50.00

The PCR protocol comprised 30 cycles of amplification, with 45 seconds each of denaturation at 96°C, primer annealing at 59°C, and elongation at 72°C.

3.2.3 Cloning of chimeric MHC class I molecules

Plasmids carrying cDNAs of full-length class I heavy chains, pCMV-HLA-A*0201 and pCMV-HLA-C*0702, were used for the construction of the HLA-chimera HLA-A2/C7 (aa -24 to 175 of HLA-A*0201 and 176 to 342 of HLA-C*0702) and its counterpart HLA-C7/A2 (aa -24 to 175 of HLA-C*0702 and 176 to 341 of HLA-A*0201).

These switched chimeras were constructed using an EcoRI restriction site within the vector backbone and an Esp3I restriction site that was located within an entirely conserved sequence near the C-terminal end of the α_2-domain. Each template was sequentially digested with the two restriction enzymes. To minimize spontaneous religation of the corresponding sticky ends, one of the two plasmids, pCMV-HLA-A*0201 or pCMV-HLA-C*0702, was dephosphorylated with Shrimp Alkaline Phosphatase (SAP, Fermentas). Fragments of both templates, dephosphorylated and not dephosphorylated, were mixed, religated with T4 DNA Ligase (Fermentas) and transformed into DH5α (Invitrogen). When analyzing the resulting plasmids with restriction enzyme PvuII (Fermentas), all 4 possible HLA constructs were obtained: the two chimeras HLA-A2/C7 and -C7/A2 as well as religated HLA-A*0201 and -C*0702.

3.3 Cell culture methods

3.3.1 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 for LCLs, mLCLs, K562 cells, Daudi cells, LL8 cells, CD40-stimulated B cells, and TR-2/293 cells was RPMI-1640 supplemented with 10% fetal calf serum (FCS), penicillin/streptomycin (100 U/mL/100 µg/mL) and 100 nM sodium selenite.

LCLs, mLCLs, K562 cells, and Daudi cells were cultivated in suspension and, depending on their proliferation rate, were expanded one or two times per week 25-fold each time. For the HLA-C*0602- or -C*0702-expressing derivatives of the MHC class I-deficient LCL L721.221, hygromycin B (0.5 mg/mL) was added to the cell culture medium to avoid loss of the transfected HLA-encoding construct.

Murine LL8 fibroblasts stably expressing CD40 ligand (CD40L) were cultivated adherently. Confluent cells were split once per week by washing with PBS and incubation with 0.5% Trypsin/EDTA for 5 minutes at 37°C. Detached cells were pelleted by centrifugation and expanded approximately 40-fold in fresh standard cell culture medium.

CD40-stimulated B cell lines were cultivated in flat-bottom 12-well plates on a layer of irradiated (180 Gy) CD40L-expressing LL8 cells (1x 10^6 cells/plate). Standard cell culture medium was

supplemented with recombinant human (rhu)IL-4 (2 ng/mL). CD40-stimulated B cell lines were replated two times per week on fresh irradiated LL8 feeder cells and thereby expanded 2- to 6-fold depending on their proliferation rate.

The EBV-packaging TR-2/293 cell line was cultivated adherently in presence of hygromycin B (100 µg/mL) in order to preserve the stably transfected maxi-EBV plasmid. Confluent cells were passaged two times per week by tapping the flask until all cells were detached. Cells were pelleted by centrifugation and expanded 5-10-fold in fresh standard cell culture medium.

BHK-21 cells and human fibroblast cell lines were cultivated in Dulbecco's modified Eagle's medium (DMEM, Gibco/Invitrogen) with the same supplements as described for the standard cell culture medium. Both cell lines were passaged twice a week by trypsinization (0.05% Trypsin/EDTA for 1–2 minutes). Fibroblast cell lines were split at around 80% confluency approximately 24-fold. BHK-21 cells were grown until reaching 100% confluency and expanded 10-fold.

In general, cells were pelleted by centrifugation for 10 minutes at 300 x g and at room temperature.

Cell count was determined using a Neubauer counting chamber. Before counting, cell suspensions were diluted 2–10-fold with a 0.5% trypan blue solution in order to visualize dead cells. Negatively charged trypan blue cannot be absorbed by viable cells with intact cell membranes. By contrast, dead cells with damaged cell membranes do not exclude this dye and appear in blue color under the microscope.

3.3.2 Cryopreservation of cells

For long-term preservation, eukaryotic cells were stored in the gas phase over liquid nitrogen in presence of dimethyl sulfoxide (DMSO). The cells were pelleted by centrifugation and resuspended in cold (4°C) RPMI supplemented with 50% FCS and 10% DMSO. The cell suspensions were aliquoted in 2 mL cryovials, which were pre-cooled on ice. To follow a gentle freezing procedure, the cryovials with cells were first cooled on ice for up to 20 minutes, then placed into styrofoam boxes and stored at -80°C for a few days, and finally transferred to liquid nitrogen tank.

Frozen cells were thawed as fast as possible by adding pre-warmed (37°C) cell culture medium to the cryovial. The DMSO-containing cell suspensions were diluted in at least 20 mL medium, pelleted by centrifugation and the supernatant was removed completely. The cell pellets were resuspended in the respective medium and taken into culture. If required, selection pressure was applied by supplementing the cell culture medium with antibiotics no earlier than two days after thawing.

3.3.3 Transfection of cells with plasmid DNA

For production of mini-EBV virions (section 3.5.1), EBV-packaging cell line TR-2/293 was transfected with mini-EBV plasmid and helper plasmids by using polyethyleneimine (PEI) as transfection agent. This cationic polymer forms complexes with the DNA, which bind to the negatively charged cell membrane and are incorporated into the cell by endocytosis (Vancha et al., 2004). For a 15-cm cell culture dish of subconfluent TR-2/293 cells, 120 µL PEI (1 mg/mL; M_W≈25 000 g/mol; Aldrich) were used to transfect a total amount of 20 µg of endotoxin-free plasmid DNA (prepared with Endofree® Plasmid Maxi Kit). Plasmid DNA and PEI were separately mixed with 4 mL serum-free OptiMEM medium each and incubated for 5 minutes at room temperature. Then, DNA and PEI mixtures were combined and incubated for 20 minutes at room temperature to enable DNA/PEI complex formation. Old medium was removed from the cells and the DNA-PEI transfection mixture was added to the cells together with 12 mL fresh serum-free OptiMEM so that the cells were covered by 20 mL medium in total. After 5 hours of incubation, medium containing the transfection mixture was replaced by fresh RPMI supplemented with 10% FCS.

Fibroblast cell lines were transfected with the plasmids encoding recombinant HLA molecules or HLA chimeras by electroporation using the Amaxa Cell Line Nucleofector Kit R and Nucleofector I Device (program V-01) according to the manufacturer's instructions for WI-38 cells (Lonza). The enhanced green fluorescent protein (EGFP) expressing plasmid pEGFP-C1 (BD Biosciences Clontech) was used as a transfection control and demonstrated transfection rates of 60–70%. After 24 hours, transfectants were kept under selection pressure by adding G-418 (200 µg/mL). On day 4 post transfection fibroblasts were infected with MVA or HCMV and used as target cells for T cell effector assays 24 or 48 hours post infection (hpi), respectively.

3.3.4 Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) of healthy adult donors were purified from whole venous blood samples of standard blood donations or from buffy coats by density gradient centrifugation using Ficoll/Hypaque (Biochrom). Blood samples were transferred to 50 mL Falcon tubes (≤ 30 mL/tube) and diluted with PBS to 40 mL/tube. Thereby, the pre-concentrated buffy coats had to be mixed with PBS in a ratio of at least 1:1. The diluted blood was carefully underlayered with 10 mL room-temperature Ficoll/Hypaque. The Falcon tubes were centrifuged at 2000 rpm (centrifuge "Multifuge 3 L-R") and room temperature for 25 minutes and with lowest breaking rate for deceleration. During centrifugation, Ficoll separates the whole blood components according to their density: erythrocytes which are more dense are able to pass the Ficoll underlayer and sediment at the bottom of the tube, whereas less dense PBMCs are trapped immediately above, at the interphase between Ficoll and diluted plasma. Using a 10 mL pipette,

PBMCs were carefully collected without aspirating any erythrocytes and transferred to a clean 50 mL Falcon tube. PBMCs were washed 3–5 times with 50 mL PBS in order to remove remaining Ficoll and to deplete thrombocytes. Freshly isolated PBMCs were either frozen or directly used for establishment of permanent B cell lines, T and NK cell cultures or monocyte-derived dendritic cells.

For determination of total PBMC number, cells were diluted in Türk's solution (0.01% crystal violet, 1% acetic acid) and counted in a Neubauer counting chamber. Türk's solution is a hypotonic acidic agent that lyses erythrocytes, which might still be present in the PBMC preparation but leaves white blood cells intact. The crystal violet dye stains cells in slight blue, thus facilitating the counting.

An aliquot of the diluted plasma was collected for determination of the EBV and HCMV IgG serostatus of the blood donors (Max von Pettenkofer-Institute, München, Germany).

3.3.5 Establishment of permanent B cell lines

3.3.5.1 CD40-stimulated B cell lines

Long-term EBV-free B cell cultures were established from PBMCs as described in Wiesner et al., 2008. Unseparated PBMCs were plated on irradiated CD40L-expressing murine fibroblasts (LL8) in flat-bottom 96-well plates. To consider varying proliferative capacity of B cells from different donors, individual microcultures with 10, 5, 2.5 and 1.25x 10^4 cells per (well in 200 µL medium and with 6 replicates per cell number were set up. Cells were cultivated in standard cell culture medium supplemented with rhuIL-4 (R&D Systems). Cyclosporin A (1 µg/mL, Novartis) was present for the first 4 weeks in order to inhibit T cell and NK cell growth. Cells were restimulated every week by replating on fresh LL8 cells. Outgrowth of B cells became prominent after approximately 4 weeks and cells were expanded stepwise to 48-, 24- and 12-well plates. For long-term cultivation and freezing, a well-proliferating culture derived from the lowest initial PBMC number that had led to rubust outgrowth was chosen from each donor. These cultures were maintained in 12-well plates by weekly replating on irradiated (180 Gy) LL8 cells in the presence of 2 ng/mL rhuIL-4.

In EBV carriers (>95% of the adult human population) one in about 100 000 B cells is latently infected with endogenous EBV (Miyashita et al., 1995). Therefore, established CD40-stimulated B cell lines were tested for the presence of wild-type EBV DNA (BXLF1 gene encoding gp85) by PCR as described before (section 3.2.2). The majority of cultures was negative for wild-type EBV.

3.3.5.2 EBV-transformed mini-lymphoblastoid cell lines

Mini-lymphoblastoid cell lines (mLCLs) stably expressing pp65 or IE-1 protein and control mLCLs (without HCMV antigen) were generated as described by Moosmann et al. (2002). PBMCs were infected with B cell-transforming mini-Epstein-Barr virus (mini-EBV) carrying a mini-EBV genome with or without the relevant HCMV transgene under control of the SV40 promoter (Moosmann et al., 2002). In a well of a flat-bottom 96-well plate, 4x 10^5 PBMCs in 100 µL medium per well were coincubated overnight with 100 µL/well of mini-EBV-containing medium. As a negative control and to detect transformation by endogenous EBV, 100 µL medium was used instead of mini-EBV supernatant. The next day, half of the supernatant was replaced by fresh medium. The cells were cultivated in standard cell culture medium and fed every week. Cyclosporin A (1 µg/mL, Novartis) was present for the first 4 weeks. Outgrowing mLCLs were visible after approximately 3 weeks and expanded stepwise from 96-well plates to cell culture flasks.

Established mLCLs were tested for the presence of a mini-EBV-specific sequence (encoding chloramphenicol acetyltransferase) and the absence of a wild-type EBV-specific sequence (BXLF1 gene encoding gp85) by PCR as described before (section 3.2.2).

3.3.6 Establishment and maintenance of T cell cultures

3.3.6.1 Generation of HCMV-specific polyclonal T cell lines

Polyclonal T cell cultures specific for pp65 or IE-1 antigen of HCMV were established from freshly isolated or cryopreserved PBMCs of HCMV-seropositive donors. IE-1- or pp65-specific T cells were reactivated and expanded *in vitro* by repeated restimulation of PBMCs with irradiated (50 Gy) autologous mLCLs expressing the appropriate antigen. Endogenously processed peptides of the HCMV antigens are loaded on MHC class I and II molecules expressed by the mLCLs and presented to T cells. By using autologous mLCLs, the whole repertoire of pp65- or IE-1-specific memory T cells existing in one donor can theoretically be reactivated and enriched without stimulating alloreactive T cell specificities.

Cocultivations of PBMCs/T cells and mLCLs were performed in 12-well plates (3 mL/well) in standard cell culture medium. On day 0, 2x 10^6 PBMC/mL were stimulated with 5x 10^4 irradiated mLCLs/mL. On day 10 and weekly thereafter, cells were collected, pelleted, resuspended in fresh medium with irradiated mLCLs and distributed to new 12-well plates. The following cell concentrations were used:

day 10: 2x 10^6 T cells/mL and 5x 10^5 mLCLs/mL;
day 17 and later: 1x 10^6 T cells/mL and 2.5 x10^5 mLCLs/mL.

From day 17, medium was supplemented with 50-100 U/mL rhuIL-2 („Proleukin S“, Novartis). Between restimulations, the T cell cultures were fed with fresh medium as necessary and expanded in case of strong proliferation. The cell cultures were stimulated over approximately 50 days. During this period, the proportion of CD4+ and CD8+ T cells was followed by FACS staining.

As a rapid alternative to mLCL-stimulation, individual T cell specificities can also be reactivated and expanded by peptide-stimulation. Thereby, PBMCs are loaded with the antigenic peptide and can function as antigen-presenting cells for T cells. This method was used for detection of low-frequency CD4+ EFF-specific T cells. PBMCs (2x 10^7) in 4 mL standard cell culture medium were loaded with 5 µg/mL EFF peptide for 2 hours at 37°C. Cells were washed 3 times with PBS, mixed with 2x 10^7 unlabeled PBMCs and adjusted to a concentration of 2.5x 10^6 cells/mL. PBMCs were incubated with EFF-peptide in standard cell culture medium supplemented with 10 U/mL rhuIL-2 for 5–6 days. Then, IL-2 concentration was raised to 25 U/mL and cells were kept until day 11–14.

3.3.6.2 Generation of HCMV-specific monoclonal T cell lines

T cell clones were obtained by limiting dilution from mLCL-stimulated polyclonal T cell lines or directly from PBMCs after peptide-stimulation and IFN-γ secretion assay (Miltenyi Biotec). T cells were seeded into round-bottom 96-well plates (200 µL/well) with 0.7 or 2.5 cells/well, so that outgrowing T cell clones should result from one single cell expressing one specific T cell receptor. Proliferation of antigen-specific T cells was stimulated with a feeder mixture containing standard cell culture medium supplemented with 1000 U/mL rhuIL-2, 1× 10^5/mL irradiated (50 Gy) HLA-matched pp65- or IE-1-expressing mLCLs 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 visible after 3–4 weeks and expanded in round-bottom 96-well plates by restimulating every 2 weeks under the same conditions. T cell clones were screened by IFN-γ ELISA for antigen and peptide specificity. Thereby, the target cells were autologous mLCLs expressing the appropriate HCMV antigen, pp65 or IE-1, autologous ctrl-mLCLs (without antigen) and autologous CD40-stimulated B cells loaded with a potential target peptide or unloaded. T cell clones identified to be antigen- or peptide-specific were expanded and aliquots of well-growing clones were cryopreserved.

3.3.7 Generation of monocyte-derived dendritic cells

The recognition of IE-1 by CD4+ T cells was analyzed in context of an HCMV infection of monocyte-derived dendritic cells (DCs). DCs were generated from whole blood of HLA-matched

healthy donors using a modified published protocol (Jonuleit et al., 2000). Freshly isolated PBMCs were incubated in standard cell culture medium in 80 cm^2 flasks (5x 10^7 cells in 15 mL/flask) for 2 hours in order to adhere the monocytes to the plastic surface. Then, supernatant containing the remaining PBMCs was discarded and residual non-adherent cells were rinsed off the plates with a 10 mL plastic pipette in two washing steps using prewarmed (37°C) RPMI + P/S/Na-Se (without FCS). Immature DCs were generated by cultivating the monocytes for 3 days in RPMI + 2% human serum + P/S/Na-Se (15 mL/flask) supplemented with rhuGM-CSF (Genzyme, 100 ng/mL) and rhuIL-4 (R&D Systems, 20 ng/mL). For maturation of DCs, cells were stimulated for another 24 hours with rhuTNF-α (Biochrom, 10 ng/mL), rhuIL-1β (Biochrom, 10 ng/mL), PGE_2 (Sigma, 1 µg/mL) and rhuIL-6 (Biochrom, 15 ng/mL), which were all directly added into the old medium. DCs were infected with MVA for 24 hours or with HCMV for 48 hours and used as targets in CD4+ T cell effector assays (IFN-γ ELISA, calcein-release assay).

3.3.8 Isolation and cultivation of primary NK cells

The inhibitory effect of HLA-C*0702 on NK cell lysis of HCMV-infected fibroblasts was investigated with NK cells expressing the HLA-C*0702-specific killer-cell immunoglobulin-like (KIR) receptor KIR2DL3. KIR2DL3-positive NK cells were enriched from PBMCs of donor AJU by immunomagnetic separation (section 3.4.1). KIR2DL3-enriched NK cells were expanded in bulk as a polyclonal NK cell line and in parallel under conditions of limiting dilution in order to obtain NK cell clones. In both cases, NK cells were cultivated in round-bottom 96-well plates (200 µL/well) in standard cell culture medium supplemented with 500 U/mL rhuIL-2 (Novartis) and restimulated every two weeks with a feeder mixture consisting of 2× 10^5/mL irradiated (50 Gy) allogeneic mLCLs and 1× 10^6/mL irradiated (50 Gy) allogeneic PBMCs from at least three different donors. NK cell lines were analyzed for expression of NK cell inhibitory receptors (KIRs and NKG2A) by flow cytometry and analyzed for cytotoxicity against target cells by calcein-release assay.

3.3.9 Cytotoxicity analysis

The cytotoxicity of CD4+ or CD8+ T cells and NK cells against target cells was measured by calcein-release assay. Target cells were mLCLs, CD40-stimulated B cells, HCMV-infected or not infected fibroblasts, HCMV-infected or not infected mo-DCs. The target cells (1–2× 10^6 per 50 mL Falcon tube) were labeled with 5 µg/mL calcein acetoxymethylester (AM) (Invitrogen) in 1 mL medium for 30 minutes at 37°C. For peptide-loading of fibroblasts and mo-DCs as positive controls, cells (in 1 mL medium) were incubated with peptide (5 µg/mL) for 1 hour at 37°C, adding calcein for the last 30 minutes. After washing three times with PBS, 5000 targets/well were coincubated with effector cells in V-bottom 96-well plates (200 µL/well) using effector/target-ra-

tios between 2/1 and 8/1. For each type of target, spontaneous release (no effector cells added, 0% lysis) and maximal release (0.5% of Triton-X100 added, 100% lysis) was determined. After 3.5 hours of incubation at 37°C and 5% CO_2, 150 µL/well of supernatant were transferred to a flat-bottom 96-well plate, and fluorescence intensity at 485/535 nm (excitation/emission) was measured in a Wallac Victor counter (Perkin-Elmer).

3.3.10 Regression assay

Cytotoxic effects of IE-1-specific CD4+ T cell clones were also analyzed by regression assay. Cocultivation of mLCLs with T cells may result in a regression of mLCL growth compared to the cultivation of mLCLs without T cells. This "outgrowth inhibition" can be due to direct cytolytic T cell activity or be mediated by released cytokines. To test whether the cytotoxic effects are dependent on the presence of the target antigen or not, outgrowth of ctrl- and IE-1-mLCLs was compared in the assay. Cocultivations of mLCLs and T cells were performed in round-bottom 96-well plates (200 µL/well, standard cell culture medium) with a constant number of 10 000 T cells/well. HLA-matched mLCLs were seeded at doubling dilutions ranging from 10 000 to 4.9 cells per well with 10 replicates per dilution. Cultures of diluted mLCLs without T cells were set up in parallel to determine unimpaired outgrowth. As additional control, T cells were incubated without mLCLs. The cells were fed weekly with fresh standard cell culture medium and monitored for mLCL outgrowth using an inverted microscope. After 4–6 weeks, wells with viable, proliferating mLCLs (mLCL "outgrowth") were detected by MTT assay.

3.3.11 MTT assay

Mammalian cell viability or proliferation was detected with a colorimetric assay using the tetrazolium salt MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Mosmann, 1983). In the mitochondria of living cells, yellow MTT is reduced by dehydrogenase enzymes to insoluble purple formazan. After solubilization of formazan into a colored solution, absorbance can be quantified in a spectrophotometer.

The MTT assay was used at the end of a regression assay to determine wells with cell "outgrowth". Cells were resuspended in the round-bottom 96-well plates used for the regression assay and 50 µL/well of the cell suspensions were transferred to flat-bottom 96-well plates. An MTT stock solution (5 mg/mL, thiazolyl blue, Sigma-Aldrich) was prepared in PBS, filtrated using a 0.22 µm filter and stored at 4°C. Prior to use, the MTT stock solution was diluted 5-fold in standard cell culture medium and 50 µL/well were added to the cell suspensions. The plates were incubated at 37°C for 2–4 hours to allow reduction of MTT to the formazan product. Then, 100 µL/well of 10% SDS (in PBS, Carl Roth) were added and the plates were incubated overnight at room temperature in order to dissolve the insoluble formazan into a purple colored

solution. The absorbance of the solution was quantified at 595 nm using a spectrophotometer. Wells were scored positive (= viable cells/outgrowth) when the absorption value was >0.2.

3.4 Immunological methods

3.4.1 Immunomagnetic separation of cell populations

Subpopulations of PBMCs like CD4+ T cells, KIR2DL3+ NK cells and peptide-specific T cells were isolated by magnetic separation using the MACS (*magnetic cell sorting*)-technology from Miltenyi Biotec (Bergisch-Gladbach). This method is based on paramagnetic particles ("MicroBeads", 50 nm), which are conjugated to highly specific antibodies directed against cell surface antigens. MicroBeads are non-toxic and biodegradable so that they do not have to be removed for any following experiment. Cell subpopulations coupled to MicroBeads were manually separated by using MACS columns placed on a magnetic separator. The magnetic field retains cells labeled with the paramagnetic beads in the column while allowing unlabeled cells to pass through. After removing the columns from the magnetic field, MicroBead-coupled cells were eluted and collected separately. The purity of the isolated cell populations was checked by flow cytometry. In all separation procedures, PBS + 2% FCS was used as washing buffer and for elution of cells from the columns.

CD4+ T cells were positively isolated from polyclonal IE-1-mLCL-stimulated T cell lines using CD4 MicroBeads according to the manufacturer's instructions. Approximately 1–2x 10^7 polyclonal T cells were used for CD4+ T cell isolation with MS columns. CD4+ T cell-enriched and CD4+ T cell-depleted fractions were collected separately and analyzed for CD4+ T cell content by flow cytometry. Isolated CD4+ T cells were immediately used for generation of T cell clones or in IFN-γ ELISpot assays.

Enrichment of KIR2DL3+ NK cells from freshly isolated PBMCs of donor AJU was performed by a two-step MACS separation procedure according to the manufacturer's instructions. First, CD3+ cells (T cells, NKT cells) were depleted from PBMCs (~1x 10^8) using CD3 MicroBeads and LD columns. Then, remaining PBMCs (~1x 10^7) were labeled with PE-conjugated anti-KIR2DL2/3 antibody (30 µL for 1x 10^7 cells; clone CH-L, BD Pharmingen) and PE MicroBeads. Labeled cells were separated using MS columns. After each separation step, resulting cell fractions were analyzed for expression of CD56, KIRs and NKG2A. Freshly isolated cells were taken into culture as described before (section 3.3.8).

CD8+ peptide-specific T cells were isolated after peptide stimulation and IFN-γ secretion assay directly from PBMCs of donors with initial high numbers of memory T cells specific for that particular peptide. Freshly isolated CD4+ T cell-depleted (CD4 MicroBeads) PBMCs were diluted to 2.5x 10^6/mL in standard cell culture medium supplemented with 50 U/mL rhuIL-2 and

stimulated with 0.5 µg/mL peptide for 12 hours at 37°C. Then, peptide-specific IFN-γ secreting cells were isolated using the IFN-γ secretion assay according to the manufacturer's instructions. IFN-γ secreting cells were magnetically labeled with a combination of an IFN-γ Catch Reagent, a PE-conjugated IFN-γ detection antibody, and anti-PE MicroBeads. Labeled cells were enriched with an MS column. The cells were analyzed for CD3, CD4 and CD8 expression and IFN-γ secretion by flow cytometry immediately before and after separation. The isolated cells were directly used for generation of T cell clones by limiting dilution. In this way, CRV-specific T cells clones were established from PBMCs of donor AJJ.

3.4.2 Phenotypic analysis of cells by flow cytometry

3.4.2.1 Staining of cell surface antigens

Cells were stained for expression of surface antigens with specific antibodies conjugated to FITC, PE, PE-Cy5 or APC. All centrifugation steps were carried out in a table centrifuge at 1000 x g, 4°C and 4 minutes. Pre-cooled (4°C) PBS + 2% FCS was used as washing buffer. The following cell numbers were stained in a 1.5 mL reaction tube (Eppendorf): PBMCs/3x 10^5, polyclonal T cells/1–3x 10^5, NK cells/1–3x 10^5, fibroblasts/5x 10^4. Before staining, cells were pelleted and remaining cell culture medium was washed away. The cells were resuspended in 30 µL PBS + 2% FCS containing the appropriate amounts of antibodies according to the manufacturer's recommendations and incubated for 20 minutes on ice. After staining, cells were washed and either resuspended in PBS + 2% FCS for direct analysis on the flow cytometer or fixed with 1% formaldehyde (Carl Roth) and stored at 4°C for up to one week.

Polyclonal T cell lines were analyzed for their CD4+ and CD8+ T cell content using CD3-PE-Cy5 (clone HIT3a, BioLegend), CD4-FITC (clone RPA-T4, BD Biosciences) and CD8α-APC (clone RPA-T8, BioLegend) antibodies.

For analysis of relative MHC class I expression levels on fibroblasts infected with wild-type HCMV or immunoevasion defective HCMV strains, HLA-ABC-PE (clone W6/32, BioLegend), HLA-A2-PE (clone BB7.2, BioLegend) and HLA-B7-PE (clone BB7.1, Millipore) antibodies and corresponding PE-labeled isotype controls (IgG2a, IgG2b and IgG1, all from BioLegend) were used. Specific mean fluorescence intensities (MFIs) were calculated by subtracting the MFIs of the isotype controls from the MFIs of the specific stainings. To calculate the relative MHC class I expression levels, specific MFI values obtained for CMV-wt or mutant CMV-infected cells (moi = 5) were standardized to the specific MFI of CMV-Δall (US2/3/6/11 deleted) infected cells (moi = 5) which was set to 100% MHC class I surface expression.

The following antibodies were used for characterization of polyclonal and clonal NK cells: CD56-PE-Cy5 (clone HCD56, BioLegend), KIR2DL1-FITC (clone HP-3E4, BD Pharmingen),

KIR2DL2/3-PE (clone CH-L, BD Pharmingen), KIR3DL1-FITC (clone DX9, BioLegend) and NKG2A-APC (clone 131411, Beckman Coulter).

3.4.2.2 Peptide/HLA class I multimer staining

Antigen-specific CD8+ T cell populations were quantified *ex vivo* using the MHC class I multimer technology. This method uses the natural ligand of the TCR, the MHC class I/peptide complex, to stain for epitope-specific T cells. As the association of TCR and MHC/peptide is characterized by a high dissociation rate, the avidity of this interaction is increased by multimerization of MHC/peptide complexes to tetramers, pentamers or even higher multimers.

For quantification of TPR-specific T cells, 5x 10^5 PBMCs were incubated with 1 µL HLA-B*0702/TPR Pentamer (Proimmune, Oxford, UK) in a total volume of 40 µL PBS + 2% FCS for 10 minutes at room temperature. After washing, cells were counterstained with Pentamer-binding Pro5 Fluorotag R-PE (Proimmune) and CD8α-APC (BioLegend) for 20 minutes on ice. Then, cells were washed and either resuspended in PBS + 2% FCS for direct analysis on the flow cytometer or fixed with 1% formaldehyde (Carl Roth) and stored at 4°C for up to one week.

CRV-specific CD8+ T cells were stained using an MHC-streptagII-StrepTactin multimer reagent (Streptamer) as first described in (Knabel et al., 2002) as a "reversible" T cell staining procedure. When using conventional MHC multimer reagents, the functional analysis of MHC multimer-stained cells is hampered by the persistence of TCR-MHC interactions and subsequently induced signaling events. Streptamers can be disassembled into MHC monomers by addition of Biotin. Since MHC monomers do not stably bind to TCRs, this results in a rapid loss of the staining reagent. CRV/HLA-C*0702 monomers were generated within a collaboration with the group of Michael Neuenhahn/Dirk Busch (Institute for Medical Microbiology, Immunology and Hygiene, Technische Universität München) and kindly provided for staining of CRV-specific CD8+ T cells. For multimerization of streptamer complexes, soluble CRV/HLA-C*0702-streptagII monomeric complexes were incubated with PE-labeled StrepTactin for 45 minutes at molar ratios of MHC molecule/StrepTactin molecule = 1/1. Then, 5x 10^5 PBMCs were incubated with an aliquot of the assembled streptamer reagent containing 2.5 µg CRV/HLA-C*0702 monomer in a total volume of 30 µL PBS + 2% FCS for 30 minutes on ice. Cells were counterstained by directly adding CD8α-APC (BioLegend) to the streptamer staining mixture and incubating for 20 minutes on ice. After washing, cells were either resuspended in PBS + 2% FCS for direct analysis on the flow cytometer or fixed with 1% formaldehyde (Carl Roth) and stored at 4°C for up to one week.

Cells were analyzed on a BD Biosciences FACSCalibur flow cytometer. Data analysis was performed using the FlowJo 9.4.11 software (Tree Star). Viable lymphocytes were gated according to their characteristic position in the two-dimensional forward/side scatter (FSC/SSC) dot plot.

The axes of the dot plot diagrams shown in this work are scaled logarithmic, comprising 10^0 to 10^4 arbitrary units of the mean fluorescence intensity (MFI).

3.4.3 HLA class I- and KIR-blocking studies with NK cells

To evaluate the role of HLA-C*0702 in NK cell-mediated killing of infected cells, the interaction of HLA class I molecules on target cells and KIR receptors on NK cells was blocked by specific antibodies. MHC class I molecules were masked at the surface of target cells using purified HLA-ABC- or HLA-A2-specific antibodies (W6/32 or BB7.2, respectively; BioLegend), which were added at 60 µg/mL to the target cells for 1 hour at 37°C prior to addition of effector cells. Similarly, to inhibit KIR2DL2/3 interaction with its MHC class I ligands, NK cells were preincubated with an antibody blocking KIR2DL2/3 (clone DX27, BioLegend) (Valiante et al., 1997) at 60 µg/mL for 1 hour at 37°C prior to addition of target cells. Purified Mouse IgG2a and IgG2b (BioLegend) were used as isotype controls.

3.4.4 ELISA for quantification of cytokine-release

The release of effector cytokines like interferon γ (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor (TNF)-α, IL-2, IL-4, or IL-10 is a marker for antigen- or peptide-specific activation of CD4+ and CD8+ T cells. In this work, polyclonal T cell lines and CD4+ or CD8+ T cell clones were stimulated for cytokine secretion by cocultivation with target cells that presented specific peptides on MHC class I or II molecules. Above mentioned cytokines were quantified by *Enzyme-Linked Immunosorbent Assay* (ELISA) using ELISA kits from the manufacturer Mabtech (Nacka Strand, Sweden). All ELISA assays performed in this work were "sandwich" ELISAs and based on alkaline phosphatase (ALP) for colorimetric detection of the cytokine concentrations.

Effector cells (1–4x 10^4/well, as indicated in the figure legends) were cocultivated overnight (16–18 hours) with target cells (1–5× 10^4/well, as indicated in the figure legends) in 200 µL/well of a V-bottom 96-well plate at 37°C and 5% CO_2. Then supernatants were harvested, and an ELISA was performed according to the manufacturer's guidelines (Mabtech, Nacka, Sweden). ELISA antibodies were diluted in sterile PBS without Mg^{2+} and added to the plates in 50 µL/well. Unless otherwise noted, all incubation steps were carried out at room temperature. For washing, plates were rinsed 4 times with PBS + 0.05% Tween. ELISA plates (Immunoplates MaxiSorp® 96-w flat-bottom) were coated with a monoclonal "capture" antibody (2 µg/mL) specific for the appropriate cytokine by overnight incubation at 4°C. The next day, unbound antibody was washed away and nonspecific binding sites on the plates were blocked by incubation with standard cell culture medium for 1 hour. Then, 50 µL of the cell supernatants were applied to

the plates and incubated for 2 hours to allow cytokine binding to the capture antibody. For quantification purposes, a cytokine standard was prepared and incubated in parallel on each plate. Then, plates were washed and incubated with a second cytokine-specific biotinylated antibody (1 µg/mL) for 1 hour. The antibody "sandwiches" were labeled for 1 hour with Streptavidin-ALP (diluted 1:1000), which binds to the second antibody via biotin. Immobilized cytokines were detected by applying 50 µL/well ALP substrate solution with *para*-nitrophenyl-phosphate (*p*-NPP). The ALP substrate stock solution contained 50% diethanolamine and 5 mg/mL *p*-NPP in sterile water (pH 9.5). Immediately before use, the stock solution was diluted 5-fold in sterile water and the ALP cofactor Mg^{2+} was added as $MgSO_4$ in a final concentration of 0.8 mM. ALP catalyzes the hydrolysis of colorless *p*-NPP to the yellow p-nitrophenol, which was detected with a spectrophotometer at 405 nm.

3.4.5 ELISpot for quantification of IFN-γ-secreting cells

Enzyme-Linked ImmunoSpot (ELISpot) assays were used to determine the frequency of peptide-specific T cells in freshly isolated PBMCs and polyclonal T cell lines by detection of IFN-γ-secretion at the single cell level. The assays were performed using the Human IFN-γ ELISpot Kit (ALP) from Mabtech (Nacka, Sweden) according to the manufacturer's protocol. ELISpot antibodies were diluted in sterile PBS without Mg^{2+} and added to the plates in 50 µL/well. Unless otherwise noted, washing was performed by rinsing the plates 5 times with PBS + 0.05% Tween. As a first step, an IFN-γ-specific monoclonal antibody (15 µg/mL) was immobilized on the membrane of 96-well MultiScreen HTC Filter Plates (Millipore) by overnight incubation at 4°C. The next day, plates were washed 5 times with PBS without Mg^{2+} and nonspecific binding sites were blocked with standard cell culture medium for 1 hour at room temperature. Then, the cells were added to the plates in 200 µL standard cell culture medium per well and incubated in presence of antigenic stimuli for 16–18 hours at 37°C to allow IFN-γ release and binding to the "capture" antibody. To detect peptide-specific T cells within PBMCs, 200 000 cells were distributed to each well and directly loaded with antigenic peptide. For characterization of polyclonal T cell lines, autologous CD40-stimulated B cells were loaded with antigenic peptides and coincubated at $5x\ 10^4$/well together with the T cells at 5000 or 10 000 cells/well (as indicated). TPA (12-O-tetradecanoylphorbol-13-acetate; 50 ng/mL) and ionomycin (0.5 µM), which both trigger strong T cell activation and IFN-γ secretion in an antigen-independent way, were added together to the PBMCs or T cells as positive control. The next day, cells were removed by washing and the plates were incubated with biotinylated anti-IFN-γ antibody (1 µg/mL) for 2 hours at room temperature. After another washing step, the antibody "sandwiches" were labeled for 1 hour at room temperature with Streptavidin-ALP (diluted 1:1000). Immobilized cytokines were detected using the AP Conjugate Substrate Kit (Bio-Rad) according to the manufacturer's instructions. The AP Conjugate Substrate Kit contains 5-bromo-4-chloro-3'-indolylphosphate

(BCIP) and nitro-blue tetrazolium chloride (NBT). BCIP is hydrolyzed by the ALP leading to the reduction of NBT and the formation of purple insoluble precipitates which appear as spots on the membrane. Spots were visually counted after scanning.

3.5 Virus-based methods

3.5.1 Epstein-Barr virus

Infectious virions carrying mini-EBV genomes were produced by transfection of mini-EBV DNA into the EBV-packaging cell line TR-2/293 which carries a non-packageable terminal repeat-deleted EBV genome that fully supports the packaging of terminal repeat-carrying EBV constructs (Delecluse et al., 1999). TR-2/293 cells were grown in 15-cm dishes to semiconfluency and transfected with a mixture of mini-EBV plasmid and two additional plasmids coding for individual EBV-derived factors. Per 15-cm dish, 10 µg mini-EBV DNA, 5 µg plasmid p509 (encoding the EBV lytic transactivator BZLF1) (Delecluse et al., 1999) and 5 µg plasmid p2670 (encoding the viral glycoprotein BALF4/gp110) (Neuhierl et al., 2002) were transfected using 120 µL PEI (described in 3.3.3). Virus-containing supernatant was harvested 3 days after transfection, freed from cellular debris by centrifugation and filtration (0.8 µm), and directly used for infection of PBMCs or stored at -80°C.

3.5.2 Modified vaccinia virus Ankara

Modified vaccinia virus Ankara (MVA) is a highly attenuated strain of vaccinia virus, which has lost its ability to replicate efficiently in primate cells. However, MVA provides endogenous expression of recombinant genes at very high levels even after infection of cells, which are non-permissive for productive virus replication.

MVA recombinants expressing pp65 or IE-1 or no HCMV antigen (Khan et al., 2005) were propagated on baby hamster kidney (BHK-21) cells. Semiconfluent BHK-21 cells were infected with MVAs at a multiplicity of infection (moi) of 0.1. Freshly thawed virus was vortexed briefly and directly added into the cell culture medium. The cells were harvested 2–3 days after infection when a widespread cytopathic effect was visible. To release virus from cells, stocks were subjected to three rounds of freeze-thaw/vortexing. Cell debris was removed by centrifugation and MVA stocks were aliquoted for storage at -80°C. Viral titers were determined by limiting dilution on BHK-21 cells seeded in flat-bottom 96-well plates. Wells displaying a cytopathic effect were visually scored one week after infection. The 50% infectious tissue culture dose ($TCID_{50}$) was calculated by the Spearman-Kaerber approximation. Typical titers reached up to 5x 10^8 $TCID_{50}$/mL. Multiplicity of infection (moi) was defined as $TCID_{50}$ units per cell. For antigen presentation assays, LCLs or fibroblasts were infected with MVAs at an moi of 10 for 24 hours.

3.5.3 Human cytomegalovirus

Stocks of HCMV AD169 and immunoevasin deletion mutants were generated and maintained by virus passage on primary human fibroblasts. HCMV replicates lytically in fibroblast cell lines leading to the release of progeny virus and pseudovirions into the cell culture supernatant.

For the preparation of high-titer HCMV stocks, semiconfluent MRC-5 cells were infected with virus at an moi of 0.1. After approximately 14–18 days, most cells displayed a cytopathic effect, and supernatants were harvested and cleared from cellular debris by 2 rounds of centrifugation for 10 minutes at 300 x g and room temperature. Virus stocks were aliquoted and stored at -80°C. Virus titers were determined by infection of subconfluent MRC-5 cells in flat-bottom 96-well plates at limiting dilution. Cytopathic effect was visually identified no earlier than two weeks after infection, when virus had spread over the whole well and the $TCID_{50}$ was calculated. Typical virus titers were between 1x 10^6 and 1x 10^7 $TCID_{50}$/mL. Again, moi was defined as $TCID_{50}$ units per cell.

For studying T cell recognition or NK cell-mediated lysis of HCMV-infected fibroblasts in effector assays, fibroblasts were seeded in cell culture flasks (80 cm^2, DMEM + 10% FCS + P/S/Na-Se, 15 mL/flask), grown to 70–80% confluency, and infected with HCMV at an moi of 5 by directly adding the virus containing supernatant into the cell culture medium. For some experiments, fibroblasts were pretreated with 300 U/mL rhuIFN-γ (PAN Biotech) for 72 hours prior to infection in order to enhance basal level of MHC expression. For use as T and NK cell targets in the effector assays, infected fibroblasts were harvested by trypsinization at 24–96 hours post infection.

HCMV immunoevasion of T cell recognition at the immediate-early (IE) phase of the viral replication cycle was analyzed by cultivating infected fibroblasts under conditions that allowed only expression of IE proteins according to a protocol from J. Hesse and B. Plachter (unpublished). MRC-5 fibroblasts were seeded in cell culture flasks (80 cm^2, DMEM + 10% FCS + P/S/Na-Se, 12 mL/flask) and grown to 70–80% confluency. 1 hour prior to infection, an inhibitor of protein translation, cycloheximide (CHX, 250 µg/mL), was added to the cell culture medium in order to accumulate IE-expressed mRNAs. Next, fibroblasts were infected with HCMV at an moi of 5 for 1.5 hours in a total of 5 mL/flask of DMEM + 10% FCS + P/S/Na-Se supplemented with CHX (250 µg/mL). After 1.5 hours, medium was filled up to 12 mL again, thereby adjusting CHX concentration to retain 250 µg/mL. 8 hours post infection, CHX was removed by washing 2 times with PBS and translation of mRNA was allowed for 13.5 hours. During this time, expression of early and late proteins was blocked by addition of the transcriptional inhibitor actinomycin D (ActD, 10 µg/mL). Then, the infected fibroblasts were harvested by trypsinization, fixed with formaldehyde to prevent any further antigen processing, and subjected to T cell recognition in IFN-γ ELISA assays using 20 000 target cells and 40 000 T cells.

4 Results

The observation that reconstitution of HCMV-specific T cell immunity and adoptive T cell transfer in immunocompromised patients correlate with protection from HCMV disease revealed that antiviral T cell responses are the predominant mechanism of HCMV control (Jackson et al., 2011). In healthy virus carriers, HCMV-specific T cell responses comprise approximately 10% of the CD4+ and CD8+ T cell memory compartments and are focused on an immunodominant subset of HCMV antigens, including the IE-1 protein (Khan et al., 2005; Sylwester et al., 2005). As a transcription activator, IE-1 is expressed among the first proteins in infected cells and functiones in driving the lytic replication cycle of HCMV by inducing viral and cellular genes. More importantly, IE-1 expression initiates virus reactivation from latency (Sinclair and Sissons, 2006). Consequently, IE-1-specific T cells and not T cells directed against late expressed structural proteins might be particularly efficient in controling HCMV reactivation and virus spread. For this reason, the IE-1 protein has to be considered as a preferable target for HCMV-specific immunotherapy including adoptive T cell transfer and vaccination. A basic prerequisite for the design of efficient immunotherapeutic strategies is the detailed knowledge about the structure of the IE-1-specific T cell repertoire. In the present work, both arms of the IE-1-specific T cell response, CD4+ and CD8+ T cells, were characterized in detail. The main challenges were: definition of IE-1 T cell epitopes, identification of immunodominant T cell specificities and evaluation of T cell effector functions in context of an HCMV infection.

4.1 Generation and analysis of IE-1-specific polyclonal T cell lines

HCMV-specific memory T cells can be reactivated and expanded *in vitro* from peripheral blood mononuclear cells (PBMCs) of HCMV carriers by antigen-specific stimulation using the well-established mini-lymphoblastoid cell line (mini-(m)LCL)-system. Mini-LCLs are permanent growing B cell lines carrying a mini-EBV vector that mediates growth transformation of B cells but does not support lytic EBV replication. Furthermore, mLCLs show a high expression of MHC class I and II molecules, as well as of costimulatory molecules (B7.1, B7.2, ICAM-1 and LFA-3) (Kilger et al., 1999; Moosmann et al., 2002). As mLCLs constitutively express EBV latent antigens (e.g. EBNA2, EBNA3a, EBNA3c, LMP1, LMP2a) (Kempkes et al., 1995), which elicit good T cell responses *in vivo*, they can be used to efficiently reactivate and expand EBV-specific T cells from PBMCs of EBV-seropositive donors (Wallace et al., 1982). In the same way, mLCLs expressing a foreign antigen can be used to reactivate CD8+ and CD4+ foreign antigen-specific T cells, as previously reported for pp65 (Moosmann et al., 2002). In this work, mini-LCLs expressing IE-1 were used to reactivate and enrich IE-1-specific T cells from PBMCs of HCMV-seropositive donors, thereby giving rise to polyclonal T cell lines. By using the autologous IE-1-mLCL for the stimulation procedure, all IE-1-specific memory T cells that recognize peptides

presented by the combination of HLA allotypes present in one donor might theoretically be reactivated. In addition, autologous stimulation avoids the expansion of alloreactive T cell species.

4.1.1 Generation of mini-lymphoblastoid cell lines

Mini-LCLs were generated from different blood donors by coincubation of isolated PBMCs with mini-EBV-containing supernatant. After establishment of stably growing cell lines approximately 8 weeks after infection, each mLCL was checked by PCR for the presence of a mini-EBV-specific sequence (chloramphenicol acetyltransferase, cam) (Fig. 4.1). In EBV-seropositive donors approximately one out of 100 000 B cells is latently infected with endogenous EBV (Miyashita et al., 1995). To verify that an established cell line is a pure culture of mini-EBV-transformed cells and not contaminated with cells transformed by endogenous wild-type EBV, each cell line was also PCR-tested for a wild-type EBV-specific sequence (viral glycoprotein gp85). All mLCLs analyzed were cam-positive. Only 1% of mLCLs tested contained wild-type EBV DNA.

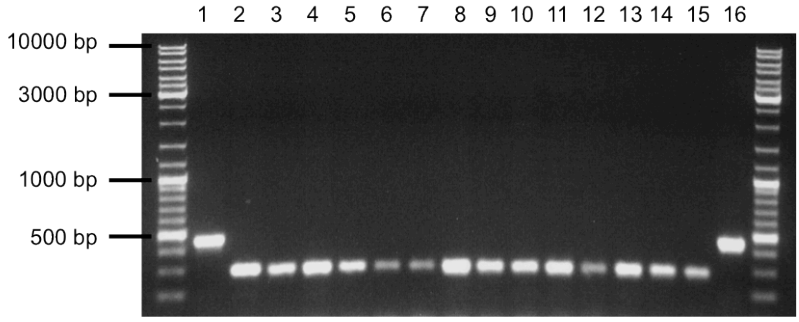

Fig. 4.1: PCR analysis of mini-EBV-infected B cell lines. Independent mLCLs (lines 2–15) were tested for the presence of a mini-EBV-specific sequence (cam) and the absence of a wild-type EBV-specific sequence (gp85) by PCR. B95.8-transformed B cells (LCLs) were used as a positive control for endogenous wild-type EBV transformation (lines 1, 16); cam: product size = 294 bp; gp85: product size = 461 bp.

Expression of foreign antigens including pp65 and IE-1 from mLCLs was verified in previous studies using intracellular staining and Western blot analysis (Moosmann et al., 2002; Wiesner, 2005). Foreign antigen expression remained stable over the whole cultivation period, which was sometimes more than 6 months (Moosmann et al., 2002). In this work, IE-1 and pp65 expression by mLCLs was tested exemplarily by using well-characterized antigen-specific CD8+ T cell clones. Upon specific stimulation with an autologous or HLA-matched mLCL, a CD8+ T cell clone produces IFN-γ, which can then be detected by ELISA. IE-1 and pp65 expression by HLA-A*0201-positive mLCLs was verified using autologous T cell clones specific for HLA-A*0201-presented peptides, the VLE peptide in IE-1 (Khan et al., 2002) and the NLV peptide in pp65 (Wills et al., 1996; Diamond et al., 1997) (Fig. 4.2). All mLCLs tested expressed the respective HCMV antigen encoded by the mini-EBV vector used for B cell transformation. Mini-

LCLs which have shown to be mini-EBV-positive and wild-type EBV-free and expressed HCMV antigens were used for the generation of polyclonal T cell lines.

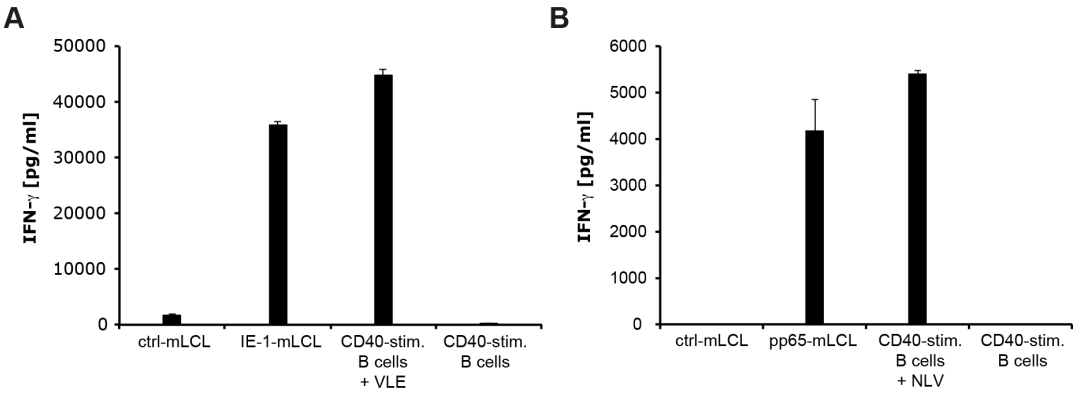

Fig. 4.2: Verification of IE-1 and pp65 expression by mLCLs using antigen-specific CD8+ T cell clones in an IFN-γ ELISA assay. A: IE-1-specific T cell clone ALTM#106 VLE. **B:** pp65-specific T cell clone ALTP#53 NLV. For each T cell clone, 20 000 T cells per well were incubated with 50 000 autologous target cells per well for 16–18 hours. Antigen-specific IFN-γ secretion was measured by ELISA. CD40-stimulated B cells pulsed with the target peptides of the T cell clones were used as positive controls. Ctrl-mLCLs expressing no foreign antigen and CD40-stimulated B cells without peptide were used as negative controls. Means and standard deviations of duplicates are shown.

4.1.2 Generation of polyclonal T cell lines by mLCL-stimulation

Twelve HCMV-seropositive donors covering a broad range of HLA types (Table 2.1) were chosen. IE-1-specific polyclonal T cell lines were established from these donors by stimulation of PBMCs with autologous IE-1-expressing mLCLs according to a previously described protocol (Moosmann et al., 2002). In addition, ctrl-mLCL-stimulated polyclonal T cell lines were generated from each donor as negative controls. Polyclonal T cells were cultivated over a period of 43–54 days by repeated stimulation with irradiated mLCLs on d0, d10 and weekly thereafter. Only the ctrl-mLCL-stimulated T cell line of donor F65 could not be maintained, due to a contamination of the mLCL. To follow the progress of relative T cell numbers with each round of stimulation, the T cell numbers counted during the restimulation procedure were set in relation to the number of initially used PBMCs (Fig. 4.3). During the first 3 rounds of stimulation, the relative T cell numbers of most T cell lines, ctrl- and IE-1-mLCL-stimulated, declined or did not change much. This might be explained by the fact that PBMCs also contain other cells than T cells, for example B cells, NK cells and monocytes, which die during the first weeks of cultivation due to a lack of specific stimulation. For the same reason, T cells, which are not stimulated by mLCL-presented peptides, are eliminated from the cultures. By contrast, T cells specific for EBV latent antigen- or HCMV IE-1-derived peptides presented by the mLCLs should be able to proliferate in response to antigen-specific stimulation. However, as these T cells comprise only few percent of total PBMCs it takes around 3 rounds of restimulation (17 days) until proliferation of antigen-specific T cells results in increase of relative T cell numbers. As most non-specific T

cells should be dead at that time due to lack of a stimulation signal, low-dose IL-2 can be added to the cultures to enhance T cell proliferation in an antigen-independent way. After the third restimulation, the relative T cell numbers of most polyclonal T cell lines increased constantly, indicating that there were EBV- or IE-1-specific T cells in the PBMCs of the donors.

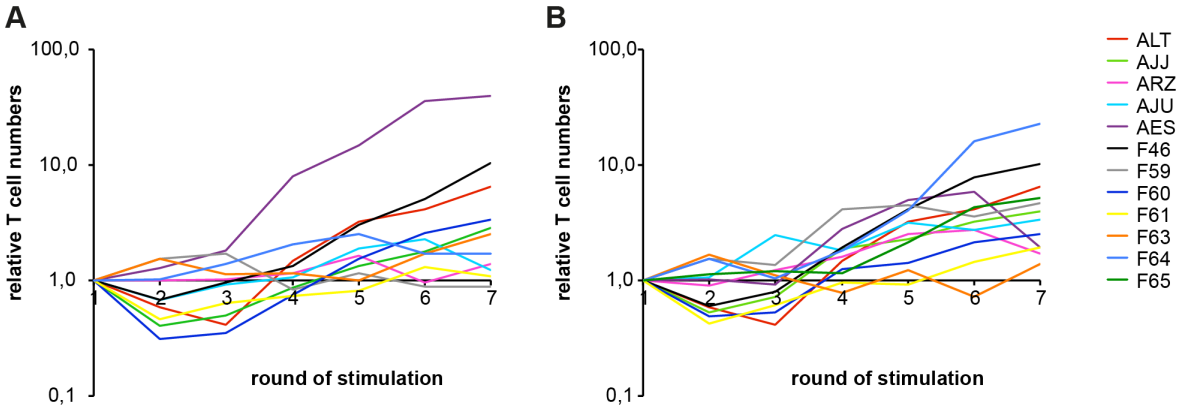

Fig. 4.3: Proliferation curves of polyclonal T cell lines stimulated with ctrl-mLCL (A) or IE-1-mLCL (B). Polyclonal T cell lines were established from PBMCs of 12 HCMV-seropositive donors by repeated stimulation with irradiated mLCLs on d0 (1st stimulation), d10 (2nd stimulation) and weekly thereafter (7 stimulations in total). T cell numbers were counted during each restimulation procedure and were set in relation to the number of initially used PBMCs. The ctrl-mLCL-stimulated T cell line of donor F65 could not be maintained and is therefore not shown in A.

The mLCL-procedure is reported to work nicely for the expansion of pp65-specific T cells (Moosmann et al., 2002; Wiesner et al., 2005). Therefore, a pp65-mLCL-stimulated T cell line was established in parallel from one donor, ALT, and used for comparative analysis (Fig. 4.4). Ctrl-, IE-1- and pp65-stimulated T cell cultures of donor ALT show similar proliferative behavior with an initial decline in relative T cell numbers followed by a constant increase. However, compared to IE-1- and pp65-stimulated cell lines, proliferation of the ctrl-mLCL-stimulated culture is slower which is probably due to the lack of stimulation of HCMV-specific T cells.

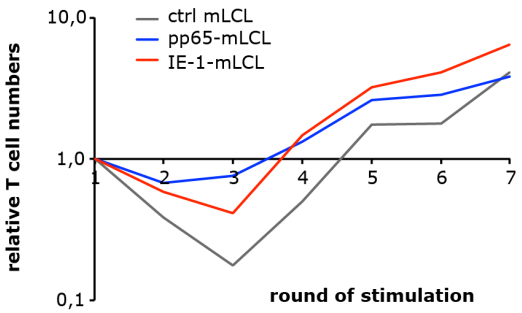

Fig. 4.4: Proliferation curves of polyclonal T cell lines of donor ALT. PBMCs of donor ALT were stimulated in parallel with irradiated ctrl-, IE-1- or pp65-mLCLs on d0 (1st stimulation), d10 (2nd stimulation) and weekly thereafter (7 stimulations in total). T cell numbers were counted during each restimulation procedure and were set in relation to the number of initially used PBMCs.

4.1.3 Characterization of polyclonal T cell lines

The polyclonal T cell lines were analyzed after 6–7 rounds of restimulation (43–54 days) for expression of the cell surface molecules CD8 and CD4 by flow cytometry (Fig. 4.5). With exception of the T cell lines from donor AES, all T cell cultures were dominated by CD8+ T cells (average 90.0%; average CD4+ T cells 6.1%; AES: 30.7% CD8+, 64.3% CD4+). AES was the only donor known to be EBV-seronegative; it is not clear whether this is causally connected to the predominance of CD4+ T cells in this donor's cell line (EBV-seropositive: ALT, AJJ, ARZ, AJU, F46, F60, F61, F64; EBV serostatus not known: F59, F63, F65). When comparing ctrl- and IE-1-mLCL-stimulated cell lines from the same donor, the CD4+ T cell fraction tended to be higher in the ctrl-mLCL-stimulated cell lines (average ctrl cell lines: 9.3 %; average IE-1-stimulated cell lines: 3.0%). One possible explanation could be that the relatively high number of IE-1-specific CD8+ T cells in PBMCs led to their dominance in the stimulated T cell lines; in contrast, there are few IE-1-specific CD4+ T cells in the peripheral blood of the donors.

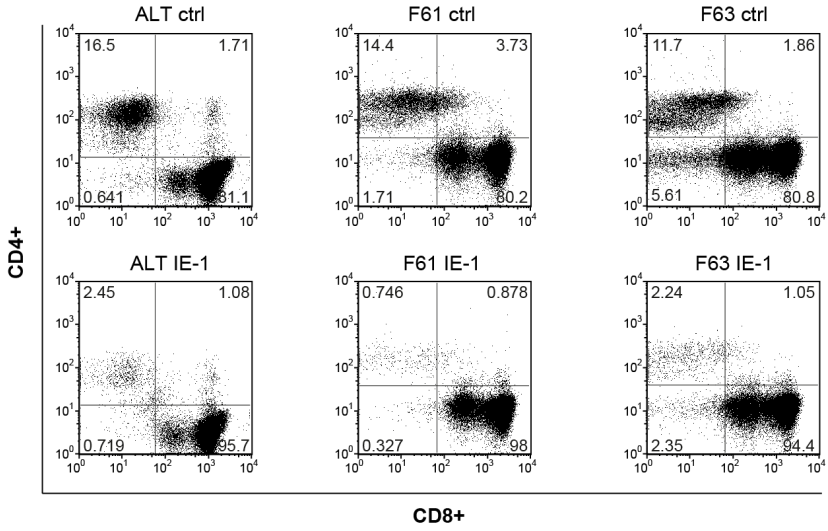

Fig. 4.5: Expression of the cell surface molecules CD8 and CD4 by the polyclonal T cell lines. IE-1-mLCL- and ctrl-mLCL-stimulated polyclonal T cell lines of 12 donors were analyzed for CD8 and CD4 surface expression by flow cytometry. FACS dot plots are shown for the ctrl-mLCL-stimulated (upper panel) and the IE-1-mLCL-stimulated (lower panel) polyclonal T cell lines of donors ALT (d43), F61 (d47) and F63 (d46).

To assess the enrichment of IE-1-specific T cells in the IE-1-mLCL-stimulated cultures, the reactivity of ctrl- and IE-1-mLCL-stimulated T cell lines (d46–54) was compared in T cell effector assays (Fig. 4.6). IFN-γ secretion upon specific stimulation is a characteristic effector function of CD8+ T cells, which largely dominated the polyclonal T cell lines. Moreover, IFN-γ secretion is also characteristic for virus-specific CD4+ T cells of the Th1 type, which have been described to dominate the CD4+ T cell response to HCMV (Rentenaar et al., 2000) and EBV (Ning et al., 2011). Antigen-specific IFN-γ secretion was measured by ELISA assay. Autologous ctrl- and IE-

1-mLCLs, as well as autologous EBV-free CD40-stimulated B cells were used as T cell targets. All ctrl-mLCL-stimulated T cell lines recognized both types of mLCL to a similar level, suggesting the enrichment of T cells specific for EBV latent antigens expressed by the mLCLs. By contrast, the IE-1-mLCL-stimulated T cell cultures from 8 donors showed a much stronger reactivity to autologous IE-1-expressing mLCLs than to ctrl-mLCLs, indicating that IE-1-specific T cells had been specifically enriched by mLCL-stimulation as intended. IE-1-mLCL-stimulated T cell cultures of 3 donors did not show a preferential recognition of the IE-1-mLCL, suggesting that low numbers of IE-1-specific T cells were present in the peripheral blood of these donors. In addition, both polyclonal T cell cultures of donor AES recognized autologous CD40-stimulated B cells, suggesting that broadly reactive or self-specific T cells were reactivated and expanded in these cultures.

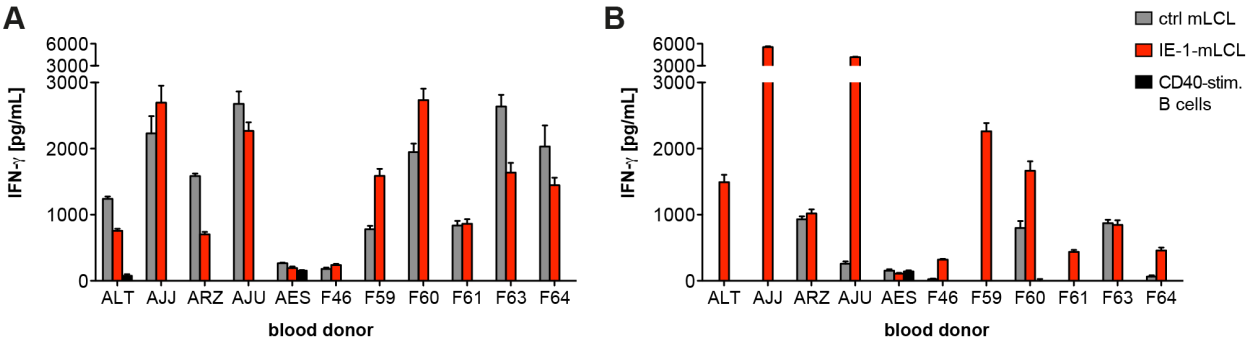

Fig. 4.6: Target specificity of ctrl-mLCL-stimulated (A) and IE-1-mLCL-stimulated (B) polyclonal T cell lines. The reactivity of ctrl-mLCL- and IE-1-mLCL-stimulated T cell lines (d46–54) in response to autologous ctrl-mLCL, IE-1-mLCL and CD40-stimulated B cells was compared in an IFN-γ ELISA assay. 10 000 T cells per well were incubated overnight with 20 000 target cells per well. Means and standard deviations of 3–4 replicates are shown.

For donor ALT, the IE-1-, pp65- and ctrl-mLCL-stimulated T cell lines were comparatively analyzed in the IFN-γ ELISA assay (Fig. 4.7 A) for reactivity against autologous ctrl-, pp65- and IE-1-mLCL as well as autologous CD40-stimulated B cells. Again, the cell line expanded with the ctrl-mLCL recognized all three types of mLCL to a similar extent, indicating recognition of EBV latent antigens expressed by the mLCLs. By contrast, IE-1- and pp65-expanded polyclonal T cell lines displayed dominant specificity for the respective HCMV antigen, suggesting the presence of pp65- as well as IE-1-specific T cells in the PBMCs of donor ALT. CD40-stimulated B cells were not recognized by any of the three T cell lines. In addition, direct cytotoxic activity of the three polyclonal T cell lines against the same target cells was assessed in a calcein-release assay (Fig. 4.7 B). Direct cytotoxicity is the most important effector function of CD8+ T cells and has also been reported for CD4+ T cells directed against EBV (Morales et al., 2012) and HCMV antigens (Elkington et al., 2004). Cytotoxicity and IFN-γ secretion were equally antigen-specific and closely correlated with each other, demonstrating that mLCL-stimulation produced T cell lines with an intact repertoire of effector functions.

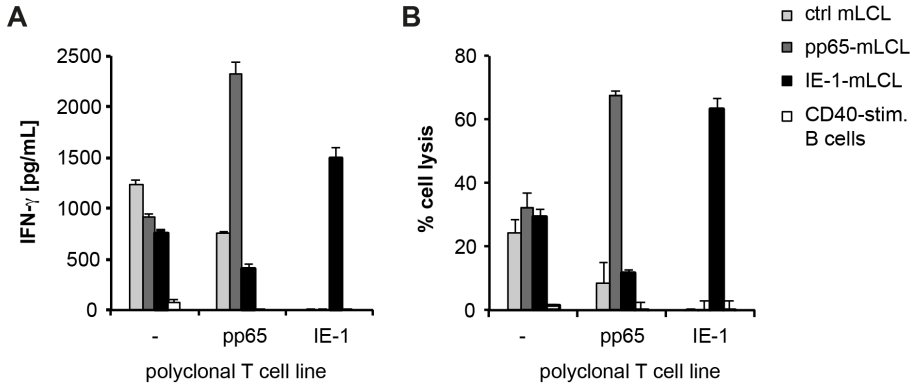

Fig. 4.7: Specific reactivity of ctrl-, pp65- and IE-1-mLCL-stimulated T cell cultures of donor ALT in IFN-γ ELISA (A) and cytotoxicity assay (B). For the ELISA, 10 000 T cells were incubated with 20 000 target cells per well. To measure cytotoxicity in a calcein-release assay, 40 000 T cells were added to 5 000 target cells per well. Means and standard deviations of 4 replicates are shown.

For donor ALT, the expansion of epitope-specific T cells in the IE-1-, pp65- and ctrl-mLCL-stimulated T cell lines was analyzed on day 43 using HLA class I/peptide multimeric complexes (Fig. 4.8). The multimers were chosen according to the HLA type of donor ALT and the bound peptides are listed in Table 4.1. T cells directed against EBV lytic antigens were also stained, to confirm that EBV lytic-cycle antigens were not expressed and presented by mLCLs. The precursor frequencies of epitope-specific T cells in PBMCs of donor ALT are also given in Table 4.1. The T cell line stimulated with the ctrl-mLCL showed a clear expansion of T cells specific for EBV latent antigens LMP2 and EBNA3A. The pp65-mLCL-stimulated cell line was strongly dominated by pp65 NLV-specific T cells. The IE-1-mLCL-stimulated T cell line showed only a slight enrichment of IE-1 VLE-specific T cells, suggesting that other IE-1-specific T cells with unknown specificity dominated the culture and led to the strong recognition of IE-1 in the ELISA and cell lysis assays. Interestingly, in both HCMV antigen-stimulated cell lines, coexpansion of EBV-specific T cells was fully suppressed. At least in the case of the pp65-mLCL, this was not a reflection of the differences in numbers of specific T cells in PBMCs, because pp65 NLV-specific T cells and EBV CLG-specific T cells were present in similar numbers in PBMCs, but the former were strongly dominant over the latter in stimulated T cell lines. Analysis of T cells stimulated with IE-1-mLCL also suggested a preferential expansion of T cells specific for the HCMV antigen. A low synthesis rate of certain EBV latent proteins, compared to pp65 and IE-1, could have resulted in a lower cell surface presentation of EBV antigens (Hill et al., 1995) and, consequently, reduced stimulation of EBV-specific T cells.

Table 4.1: Antigenic peptides used for staining of epitope-specific T cells in the polyclonal T cell lines of donor ALT and frequencies of peptide-specific T cells in the PBMCs of donor ALT. T cell frequencies were determined by staining with specific HLA/peptide multimers and are indicated as % lymphocytes.

peptide	antigen	HLA restriction	% of lymphocytes
NLV	HCMV pp65	A*0201	0.05
TPR	HCMV pp65	B*0702	0.02
VLE	HCMV IE-1	A*0201	0.25
CLG	EBV LMP2	A*0201	0.05
RPP	EBV EBNA3A	B*0702	0.01
GLC	EBV BMLF1	A*0201	0.01
FLY	EBV LMP2	A*0201	0.00 (3/75 000)
IED	EBV LMP2	B*4002	0.00 (1/91 300)
YVL	EBV BRLF1	A*0201	0.00 (3/95 000)

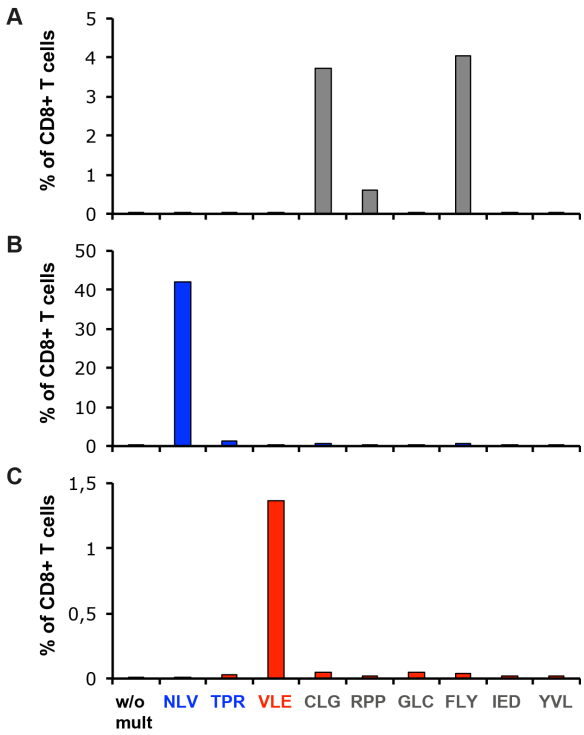

Fig. 4.8: Staining of epitope-specific T cells in the polyclonal T cell lines of donor ALT with HLA/peptide multimeric complexes. A: ctrl-mLCL-stimulated T cell line; **B:** pp65-mLCL-stimulated T cell line; **C:** IE-1-mLCL-stimulated T cell line. EBV- (grey), pp65- (blue) and IE-1- (red) peptide multimers were matched to the HLA type of donor ALT. HLA multimers are identified by the first three amino acids of the incorporated antigenic peptide, in one-letter amino acid code. The polyclonal T cell lines were stained on day 43. Stainings without multimer and stainings for T cells specific for EBV lytic antigens served as negative controls.

4.2 IE-1-specific CD4+ T cell repertoire

Much emphasis has been placed on the importance of cytotoxic CD8+ T cells in the recognition of HCMV-infected cells (section 4.3). However, there is evidence that CD4+ T cells also play a crucial role in the control of HCMV infection. Adoptive T cell transfer studies in immunosuppressed patients have shown that the development of an HCMV-specific CD4+ helper T cell response is essential for maintaining a long-term virus-specific CD8+ T cell immunity (Walter et al., 1995). Several HCMV antigens have been described as CD4+ T cell targets, among them the IE-1 protein (Sylwester et al., 2005). In fact, IE-1-specific T cells were among the first HCMV-specific CD4+ T cells to be characterized in detail (Alp et al., 1991; Davignon et al.,

1995), but nontheless there is little information on the structure of the CD4+ T cell repertoire against IE-1 available to date. For this reason, one aim of the present work was to characterize IE-1-specific CD4+ T cells in terms of frequency, target peptide specificity and effector functions. As previously reported for pp65, mLCLs expressing a foreign antigen can be used to reactivate CD4+ antigen-specific T cells (Moosmann et al., 2002). Therefore, the IE-1-mLCL-stimulated polyclonal T cell lines were considered to be an appropriate source of IE-1-specific CD4+ T cells.

4.2.1 Analysis of the CD4+ fraction of the polyclonal T cell lines

As shown before, the IE-1-specific polyclonal T cell lines were largely dominated by CD8+ T cells which makes it difficult to detect specific effects of CD4+ T cells within these cultures. Therefore, the CD4+ T cell fraction was positively isolated from the IE-1-stimulated T cell lines of eight different donors (ALT, ARZ, AJU, F46, F61, F63, F64, F65) by using CD4 MicroBeads. The two T cell populations, depleted and enriched for CD4+ T cells, obtained by immunomagnetic separation were analyzed for CD4 and CD8 surface expression by flow cytometry. FACS plots for two representative T cell lines (from donors AJU and F61) are shown in Fig. 4.9. The fraction enriched for CD4+ T cells had an average CD4+ T cell content of 96.6% and a reduction of CD8+ T cells to 1.4%.

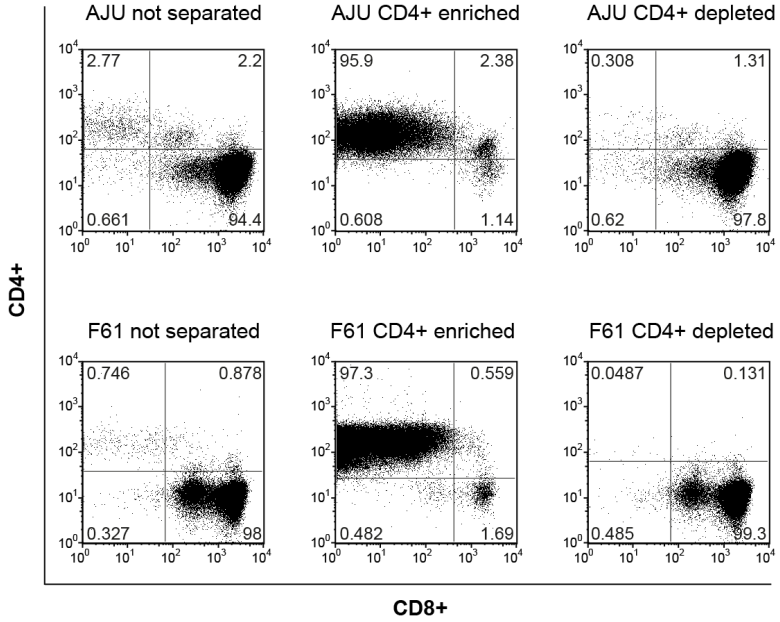

Fig. 4.9: IE-1-mLCL-stimulated polyclonal T cell lines before and after immunomagnetic separation. CD4+ T cells were positively isolated from the polyclonal T cell lines using anti-CD4 immunomagnetic beads (CD4 MicroBeads). The polyclonal T cell lines (not separated) as well as the resulting cell fractions from separation, CD4+ enriched and CD4+ depleted, were stained for CD4 and CD8 cell surface expression and analyzed by flow cytometry. Data are shown for the polyclonal T cell lines of two representative donors, AJU (d54) and F61 (d47).

The isolated CD4+ T cell fractions from the IE-1-stimulated T cell lines were screened for the presence of IE-1 epitope-specific CD4+ T cells in IFN-γ ELISpot assays by using an overlapping 15-meric peptide library covering IE-1 (Fig. 4.10). In previous studies on the pp65 CD4+ T cell response, 15-meric peptide libraries have shown to be suitable tools for the stimulation of CD4+ T cell responses, which were approximately equal to those elicited with soluble protein antigen (Maecker et al., 2001). The peptide library used in this work contained 120 peptides spanning the entire IE-1 sequence of HCMV strain AD169, with subsequent peptides overlapping 11 amino acids (Table 2.2). Nine additional 15-meric peptides containing sequence variants present in the HCMV strains Toledo and TB40E were included in the study (Table 2.2, peptides #121-129). For convenience, the 129 peptides were subdivided into 23 subpools in a cross-matrix fashion, such that every single peptide was contained in one "vertical" subpool (#1–12) and one "horizontal" subpool (#13–23) (Fig. 2.1).

In the ELISpot assays, autologous CD40-stimulated B cells were used as antigen-presenting cells (APCs). ELISpot results are shown in Fig. 4.10. The CD4+ T cell cultures of all eight donors contained IE-1 peptide-specific T cells. The T cells of donors AJU, F63, F64 and F65 recognized a very broad panel of IE-1 peptide subpools indicating the presence of T cells directed against various IE-1 peptides. However, the complex subpool recognition patterns prevented direct identification of individual T cell epitopes from the peptide cross-matrix. By contrast, the CD4+ T cells of donors ALT, ARZ, F46 and F61 recognized only a limited number of peptides. CD4+ T cells of donors ARZ and F46 were predominantly stimulated by subpools #5, 6 and 17, which only overlap in the individual IE-1 peptides #53 and 54. For donors ALT and F61, the subpool recognition pattern of the CD4+ T cells largely overlapped with the recognition pattern of the CD8+ T cells (Fig. 4.23), indicating that some signals might be caused by remaining CD8+ T cells within the positively isolated CD4+ T cells (Fig. 4.9). After subtraction of potential CD8+ T cell signals, peptides #53 and 54 were identified as CD4+ T cell targets also for donor ALT. For donor F61, epitope mapping was not possible from this assay.

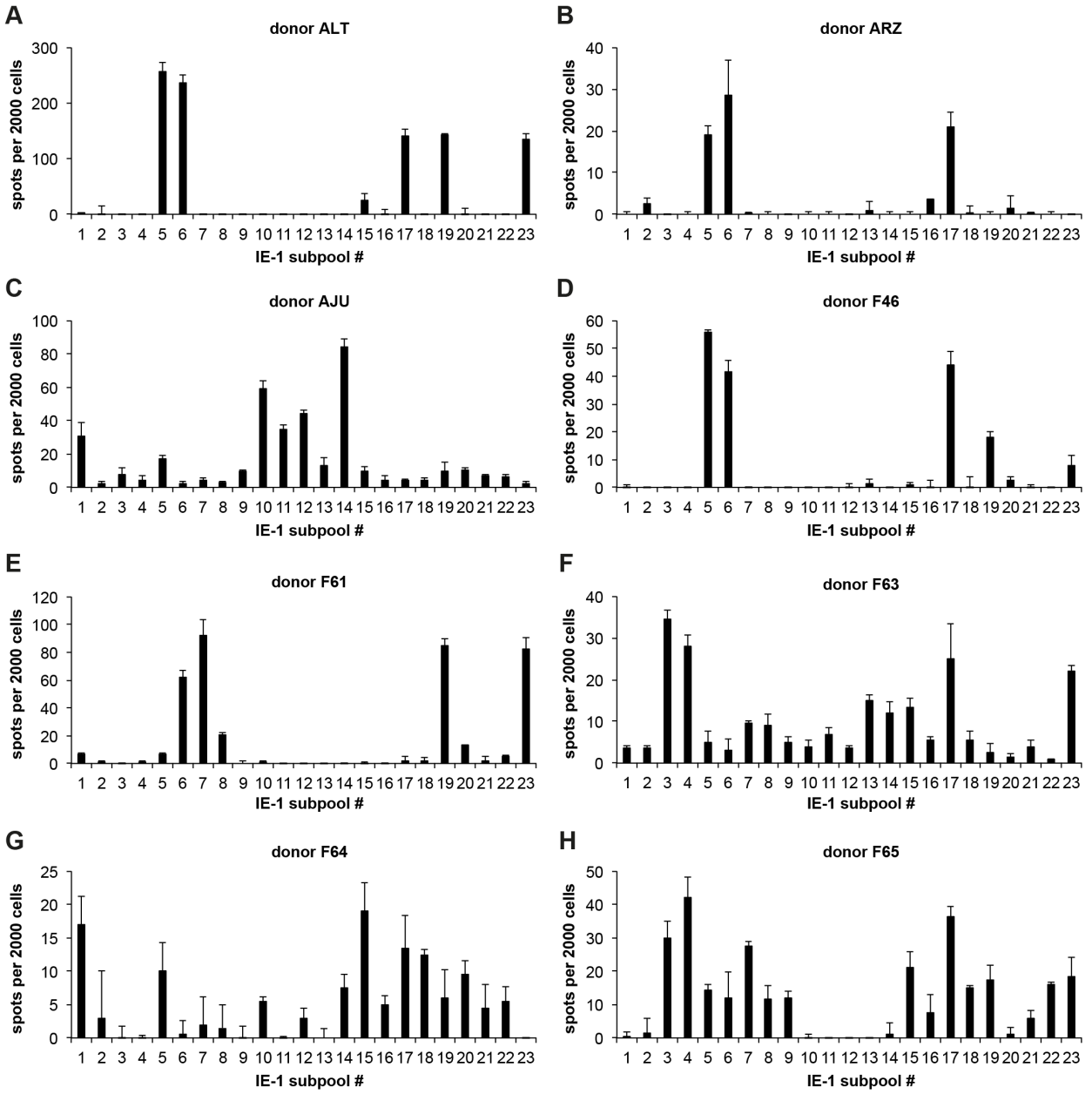

Fig. 4.10: Frequency and epitope specificity of IE-1-specific CD4+ T cells. The CD4+ T cell fraction of the IE-1-stimulated polyclonal T cell lines of eight different donors (A–H) was analyzed for IE-1 reactivity in IFN-γ ELISpot assays. IE-1-epitope-specific T cells were detected using a 15-meric peptide library consisting of peptides covering the IE-1 sequence from AD169 and sequence variants from strains Toledo and TB40E. Peptides were pooled in a cross-matrix orientation ("vertical" subpools: #1–12; "horizontal" subpool: #13–23) and loaded on CD40-stimulated B cells (50 000/well). Means and standard deviations of duplicates are shown.

4.2.2 Generation of T cell clones and screening for IE-1 recognition

As demonstrated above, mLCL-stimulated T cell lines contained IE-1-specific CD4+ T cells. In the following, more detailed information was obtained about the IE-1-specific CD4+ T cell repertoires present in the blood donors by analyzing T cell clones generated from the IE-1-stimulated polyclonal T cell lines. A T cell clone arises from a single T cell and therefore expresses only

one TCR with one specificity. Due to this attribute, T cell clones are precise tools to identify target peptides of IE-1-specific CD4+ T cells as well as the HLA class II molecules that present them to the T cells. T cell clones were established from CD4+-enriched polyclonal T cell lines by limiting dilution and were tested for their reactivity against autologous ctrl- and IE-1-mLCLs as well as autologous CD40-stimulated B cells. CD4+ helper T cell responses are classically divided into T helper 1 (Th1) and T helper 2 (Th2) responses. Th1 cells produce effector cytokines such as IFN-γ and TNF-α, whereas Th2 cells secrete IL-4, IL-5, IL-10, and IL-13. It is reported that most HCMV-specific memory CD4+ T cells secrete the Th1 cytokine IFN-γ, whereas the Th2 cytokine IL-4 is secreted by only very few HCMV-specific CD4+ T cells (Rentenaar et al., 2000). Therefore, reactivity of the generated T cell clones against mLCLs and CD40-stimulated B cells was determined by measuring antigen-specific IFN-γ secretion by ELISA. For some donors, IL-4 or IL-10 ELISAs were performed in parallel to the IFN-γ ELISA with the same cell supernatants. However, clones producing only IL-4 or IL-10 but no IFN-γ were not identified. An extract of a T cell clone screening is shown in Fig. 4.11. The number of T cell clones screened per donor was dependent on the number of available T cell clones and was at least 100. Between 3 and 33.5% of the clones exhibited clear IE-1 specificity. As the CD4+ fraction of the T cell lines contained residual CD8+ T cells, identified IE-1-specific T cell clones were further screened for CD4 and CD8 surface expression by flow cytometry (data not shown) in order to exclude CD8+ T cell clones from the following experiments.

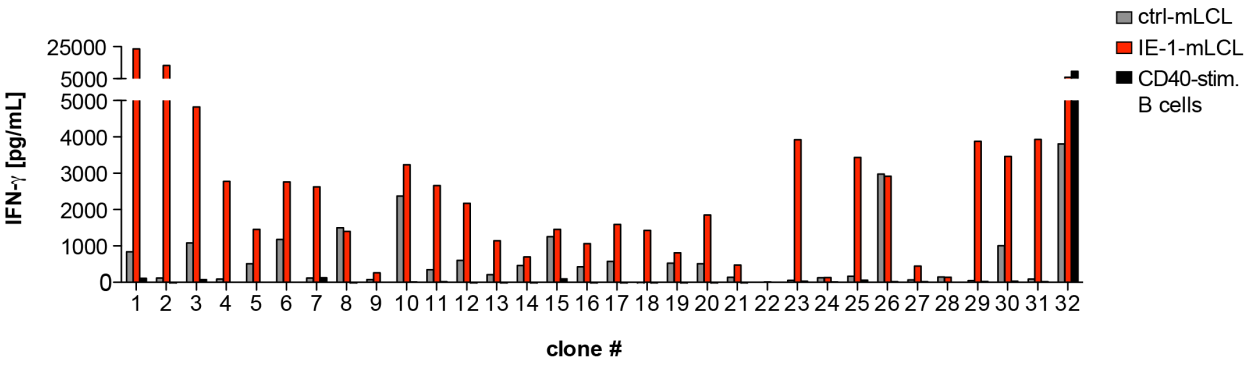

Fig. 4.11: Screening of CD4+ T cell clones for IE-1 reactivity. T cell clones of donor F65 were tested for their reactivity towards autologous ctrl-mLCL, IE-1-mLCL, and CD40-stimulated B cells in an IFN-γ ELISA assay (50 000 target cells/well, T cells not counted). Only T cell clones that displayed clear IE-1-mLCL reactivity and no reactivity against ctrl-mLCL and CD40-stimulated B cells, such as clones #2, 4, 7, 18, 23, 25, 29, 31 in this experiment, were used for subsequent experiments.

4.2.3 Definition of IE-1 CD4+ T cell epitopes

T cell clones displaying clear IE-1 specificity in the ELISA assays were used for the definition of IE-1 epitopes. In a first round of experiments, the peptides targeted by the T cell clones were mapped within the IE-1 sequence using the same 23 subpools containing 15-meric peptides of IE-1 as described before in 4.2.1. Autologous ctrl-mLCLs or CD40-stimulated B cells were

loaded with the peptide subpools and used as APCs for T cells. IFN-γ secretion upon T cell stimulation was measured by ELISA assay. For almost all T cell clones tested, a clear peptide subpool recognition pattern was obtained which could be used to fine map targeted peptides within the IE-1 sequence using the peptide "cross-matrix" (Fig. 2.1). Representative examples of subpool recognition patterns are shown in Fig. 4.12 for CD4+ T cell clones of four different donors. Most patterns displayed recognition of three subpools, two "vertical" and one "horizontal" (Fig. 4.12 A and B), which corresponds to the recognition of two adjacent overlapping 15-meric peptides. If the two "vertical" subpools were recognized with similar intensity, the 11 amino acid overlap between the peptides was defined as the putative minimal epitope. If one of the adjacent peptides was preferentially recognized, this 15-mer was considered to largely contain the minimal epitope. In some cases, the subpool recognition pattern contained only one "horizontal" and one "vertical" subpool (Fig. 4.12 C); in this case the corresponding 15-meric sequence was defined as the epitope. Finally, a few subpool patterns contained subpool #23 (Fig. 4.12 D), which comprises 9 variant peptides with single amino acid exchanges found in HCMV strains Toledo and TB40E. A signal for subpool #23 indicates that the clone was able to recognize an IE-1 sequence variant in addition to the sequence of the AD169 IE-1 protein, which was expressed in the mLCLs used for expansion of the polyclonal T cell lines, and should therefore be recognized by default. Subpool #23 contained only some of the sequences that vary between AD169 and other strains; there are many more such variations in IE-1, so a lack of reactivity with this subpool did not necessarily indicate a lack of reactivity to IE-1 from other HCMV strains.

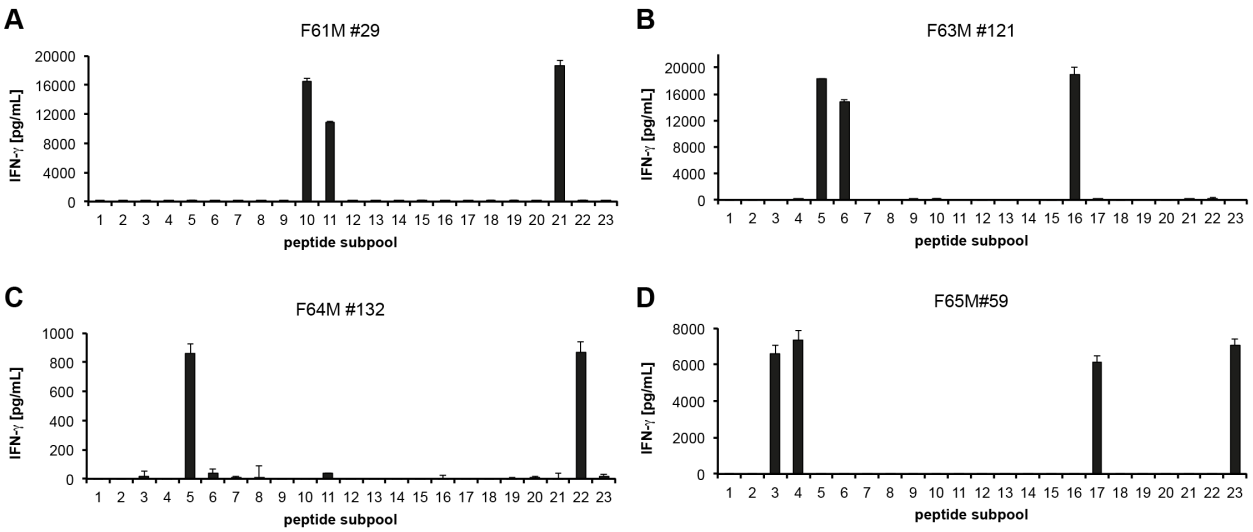

Fig. 4.12: Mapping of CD4+ T cell epitopes within the IE-1 sequence. Autologous ctrl-mLCLs or CD40-stimulated B cells were used as APCs for T cells and loaded with 23 peptide subpools containing 120 IE-1 AD169 15-meric peptides and 9 sequence variants of strains Toledo and TB40E (Fig. 2.1). APCs (20 000/well) and T cells (10 000/well) were coincubated for 16–18 hours and IFN-γ secretion was measured by ELISA. Respresentative recognition patterns are shown for T cell clones of four different donors (A–D). Means and standard deviations of duplicate samples are shown.

In a second round of experiments, the HLA class II molecules were identified that presented the IE-1 peptides targeted by the CD4+ T cell clones. HLA class II molecules exist in three different isoforms, termed HLA-DR, -DQ and -DP, which are simultaneously expressed in every donor. Each isoform consists of two polypeptide chains, α and β, which are encoded by separate genes in the HLA class II region and are both involved in presentation of the antigenic peptide. With exception of the gene encoding the DR α chain, all of these genes are polymorphic. The exact number of different HLA-DR, -DQ and -DP allotypes expressed in one donor depends on the number of α and β chain genes and alleles present on each chromosome (described in detail in 1.1.1). The available HLA class II types of the donors are listed in Table 2.1. To deal with the frequent association of certain α or β chain alleles of different isoforms, the presenting class II isoform was determined in a first experiment, by assessing T cell reactivity to autologous IE-1-expressing mLCL in the presence of blocking antibodies directed against HLA-DR, -DQ or -DP. For most T cell clones, a clear impairment of IE-1 recognition by exactly one of the anti-HLA-DR, -DQ or -DP antibodies could be detected in IFN-γ ELISA assays, identifying the presenting HLA class II isoform. Examples for T cell clones restricted through HLA-DR, -DQ and -DP are shown in Fig. 4.13.

In further experiments, it was attempted to determine the exact combination of presenting α and β chains for each target peptide by testing the reactivity of the T cell clones towards IE-1-mLCLs. The respective ctrl-mLCLs were also included in the experiment in order to identify IE-1-independent alloreactive mLCL recognition. However, alloreactivity was only detected for very few T cell clones and was not characterized in more detail. An exact HLA class II restriction could be determined for approximately half of the T cell clones tested. For some T cell clones, (highly resolved) HLA class II types were not available for all HLA class II loci. Some T cell clones showed IE-1 recognition of mLCLs that were not matched for the relevant HLA loci. This indicates that the TCRs of such T cell clones are able to recognize their target peptides on different HLA class II allotypes. Further studies with a broader panel of mLCLs that are typed in high resolution for all MHC class II loci, complemented by experiments with transgenic targets expressing single MHC class II allotypes, will help to determine the HLA restrictions of such T cell clones and to identify the different HLA class II allotypes that are able to promiscuously present the same peptides. For some T cell clones, the HLA restriction was narrowed down to two different HLA class II allotypes encoded by separate DRB genes, e.g. DRB1*1501 and DRB5*0101. These genes are closely associated, thus preventing the generation of mLCLs that express only one of them. Consequently, only transfection experiments with recombinant HLA-DR β chains will allow the identification of the relevant HLA class II allotype.

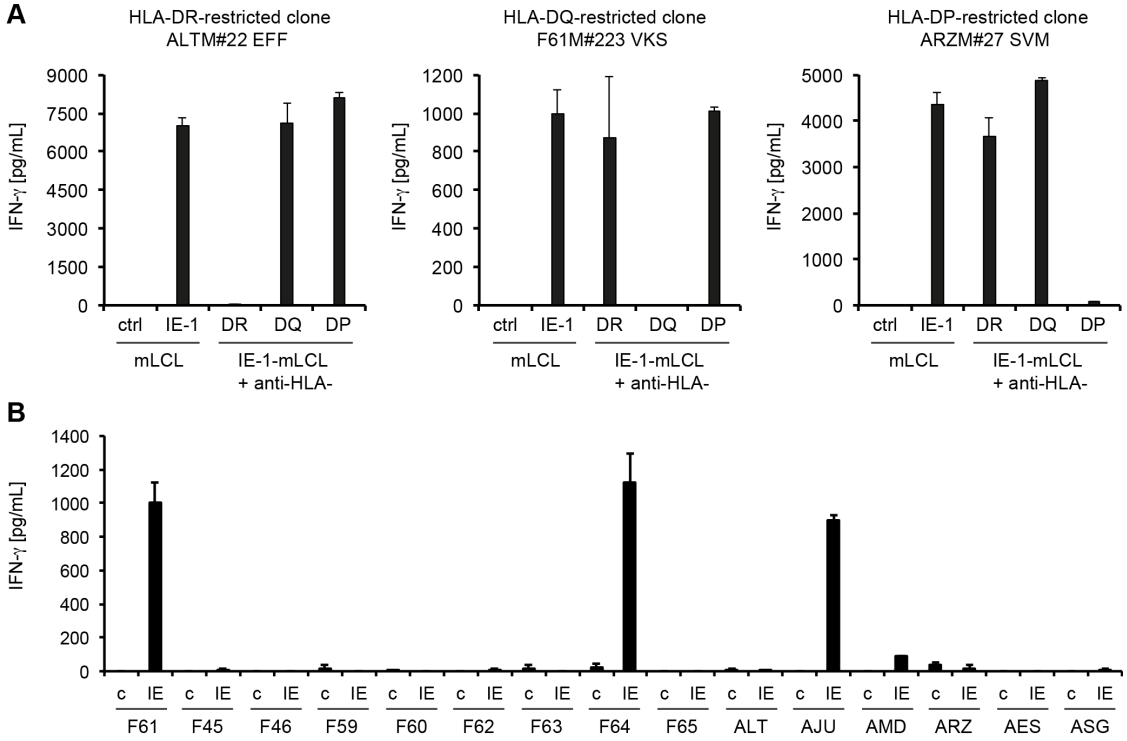

Fig. 4.13: Determinition of HLA restriction. A: For identification of the peptide-presenting HLA class II isoform, autologous IE-1-expressing mLCLs (20 000/well) were incubated with blocking antibodies (10 µg/mL) directed against HLA-DR, -DQ or -DP prior to addition of T cells (10 000/well). After overnight coincubation, impairment of IE-1 recognition by the antibodies was detected by IFN-γ ELISA. T cell clones restricted through each HLA class II isoform are shown as examples. **B:** The exact combination of an HLA class II α and β chain presenting a certain target peptide was determined by testing the reactivity of the respective T cell clone towards IE-1 ("IE")-mLCLs in an IFN-γ ELISA assay. Ctrl ("c")-mLCLs were included in the experiment to control for alloreactive mLCL recognition. HLA-DQ-restricted T cell clone F61M#223 VKS is shown as an example. This clone recognizes its target peptide on IE-1-mLCLs of donors F61, F64 and AJU, which have only DQA1*0201 and DQB1*0202 in common.

Table 4.2 summarizes the CD4+ IE-1-specific T cell clones analyzed for each donor and the peptides identified as T cell targets. For all donors except F46, T cell clones were selected at random for unbiased determination of specificity. For donor F46, CD4+ T cell clones were selectively screened with a peptide that was identified before as an epitope candidate in other donors (Fig. 4.10). Therefore, unbiased information on the composition of the CD4+ IE-1-specific T cell repertoire of donor F46 is not available. Fig. 4.14 gives a simplified overview of the T cell specificities and HLA-restrictions found in the other 7 donors. A range of 3 to 7 different peptides was targeted by the T cell clones of each donor. With exception of the clone F64M#228 KDE, all T cell clones recognized the IE-1 sequence variants of HCMV strains Toledo and TB40E in addition to the AD169 IE-1 sequence. T cell clones restricted through either one of the three HLA class II isoforms, HLA-DR, -DQ and -DP, could be identified, even in the same donor (AJU). Two peptides, **EFFTKNSAFPKTT** and **VKSEPVSEIEEVAPE,** were found to be targeted by T cell clones of 4 and 2 donors, respectively. Both peptides were presented by different donors on the same HLA class II molecules. By contrast, some of the

peptides were also recognized by T cell clones of different donors and with different HLA restrictions. Interestingly, these clones frequently showed recognition of IE-1-mLCLs from the other donors harboring T cells against the same peptide. For example, the HLA-DP-restricted peptide **SVMKRRIEEICMKVF**AQYI was recognized by clones of donors ARZ, AJU, and F61 which do only share DPA1*0103 allele encoding the α chain, but no DPB allele encoding the β chain. SVM-specific clones of all three donors do recognize the IE-1-mLCLs of the other donors (data not shown).

Table 4.2: Overview of identified IE-1 CD4+ T cell epitopes. Single 15-mers or two adjacent 15-mers of the AD169 IE-1 peptide library were identified as T cell targets in the ELISA assays. For adjacent 15-mers, putative minimal epitopes were defined based on the magnitude of IFN-γ secretion in response to the subpools containing the individual 15-mers and are denoted in bold letters. Peptides recognized by T cell clones of different donors on the same or on different HLA molecules are highlighted with colors. T cell clones that recognized a sequence variant of IE-1 found in HCMV strains Toledo and TB40E in addition to an AD169 IE-1 peptide are highlighted in grey. T cell clones that did not recognize such sequence variants are denoted in red (only F64M#228 KDE).

donor	clone #	recognized peptide	HLA-restriction
ALT	19, 22, 37, 65, 80, 82	YRNI**EFFTKNSAFPK**TTNG	DRB1*1501 or DRB5*0101
	51	CSPD**EIMAYAQKIFK**ILDE	DR
	70	EKFT**GAFNMMGGCLQ**NALD	DR
	47, 53, 66, 114	FPKT**TNGCSQAMAAL**QNLP	DQ
	63	**FPKTTNGCSQAMAAL**	n. d.
ARZ	50, 93, 94, 101, 155, 158, 159	YRNI**EFFTKNSAFPK**TTNG	DRB1*1501 or DRB5*0101
	105	**KDELRRKMMYMCYRN**	DRB1*1501 or *0501
	18, 37, 73, 123, 125	ARAK**KDELRRKMMY**MCYRN	DPA1*0103 + DPB1*0201
	98	**KDELRRKMMYMCYRN**IEFF	DPA1*0103 + DPB1*0201
	27	**SVMKRRIEEICMKVF**AQYI	DPA1*0103 + DPB1*0201 or *0301
AJU	11, 17, 34, 64, 112, 118, 137, 153, 155, 159, 179, 190, 200	LVKQ**IKVRVDMVRHRIKEH**	DR
	31	IKVR**VDMVRHRIKEHMLKK**	DRB1*1301
	120	LITK**PEVISVMKRRIEEIC**	DR
	52	**AIAEESDEEEAIVAY**	DQ
	6, 10, 50, 76, 81, 90, 106, 109, 115, 139	**SVMKRRIEEICMKVF**AQYI	DPA1*0103 + DPB1*0401 or *2001
F46	1, 48, 60, 66, 73, 74, 79	YRNI**EFFTKNSAFPK**TTNG	DRB1*1501 or DRB5*0101
F61	29	ENSD**QEESEQSDEEE**EEGA	DQA1*0201 + DQB1*0202
	223	**VKSEPVSEIEEVAPE**	DQA1*0201 + DQB1*0202
	94, 97, 126, 154	**SVMKRRIEEICMKVF**AQYI	DP
F63	6, 25, 27, 46, 65	**RRKMMYMCYRNIEFF**TKNS	DR
	41, 58, 84	CLQN**ALDILDKVHEP**FEEM	DR
	99, 108, 121	EDKR**EMWMACIKELH**DVSK	DR
	31	**HEPFEEMKCIGLTMQ**SMYE	n. d.
F64	58, 69, 168, 189	**VDMVRHRIKEHMLKK**	DRB1*1301
	65, 169, 181	**RHRIKEHMLKKYTQT**	DRB1*1301
	138	**YRNIEFFTKNSAFPK**	DRB1*1301
	139	**LVKQIKVRVDMVRHR**IKEH	DRB1*1301
	228	**KDELRRKMMYMCYRN**	DRB1*1301
	236	CSPD**EIMAYAQKIFK**ILDE	DRB1*1301
	51, 115, 124, 132, 231	**VKSEPVSEIEEVAPE**	DQA1*0201 + DQB1*0202
F65	11, 18, 21, 42, 48, 50, 59, 61, 64, 91, 97, 123, 124, 146, 148, 150, 156, 159	**RRKMMYMCYRNIEFF**TKNS	DRB3*0202
	84, 92	YRNI**EFFTKNSAFPK**TTNG	DRB1*1501 or DRB5*0101
	128, 148	**GAFNMMGGCLQNALD**	DRB1*1501 or DRB5*0101
	63, 87, 138, 140, 151, 160	EGAQ**EEREDTVSVKS**EPVS	n.d.
	107	LDEE**RDKVLTHIDHIFMDI**	n.d.
	101	**LVKQIKVRVDMVRHR**IKEH	n.d.

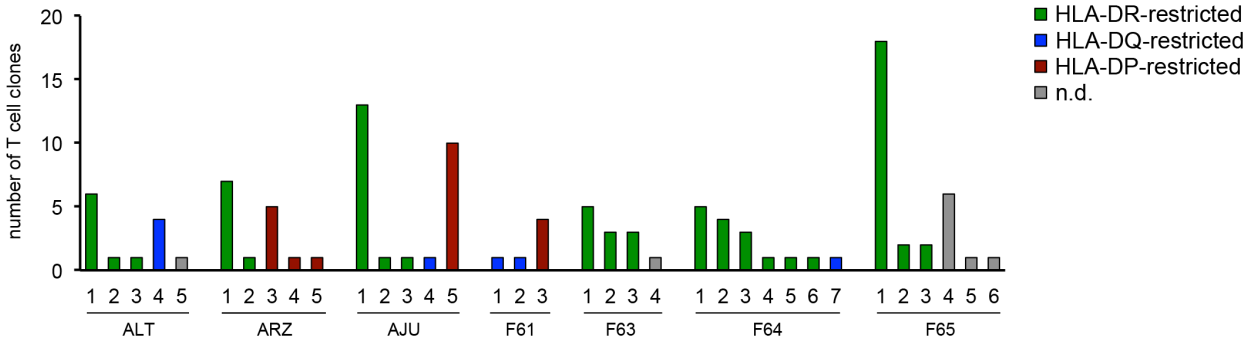

Fig. 4.14: T cell specificities and HLA-restrictions found in the donors ALT, ARZ, AJU, F61, F63, F64 and F65. This graph gives a simplified summary of the complex data listed in Table 4.2. T cell clones with 3 to 7 different peptide specificities (x-axis) and HLA-DR, -DQ and -DP restrictions were found in the donors. The number of T cell clones analyzed per target peptide is represented by the y-axis.

One major goal of HCMV research is the establishment of an active immune prophylaxis by vaccination in order to prevent congenital HCMV infection, which is the leading cause of mental retardation in children. One strategy of vaccine development is the generation of peptide vaccines for stimulation of HCMV-specific T cells. The prerequisite for the design of an effective peptide vaccine is the identification of immunodominant regions within HCMV T cell antigens such as the IE-1 protein. The detailed characterization of the IE-1-specific CD4+ T cell repertoire within this study enables the evaluation of the IE-1 primary structure with regard to immunogenicity towards CD4+ T cells. The identified IE-1 CD4+ T cell epitopes are highlighted in the IE-1 amino acid sequence shown in Fig. 4.15. At first glance, with exception of the first 80 amino acids, the whole IE-1 sequence is well accessible for recognition by CD4+ T cells. However, a more precise analysis revealed that some IE-1 sections are more frequently targeted by T cells than others (Fig. 4.15 in bold letters). Such hotspots of immunogenicity are mainly found in the first half of the protein. For example, 6 different epitopes were mapped to aa 85–107 and 8 different epitopes to aa 197–235.

```
  1 MESSAKRKMD PDNPDEGPSS KVPRPETPVT KATTFLQTML RKEVNSQLSL
 51 GDPLFPELAE ESLKTFEQVT EDCNENPEKD VLAELVKQIK VRVDMVRHRI
101 KEHMLKKYTQ TEEKFTGAFN MMGGCLQNAL DILDKVHEPF EEMKCIGLTM
151 QSMYENYIVP EDKREMWMAC IKELHDVSKG AANKLGGALQ AKARAKKDEL
201 RRKMMYMCYR NIEFFTKNSA FPKTTNGCSQ AMAALQNLPQ CSPDEIMAYA
251 QKIFKILDEE RDKVLTHIDH IFMDILTTCV ETMCNEYKVT SDACMMTMYG
301 GISLLSEFCR VLCCYVLEET SVMLAKRPLI TKPEVISVMK RRIEEICMKV
351 FAQYILGADP LRVCSPSVDD LRAIAEESDE EEAIVAYTLA TAGVSSSDSL
401 VSPPESPVPA TIPLSSVIVA ENSDQEESEQ SDEEEEEGAQ EEREDTVSVK
451 SEPVSEIEEV APEEEEDGAE EPTASGGKST HPMVTRSKAD Q
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Fig. 4.15: Distribution of CD4+ T cell epitopes within the IE-1 sequence. Sequences recognized by CD4+ T cell clones are denoted in red letters. Sequences containing overlapping T cell epitopes are denoted in bold letters.

The characterization of the CD4+ T cell repertoire against IE-1 revealed that all donors carrying HLA-DRB1*1501 and DRB5*0101 (which are regularly associated) have CD4+ T cells recognizing the adjacent peptides #53 and 54 of the IE-1 peptide library. In the polyclonal T cell lines of 2 out of 3 DRB1*1501/DRB5*0101-positive donors comprehensively analyzed for IE-1 epitopes, such T cells represented the dominant IE-1 CD4+ T cell specificity. The comparatively high frequency of these T cells and the fact that DRB1*1501 is the most frequent HLA-DRB1 allele in the German population (~15%) (Schmidt et al., 2009) renders this T cell species a good candidate for adoptive T cell therapy. For this reason, these T cells were submitted to closer analysis in terms of minimal target peptide and precursor frequency in unstimulated PBMCs. T cell clones of donors ALT, ARZ, F46 and F65 were available for definition of the minimal epitope. These clones recognized peptides #53 and 54 equally well in the IFN-γ ELISA assays (data not shown). Consequently, the minimal epitope should largely comprise the oberlapping region of the two peptides (YRNI**EFFTKNSAFPK**TTNG). For definition of the exact epitope residues, T cell recognition of various peptides, 11 to 15 aa in length and all contained in peptides #53 and 54, was compared in peptide titration studies (Fig. 4.16). Autologous CD40-stimulated B cells were used as APCs and pulsed with 10-fold serially diluted peptides. T cell stimulation was measured by IFN-γ ELISA. With peptide recognition down to a concentration of 10^{-10} mol/L, the T cell clones showed the highest avidity for peptides that minimally contained the central 11 amino acids of the sequence (E)FFTKNSAFPKT(T) plus at least one additional flanking amino acid at one end. Thus, the definition of a minimal epitope was not without ambiguity for this specificity. As the 13-meric peptide EFFTKNSAFPKTT triggered highest IFN-γ secretion at the lowest peptide concentrations able to stimulate T cell activation (10^{-10}–10^{-8} mol/L), this peptide was operationally defined as the minimal epitope, abbreviated EFF.

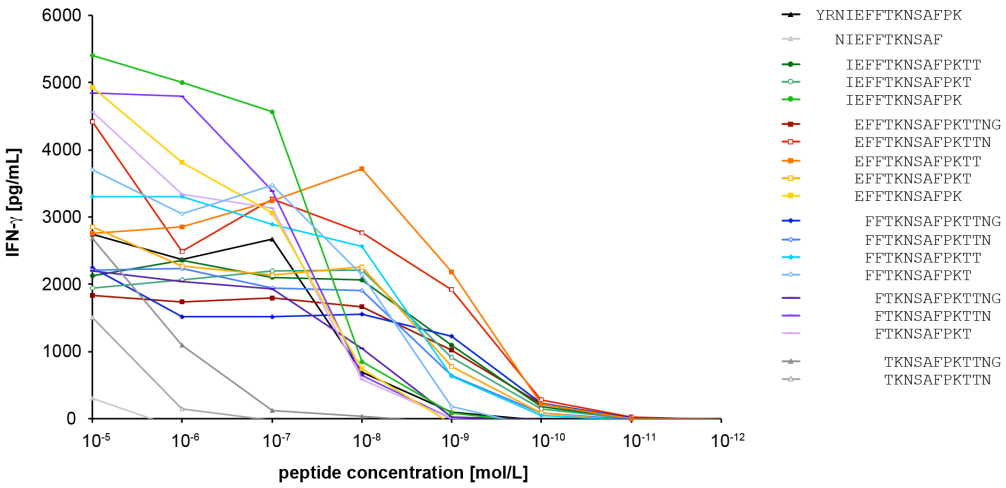


Fig. 4.16: Determination of the minimal CD4+ T cell epitope contained within peptides #53 and 54 of the IE-1 peptide library. The avidity of the T cell clone F46M#1 towards 11–15-meric peptides contained within peptides #53 and 54 was assessed in peptide titration studies. Autologous CD40-stimulated B cells (50 000/well) were loaded with 10-fold diluted peptides and coincubated with T cells (20 000/well). T cell recognition was measured by IFN-γ ELISA. Means of triplicate samples are shown. Standard deviations are not shown in order to preserve readability of the diagram.

EFF-specific T cells were further analyzed for their precursor frequency in unstimulated PBMCs of DRB1*1501/DRB5*0101-positive donors. PBMCs were tested for recognition of the 23 IE-1 subpools using IFN-γ ELISpot assays. A clear signal for subpools #5, 6 and 17 (containing the EFF peptide) could not be detected in total PBMCs of such donors. Only in donor ALT, a signal of 15 spots in 200 000 PBMCs was visible for subpool #17. Spots caused by EFF-specific T cells in wells with subpools #5 and 6 could not be identified due to the presence of a very dominant CD8+ T cell population identified in donor ALT (described in 4.3.). This result indicates that EFF-specific T cells are rather rare in unstimulated PBMCs of HCMV carriers. A subsequent experiment was performed to test whether EFF-specific T cells can be specifically enriched by peptide stimulation of PBMCs of DRB1*1501/DRB5*0101-positive donors. PBMCs of ALT, ARZ, AES and F65 were coincubated with EFF peptide for 11–14 days. EFF-specific T cells could be strongly enriched in three (ALT, ARZ, F65) out of the four donors tested. Frequencies of EFF-specific T cells in unstimulated PBMCs and peptide-stimulated T cell lines of donor ARZ were qualitatively compared by IFN-γ ELISpot assays (Fig. 4.17).

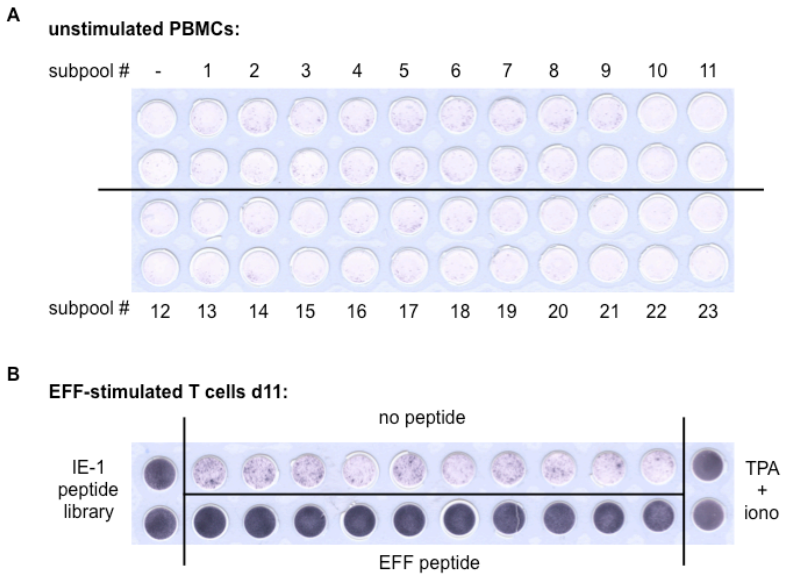


Fig. 4.17: Frequencies of EFF-specific T cells in unstimulated PBMCs (A) and an EFF peptide-stimulated T cell line (B) of DRB1*1501/DRB5*0101-positive donor ARZ. EFF-specific T cells were identified with IFN-γ ELISpot assays. Therefore, 200 000 cells/well were incubated overnight either with the 23 IE-1 subpools (A) or with the total IE-1 peptide library and the EFF peptide (B). As a positive control, cells were incubated with global T cell activators TPA and ionomycin (only shown in B).

4.2.4 Analysis of effector functions of IE-1-specific CD4+ T cells

CD4+ T cell responses may play an important role in the defense of viruses by providing diverse antiviral effector functions. CD4+ T cells primarily mediate helper function for both humoral and cell-mediated immune mechanisms. According to their distinct patterns of cytokine secretion, CD4+ T cells are classically categorized into Th1 and Th2 cells (Cousins et al.,

2002). Th1 cells (IL-2, IFN-γ, TNF-α) are critical for activation of APCs, increase in antigen presentation, and induction of CTL proliferation. Furthermore, the Th1 cytokines IFN-γ and TNF-α mediate direct antiviral and growth-inhibitory effects. Th2 cytokines (IL-4, IL-5, IL-6, IL-10, IL-13 and IL-25) stimulate naive B cells to become plasma cells that secrete specific antibodies with particular isotypes. In addition to Th1 and Th2 cells, CD4+ T cell subsets have been identified with acquired cytotoxic activity, thus being able to directly kill infected target cells in an MHC class II-dependent manner (Marshall and Swain, 2011). HCMV-specific CD4+ T cells have been primarily associated with the Th1 type due to secretion of IFN-γ and TNF-α (Rentenaar et al., 2000; van Leeuwen et al., 2006), and due to their crucial role in maintaining adoptively transferred HCMV-specific CD8+ T cell immunity (Walter et al., 1995). However, as HCMV infection also elicits a broad antiviral neutralizing antibody response, it should be considered that Th2 cells might also be involved in anti-HCMV immunity (Jackson et al., 2011). Interestingly, a panel of direct cytolytic CD4+ T cells has been identified to target HCMV antigens pp65, gB and gH (Hopkins et al., 1996; Elkington et al., 2004; Weekes et al., 2004).

In this context, the functional characteristics of anti-IE-1 CD4+ T cell responses were evaluated here. Two CD4+ T cell effector functions, cytokine secretion and direct cytotoxicity, were assessed using CD4+ IE-1-specific T cell clones. Mini-LCLs and monocyte-derived dendritic cells (mo-DCs) served as T cell targets, as these cells constitutively express high levels of MHC class II molecules. Amongst these professional APCs, DCs deserve particular attention because they are natural targets of HCMV infection *in vivo*. Furthermore, precursor cells of the myeloid lineage are considered to be reservoirs of latent virus *in vivo* and their differentiation into monocytes, DCs or macrophages triggers virus reactivation. Mature DCs were generated from peripheral blood monocytes using a modified protocol from Jonuleit et al. (2000). CD4+ IE-1-specific T cell clones were analyzed for secretion of Th1 (IL-2, IFN-γ, TNF-α) and Th2 (IL-4, IL-10) cytokines in response to peptide-stimulation using ELISA assays. As Th1 and Th2 cells reportedly produce GM-CSF upon stimulation (Cousins et al., 2002), GM-CSF ELISAs were performed as positive controls. Mo-DCs were loaded with the IE-1 peptides identified as targets of the CD4+ T cell clones (Table 4.2). The cytokine profiles obtained from four different T cell clones are shown in Fig. 4.18. All T cell clones tested secreted the Th1 cytokines IL-2, IFN-γ and TNF-α. Production of the Th2 cytokine IL-10 could not be detected for any of the four T cell clones tested. Interestingly, two of the T cell clones, ALTM#22 EFF and ARZM#37 ARA, showed secretion of the Th2 cytokine IL-4 in addition to the Th1 cytokines. T cell subsets with less differentiated cytokine profiles than Th1 and Th2 cells have been reported before, for example for HSV (Yasukawa et al., 1991), and are designated as Th0 cells. Th0 cells arise in the absence of clear polarizing signals and can mediate intermediate effector functions of Th1 and Th2 types (Romagnani, 1994). However, it cannot be excluded that cytokine secretion phenotypes may be modulated during *in vitro* cultivation.

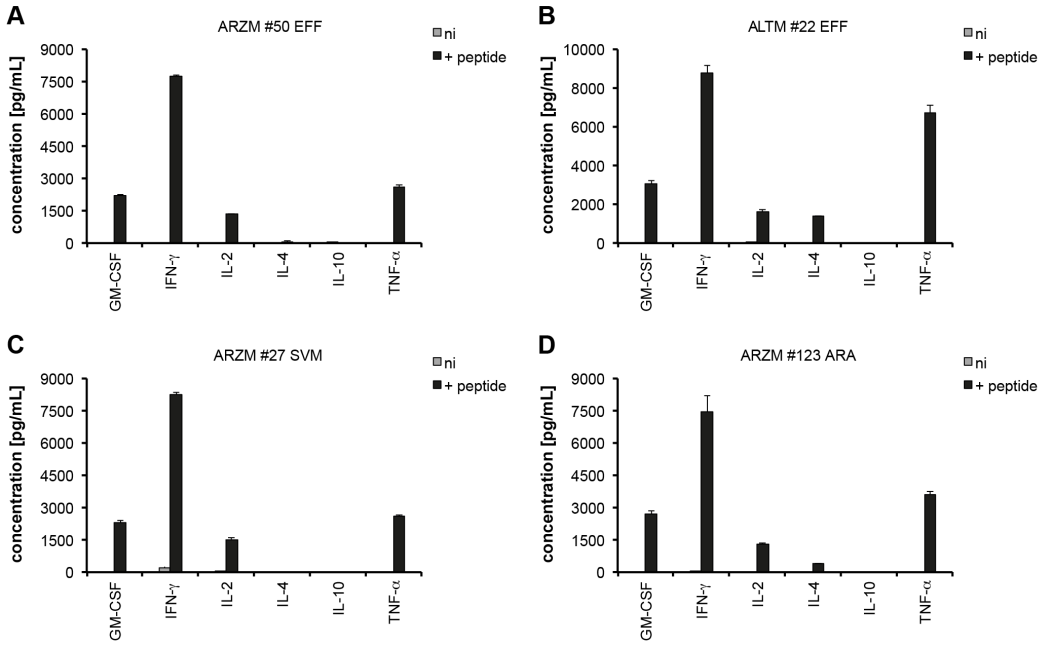


Fig. 4.18: Cytokine profiles of CD4+ IE-1-specific T cell clones. Peptide-pulsed monocyte-derived (mo)-DCs (generated from donor ARZ, 10 000/well) were coincubated overnight with T cells (10 000/well). Secretion of GM-CSF, IFN-γ, IL-2, IL-4, IL-10 and TNF-α was detected by ELISA. Means and standard deviations of triplicate samples are shown.

The less common effector function of CD4+ T cells, direct cytotoxicity, was evaluated for a broad panel of CD4+ IE-1-specific T cell clones using calcein-release assays (Fig. 4.19). The T cell clones were tested for antigen-specific lysis of B cell lines and mo-DCs in separate experiments. In the first experiment (Fig. 4.19 A), autologous or HLA-matched IE-1-mLCLs, ctrl-mLCLs and CD40-stimulated B cells were used as T cell targets, in the second experiment (Fig. 4.19 B), autologous or HLA-matched mo-DCs, peptide-loaded or without peptide, were used to detect antigen-specific lysis. The panel of CD4+ T cell clones tested in the two separate experiments was largely overlapping. As a positive control for T cell lysis, both experiments were performed in parallel with CD8+ cytotoxic T cell clones recognizing the well-described IE-1 epitope VLE (Khan et al., 2002). In both experiments, the majority of CD4+ T cell clones did not show clear IE-1-specific cell lysis. However, each experiment identified a few T cell clones displaying IE-1-dependent cytotoxicity. In the B cell experiment, two clones of donor AJU (AJUM#17 LVK and #118 LVK) recognizing the same epitope, and two clones of donor F64 (F64M#115 VKS and #69 VDM) recognizing different epitopes, showed preferential lysis of the IE-1-mLCL. In the DC experiment, only T cell clones of donor F64 (including F64M#69 VDM tested in the B cell experiment) showed clear IE-1 peptide-specific target lysis. The T cell clones of donor AJU identified as cytotoxic in the B cell experiment did not show any significant lysis in the DC experiment. Further experiments will be necessary to define the mechanisms that give rise to these differences in cytotoxic potential of IE-1-specific CD4+ T cell clones against different types of target cells. Potential factors might be differences in the expression of different HLA

class II molecules and allotypes, or a modulation of CD4+ T cell reactivity by cell-type-specific costimulatory or coinhibitory molecules on APCs. In summary, from a broad panel of CD4+ T cell clones tested, only a minority displayed antigen-dependent cell lysis. Furthermore, levels of specific lysis detected for these CD4+ T cell clones were smaller than for CD8+ T cell clones specific for epitopes from the same antigen.

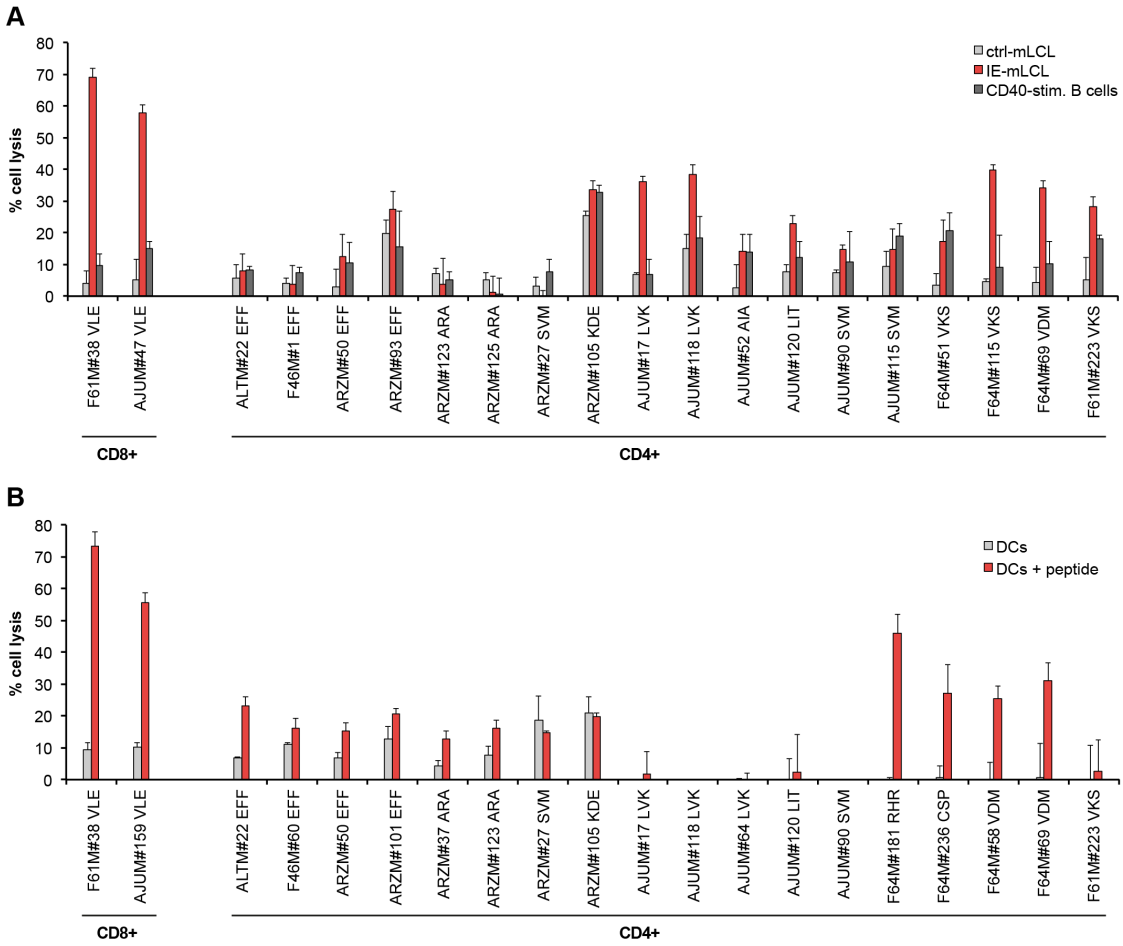


Fig. 4.19: Analysis of direct cytotoxic effects of IE-1-specific CD4+ T cell clones. CD4+ T cell clones from donors ALT, F46, ARZ, AJU, F64 and F61 were tested for IE-1-specific lysis of autologous (ALT, ARZ, AJU) or HLA-matched (F46, F64, F61) B cell lines (IE-1-mLCLs, ctrl-mLCLs and CD40-stimulated B cells) (**A**) and autologous (ARZ, AJU) or HLA-matched (ALT, F46, F64, F61) monocyte-derived DCs (pulsed with IE-1 peptides or without peptides) (**B**) in separate experiments. In both experiments, cytotoxicity was detected by calcein-release assay using 5000 targets/well and two different effector/target-ratios: 8/1 and 2/1. Only the data for E/T = 2/1 are shown. Means and standard deviations of triplicate samples are shown.

Although the majority of IE-1-specific CD4+ T cell clones did not display significant direct cytotoxic capacity, it has to be considered that these clones might still provoke indirect cytotoxic or growth-inhibitory effects towards surrounding cells *in vivo* via the secreted cytokines. For example, the Th1 cytokine IFN-γ is described to mediate broad anti-proliferative and pro-apoptotic effects (Schroder et al., 2004). In this study, two EFF-specific T cell clones that did not display clear direct cytotoxicity in the calcein-release assays were analyzed for indirect cytotoxicity and

growth-inhibitory effects by regression assay (Fig. 4.20). In this assay, T cells were cocultivated with different amounts of HLA-matched mLCLs per well. Wells with proliferating mLCLs (mLCL "outgrowth") were detected after 5–6 weeks by MTT assay. To know whether potential inhibitory effects of T cells are IE-1-dependent, proliferation of ctrl- and IE-1-mLCLs was examined in parallel. A CD8+ cytotoxic T cell clone directed against the IE-1 VLE-epitope was included in the experiment as a control. Fig. 4.20 A shows the proliferative behavior of mLCLs in absence of T cells. 100% outgrowth of IE-1- and ctrl-mLCLs can be achieved with a minimal number of 625 and 2500 mLCLs/well, respectively. Partial outgrowth of IE-1- and ctrl-mLCLs can already be detected at lower cell numbers, 156 and 625 cells/well, respectively. Cocultivation of mLCLs with the IE-1-specific CD8+ cytotoxic T cells (Fig. 4.20 B) resulted in IE-1-dependent inhibition of mLCL proliferation. Inhibitory effects on ctrl-mLCL proliferation were minimal. By contrast, the two IE-1-specific CD4+ T cell clones could not completely inhibit outgrowth of either mLCL (Fig. 4.20 C and D). However, a clear increase of the minimal cell number necessary for 100% or partial outgrowth was detectable for both mLCLs, indicating that the T cells possess growth-inhibitory properties. Furthermore, IE-1-mLCL outgrowth was reduced much more strongly than ctrl-mLCL outgrowth, suggesting that the anti-proliferative effects mediated by the T cells were largely IE-1-specific.

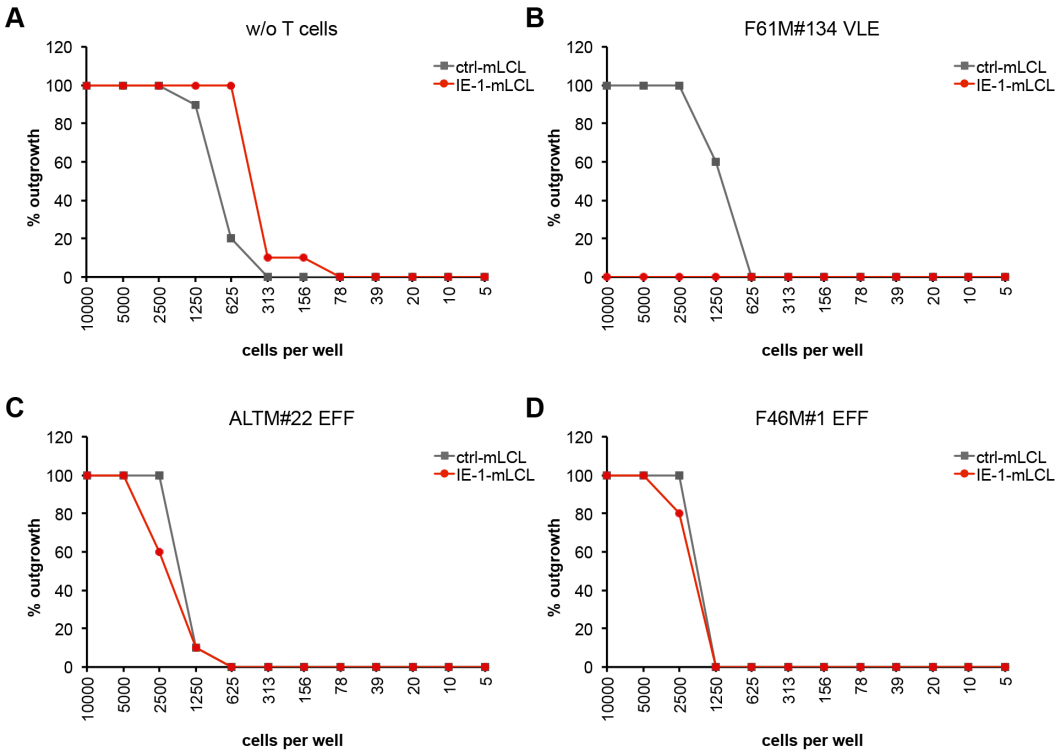


Fig. 4.20: Analysis of indirect cytotoxic and growth-inhibitory effects of IE-1-specific CD4+ T cell clones by regression assay. Outgrowth of IE-1- and ctrl-mLCLs (from donor ALT) was measured in absence (**A**) and presence (**B–C**) of T cells (10 000/well). Effects of IE-1-specific CD4+ T cell clones ALTM#22 EFF (**C**) and F46M#1 EFF (**D**) on mLCL outgrowth were compared to the direct cytotoxic effects of IE-1-specific CD8+ T cell clone F61M#134 VLE (**B**). The assay was performed with 10 replicates for each number of mLCLs/well. Mini-LCL outgrowth was detected by MTT assay after 5–6 weeks.

4.2.5 T cell recognition of HCMV-infected target cells

In previous experiments, CD4+ IE-1-specific T cell clones were shown to produce high amounts of cytokines, especially IFN-γ (Fig. 4.13 and Fig. 4.18), in response to cells endogenously expressing IE-1 or exogenously loaded with IE-1 peptides. This potent effector function suggests an important role of these T cells in the immune response to HCMV. However, IE-1 presentation in HCMV-infected cells might be altered due to lower availability of target peptides or immunomodulatory functions of the virus. Hence, the actual role of CD4+ IE-1-specific T cells in the HCMV immune response is determined by their ability to recognize IE-1 in context of an HCMV infection. A commonly used cellular system for studying HCMV infection *in vitro* are primary human fibroblasts. These cells show a high permissiveness for productive infection by HCMV laboratory strain AD169, which was the available HCMV strain for this study. However, fibroblasts do not constitutively express MHC class II molecules. MHC class II expression was induced in fibroblasts by treatment of the cells with IFN-γ (data not shown). However, although the fibroblasts thereby acquired the ability to present exogenously loaded IE-1-peptides to the CD4+ T cell clones, recognition of HCMV-infected fibroblasts by the CD4+ T cells could hardly be detected. In search of an appropriate cell system with high constitutive expression of MHC class II molecules and permissiveness for HCMV infection, mo-DCs were evaluated as T cell targets. As shown above, mo-DCs presented exogenously loaded IE-1 peptides to CD4+ T cell clones (Fig. 4.18). Mature mo-DCs permit, to some extent, the infection with the fibroblast-adapted HCMV strain AD169, and infected mo-DCs support all phases of viral gene expression, but do not release infectious progeny virus (Senechal et al., 2004). Autologous or HLA-matched mature mo-DCs were generated from peripheral blood monocytes and infected with AD169 at an moi = 5 for 48 hours. IFN-γ released during cocultivation of infected mo-DCs and T cells was measured by ELISA. Fig. 4.21 summarizes the outcome of several infection experiments with a broad panel of CD4+ T cell clones. Strikingly, all T cell clones tested recognized AD169-infected mo-DCs. Antigen-specific IFN-γ secretion varied between the T cell clones tested. Only the HLA-DP-restricted SVM-specific T cell clones of donors ARZ and F61 showed similar IFN-γ secretion in response to infected cells and peptide-loaded cells. This could reflect different avidities of the T cell clones or different availabilities of the target peptides. However, also virus-mediated immunomodulatory effects on MHC class II presentation have to be considered. HCMV encodes at least two proteins, gpUS3 and gpUS2, which are able to interfere with the MHC class II pathway of antigen presentation (Tomazin et al., 1999; Hegde et al., 2002). Such immunomodulatory proteins might differentially affect antigen presentation on different MHC class II isoforms or allotypes. Peptide titration studies and experiments using HCMV immunoevasin deletion mutants might clarify if the HLA-DP-restricted SVM-specific T cell clones possess particular high avidity or are less affected by viral immunoevasion compared to the other T cell clones. In summary, this experiment showed two things: first, mature mo-DCs are

an adequate system to study CD4+ T cell responses to HCMV infection; second, owing to their good recognition of infected DCs, which are also naturally targeted by HCMV *in vivo*, CD4+ IE-1-specific T cells are likely to play an important role during the anti-HCMV immune response.

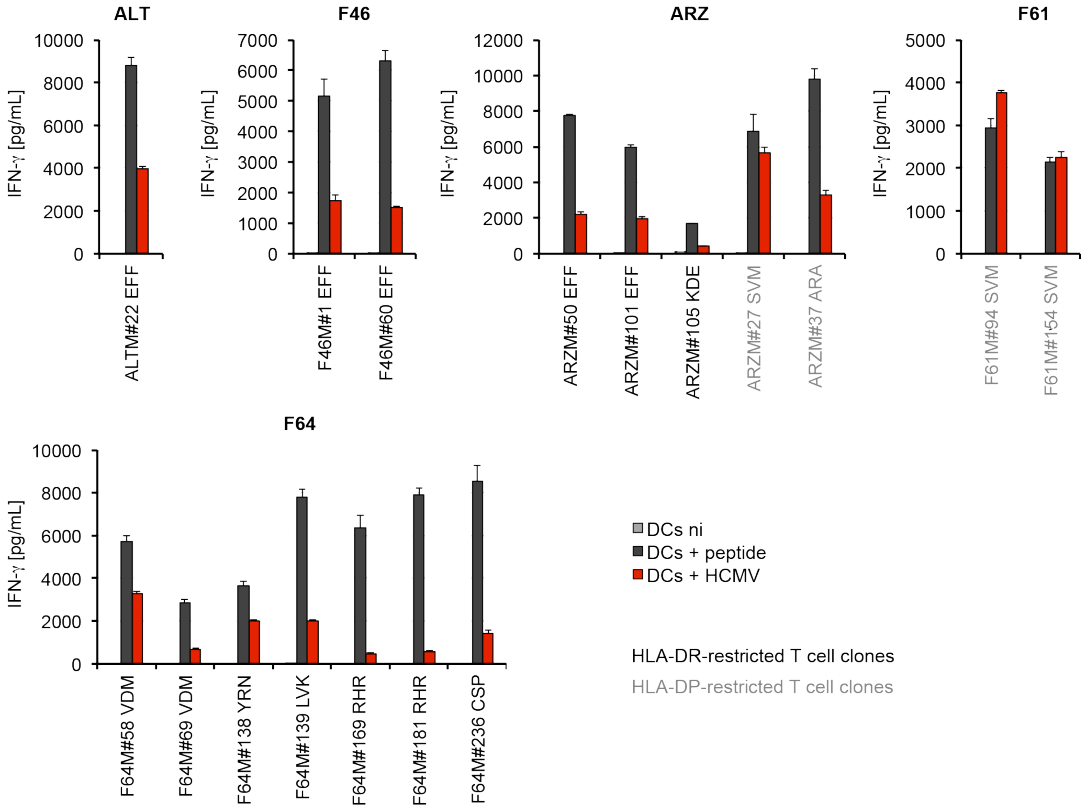


Fig. 4.21: Recognition of HCMV-infected monocyte-derived (mo-)DCs by CD4+ IE-1-specific T cell clones. CD4+ T cell clones of 5 different donors were tested for recognition of HCMV-infected (48 hpi) mo-DCs by IFN-γ ELISA. DCs that were not infected (ni) and peptide-loaded DCs were used as negative and positive controls, respectively. Depending on the donor, autologous (ARZ) or HLA-matched (ALT, F46, F61, F64) mo-DCs were available. 10 000 T cells/well were incubated overnight with 10 000 mo-DCs/well. Means and standard deviations of triplicate samples are shown.

4.3 IE-1-specific CD8+ T cell repertoire

CD8+ cytotoxic T cells (CTLs) are considered to be pivotal for defense against virus infections because they can directly interfere with virus spread by killing of infected cells upon cell-to-cell contact. IE-1-specific CD8+ T cells can be detected in the majority of healthy virus carriers (Khan et al., 2005; Sylwester et al., 2005) and are associated with control of HCMV. For example, after allogeneic SCT a delay in IE-1-specific CD8+ T cell reconstitution correlated with HCMV disease (Sacre et al., 2008). Another study on solid organ transplant patients indicated an outstanding role of IE-1-specific CD8+ T cells, as CTLs targeting IE-1 but not T cells directed against an immunodominant structural protein, pp65, were correlated with protection from viral disease (Bunde et al., 2005). IE-1-specific CTLs might control HCMV infection with particular efficiency because their target protein, IE-1, is expressed very early during lytic infection but

also during reactivation from latency, thereby enabling the eradication of HCMV-infected cells before progeny virus is released. Within this work, IE-1-specific CD8+ T cells were isolated from the IE-1-stimulated polyclonal T cell lines and reevaluated with a focus on identification of efficient candidate epitopes for adoptive T cell therapy.

4.3.1 Analysis of the CD8+ fraction of the polyclonal T cell lines

As shown above, IE-1-stimulated polyclonal T cell lines were largely dominated by CD8+ T cells and preferentially recognized IE-1-expressing mLCLs, compared to ctrl-mLCLs and CD40-stimulated B cells, in IFN-γ ELISA assays (Fig. 4.6). These findings strongly suggest an enrichment of IE-1-specific CTLs in the polyclonal T cell lines stimulated with IE-1. To obtain information on the frequency of IE-1-specific CD8+ T cells, IFN-γ ELISpot assays were performed on CD4+ T cell-depleted polyclonal T cell lines of eleven donors at day 46–54 of cultivation (Fig. 4.22). In this assay, T cells were stimulated with autologous ctrl- or IE-1-mLCL. Most T cell lines showed preferential reactivity against the IE-1-mLCL, indicating a predominance of IE-1-specific CD8+ T cells in the cultures. In the T cell lines of donors ALT, AJJ, F46, F59 and F61, more than 10% of polyclonal T cells selectively recognized the IE-1-mLCL, whereas recognition of other mLCL determinants was minor, as shown by the control. It is well established for virus-specific T cells that ELISpot assays underestimate the frequency of antigen-specific T cells, because not all antigen-specific T cells secrete sufficient amounts of cytokine within the time frame of an ELISpot assay to be detected; in peripheral blood, multimer staining typically yields four times higher numbers of specific T cells than ELISpot assay (Tan et al., 1999). Therefore, the actual proportion of IE-1-specific T cells in the polyclonal T cell cultures is supposedly higher than the proportion of cells that reacted in the ELISpot assay.

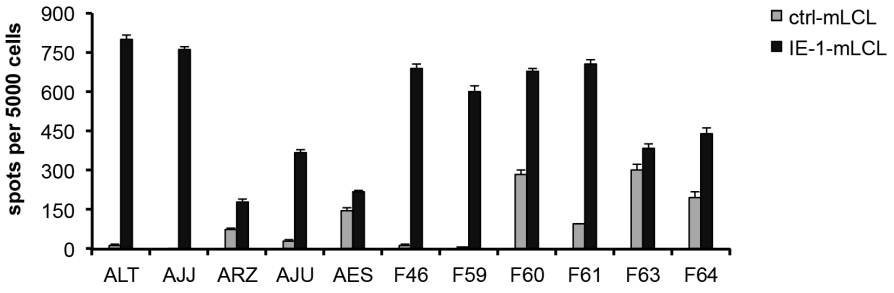
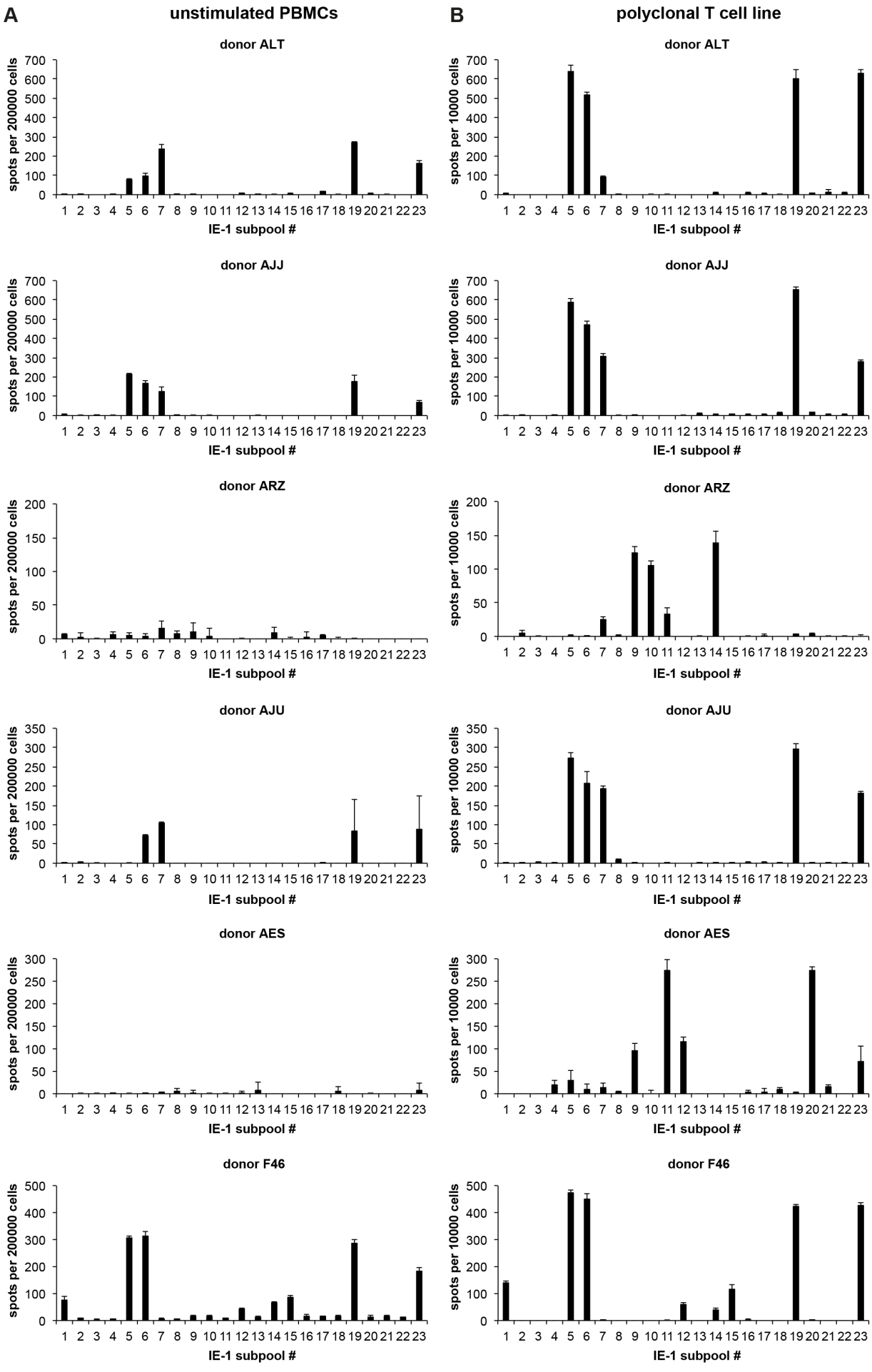


Fig. 4.22: Frequency of IE-1-specific T cells in the IE-1-stimulated polyclonal T cell lines. The reactivity of CD4+ T cell-depleted IE-1-stimulated polyclonal T cell lines (day 46-54) of 11 different donors in response to autologous IE-1- and ctrl-mLCL was analyzed by IFN-γ ELISpot assays (5000 T cells and 50 000 mLCLs per well). Means and standard deviations of duplicate samples are shown.

Next, the IE-1-stimulated polyclonal T cell lines were evaluated for the presence and frequency of T cells specific for individual IE-1 epitopes in IFN-γ ELISpot assays (Fig. 4.23 B). For epitope mapping, the polyclonal T cell lines were challenged with autologous CD40-stimulated B cells

loaded with each of the 23 IE-1 peptide subpools (described in 4.2.1). To estimate the enrichment rate of IE-1 epitope-specific T cells, ELISpot assays were performed in parallel with unstimulated PBMCs of the same donors (Fig. 4.23 A). An enrichment of IE-1 peptide-specific T cells could be detected in the polyclonal T cell lines of all donors tested. Interestingly, with the only exception of donor F64, the enrichment of IE-1-specific T cells in the polyclonal T cell lines inversely correlated with the T cell precursor frequency in unstimulated PBMCs. The highest enrichment rates (above 300-fold) of IE-1 peptide-specific T cells were detected for donors ARZ, AES and F63, where the frequency of precursor cells in PBMCs was below the detection limit of IFN-γ ELISpot assays (approximately 10 spots). Conversely, the lowest T cell enrichment rates (40–60-fold) were achieved in donors AJJ, AJU and F59, which were among the donors with the highest precursor frequencies of IE-1-specific T cells in unstimulated PBMCs (0.05–0.15% of PBMCs). This finding might indicate two things: first, a preceding extensive enrichment of IE-1-specific T cells *in vivo* might hamper subsequent strong expansion *in vitro*; second, T cell specificities with low precursor frequencies in PBMCs should still be considered for adoptive T cell therapy because these T cells can be strongly expanded *in vitro,* resulting in similarly high T cell numbers in polyclonal T cell lines as found for T cells with high precursor frequencies. Furthermore, for donors ALT, AJU and F60, the ratios of T cell numbers recognizing different peptide subpools were not maintained during expansion. T cells directed towards peptides in subpools #5 and/or 6 were expanded much more strongly than T cells specific for subpool #7, suggesting that certain T cell specificities possess higher proliferative capacity than others.

In the following, the IE-1 peptides recognized by the polyclonal T cell lines were predicted from the peptide subpool recognition patterns using the peptide cross-matrix (Fig. 2.1). For donors with complex recognition patterns (e.g. F63) due to the presence of T cells against more than one IE-1 peptide, published target peptides could be identified in most cases. IE-1 epitopes have been described for frequent HLA-A and -B allotypes including HLA-A*0201 and -B*1801 which are present in some of the donors analyzed. T cells towards published IE-1 epitopes were confirmed by challenging PBMCs of the donors with the minimal epitope sequences (usually 9 amino acids, data not shown). Table 4.3 gives an overview of the identified IE-1 peptides targeted by the polyclonal T cell lines of the 11 donors tested. Only one IE-1-specific T cell species was found in donors ARZ, AES, F61 and F64. Two equally dominant or one dominant and one subdominant T cell species were identified in donors ALT, AJJ, AJU, F46, F59 and F60. For donor F63, two published IE-1 epitopes were identified, however, the subpool recognition pattern indicated that a third T cell population with so far unknown specificity was also present. In total, four new IE-1 CD8+ T cell epitopes were identified within this study. T cells targeting these IE-1 epitopes were characterized in more detail, as described below, using specific T cell clones established by limiting dilution from the polyclonal T cell lines.


A
unstimulated PBMCs
B
polyclonal T cell line
donor ALT
donor AJJ
donor ARZ
donor AJU
donor AES
donor F46
spots per 200000 cells
spots per 10000 cells
IE-1 subpool #

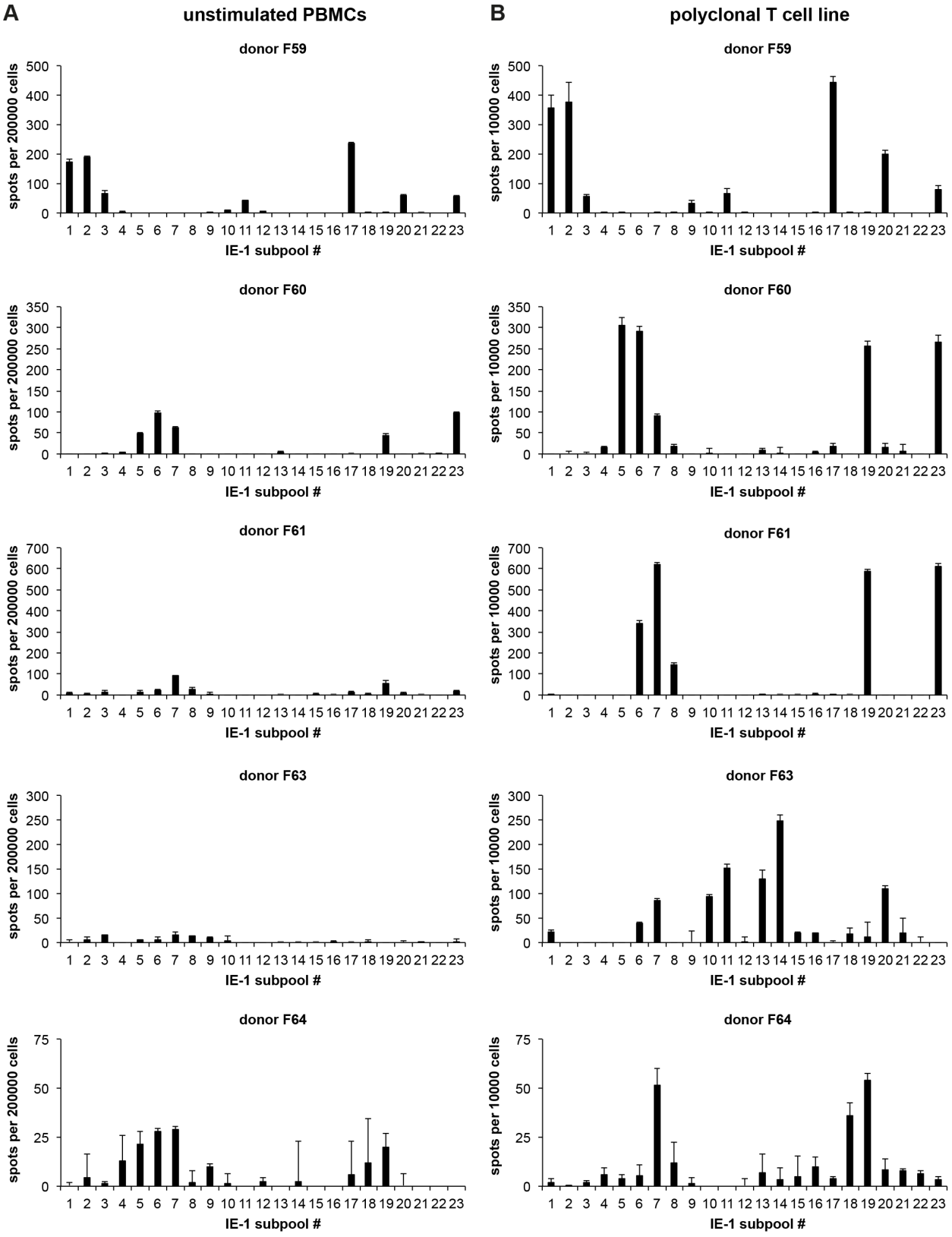


Fig. 4.23: Frequency and epitope specificity of IE-1-specific T cells in PBMCs and polyclonal T cell lines of 11 donors. The unstimulated PBMCs (**A**) and IE-1-stimulated CD8+ polyclonal T cell lines (**B**) of 11 different donors were analyzed for IE-1 reactivity in IFN-γ ELISpot assays. IE-1 epitope-specific T cells were detected using a 15-meric peptide library covering the IE-1 sequence from AD169 and sequence variants from strains Toledo and TB40E. Peptides were subpooled in a cross-matrix orientation ("vertical" subpools: #1–12; "horizontal" subpool: #13–23) and directly loaded on PBMCs (200 000/well) (A) or on autologous CD40-stimulated B cells as APCs (5x 10^4/well) (B). Means and standard deviations of duplicate samples are shown.

Table 4.3: IE-1 CD8+ T cell epitopes recognized by the polyclonal T cell lines of 11 donors. IE-1 peptides targeted by the polyclonal T cell lines were predicted from the peptide subpool recognition patterns of the ELISpot assays (Fig. 4.23) using the peptide cross-matrix (Fig. 2.1). For published epitopes, the references are denoted. Newly identified IE-1 epitopes targeted by the T cells of only one donor or several donors are highlighted in blue or red color, respectively. New IE-1 epitopes were characterized further below in more detail.

donor	target peptides in IE-1	aa position minimal epitope	HLA-restriction	reference
ALT	**VLE**ETSVML	316–324	A*0201	(Khan et al., 2002)
	CRVLCCYVL	309–317	C*0702	this study, section 4.3.2
AJJ	**VLE**ETSVML	316–324	A*0201	(Khan et al., 2002)
	CRVLCCYVL	309–317	C*0702	this study, section 4.3.2
ARZ	VLAELVKQIKVRVDMVRHR	n.d.	C*0602	this study
AJU	**VLE**ETSVML	316–324	A*0201	(Khan et al., 2002)
	CRVLCCYVL	309–317	C*0702	this study, section 4.3.2
AES	ESDEEEAIVAYTLATAGVS	n.d.	n.d.	this study
F46	**CRV**LCCYVL	309–317	C*0702	this study, section 4.3.2
	RHRIKEHMLK	97–106	A*0301	this study
F59	**ELR**RKMMYM	199–207	B*1801	(Retiere et al., 2000)
	DEEEAIVAY	379–387	B*1801	(Retiere et al., 2000)
F60	**VLE**ETSVML	316–324	A*0201	(Khan et al., 2002)
	CRVLCCYVL	309–317	C*0702	this study, section 4.3.2
F61	**VLE**ETSVML	316–324	A*0201	(Khan et al., 2002)
F63	**KEV**NSQLSL	42–50	B*4001	(Khan et al., 2007)
	DEEEAIVAY	379–387	B*1801	(Retiere et al., 2000)
F64	**VLE**ETSVML	316–324	A*0201	(Khan et al., 2002)

Three of the four newly identified IE-1 epitopes within this study were only found in one donor each (ARZ, AES and F46). T cell clones could be established from the polyclonal T cell lines of two of the donors, ARZ and F46, and were used to determine the HLA restriction of the corresponding two new IE-1 epitopes in IFN-γ ELISA assays (Fig. 4.24). The T cell clones were challenged with IE-1- and ctrl-mLCLs of several donors partially matched or not matched for various HLA class I allotypes (Fig. 4.24 A and C). The T cell clone of ARZ recognized only IE-1-mLCLs of donors expressing HLA-C*0602 (Fig. 4.24 A). This HLA restriction could be molecularly confirmed using class I-deficient L721.221 LCLs that were stably transfected with HLA-C*0602, thus expressing HLA-C*0602 as their only HLA class I molecule. These cells were infected with IE-1-expressing modified vaccinia virus Ankara (MVA) and used as T cell targets in an IFN-γ ELISA assay (Fig. 4.24 B). T cell clones of ARZ recognized IE-1 only in context of HLA-C*0602, thus confirming the HLA-C*0602 restriction of these T cells, whereas control-transfected and control MVA-infected targets were not recognized.

The T cell clones of donor F46 showed IE-1-specific IFN-γ secretion only upon stimulation with mLCLs of donors expressing HLA-A*03(01) (Fig. 4.24 C), thus defining HLA-A*03(01) as the IE-1 peptide-presenting HLA allomorph. In addition, these T cell clones showed IE-1-independent recognition of mLCLs of donors ARZ, ASG and F64, which are completely mismatched with F46 in all HLA-A, -B and -C allotypes. This alloreactive T cell recognition appeared to depend on HLA-A*29(02), which was the only HLA molecule shared by these donors and absent in all other donors tested. Cross-reactivity of HCMV-specific T cells against alloantigens is no

unusual phenomenon (Amir et al., 2011). Alloreactive T cells are associated with graft rejection and graft-versus-host disease after HLA-mismatched transplantation. Especially with regard to adoptive T cell therapy in transplant patients, further experiments should be considered to characterize the basis of this alloreactive T cell recognition. For example, it would be interesting to know whether the T cell clones recognize a particular self peptide on HLA-A*29(02) or if the TCR/MHC interaction is peptide-independent. Based on common amino acid residues of well-described HLA-A*03(01)-presented peptides (Hill et al., 1995; Pepperl et al., 1998), the 10-mer RHRIKEHMLK was defined as the minimal T cell epitope.

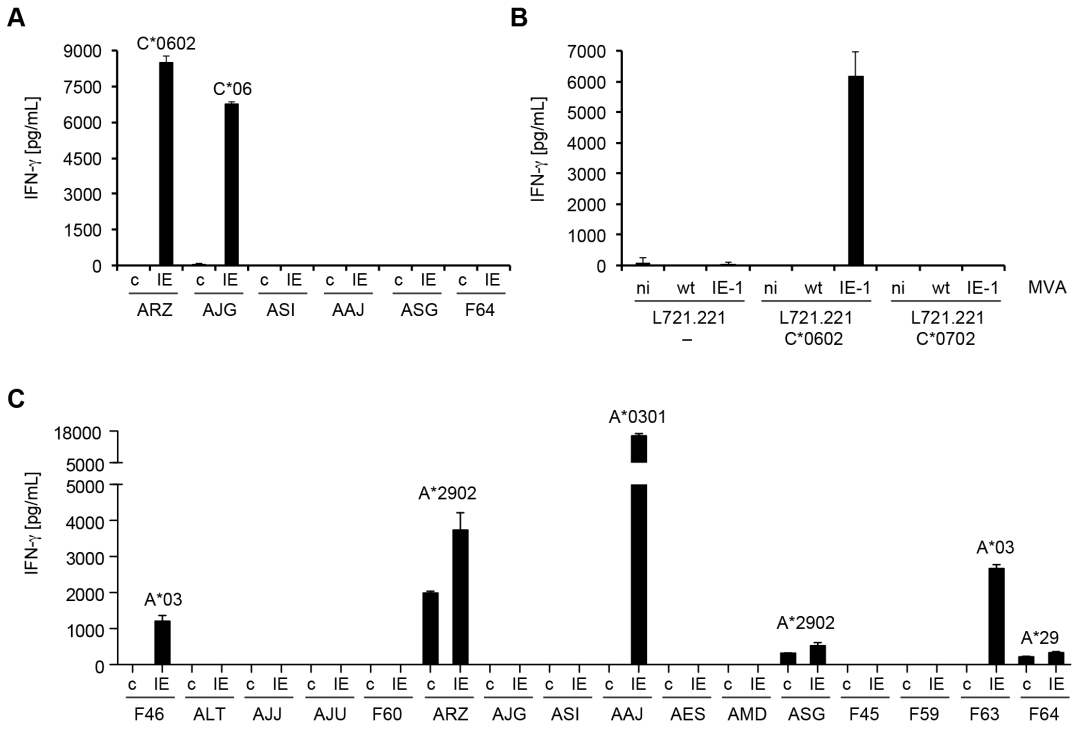


Fig. 4.24: HLA restriction of T cell clones of donors F46 and ARZ. T cell clones of donors ARZ (**A**, clone ARZM#161 VLA) and F46 (**C**, clone F46M#131 RHR) were challenged with IE-1 ("IE")- and ctrl ("c")-mLCLs of several donors partially matched or not matched for different HLA-A, -B and -C allotypes. HLA restriction found in A for the T cell clones of donor ARZ was confirmed by stimulating T cell clones (**B**, ARZM#19 VLA) with L721.221 cells transfected with HLA-C*0602 or C*0702 and infected, or not, with IE-1-expressing modified vaccinia virus Ankara (MVA). For all experiments, 10 000 T cells were incubated overnight with 20 000 target cells and specific IFN-γ secretion was measured by ELISA.

4.3.2 Identification of a dominant HLA-C-restricted T cell species

Interestingly, among the donors with the highest IE-1-specific T cell numbers in the IFN-γ ELISpot assays (Fig. 4.23), 5 donors (ALT, AJJ, AJU, F46 and F60) shared a pattern in which subpools #5, 6, (7), 19 and 23 were dominantly recognized by their polyclonal T cell lines (Fig. 4.23 B). According to the peptide cross-matrix, the IE-1 region containing the T cell epitope comprised individual peptides #76, 77 and 78 and their sequence variants #125, 126 and 127. To define a minimal epitope, these peptides were individually tested for recognition by the

polyclonal T cell line of donor ALT in an IFN-γ ELISpot assay (Fig. 4.25 A). Two earlier described nonameric IE-1 T cell epitopes located in this region were included in the assay: VLEETSVML (VLE, IE-$1_{aa316-324}$) (Khan et al., 2002) and CRVLCCYVL (CRV, IE-$1_{aa309-317}$) (Kern et al., 1999). The VLE epitope was described as A*0201-restricted by Khan et al., 2002 and confirmed as a dominant IE-1 epitope in several studies (Elkington et al., 2003; Khan et al., 2007). The CRV epitope was identified in early studies as a major target of T cells from B*07-positive donors (Kern et al., 1999). In the ELISpot assay, the CRV nonamer and the 15-mers containing it or its variant CRVLCCY**I**L, but not the other peptides tested in the assay, were dominantly recognized by the T cells from donor ALT.

For further analyses, CD8+ T cell clones were established by limiting dilution of IE-1 mini-LCL-stimulated cultures of donors ALT, AJU and F46. Between 200 and 240 clones from each donor were tested for reactivity towards autologous IE-1-mLCL, ctrl-mLCL and CRV nonamer-loaded CD40-stimulated B cells in IFN-γ ELISA assays (data not shown). Of the T cell clones screened from donors ALT, AJU and F46, 96%, 78% and 84% were specific for IE-1, and among these 87%, 24% and 88% were specific for the CRV nonamer. CRV-specific T cell clones from a fourth donor, AJJ, were obtained by overnight stimulation of PBMCs with the CRV peptide, followed by IFN-γ capture-based immunomagnetic isolation and limiting dilution. Using these T cell clones, the minimal epitope was verified in an IFN-γ ELISA assay by comparing the avidity of the T cells against 10-fold diluted candidate peptides, 8 to 10 amino acids in length and loaded on autologous CD40-stimulated B cells (Fig. 4.25 B). The T cell clones reacted more strongly to CRVLCCYVL than to each longer, shorter or shifted peptide, confirming that this peptide was indeed the minimal epitope. Furthermore, the peptide CRVLCCY**I**L, which is the only known existing variant of CRVLCCY**V**L, was recognized with similar sensitivity by the T cell clones tested. CRVLCCY**V**L is present in HCMV strains including AD169, Towne, strain W and Merlin, its variant CRVLCCY**I**L in strains including Toledo, Davis and TB40E (Fig. 4.25 C).

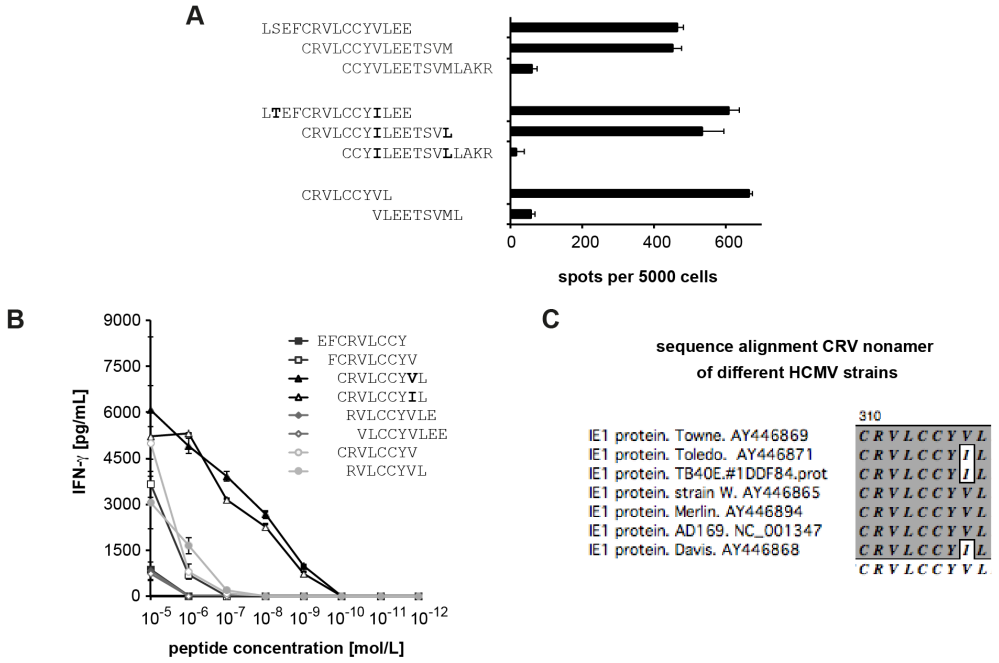


Fig. 4.25: Definition of the CRV nonamer as dominant IE-1 epitope. A: The minimal IE-1 epitope dominantly recognized by the polyclonal T cell line of donor ALT was determined in IFN-γ ELISpot assays. T cells (5000/well) were incubated overnight with autologous CD40-stimulated B cells (50 000/well) loaded with single IE-1-derived 15-aa or 9-aa peptides. Means and standard deviations of triplicate samples are shown. **B:** To confirm the minimal epitope, T cell clones of donor ALT were tested for their reactivity against titrated IE-1 peptides of 8–10 amino acids in length in IFN-γ ELISA assays. 20 000 T cells were incubated overnight with 50 000 autologous peptide-loaded CD40-stimulated B cells per well in duplicates. Data are shown for clone ALTM#127 CRV. **C:** Alignment of the CRV nonameric sequences of different HCMV strains.

The postulated HLA-B*0702 restriction of the CRV epitope was deduced from an observed correlation of CRV peptide-specific T cells with the donors' HLA-B type (Kern et al., 1999). However, subsequent studies observed that the CRV peptide could not be refolded with HLA-B7 in order to produce HLA/peptide multimers (Khan et al., 2004). Indeed, the sequence of the CRV nonamer deviates from confirmed HLA-B*0702-restricted peptides with the latter having a proline (P) in amino acid position two as a conserved anchor residue (Rammensee et al., 1999; Weekes et al., 1999). Therefore, the HLA restriction of the CRV epitope was reconsidered in this study. CRV-specific T cell clones from donors ALT, AJJ, AJU and F46 were challenged in an IFN-γ ELISA assay with IE-1-expressing and ctrl-mLCLs from various donors sharing different HLA alleles with the T cell donors (Fig. 4.26 A). The T cell clones responded only to IE-1-mLCLs coexpressing HLA-B*0702 and -C*0702. Due to the close association of HLA-B*0702 and -C*0702 in the German population, donors with only one of these two HLA alleles were not available. Therefore, the HLA restriction of the CRV epitope was determined by transfecting WI-38 fibroblasts (HLA-B*0702/-C*0702-negative) with plasmids encoding single candidate HLA allotypes, HLA-B*0702 or -C*0702. IE-1 was endogenously expressed in the transfected fibroblasts by infection with IE-1-encoding MVA. As a positive control for recognition in context of HLA-B*0702, a T cell clone specific for the HLA-B*0702-restricted pp65 epitope TPR (Wills et

al., 1996; Weekes et al., 1999) was challenged with transfected fibroblasts infected with MVA-pp65. CRV-specific T cell clones from three different donors recognized IE-1 in the context of HLA-C*0702, but not HLA-B*0702 (Fig. 4.26 B). Hence, the postulated HLA-B*0702-restriction of the CRV epitope has to be considered as an error.

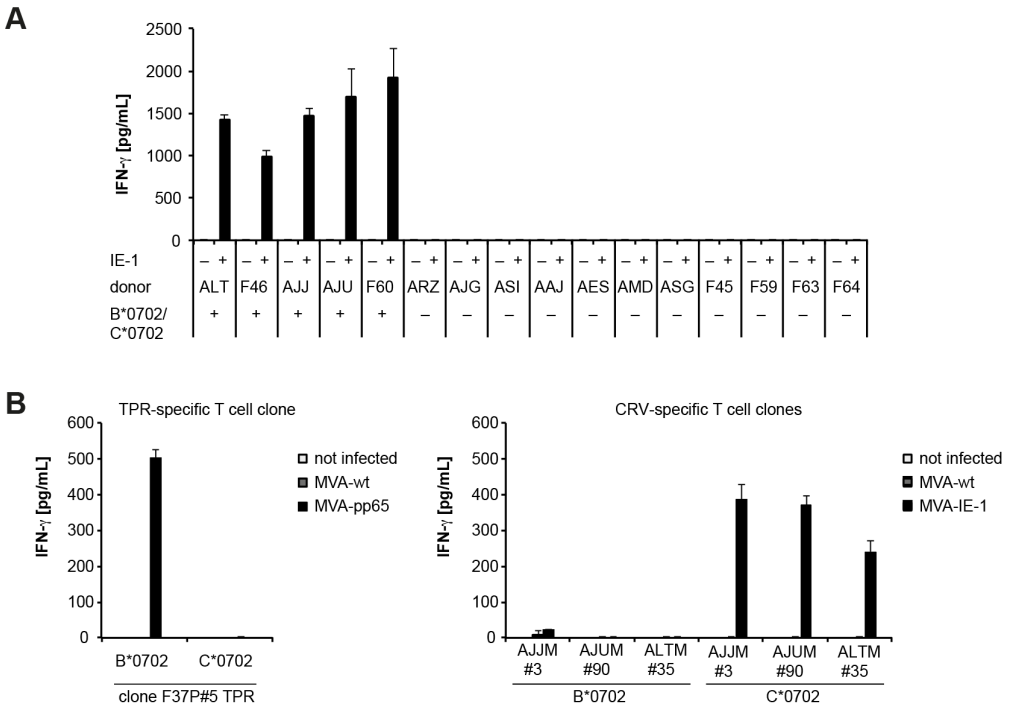


Fig. 4.26: Determination of the HLA restriction of the CRV epitope. A: CRV-specific T cell clones from donors ALT, AJJ, AJU and F46 were challenged in an IFN-γ ELISA assay with IE-1- and ctrl-mLCLs from various donors sharing different HLA alleles with the T cell donors. 10 000 T cells/well were incubated overnight with 20 000 mLCLs/well in two replicates. Data are shown for one representative T cell clone, AJU#90 CRV. **B:** CRV-specific T cell clones of donors AJJ, AJU and ALT (10 000 T cells/well) were tested in an IFN-γ ELISA assay for recognition of the CRV peptide on WI-38 fibroblasts transfected with candidate HLAs, B*0702 or C*0702, and infected with IE-1-encoding MVA (10 000 fibroblasts/well). No infection and wild-type (wt) MVA infection served as negative controls. T cell clone F37#5 specific for the HLA-B*0702-presented pp65 epitope TPR served as positive control for HLA-B*0702 restriction. Means and standard deviations of 3 replicates are shown.

4.3.3 Frequency of HLA-C*0702-specific T cells in PBMCs of HCMV carriers

In the following, the frequency of CRV-specific memory T cells in PBMCs of 15 HLA-C*0702-carrying donors was investigated in IFN-γ ELISpot assays (Fig. 4.27 A). Strikingly, CRV nonapeptide-specific T cells could be detected in all donors tested, with an average of 345 IFN-γ spots (range 29-837 spots) in 200 000 PBMCs. For selected donors, these ELISpot data were correlated with phenotypic analyses using HLA-C*0702/CRV-streptamer complexes obtained from a cooperation with the group of Michael Neuenhahn/Dirk Busch from Technische Universität München (Fig. 4.27 B). T cells directed towards the pp65-derived TPR epitope reported to be immunodominant in HLA-B*0702-positive HCMV carriers (Wills et al., 1996; Weekes et al., 1999; Hebart et al., 2002) were stained for comparison. With 0.74–4.48% of total lymphocytes,

HLA-C*0702/CRV streptamer-positive cells are of high frequency in all three donors tested. The CRV-specific T cell numbers detected by multimer staining exceeded the number of IFN-γ-secreting T cells in the ELISpot assays by several times. HLA-C*0702/CRV streptamer-positive cells were also more frequent than HLA-B*0702/TPR pentamer-positive cells. PBMCs of HCMV-positive/HLA-C*0702-negative donor SA05 and HCMV-negative/HLA-C*0702-positive donor ASM were stained as negative controls and did not show significant multimer-positive T cell populations.

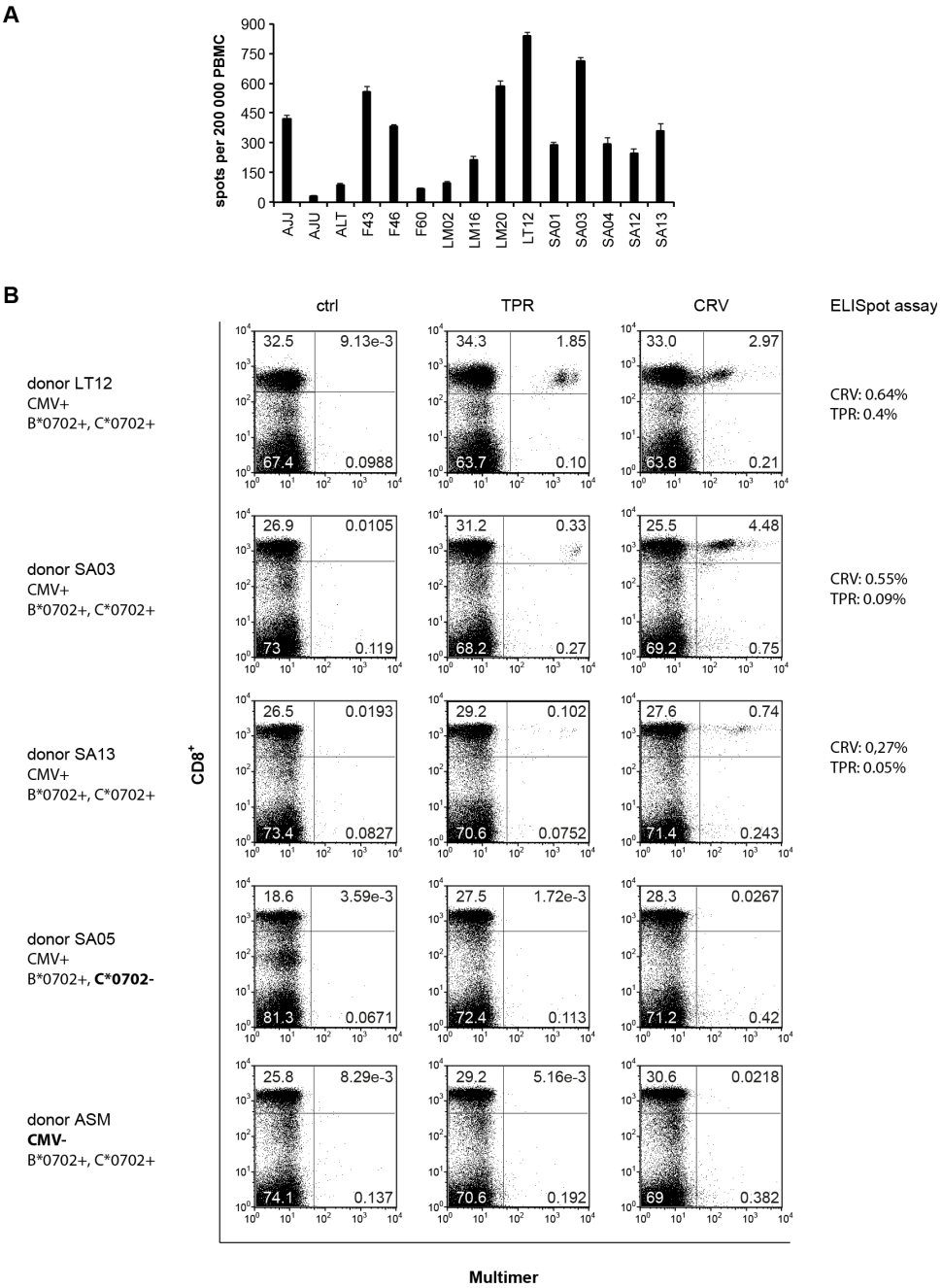


Fig. 4.27: Frequency of CRV-specific T cells in PBMCs. A: CRV nonamer-specific T cells were detected in 15 HCMV-seropositive donors by ELISpot assays (200 000 PBMCs/well in 2–4 replicates). **B:** For selected donors, CRV-specific T cells were quantified by fluorescent staining with HLA-C*0702/CRV streptamer and anti-CD8 antibody. For comparison, HLA-B*0702/TPR pentamer staining was performed for the same donors. For each donor, stainings without multimers (ctrl) served as controls.

For the 15 HLA-C*0702-positive donors, the entire IE-1 sequence was screened for the T cell epitopes recognized by these donors in ELISpot assays using 120 IE-1 AD169 peptides distributed to 10 subpools, each comprising 12 successive 15-mers with 11 aa overlaps (Fig. 4.28 A). In most donors, the highest reactivity was detected against the subpool containing CRV (encompassing IE-1 amino acids 288-347). PBMCs were further tested for recognition of published single IE-1 epitopes matching the donors' HLA types (data not shown). In 11 out of 15 donors, CRV was the dominant IE-1 T cell epitope. In the remaining 4 donors (ALT, AJU, LM02 and LM16), CRV was subdominant, and T cells were mainly directed against VLE (A*0201-restricted) (Khan et al., 2002), DEEEAIVAY (B*1801-restricted) (Gavin et al., 1993; Retiere et al., 2000) or ELKRKMIYM (B*0801-restricted) (Retiere et al., 2000; Saulquin et al., 2000). Six of the 15 HLA-C*0702-positive donors analyzed also expressed HLA-A*0201, thus allowing the comparison of CRV- and VLE-specific T cell numbers (Fig. 4.28 B). With an average of 247 spots per 200 000 PBMCs, CRV-specific T cells were 3-fold more frequent than VLE-specific T cells (average 84 spots). Finally, numbers of total IE-1-reactive T cells were compared between HLA-C*0702-positive and randomly chosen HLA-C*0702-negative donors using a full-length IE-1 15-meric peptide library (Fig. 4.28 C). Total pp65-specific T cell numbers were determined in these donors for comparison. The HLA-C*0702-positive donors exhibited much higher average IE-1-specific T cell numbers, thus confirming the dominance of the HLA-C*0702-restricted epitope CRV within the IE-1-specific T cell repertoire. In contrast, pp65-specific T cells did not significantly differ in number between the two groups of donors.

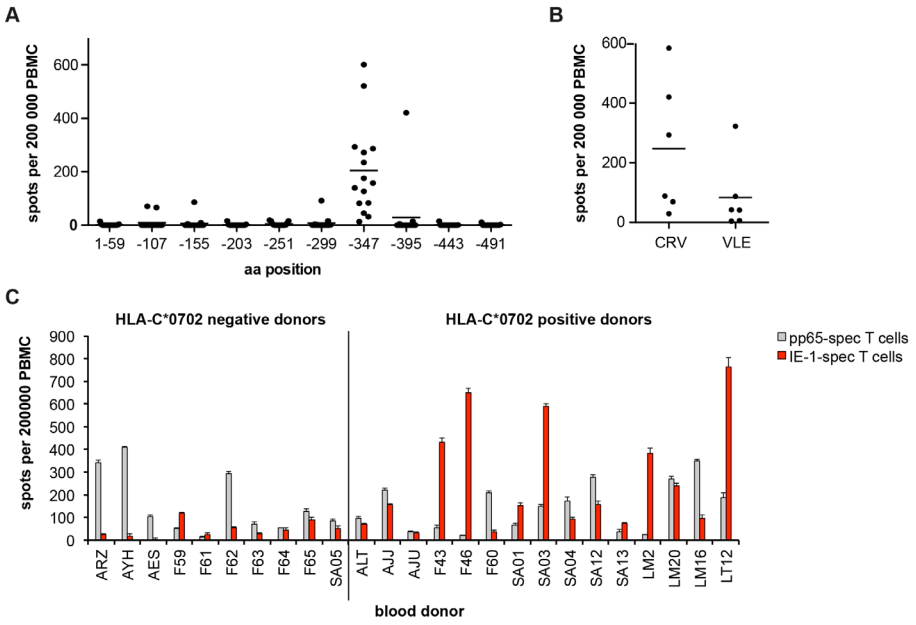


Fig. 4.28: Comparison of IE-1 epitope-specific T cell frequencies in IFN-γ ELISpot assays. A: For 15 HLA-C*0702-positive donors, the IE-1 sequence was assessed for T cell epitopes using 120 IE-1 AD169 peptides distributed to 10 subpools, each comprising 12 successive 15-mers with 11 aa overlaps. The last amino acid position represented in each subpool is indicated. **B:** CRV- and VLE-specific T cell numbers were compared in HLA-C*0702/-A*0201-positive donors. **C:** IE-1-specific T cell frequencies were compared in HLA-C*0702-positive and HLA-C*0702-negative donors. All IFN-γ ELISpot assays were performed with 200 000 peptide-loaded PBMCs/well and with 2-4 replicates per condition.

4.4 T cell recognition of IE-1 in context of an HCMV infection

Despite the identification of IE-1-specific CD8+ T cells in a majority of HCMV carriers (Sylwester et al., 2005), the importance of these T cells has remained doubtful because some studies have described that IE-1-specific CTL recognition of HCMV-infected fibroblasts is strongly impaired *in vitro* (Gilbert et al., 1993; Besold et al., 2007). This observation was mainly attributed to the action of HCMV-encoded immunoevasive proteins that interfere with MHC class I antigen presentation (Reddehase, 2002; Manley et al., 2004). Furthermore, a selective abrogation of IE-1 peptide presentation by the HCMV matrix protein pp65 has been reported (Gilbert et al., 1996). So far, T cell recognition of HCMV-infected fibroblasts was mainly studied with T cell clones directed against described HLA-A*0201-restricted IE-1 epitopes (Khan et al., 2005; Besold et al., 2007; Besold et al., 2009). Therefore, it was now investigated whether recognition of infected fibroblasts by HLA-C*0702-restricted CRV-specific T cells was similarly impaired.

4.4.1 Time course of IE-1 recognition on HCMV-infected fibroblasts by specific CD8+ T cell clones

IE-1 recognition in context of an HCMV infection of fibroblast cells was comparatively analyzed with VLE- and CRV-specific T cell clones in IFN-γ ELISA assays. T cell clones specific for A*0201-restricted epitope NLV from pp65 were tested in parallel. Before infection with HCMV, fibroblasts were cultivated in standard culture medium (Fig. 4.29 A) or in IFN-γ-supplemented medium (Fig. 4.29 B) for 72 hours. The proinflammatory cytokine IFN-γ is abundantly produced during virus defense *in vivo* by CD8+ T cells, Th1 cells and NK cells. It enhances antigen presentation by infected cells and, accordingly, CD8+ T cell recognition, by increasing the expression of TAP components and MHC class I molecules (Benz and Hengel, 2000). *In vitro* T cell recognition experiments on infected fibroblasts provided evidence for an IFN-γ-dependent conditional presentation of an IE-1 peptide of MCMV (Hengel et al., 1994; Geginat et al., 1997). T cell clones were challenged with infected cells pretreated or not pretreated with IFN-γ, at 24, 48, 72 and 96 hours post infection. In accordance with the literature, the structural protein pp65, which is introduced into cells upon infection by viral particles, was strongly recognized by NLV-specific T cells already at 24 hours post infection (McLaughlin-Taylor et al., 1994; Varnum et al., 2004). With IFN-γ pretreatment, recognition of NLV-specific T cells proportionately increased with the amount of virus applied (moi 5 vs. 10). Without IFN-γ pretreatment this effect was not detected at the 24-hour time point, probably because HLA molecules, not antigen, is limiting in this condition. T cell recognition of pp65/NLV decreased over time after infection, which correlates with a progressive impairment of antigen presentation by viral immunoevasins (McLaughlin-Taylor et al., 1994; Besold et al., 2007). T cell recognition of the HLA-A*0201-restricted IE-1 epitope VLE was abolished at all time points post infection and in both pretreatment conditions.

By contrast, the HLA-C*0702-restricted IE-1 epitope CRV was recognized on infected fibroblasts during the whole 96 hours tested. The pretreatment of fibroblasts with IFN-γ led to a particularly strong recognition of CRV in infected cells, similar to peptide-loaded target cells. Furthermore, CRV recognition closely followed the expected kinetics of IE-1 expression in infected fibroblasts (Besold et al., 2009) with a peak at 48–72 hours post infection. Thus, the HLA-C*0702-restricted CRV epitope escapes the mechanisms that prevent the presentation of other IE-1 epitopes in infected cells.

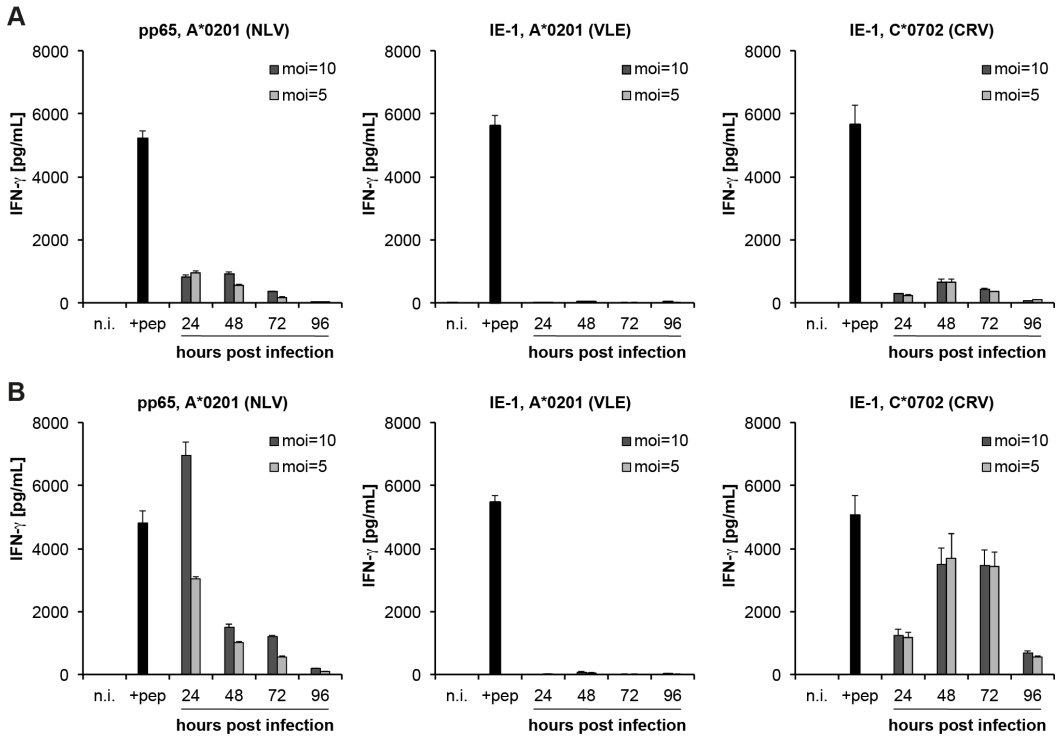


Fig. 4.29: Time course of pp65- and IE-1-specific CD8$^+$ T cell recognition of infected fibroblasts. IE-1 and pp65 T cell epitopes were analyzed for their HLA-A*0201- or C*0702-restricted presentation to T cell clones at 24, 48, 72 and 96 hours post infection by IFN-γ ELISA assay. MRC-5 fibroblasts were (**B**) or were not (**A**) pretreated with IFN-γ 72 hours prior to infection at moi of 5 or 10. At the indicated time points post infection, 10 000 fibroblasts were incubated with 10 000 T cells for 16–18 hours. Not infected (n.i.) and peptide-loaded (+pep) cells served as controls at each time point and are depicted for 48 hours post infection. Means and standard deviations of triplicate samples are shown. One out of three independent experiments with clones ALTP#21 NLV, F61M#38 VLE and AJJM#7 CRV is shown. Experiments were performed with a total of 3 NLV-, 6 VLE- and 12 CRV-specific T cell clones, from 3 different donors for each specificity.

To exclude that the observed differential recognition of NLV, VLE and CRV epitopes was due to avidity differences of the specific T cell clones, peptide titration experiments were performed (Fig. 4.30). In IFN-γ ELISA assays, NLV-, VLE- and CRV-specific T cell clones were challenged with autologous or HLA-matched CD40-stimulated B cells loaded with serially 10-fold diluted NLV, VLE and CRV peptides. Among the T cells tested, the NLV-specific T cell clones showed lowest avidity for their target peptides, with a recognition of NLV peptide down to 10^{-8} mol/L. VLE- and CRV-specific T cell clones showed similarly high avidity for their target peptides with

recognition down to 10^{-9} mol/L. Hence, avidity differences of the T cell clones were not responsible for the observed differential T cell recognition of HCMV-infected fibroblasts.

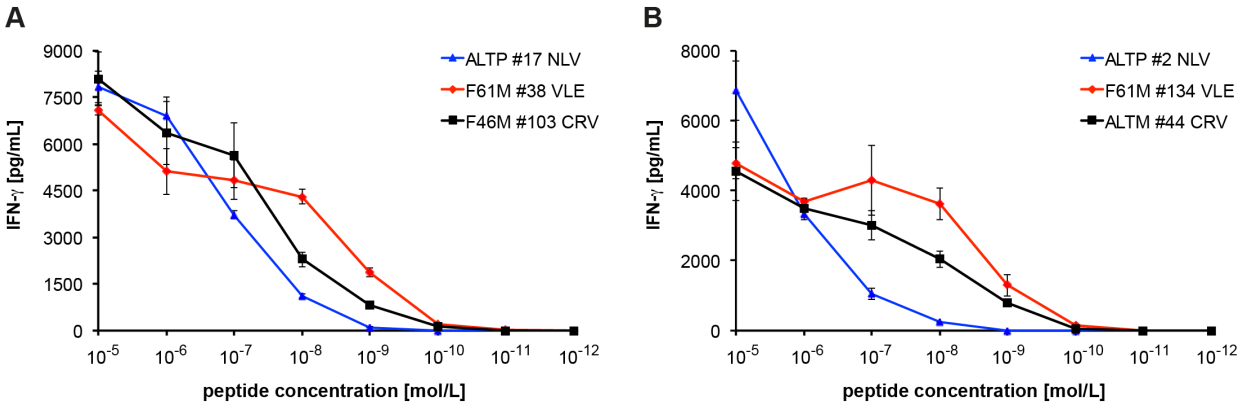


Fig. 4.30: Avidities of NLV-, VLE- and CRV-specific T cell clones for their target peptides. Two sets of NLV-, VLE- and CRV-specific T cell clones (**A** and **B**) were compared for their reactivity against 10-fold diluted target peptides in IFN-γ ELISA assays. T cells (10 000/well) were incubated overnight with CD40-stimulated B cells (20 000/well) loaded with their target peptides. Means and standard deviations of three replicates are shown.

4.4.2 Influence of pp65 on T cell recognition of IE-1

Early studies described that a pp65-associated kinase activity, potentially polo-like kinase 1 (Gallina et al., 1999), selectively blocks presentation of IE-1-derived peptides, associated with phosphorylation of the IE-1 protein (Gilbert et al., 1996). However, most of their experiments employed overexpression of the two HCMV proteins by vaccinia vectors, not genuine HCMV infection. The mechanism by which phosphorylation of IE-1 purportedly interferes with T cell recognition is unclear. Gilbert and colleagues suggested that the modification of IE-1 might limit its access to the antigen processing machinery (Gilbert et al., 1996). To test whether pp65 interferes with VLE and CRV peptide presentation to T cells, VLE- and CRV-specific T cell clones were challenged with fibroblasts infected with a pp65-deleted HCMV strain or wild-type HCMV. Not infected and peptide-loaded fibroblasts served as controls. NLV-specific T cell clones were used to verify presence or absence of the pp65 protein. IFN-γ secretion and direct cytotoxicity were analyzed as effector functions of the T cell clones by ELISA (Fig. 4.31 A) or calcein-release assay (Fig. 4.31 B), respectively. NLV-specific T cell clones recognized target cells only in the context of wild-type HCMV infection. Concerning IE-1 recognition, deletion of pp65 did not restore VLE recognition nor enhance CRV recognition by specific T cell clones. Hence, pp65 is not responsible for the observed differential recognition of IE-1 by VLE- and CRV-specific T cell clones.

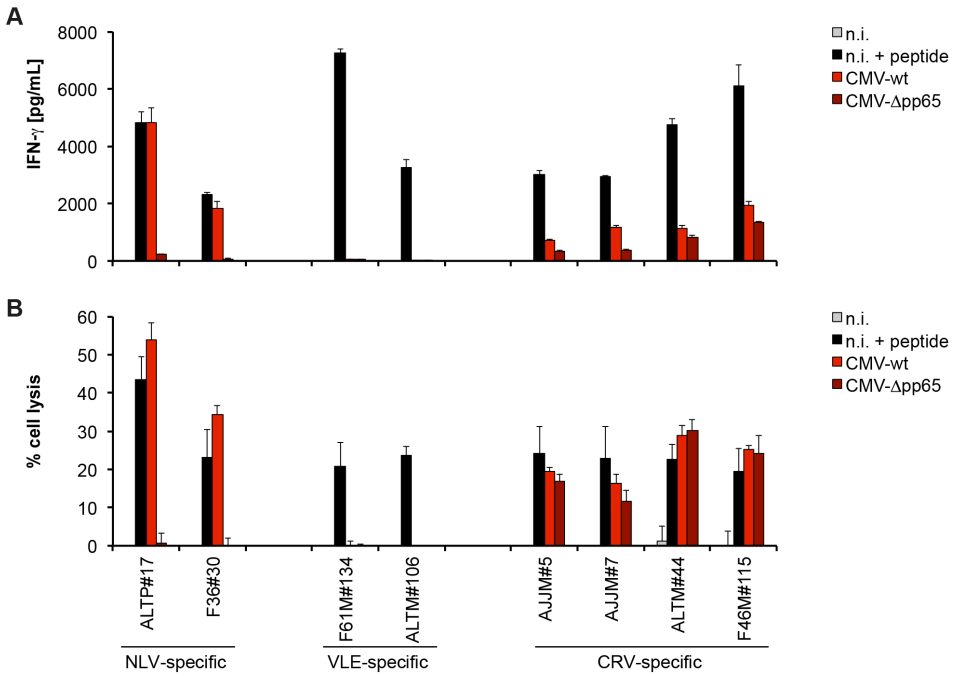


Fig. 4.31: Evaluation of a possible interference of pp65 with IE-1 presentation. To evaluate the influence of pp65 on IE-1 presentation, VLE- and CRV-specific T cell clones, and for comparison pp65 NLV-specific T cell clones, were challenged with IFN-γ-pretreated fibroblasts infected with a pp65-deleted HCMV strain or wild-type HCMV for 24 hours. Not infected and peptide-loaded fibroblasts served as controls. IFN-γ secretion and direct cytotoxicity were analyzed by ELISA (**A**, 10 000 T cells and 10 000 target cells per well in 3 replicates) and calcein-release assay (**B**, 5000 target cells and 20 000 T cells per well in 5 replicates), respectively.

Gilbert et al. hypothesized that phosphorylation of IE-1 was responsible for abrogation of IE-1 presentation by preventing IE-1 protein from being processed to generate appropriate HLA class I peptides (Gilbert et al., 1996). Alternatively, amino acids that are part of the T cell epitope peptides themselves could be phosporylated, and this might affect peptide binding to HLA class I or T cell recognition. Indeed, the VLE nonamer (VLEE**TS**VML) contains two potential phosphorylation sites, a threonine at position five and a serine at position six. These residues are plausible target sites of Polo-like kinases, which prefer acidic residues at the –2 position relative to the phosphorylation site (Nakajima et al., 2003). Therefore, the recognition of phosphorylated and not phosphorylated VLE peptide by VLE-specific T cell clones was comparatively assessed in peptide titration studies (Fig. 4.32). The reactivity of VLE-specific T cell clones against CD40-stimulated B cells loaded with the VLE variants in different concentrations was analyzed in IFN-γ ELISA assays. All VLE-specific T cell clones tested showed similar avidity to VLE and its phosphovariants. This result indicates that phosphorylation of the VLE peptide, whether mediated by pp65 or not, is not the mechanism responsible for impairing VLE peptide presentation.

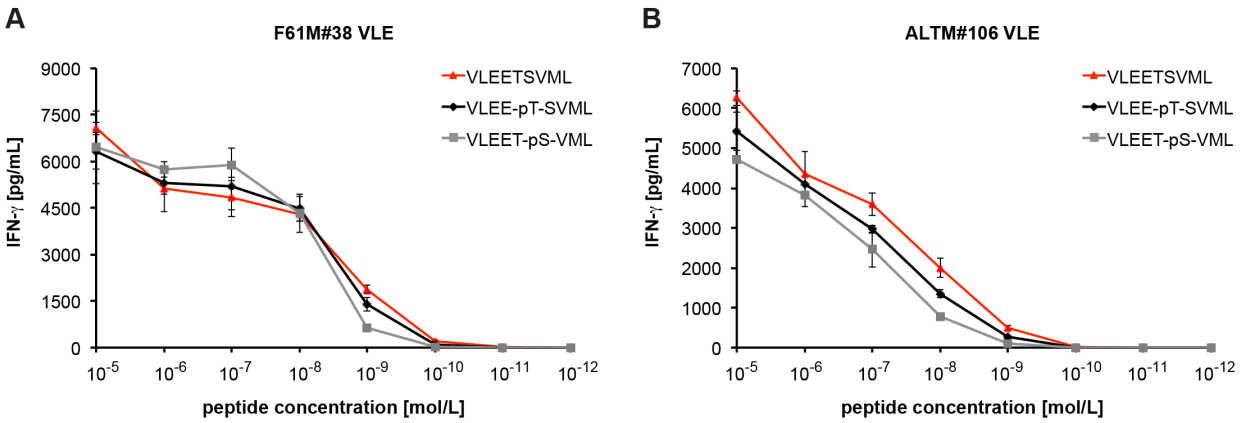


Fig. 4.32: Avidity of VLE-specific T cell clones for phosphorylated target peptides. Two VLE-specific T cell clones (**A** and **B**) were tested for their reactivity against VLE peptide and phosphorylated VLE variants in IFN-γ ELISA assays. CD40-stimulated B cells were loaded with 10-fold diluted peptides. Excessive peptides were washed away in order to prevent dephosphorylation of free peptides by serum phosphatases and subsequent presentation to T cells. 10 000 T cells/well were incubated overnight with 20 000 target cells/well. Means and standard deviations of three replicates are shown.

4.4.3 Influence of HCMV immunoevasins on T cell recognition of IE-1

HCMV encodes at least four immunoevasive proteins, gpUS2, 3, 6 and 11, that prevent the presentation of viral peptides by MHC class I molecules to CD8+ T cells (Manley et al., 2004; Khan et al., 2005; Besold et al., 2009). Each of these immunoevasins acts via downregulation of MHC class I surface expression, however, by different molecular mechanisms: gpUS6 interferes with peptide transport into the endoplasmatic reticulum (ER), gpUS3 retains MHC class I molecules in the ER, and gpUS2 and 11 target MHC class I heavy chains for cytoplasmic degradation (Ahn et al., 1996; Jones et al., 1996; Wiertz et al., 1996a; Wiertz et al., 1996b; Ahn et al., 1997). In the following experiments, the influence of gpUS2–11 on the presentation of IE-1 peptides by different HLA allomorphs was investigated in detail for a larger panel of allomorphs than previously studied.

First, the time course of HLA class I expression on the surface of infected fibroblasts was reevaluated by flow cytometry (Fig. 4.33). Antibodies were available against total HLA-A/B/C as well as HLA-A2 and -B7 which are expressed on MRC-5 fibroblasts in addition to HLA-C*0702. Specific mean fluorescent intensities (MFIs) were generally higher after IFN-γ pretreatment (data not shown) confirming an enhancement of HLA expression under this condition. To assess specific effects of HCMV immunoevasins, HLA expression data were normalized to the data obtained after infection with an HCMV mutant deleted for the four HLA class I immunoevasins gpUS2, 3, 6, and 11 (CMV-Δall) (Besold et al., 2009). Infection with wild-type HCMV reduced HLA-A2 and HLA-B7 expression to very low levels, irrespective of pretreatment with IFN-γ. In contrast, total HLA-A/B/C expression was less strongly reduced, suggesting that certain HLA molecules, potentially including HLA-C*0702, are more resistant to HCMV-mediated

downregulation than HLA-A2 and -B7. Unfortunately, an HLA-C7-specific antibody was not available to confirm this hypothesis.

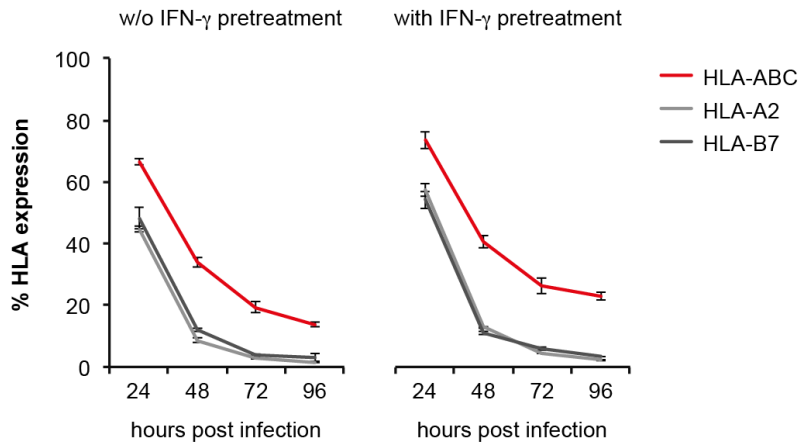


Fig. 4.33: Time course of MHC class I surface expression of infected fibroblasts. HLA-A/B/C, HLA-A2 and HLA-B7 surface expression on infected fibroblasts was analyzed over time by flow cytometry. MRC-5 cells that had or had not been pretreated with IFN-γ for 72 hours were infected with CMV-wt or CMV-Δall (US2/3/6/11 deleted) at an moi of 5. The specific mean fluorescence intensities (MFIs) were calculated by subtracting the MFIs of isotype controls from the MFIs of specific stainings. Values were standardized to the specific MFIs of CMV-Δall-infected cells set to 100%. Means and standard deviations of three (HLA-ABC and -A2) or two (HLA-B7) independent experiments are shown.

In previous studies, deletion of gpUS2–11 from HCMV AD169 restored efficient HLA-A*0201-restricted IE-1 peptide presentation to specific T cell clones (Khan et al., 2005; Besold et al., 2007). However, comparative analyses with IE-1-specific T cell clones of different HLA restrictions have not been performed so far. Therefore, the influence of gpUS2–11 on the T cell recognition of IE-1 epitopes presented by different HLA class I allomorphs was studied in the next experiment (Fig. 4.34). T cell clones of four HLA restrictions other than HLA-A*0201 and -C*0702 were established from IE-1-mLCL-stimulated polyclonal T cell cultures by limiting dilution: the epitopes presented by HLA-A*6801 (ATT), B*0801 (QIK) and B*4001 (KEV) were already described (Elkington et al., 2003; Khan et al., 2007); the A*0301 epitope was identified within this study (section 4.3.1). IFN-γ pretreated fibroblast cells were infected with wild-type HCMV or CMV-Δall and subjected to T cell recognition 48 hours post infection. Not infected and peptide-loaded cells served as controls. T cell recognition was measured by IFN-γ ELISA. Among the 15 T cell clones with 6 different HLA restrictions, only the four HLA-C*0702-restricted clones showed a distinct reactivity to fibroblasts infected with wild-type HCMV. In contrast, CMV-Δall-infected cells were recognized by all T cell clones. T cell reactivities against CMV-Δall were very similar to reactivities against target cells exogenously loaded with peptide, suggesting that CMV-Δall was devoid of any relevant immunoevasive function. In summary, viral immunoevasins completely blocked antigen presentation by each of the HLA-A and HLA-B alleles tested, but not by HLA-C*0702.

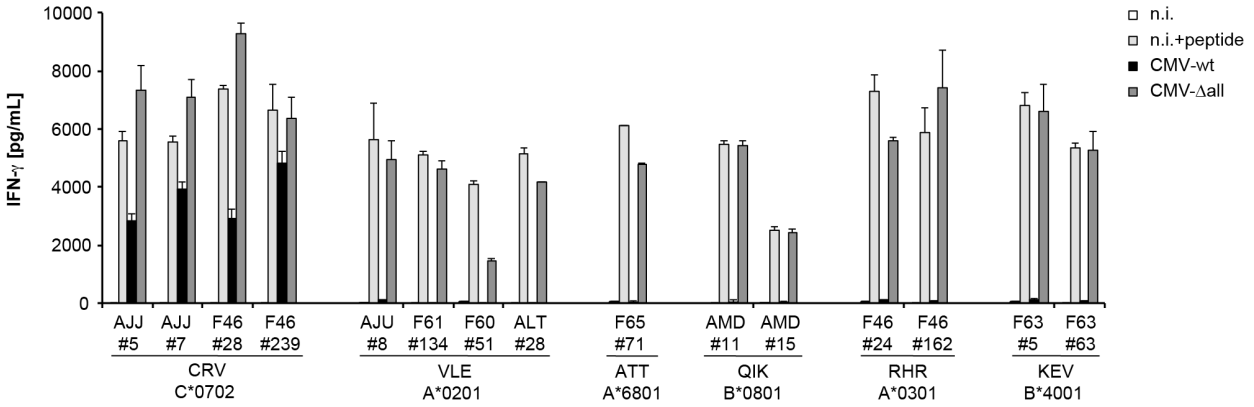


Fig. 4.34: Impact of HCMV immunoevasins on the T cell recognition of IE-1 epitopes presented by different HLA allomorphs. IE-1-specific T cell clones with different HLA restrictions were used in IFN-γ ELISA assays to analyze the influence of gpUS2–11 on IE-1 recognition. To cover every required HLA allotype, three primary fibroblast cell lines were used: MRC-5 for CRV (C*0702) and VLE (A*0201), WI-38 for ATT (A*6801) and QIK (B*0801), BFF2 for RHR (A*0301) and KEV (B*4001). Fibroblasts were pretreated with IFN-γ for 72 hours and infected with CMV-wt or CMV-Δall at moi = 5 for 48 hours. Not infected (n.i.) and peptide-loaded (n.i.+ peptide) cells served as negative and positive controls, respectively. 10 000 fibroblasts were incubated overnight with 10 000 T cells per well in triplicates. One to four representative T cell clones of each epitope specificity are shown from one out of three independent experiments.

4.5 Allotype-specific action of HCMV immunoevasins

As shown in the previous section, recognition of IE-1 epitopes by specific T cell clones of different HLA-A and -B restrictions is severely suppressed by the action of HCMV immunoevasins gpUS2–11, even after pretreatment of cells with IFN-γ. By contrast, CRV-specific T cell recognition is largely intact, suggesting that HLA-C*0702 is spared by individual immunoevasins. Several studies have addressed HLA class I allele-specific effects of HCMV immunoevasins, in particular of US2 and US11 (Schust et al., 1998; Barel et al., 2006). However, most of these studies have been limited to molecular or phenotypic investigations of transfected or transduced cell systems. Consequently, the impact of observed HLA class I allotype-specific effects of individual immunoevasins on the T cell recognition of infected cells has remained unexplored.

Therefore, this study aimed at providing a first systematic analysis of HLA class I allotype-specific effects of individual immunoevasins by using a set of HCMV strains, each deleted for three of the four virus-encoded immunoevasins US2, US3, US6 and US11. CD8+ T cell clones recognizing IE-1- and pp65-derived peptides on different HLA allomorphs were challenged with fibroblasts infected with the HCMV deletion mutants. In addition to investigate their allele-specific action, the following part of this work was also dedicated to characterize HCMV class I-specific immunoevasins in terms of molecular target structures, cooperation among each other, specialization for different phases of the viral replication cycle and compensation of their effects by IFN-γ.

4.5.1 Glycoproteins US2 and US11

The viral early proteins gpUS2 and gpUS11 both independently target HLA class I molecules for proteasomal degradation (Wiertz et al., 1996a; Wiertz et al., 1996b; Jones and Sun, 1997) but differ in their specificity for HLA class I alleles (Schust et al., 1998; Barel et al., 2003a; Barel et al., 2003b). In the following experiments, HLA class I allotype-specific patterns of US2- and US11-mediated interference with CD8+ T cell recognition were determined (Fig. 4.35). Fibroblasts pretreated (Fig. 4.35 B) or not pretreated (Fig. 4.35 A) with IFN-γ were infected with HCMV strains expressing only US2 (CMV-US2) or only US11 (CMV-US11), but not the other three proteins of the US2/3/6/11 group (Besold et al., 2009), and used to challenge CD8+ T cell clones with different HLA restrictions in IFN-γ ELISA assays. The analyses included T cell clones recognizing IE-1-derived peptides presented by HLA-A*0201 (VLE), C*0702 (CRV), A*6801 (ATT), B*0801 (QIK), A*0301 (RHR) and B*4001 (KEV). The recognition of IE-1 by T cell clones restricted through B*0801 was not impaired under both pretreatment conditions when US2 or US11 was present. HLA-B*4001- and C*0702-dependent presentation was also largely intact after IFN-γ pretreatment. Without IFN-γ pretreatment, HLA-C*0702-mediated T cell recognition was moderately reduced by US2 and US11, with some differences between the four CRV-specific T cell clones tested. HLA-A*0201-restricted recognition was fully abolished for most T cell clones by each of the immunoevasins US2 and US11 and in both pretreatment conditions. The allomorphs HLA-A*0301, A*6801 and B*4001 were differentially affected by US2 and US11: A*6801 and B*4001 were more strongly affected by US2, A*0301 by US11. As expected, the impairment of T cell recognition was more pronounced without IFN-γ pretreatment for all three allomorphs.

These ELISA data were compatible with HLA expression data obtained by surface staining of HLA-A/B/C, A2 and B7 (Fig. 4.33 and 4.36). Each of US2 and US11 alone led to a strong downregulation of HLA-A2 surface expression in infected MRC-5 fibroblasts (Fig. 4.36), indistinguishable from wild-type HCMV-infected cells. By contrast, surface expression of an MRC-5-encoded HLA-B allele, HLA-B7, and of total HLA-A/B/C was less affected by US2 and US11. For all HLA allomorphs investigated, the decrease in the relative expression levels was largely independent of pretreatment with IFN-γ. However, the specific mean fluorescent intensities (MFIs) obtained for IFN-γ-pretreated cells (data not shown) were generally higher compared to untreated cells confirming an enhancement of HLA expression under this condition. These higher MHC class I expression levels appeared to compensate for some effects of US2 and US11 (Fig. 4.35 B; C*0702, B*4001, A*0301). In summary, infection with CMV-wt strongly reduced HLA-A2 and -B7, but less so total HLA-A/B/C indicating that MHC allomorphs other than HLA-A2 and -B7 are more resistant to HCMV immunoevasins.

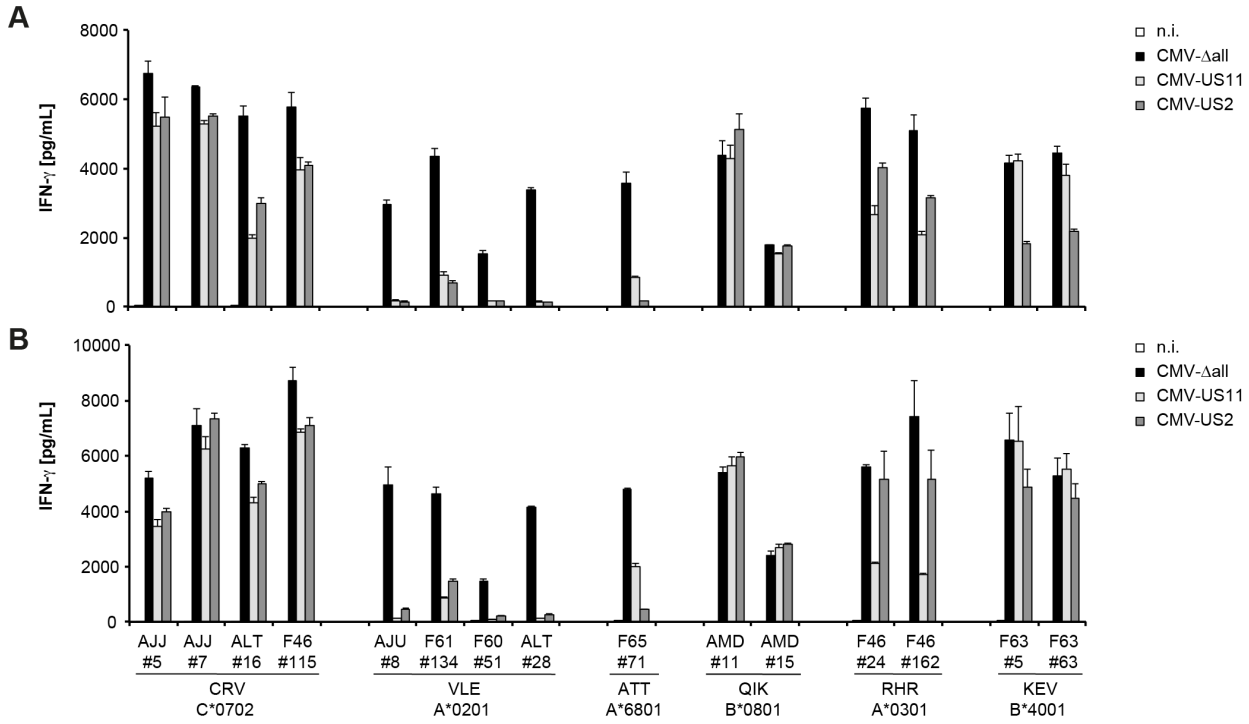


Fig. 4.35: Impact of HCMV immunoevasins US2 and US11 on the recognition of IE-1 T cell epitopes with different HLA restrictions. IE-1-specific T cell clones were used in IFN-γ ELISA assays to analyze antigen recognition on fibroblast cells infected with immunoevasion-defective HCMV strains CMV-Δall (US2/3/6/11 deleted), CMV-US2 (US3/6/11 deleted) and CMV-US11 (US2/3/6 deleted). To cover every required HLA allotype, three primary fibroblast cell lines were used: MRC-5 for CRV (C*0702) and VLE (A*0201), WI-38 for ATT (A*6801) and QIK (B*0801), BFF2 for RHR (A*0301) and KEV (B*4001). Fibroblasts were pretreated with IFN-γ for 72 hours (**B**) or not pretreated (**A**) prior to infection with the different HCMV strains at moi = 5 for 48 hours. Not infected (n.i.) fibroblasts served as controls. 10 000 fibroblasts were incubated overnight with 10 000 T cells per well in triplicates. One to four representative T cell clones of each epitope specificity are shown from one out of three independent experiments.

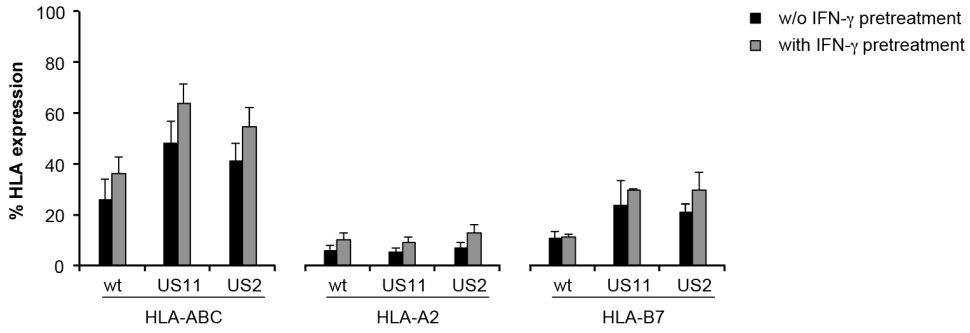


Fig. 4.36: Impact of US2 and US11 on HLA class I surface expression of infected fibroblasts. HLA class I expression was analyzed by flow cytometry of HCMV-infected MRC-5 cells that had or had not been pretreated with IFN-γ for 72 hours before infection. MRC-5 cells were infected at moi = 5 with wild-type HCMV ("wt") or recombinant viruses CMV-US2 (US3/6/11-deleted, "US2") and CMV-US11 (US2/3/6-deleted, "US11") for 48 hours. The specific mean fluorescence intensities (MFIs) were calculated by subtracting the MFIs of isotype controls from the MFIs of specific stainings. Values were standardized to the specific MFIs of CMV-Δall (US2/3/6/11 deleted) infected cells set to 100%. Means and standard deviations of two (HLA-B7 and -A2) or three (HLA-ABC) independent experiments are shown.

Furthermore, HLA allomorph-specific effects of US2 and US11 on target cell killing, another important effector function of CD8+ T cells, were analyzed by calcein-release assay (Fig. 4.37). MRC-5 fibroblasts pretreated with IFN-γ (Fig. 4.37 B) or not pretreated (Fig. 4.37 A) were infected with wild-type HCMV or mutant viruses deleted for all immunoevasins (CMV-Δall) or expressing only US11 (CMV-US11) or US2 (CMV-US2). Not infected and peptide-loaded fibroblasts served as controls. T cell clones were specific for NLV/pp65 (HLA-A*0201), VLE/IE-1 (HLA-A*0201) and CRV/IE-1 (HLA-C*0702). All T cell clones efficiently killed fibroblasts infected with CMV-Δall or loaded with peptide. HLA-C*0702-restricted IE-1-specific T cell clones showed unimpaired lysis of cells infected with CMV-US2 or CMV-US11, and were capable to lyse wild-type HCMV-infected cells under both pretreatment conditions. By contrast, HLA-A*0201-restricted IE-1-specific T cell clones only marginally lysed wild-type HCMV, CMV-US2 or CMV-US11-infected cells. As expected (Fig. 4.29), T cells specific for pp65 (NLV) were capable of killing wild-type HCMV-infected cells, however, to a lesser extent than peptide-loaded cells when the fibroblasts were not pretreated with IFN-γ. In contrast to HLA-A*0201-restricted VLE-specific T cells, A*0201-restricted NLV-specific T cell killing of infected cells was not significantly affected by either US2 and US11, which might be due to the high abundance of pp65 in infected fibroblasts before *de novo* expression of viral genes including US2 and US11. In summary, allotype-specific interference with T cell recognition of the IE-1 antigen similarly affected cytokine secretion and T cell-mediated killing.

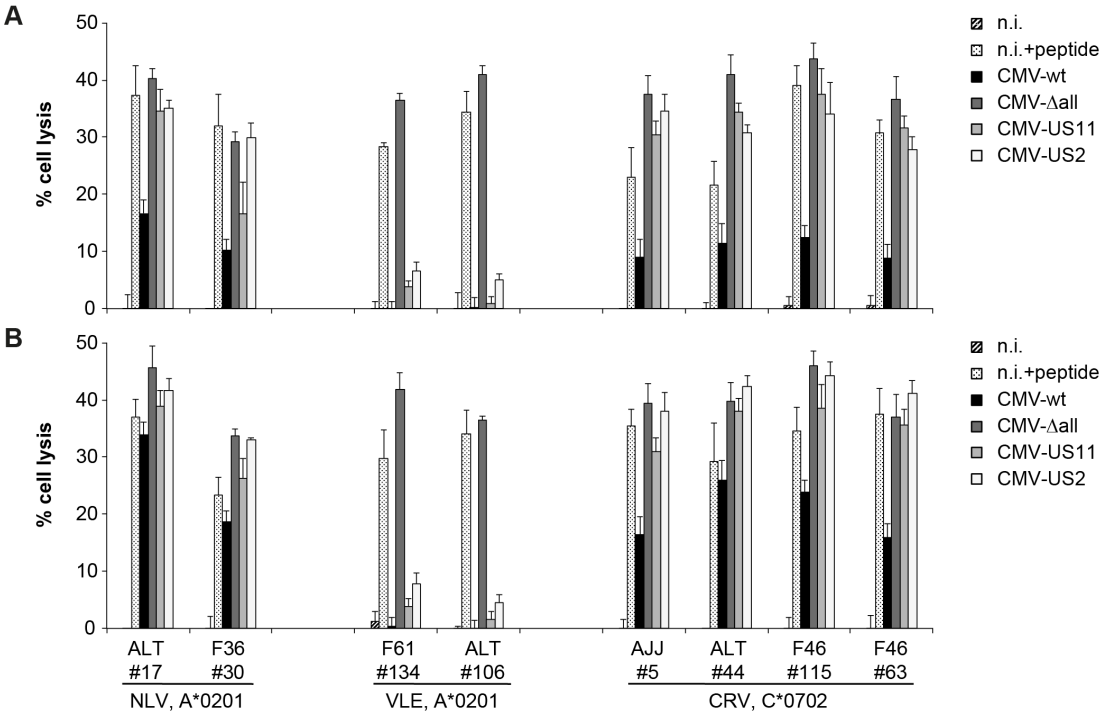


Fig. 4.37: Effect of HCMV immunoevasins on IE-1 epitope-specific T cell cytotoxicity. MRC-5 fibroblasts were pretreated with IFN-γ for 72 hours (**B**) or not pretreated (**A**) and infected at moi = 5 with viral strains CMV-wt, CMV-Δall (US2/3/6/11 deleted), CMV-US11 (US2/3/6 deleted) or CMV-US2 (US3/6/11 deleted). Cytotoxicity was determined at 48 hours post infection in a calcein-release assay using an effector/target ratio of 4. Fibroblasts that were not infected (n.i.) or peptide-loaded (n.i.+peptide) were negative and positive controls, respectively. Means and standard deviations of three to four replicates are shown.

4.5.2 Function of domains of HLA class I molecules in immunoevasion

The T cell experiments showed that HLA-C*0702- and A*0201-mediated antigen presentation were affected to a very different degree by US2 and US11. These viral glycoproteins target MHC class I molecules for intracellular degradation by direct interactions with different parts of the MHC heavy chain: US2 interacts with amino acids near the boundary of the HLA α_2 and α_3 domains, US11 with regions in the α_1/α_2 domains and, more importantly, in the cytosolic tail of the HLA molecule (Barel et al., 2003a; Barel et al., 2006). The aim of the following experiment was to assess whether antigen presentation to CD8+ T cells, and destabilizing interactions with HCMV immunoevasins, were mediated by separate domains of HLA class I molecules. To this purpose, chimeric HLA class I heavy chains containing the N-terminal part of one of these HLA allomorphs (comprising the α_1/α_2 domains that present antigen) and the C-terminal part of the other allomorph (comprising the α_3 domain, transmembrane region and cytosolic tail, supposedly targeted by viral destabilization) were constructed. To test the functionality of the constructs, WI-38 fibroblasts, which are negative for HLA-A*0201 and -C*0702, were transfected with plasmids encoding parental or chimeric HLA, infected with MVA-IE-1, and tested for recognition by IE-1-specific T cells in an IFN-γ ELISA assay (Fig. 4.38). The HLA-A*0201-restricted VLE peptide was only recognized on HLA-A*0201 and chimeric HLA-A2/C7 (A*0201 N-terminus, C*0702 C-terminus) (Fig. 4.38 A), whereas the HLA-C*0702-restricted CRV peptide was only recognized on HLA-C*0702 and chimeric HLA-C7/A2 (C*0702 N-terminus, A*0201 C-terminus) (Fig. 4.38 B), confirming that the chimeric molecules had full capability to present antigen.

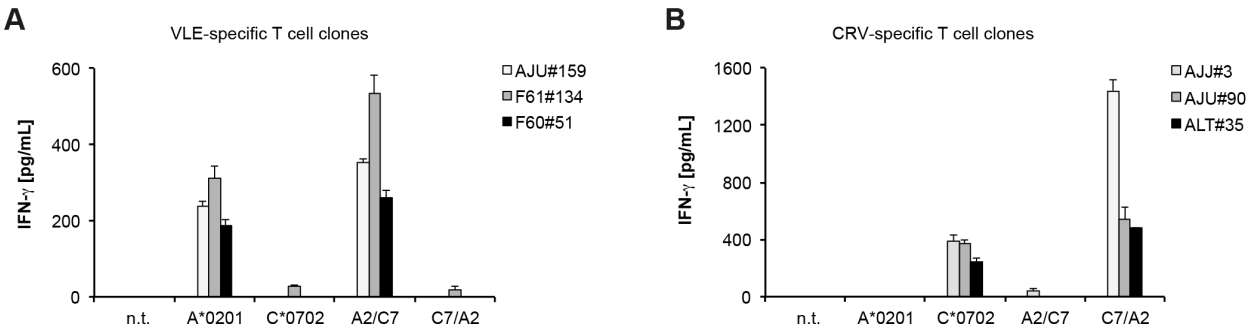


Fig. 4.38: Functional evaluation of chimeric HLA class I molecules. WI-38 fibroblasts (HLA-A*0201/C*0702-negative) were transfected with plasmids encoding HLA-A*0201, HLA-C*0702, or chimeric HLA class I heavy chains HLA-A2/C7 (A*0201 N-terminus, C*0702 C-terminus) or HLA-C7/A2 (C*0702 N-terminus, A*0201 C-terminus), and subsequently infected with MVA-IE-1 at moi = 10 for 24 hours. Presentation of IE-1 epitopes was detected by VLE- and CRV-specific T cell clones in an IFN-γ ELISA assay (10 000 T cells and 10 000 target cells per well). Means and standard deviations of three replicates are shown for 3 T cell clones generated from 3 different donors for each specificity.

Then, the effects of US2 and US11 on antigen presentation by native and chimeric molecules were tested in the context of an HCMV infection (Fig. 4.39). Again, WI-38 fibroblasts were trans-

fected with plasmids encoding parental or chimeric HLAs, infected with CMV-wt, CMV-Δall, CMV-US11 or CMV-US2 and used to stimulate VLE- and CRV-specific T cell clones in an IFN-γ ELISA assay. As expected, T cell recognition of the HLA-A*0201 and HLA-C*0702 epitopes was differentially affected by different HCMV strains in cells transfected with native MHC class I genes: recognition of A*0201/VLE was strongly reduced by wild-type HCMV, CMV-US2, or CMV-US11, whereas recognition of C*0702/CRV was not impaired by CMV-US2 or CMV-US11 and only partially by wild-type HCMV infection. Strikingly, in the chimeric HLA-A2/C7 or -C7/A2 molecules a complete exchange of the patterns of sensitivity to HCMV immunoevasins was observed. Thus, while the α_1/α_2 domains present antigen to T cells, the C-terminal part including the α_3 domain largely governs the sensitivity of MHC class I molecules to HCMV immunoevasins, and these functions are separable and exchangeable.

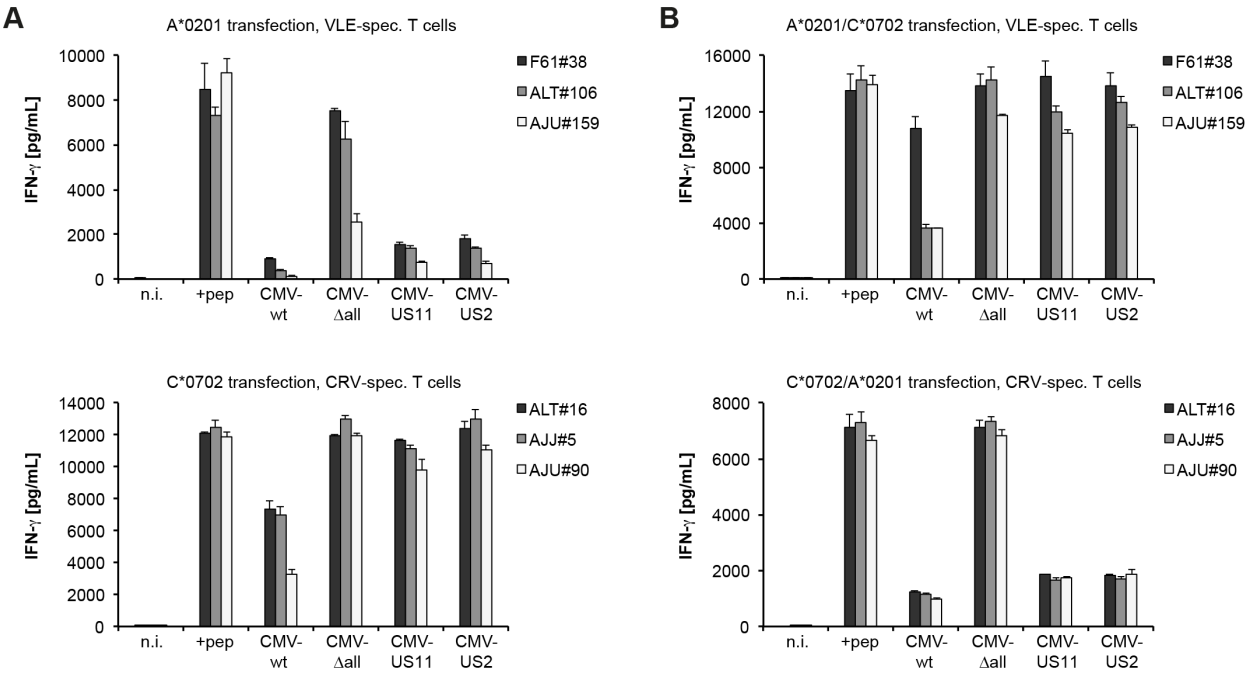


Fig. 4.39: Functional separation of epitope presentation and US2 and US11 immunoevasion. WI-38 fibroblasts (HLA-A*0201/C*0702-negative) were transfected (**A**) with plasmids encoding HLA-A*0201 or HLA-C*0702, or (**B**) with chimeric HLA class I heavy chains HLA-A2/C7 (A*0201 N-terminus, C*0702 C-terminus) or HLA-C7/A2 (C*0702 N-terminus, A*0201 C-terminus), and subsequently infected with CMV-wt, CMV-Δall, CMV-US2 or CMV-US11. Not infected (n.i.) and peptide-loaded (+pep) fibroblasts served as negative and positive controls, respectively. Antigen-specific T cell recognition was detected by measuring IFN-γ secretion by ELISA after overnight incubation of 10 000 clonal T cells with 10 000 target cells per well. Three T cell clones generated from 3 different donors were used as effectors for each specificity. Means and standard deviations of triplicate samples from one of two independent experiments are shown.

4.5.3 Time course of interference with IE-1 and pp65 epitope presentation

In the presence of US2 and US11, CD8+ T cell recognition of IE-1 peptides presented on HLA-B and -C allotypes was not significantly affected (Fig. 4.36). Likewise, HLA-A*0301 epitope

presentation was largely intact. This implies that the other two HCMV-encoded immunoevasins, US3 and US6, largely prevented peptide presentation by the B alleles and largely contributed to prevent A*0301-mediated presentation. To guarantee an optimal protection of infected cells against T cell recognition, HCMV immunoevasins are expressed at overlapping phases during the viral replication cycle. The US3 protein has been identified as an IE-gene product, with the US3 transcripts appearing as early as one hour post infection (Weston, 1988; Tenney and Colberg-Poley, 1991; Liu et al., 2002). US2 protein expression was detected by 4 hours post infection, was most abundant at 6 to 10 hours post infection, and persisted at lower but constant levels through the remainder of the replicative cycle, thus indicating early-late expression kinetics (Jones and Sun, 1997). Similarly, US11 transcripts appeared at early times post infection and then progressively decreased in abundance at late times post infection (Jones and Muzithras, 1991). US6 mRNA is most abundant at late times post infection (Jones and Muzithras, 1991), resulting in a decrease of peptide transport rates to background levels at 96 hours post infection (Hengel et al., 1996). To get an overall picture of the concerted action of all four immunoevasins over time, CD8+ T cell recognition of pp65 and IE-1 epitopes on fibroblasts infected with CMV-wt, CMV-Δall, CMV-US11, CMV-US2, CMV-US6, or CMV-US3 was assessed at 24, 48, 72 and 96 hours post infection in IFN-γ ELISA assays (Fig. 4.40 and Fig. 4.41). By using fibroblasts pretreated with IFN-γ for 72 hours prior to infection (Fig. 4.40 B and Fig. 4.41 B) and not pretreated fibroblasts (Fig. 4.40 A and Fig. 4.41 A), the compensatory effects of the proinflammatory cytokine IFN-γ on the action of individual immunoevasins were also analyzed. The experiment included IE-1-specific T cell clones specific for VLE (A*0201), RHR (A*0301), ATT (A*6801), QIK (B*0801), or CRV (C*0702) as before, and for an additional epitope restricted through HLA-B*1801, **ELR**RKMMYM (Retiere et al., 2000) (Fig. 4.40). Also included were pp65-specific T cell clones that recognized NLV (A*0201), TPR (B*0702), as described in previous parts of this work, RPH (B*0702) (Weekes et al., 1999), or IPS (B*3501) (Gavin et al., 1993) (Fig. 4.41).

Consistent with previous results of this study (Fig. 4.29 and Fig. 4.34), recognition of IE-1 epitopes on CMV-wt-infected cells was severely suppressed for all HLA allomorphs except C*0702 over the whole time period tested. This suppression was complete in the absence of IFN-γ pretreatment, and nearly so in its presence. HLA-C*0702 was the only allotype whose recognition was still clearly detectable in the absence of IFN-γ treatment, and was largely unimpaired after such treatment. With the mutant viruses, distinct patterns of allotype-specific interference with IE-1 presentation were detected for each of the four immunoevasins. Differential effects of individual immunoevasins were also observed for pp65 epitopes, despite a generally better presentation of pp65 epitopes even in wild-type HCMV infection. As mentioned above, pp65 presentation is expected to be partially resistant to immunoevasion, due to the

exceptionally high abundance of this protein in infected fibroblasts and its transfer to cells by viral particles before the onset of any viral gene expression.

The sequences of all pp65 and IE-1 epitopes included in this experiment are not expected to be of high hydrophobicity, because all of them contain charged side chains (except NLV, which contains several polar side chains). Thus, they are unlikely candidates for TAP-independent MHC class I presentation (Lautscham et al., 2003). Consistent with this observation, TAP-inhibition by US6 strongly affected presentation of all peptides from both antigens, IE-1 or pp65, by each HLA allomorph. Furthermore, effects of US6 were apparent during the whole 96 hours, which is in accordance with its early-late expression kinetics. Strikingly, US6-mediated impairment of T cell recognition was largely or, at the earlier time points, even completely compensated by IFN-γ pretreatment of fibroblasts.

For the other three immunoevasins gpUS11, gpUS2, and gpUS3, clear patterns of allele-specific effects were detected. Among the HLA allomorphs tested, only A*0301 and C*0702 were completely resistent to US3. Consistent with its expression at IE times post infection, US3 impaired peptide presentation by the other allomorphs mainly at the earlier time-points tested. HLA-B*0702 and B*0801 were particularly susceptible to US3, which suppressed T cell recognition by these allomorphs almost to CMV-wt level at 24 hours post infection. Strikingly, recognition of CMV-US3-infected fibroblasts was fully restored for all HLA allomorphs after IFN-γ pretreatment.

The HLA-B*0801 and B*0702 epitopes largely escaped both US2 and US11, whereas all other HLA allomorphs included in the experiment were affected by these immunoevasins to a various extent. The US2/11-sensitive HLA allomorphs differed in their susceptibility to individual immunoevasins US2 and US11: for each of the allomorphs HLA-A*0201, C*0702, and B*3501, the effect of US2 was similar in size to the effect of US11, although A*0201-dependent presentation was much more strongly suppressed than presentation by HLA-C*0702 or B*3501. Other allotypes showed a different behavior: HLA-A*6801 and B*1801 were more susceptible to US2, and A*0301 more to US11. Impairment of T cell recognition by US2 or US11 was apparent during the whole 96 hours analyzed and decreased with time for many T cell clones tested, which is in accordance with the reported US2/11 expression peaking at early times post infection. In contrast to US6 and US3, the effects of US2 and US11 on T cell recognition were fully reversed by IFN-γ pretreatment only for those allomorphs that were not highly US2/US11-sensitive in the absence of IFN-γ.

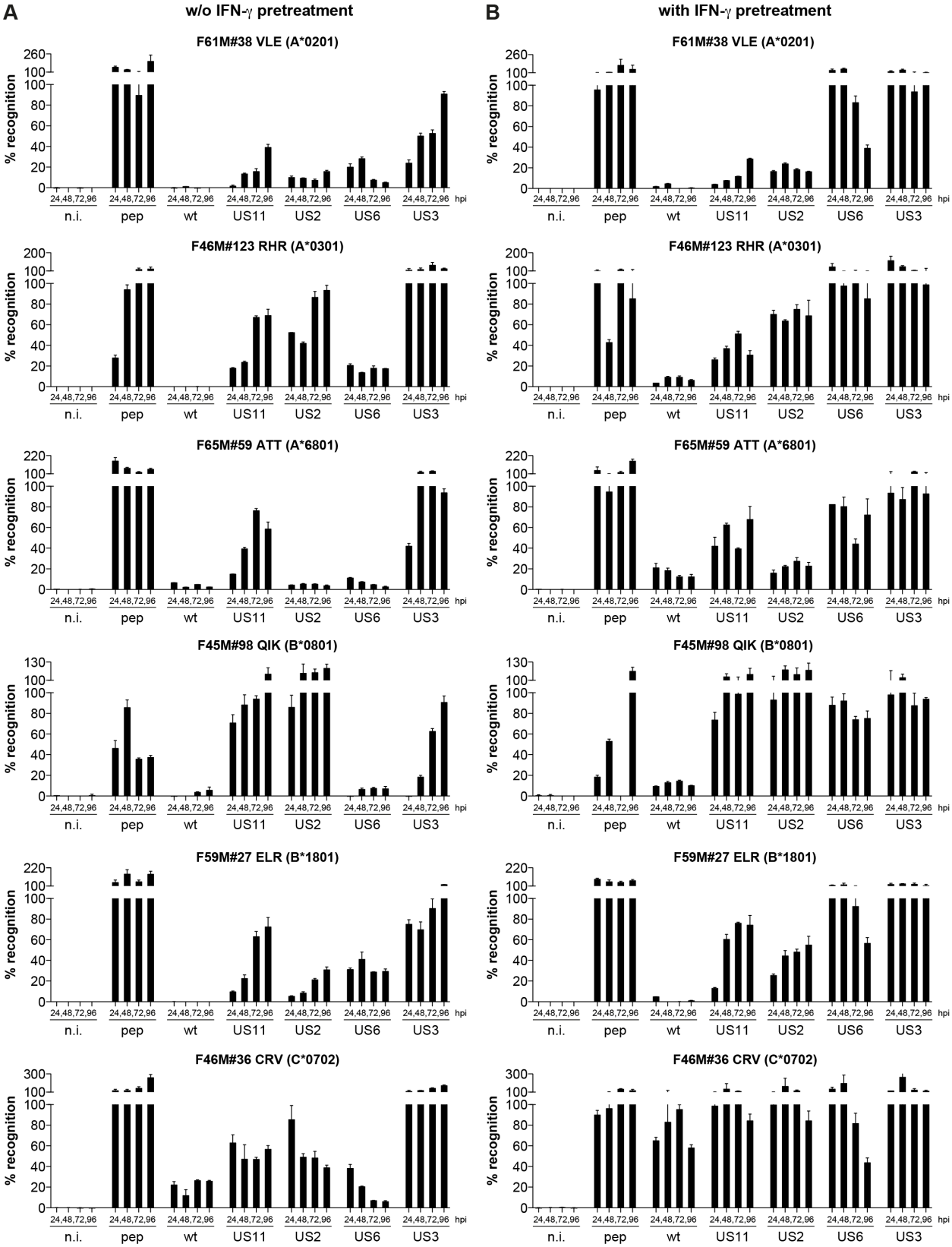


Fig. 4.40: Time course of HCMV immunoevasion of IE-1 T cell recognition by US2, 3, 6, and 11. MRC-5 (A*0201, C*0702), WI-38 (A*6801, B*0801), BFF2 (A*0301) or CZF (B*1801) fibroblasts were pretreated with IFN-γ (**B**) or not pretreated (**A**) and infected at moi = 5 with CMV-wt (wt) or recombinant HCMV strains expressing no immunoevasin (CMV-Δall) or only one of US11, US2, US3, or US6. At 24, 48, 72 and 96 hours post infection (hpi), IE-1 presentation was detected by specific T cell clones and analyzed in IFN-γ ELISAs (10 000 fibroblasts and 10 000 T cells per well in triplicates). Data were normalized to recognition of CMV-Δall-infected cells set to 100%. One representative T cell clone is shown for each epitope specificity from one out of 1–3 independent experiments.

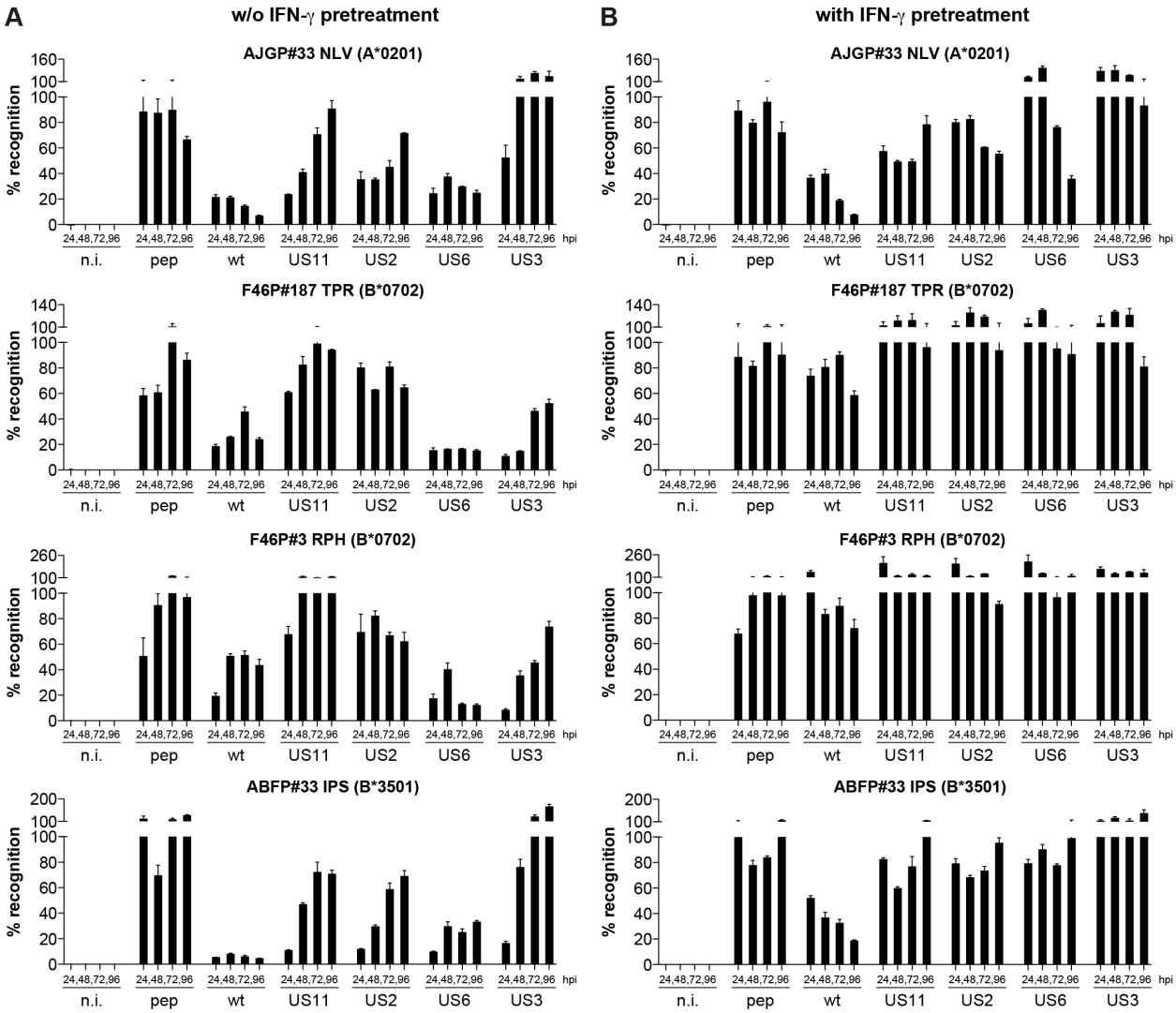


Fig. 4.41: Time course of HCMV immunoevasion of pp65 T cell recognition by US2, 3, 6, and 11. MRC-5 (A*0201, B*0702) or BFF2 (B*3501) fibroblasts were pretreated with IFN-γ (**B**) or not pretreated (**A**) and infected with CMV-wt (wt) or recombinant HCMV strains expressing no immunoevasin (CMV-Δall) or only one of US11, US2, US3, or US6 at moi = 5. Not infected (n.i.) and peptide-loaded (pep) fibroblasts served as controls. At 24, 48, 72 and 96 hours post infection (hpi), pp65 presentation was detected by specific T cell clones and analyzed in IFN-γ ELISAs (10 000 fibroblasts and 10 000 T cells per well in triplicates). Data were normalized to recognition of CMV-Δall-infected cells set to 100%. One representative T cell clone is shown for each epitope specificity from one out of 1–3 independent experiments.

The observed effects of individual immunoevasins on T cell recognition over time were compared to their effects on HLA surface expression, analyzed by flow cytometry. Again, fibroblasts were infected with wild-type HCMV or the single-immunoevasin HCMV strains (Fig. 4.42) after cultivation with or without IFN-γ for 72 hours. Specific antibodies were available for total HLA-A/B/C, A2 and B7. Relative HLA expression levels were calculated in relation to CMV-Δall-infected cells set to 100%. Specific MFIs of IFN-γ-pretreated cells were generally higher than specific MFIs of not pretreated cells (data not shown). As already shown before (Fig. 4.33), infection with CMV-wt downregulated HLA-A2 and -B7 expression to low residual levels in both pretreatment conditions, less so total HLA-A/B/C. Downregulation of HLA-A2 could be detected for all four immunoevasins, which is in accordance with the T cell recognition data. However,

US3 effects appeared more pronounced on the HLA expression level (compared to the T cell level) and could not be fully compensated by IFN-γ. Consistent with the abolishment of TPR and RPH epitope recognition without IFN-γ pretreatment, US6 and US3 had strong effects on HLA-B7 expression. However, whereas T cell recognition could be fully restored by IFN-γ, the relative B7 downregulation was similar in both pretreatment conditions. This suggests that the absolute number of B*0702/antigen complexes, and not their relative downregulation, was decisive for T cell recognition. More unexpected was the observation that US2 and US11 reduced HLA-B7 surface expression as efficiently as US3 and US6, but did not significantly impair B7-dependent T cell recognition, in contrast to US3 and US6. Total HLA-A/B/C expression was affected by all four immunoevasins but to lesser extent than HLA-A2 and B7, indicating the presence of HLA allomorphs on MRC-5 cells which are more resistent to individual immunoevasins than A2 and B7. In summary, this result suggests that a reduction of the relative HLA expression levels does not necessarily impact on T cell recognition. T cell recognition might be largely intact as long as sufficient HLA molecules are still available for antigen presentation, which is especially the case after IFN-γ pretreatment. Furthermore, T cell recognition may also depend on the availability of target peptides as well as the avidity of the T cells. This result also indicates that biochemical analyses of immunoevasins' relative effects on HLA expression do not allow to predict their impact on T cell recognition with certainty.

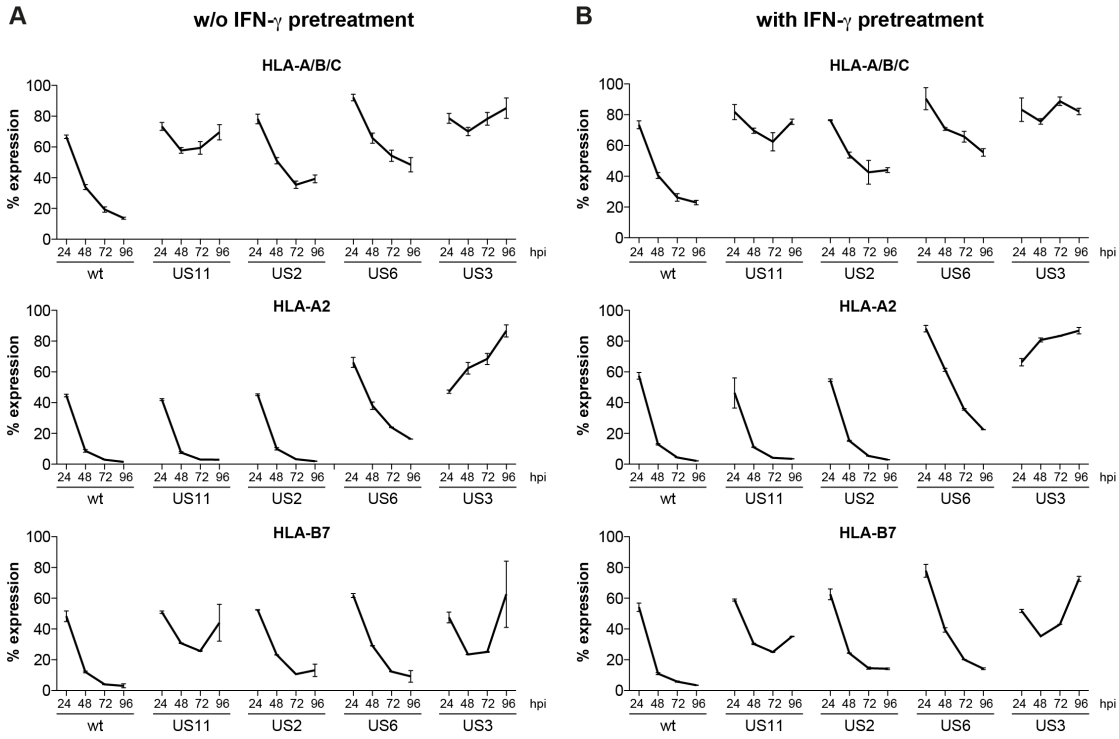


Fig. 4.42: Impact US2, 3, 6, and 11 on HLA class I surface expression of infected fibroblasts. MRC-5 cells that had (**B**) or had not (**A**) been pretreated with IFN-γ were infected at moi = 5 with CMV-wt or recombinant HCMV strains expressing no immunoevasin (CMV-Δall) or only one of US11, US2, US3, or US6. At 24, 48, 72 and 96 hpi, HLA-A/B/C, A2 and B7 surface expression was analyzed by flow cytometry. The specific MFIs were calculated by subtracting the MFIs of isotype controls from the MFIs of specific stainings. Values were normalized to specific MFIs of CMV-Δall-infected cells set to 100%. Means and standard deviations of two (HLA-B7, A2) or three (HLA-A/B/C) independent experiments are shown.

4.5.4 Immunoevasion of T cell recognition under immediate-early conditions

The immediate-early phase is a critical time during the lytic cycle of HCMV, as both exogenously introduced structural proteins and regulatory IE proteins are present in abundance, thus sensitizing infected cells to CD8+ T cell recognition. US3 is the only member of the group of MHC class I-specific immunoevasin genes that has been described as an IE gene, with its transcription initiated following infection in the absence of *de novo* protein synthesis (Weston, 1988). US3 transcripts appear as early as 1 hour post infection, with their accumulation peaking at 3–4 hours post infection, followed by a decline in transcript levels by 5 hours post infection (Biegalke, 1995; Liu et al., 2002). As shown before in the T cell recognition experiments (Fig. 4.40 A and Fig. 4.41 A), US3 suppresses T cell recognition in an allele-specific manner, most strongly so at the earliest time point analyzed (24 hours post infection). Interestingly, the other three immunoevasins also showed strong effects already at this early time. However, the T cell recognition assays performed in Fig. 4.40 and Fig. 4.41 did not address immunoevasion in the IE phase. Therefore, T cell recognition of infected fibroblasts was investigated under stringent IE conditions that allowed expression only of the first wave of viral genes, the IE genes (optimized conditions according to Hesse et al., unpublished). MRC-5 fibroblasts (not pretreated with IFN-γ) were infected with wild-type HCMV or HCMV immunoevasion-defective strains CMV-Δall, CMV-US11, CMV-US2, CMV-US6 or CMV-US3. During the first 8 hours post infection, fibroblasts were treated with an inhibitor of protein translation, cycloheximide (CHX), in order to accumulate IE mRNAs while preventing their translation. Then, CHX was removed, and translation of mRNA was allowed for 13.5 hours. During this time, expression of early and late proteins was blocked by the transcriptional inhibitor actinomycin D (ActD). The cells were fixed with formaldehyde to prevent any further change in viral gene expression or antigen processing, and were subjected to recognition by pp65 (NLV/A*0201, TPR and RPH/B*0702) and IE-1 (VLE/A*0201, CRV/C*0702) specific T cell clones, measured in IFN-γ ELISA assays (Fig. 4.43 B). For comparison, the same experiment was performed in parallel without CHX/ActD treatment of fibroblasts, thus allowing the expression of early and late genes (Fig. 4.43 A). The data were again normalized to CMV-Δall controls set to 100% recognition.

Without CHX/ActD treatment, the patterns of US3-mediated suppression of antigen presentation by the three HLA class I allomorphs tested was in accordance with previous results of this work: strong impact of US3 on HLA-A*0201 and B*0702-mediated presentation, but no significant suppression of HLA-C*0702-mediated presentation (Fig. 4.43 A). Contrary to expectation, under stringent IE conditions (CHX/ActD treatment) US3 expression in infected cells had no significant impact on HLA-A*0201 and B*0702-mediated peptide presentation (Fig. 4.43 B). Expression of US6 affected T cell recognition only under non-IE conditions, which is in accordance with its expression at early and late time-points post infection. Under IE conditions, IE-1 peptide presentation, especially of VLE, was even enhanced in CMV-US6-infected cells, an observation

that cannot currently be explained. Consistent with the data above (Fig. 4.40 A and Fig. 4.41 A), US2 and US11 impaired the presentation of peptides on either HLA allomorph tested, most strongly HLA-A*0201, under non-IE conditions. Surprisingly, effects of US2 and US11 on NLV, VLE and CRV presentation were also seen under IE conditions. In particular, VLE presentation was completely abolished by each of US2 or US11. This observation suggests that both proteins, gpUS2 and gpUS11, are already expressed and functional at IE times, and contribute to enhanced protection of infected cells from CD8+ T cell recognition at this time.

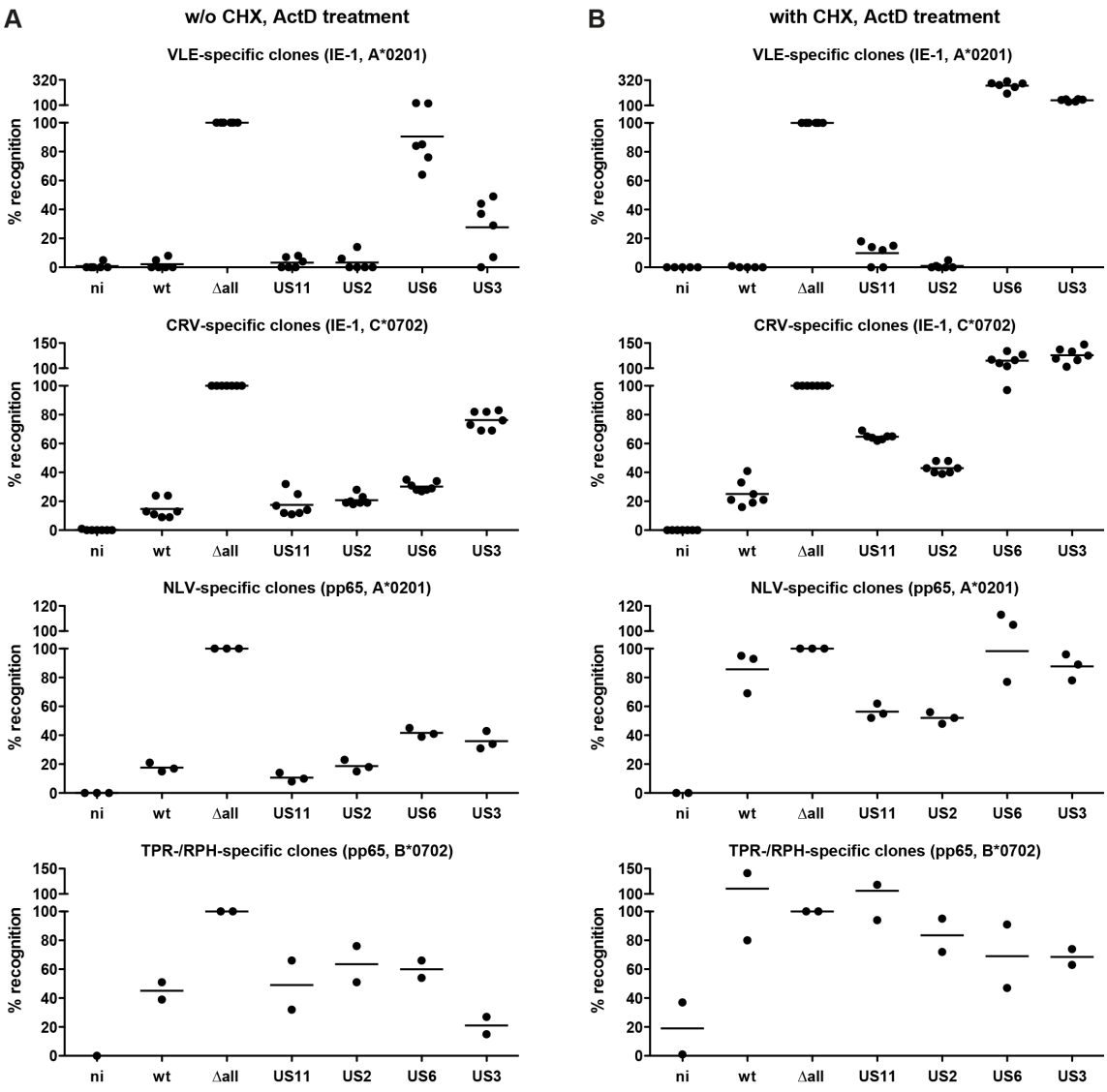


Fig. 4.43: Effects of HCMV immunoevasins on T cell recognition under IE conditions. MRC-5 fibroblasts were infected at moi = 5 with CMV-wt (wt) or recombinant HCMV strains expressing no immunoevasin (CMV-Δall) or only one of US11, US2, US3, or US6. After infection, cells were kept under cycloheximide (CHX)/actinomycin D (ActD) blocking conditions to solely allow IE gene expression (**B**) or without CHX/ActD (**A**). Not infected (ni) cells served as controls. IE-1 and pp65 epitope presentation was detected by specific T cell clones in IFN-γ ELISAs (20 000 fibroblasts and 40 000 T cells per well in triplicates). Data were normalized to recognition of CMV-Δall-infected cells set to 100%. One representative T cell clone is shown for each epitope specificity from one out of 3 independent experiments.

4.6 The role of HLA-C*0702 in context of NK cell recognition of HCMV infection

The selective preservation of HLA-C*0702 on infected cells by HCMV-encoded immunoevasins is unexpected, because it should be deleterious to the virus due to facilitated T cell attack *in vivo*. Therefore, it has to be considered that the maintenance of HLA-C*0702 might confer an independent advantage to HCMV. Another important function of a subset of HLA class I molecules, including HLA-C allotypes, is the inhibition of NK cell-mediated killing of intact body cells via inhibitory killer-cell immunoglobulin-like receptors (KIRs) (Lanier, 2005). As a component of the innate immune system, NK cells are part of the first-line defense against viruses like HCMV, capable to act before adaptive immunity has risen to sufficient levels. Downregulation of MHC class I molecules during HCMV immunoevasion should render infected cells susceptible to lysis by NK cells. However, several *in vitro* studies described that AD169-infected human fibroblasts were not highly permissive to NK cell killing (Cerboni et al., 2000; Wang et al., 2002). The following experiments tested the hypothesis that preservation of HLA-C*0702 expression by HCMV is sufficient to prevent NK cell-mediated killing of infected cells.

4.6.1 Generation and analysis of HLA-C*0702-specific NK cell lines

The HLA-C/KIR system represents one of the most general mechanisms of human NK cell control. Every HLA-C allotype can provide an inhibitory signal through at least one KIR: group 2 HLA-C allotypes through KIR2DL1, group 1 HLA-C allotypes including HLA-C*0702 through KIR2DL2 or 3 (Lanier, 2005). However, an individual NK cell typically expresses only a subset of KIRs and other MHC class I-binding inhibitory receptors, such as NKG2A that is specific for HLA-E, and the reactivity of the NK cell depends on its particular repertoire of receptors (Valiante et al., 1997). Furthermore, human fibroblasts coexpress several HLA class I allotypes that are ligands for NK cell inhibitory receptors and might potentially hamper the detection of HLA-C*0702-specific effects on NK cells expressing several HLA class I-specific inhibitory receptors. To circumvent this problem, NK cell lines were established that uniformly expressed KIR2DL3, the receptor for HLA-C*0702, and were largely devoid of any other relevant MHC class I-specific inhibitory receptors.

Donor AJU was chosen for the isolation of KIR2DL3-expressing NK cells for two reasons: this donor is homozygous for KIR haplotype A and therefore lacks several activating KIR genes that might diversify the patterns of NK cell reactivity; furthermore, AJU is carrier of two HLA-C group 1 alleles, which ensures an increased functional competence of KIR2DL3 in this donor's NK cells (Kim et al., 2008). To minimize any potential functional "re-education" in culture, the NK cells were cultivated in the continuous presence of allogeneic feeder cells that were biallelic for HLA-C group 1.

KIR2DL3-positive primary NK cells were enriched from PBMCs of donor AJU by immunomagnetic separation (CD3+ depletion followed by KIR2DL3 positive isolation). These receptor-selected NK cells were cultivated in bulk as polyclonal T cell lines. After five weeks of cultivation, the polyclonal NK cells contained 96.4% CD56-positive/KIR2DL3-positive cells, which were mostly negative for several other relevant NK receptors like KIR2DL1 (1.1% positive cells, ligands: HLA-C 2 group), KIR3DL1 (3.9% positive cells, ligands: HLA-Bw4 group) and NKG2A (1.7% positive cells, ligand: HLA-E) (Fig. 4.44). In parallel, the primary KIR2DL3-enriched NK cells were plated under limiting dilution, and NK cell clones were established from these cultures and were screened by flow cytometry for expression of KIR2DL1, KIR3DL1 and NKG2A. Among the 23 NK cell clones analyzed, all were positive for KIR2DL3. One additional receptor was expressed by eight clones, two additional receptors by three clones, and three additional receptors by one clone. FACS analyses are shown for two NK cell clones, a KIR2DL3 single-positive clone (Fig. 4.44 B) and a clone expressing four NK cell receptors (Fig. 4.44 C).

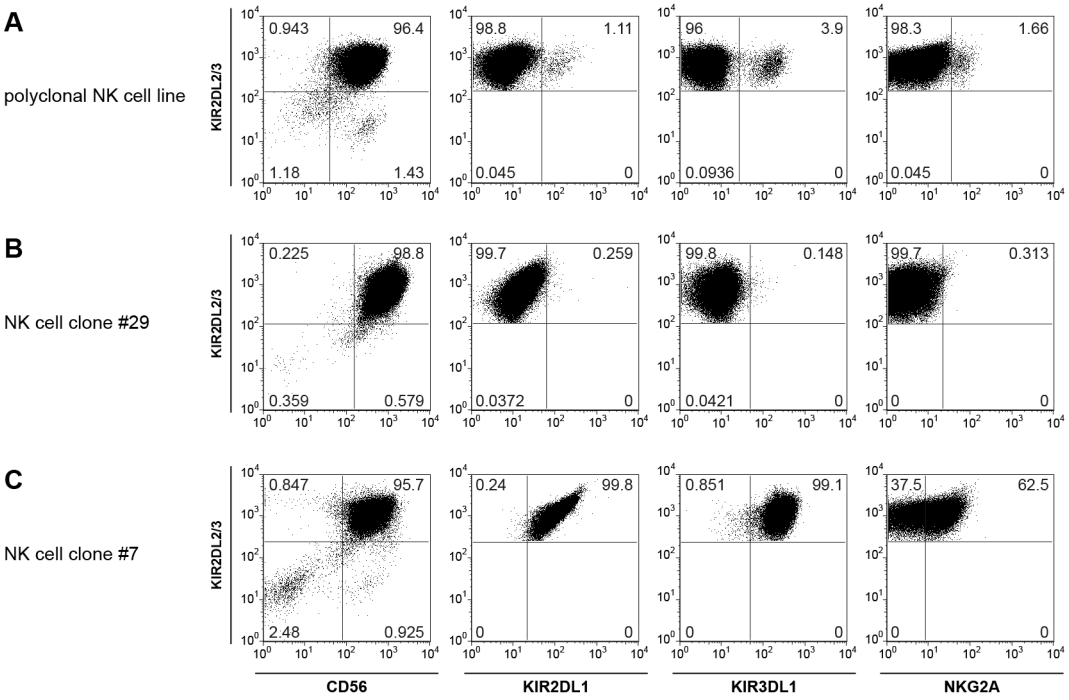


Fig. 4.44: Analysis of KIR expression by NK cell lines. NK cell lines were established from donor AJU and analyzed for expression of KIR2DL1, KIR2DL2/3, KIR3DL1 and NKG2A by flow cytometry. At the time of FACS analysis, the NK cell lines were approximately 5–6 weeks in culture. Dot plots are shown for the polyclonal NK cell line (**A**) and two NK cell clones, #29 (**B**) and #7 (**C**), which differ in their KIR expression patterns.

Next, the NK cell lines were evaluated for their potential to kill MHC class I-deficient (K562, Daudi, L721.221) and various MHC class I-expressing target cells. These included L721.221 cells transgenic for HLA-C*0702, which is a KIR2DL3 ligand, or for HLA-C*0602, which is a ligand of KIR2DL1 but not KIR2DL3, and MRC-5 fibroblasts that express a natural configuration of HLA class I molecules, including HLA-C*0702 as their only KIR2DL3 ligand (Fig. 4.45). For

MRC-5 cells, the influence of an IFN-γ pretreatment on their susceptibility to NK cells was also evaluated. Direct target cell lysis is a major effector function of NK cells and was measured by calcein-release asssay. Polyclonal and clonal NK cells killed MHC class I-deficient cell lines K562, Daudi and L721.221, but not L721.221 cells expressing HLA-C*0702. As expected, KIR2DL1-positive clone #7 was also inhibited from killing L721.221 cells expressing HLA-C*0602. KIR2DL1-negative NK cell clone #29 and the polyclonal NK cell line, which contained only a small fraction of KIR2DL1-positive cells, were largely insensitive to HLA-C*0602. Hence, these two cell lines were suitable tools to detect specific effects of HLA-C*0702 even on target cells that coexpress HLA-C group 2 KIR ligands, such as MRC-5 fibroblasts (C*0501).

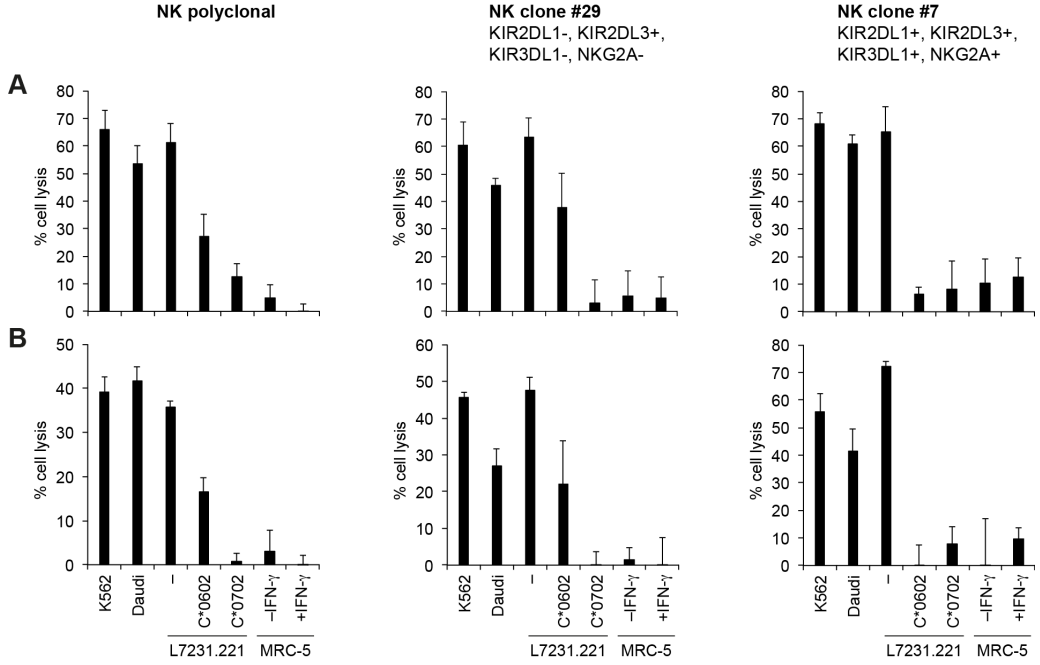


Fig. 4.45: Sensitivity of polyclonal and clonal NK cell lines for HLA-C*0702. Polyclonal and clonal (clone #29 and #7) NK cell lines of donor AJU were analyzed by calcein-release assay for killing of C*0702-transfected L721.221 cells and uninfected C*0702-positive MRC-5 fibroblasts that were or were not pretreated with IFN-γ. MHC class I-deficient cell lines K562, Daudi and LCL L721.221 served as positive controls for HLA class I-inhibited cell lysis. Killing of L721.221 cells stably transfected with HLA-C*0602, an HLA-C allotype belonging to the HLA-C group 2 of KIR2DL1 ligands, was assessed in parallel. The effector/target ratios were 8/1 (**A**) and 2/1 (**B**). Means and standard deviations of four replicates from one representative experiment out of four independent experiments are shown.

4.6.2 Analysis of NK cell recognition of HCMV-infected fibroblasts

In the T cell experiments, HLA-C*0702-mediated antigen presentation in context of a wild-type HCMV infection was somewhat reduced (Fig. 4.40), indicating that HLA-C*0702 is affected by HCMV immunoevasion to some extent. To test whether remaining HLA-C*0702 at the cell surface of infected cells is sufficient to inhibit NK cell killing, wild-type HCMV-infected MRC-5 cells were used as targets of polyclonal and clonal NK cells in a calcein-release assay (Fig. 4.46). NK cell cytotoxicity towards infected cells was assessed under both pretreatment conditions (with or

without IFN-γ) and at 24, 48, 72, and 96 hours post infection. Killing of uninfected cells was assessed in parallel. MHC class I-deficient K562 cells were used as positive control for HLA class I-dependent cell lysis. Compared to K562, killing of HCMV-infected MRC-5 fibroblasts was low during the whole time period tested, independent of pretreatment with IFN-γ. This suggests that sufficient inhibitory ligands for KIR2DL3 were present on these target cells. A slight decrease of NK cell killing over time, particularly at higher effector/target ratios, might reflect an accumulation of HCMV-encoded NK cell evasion mechanisms.

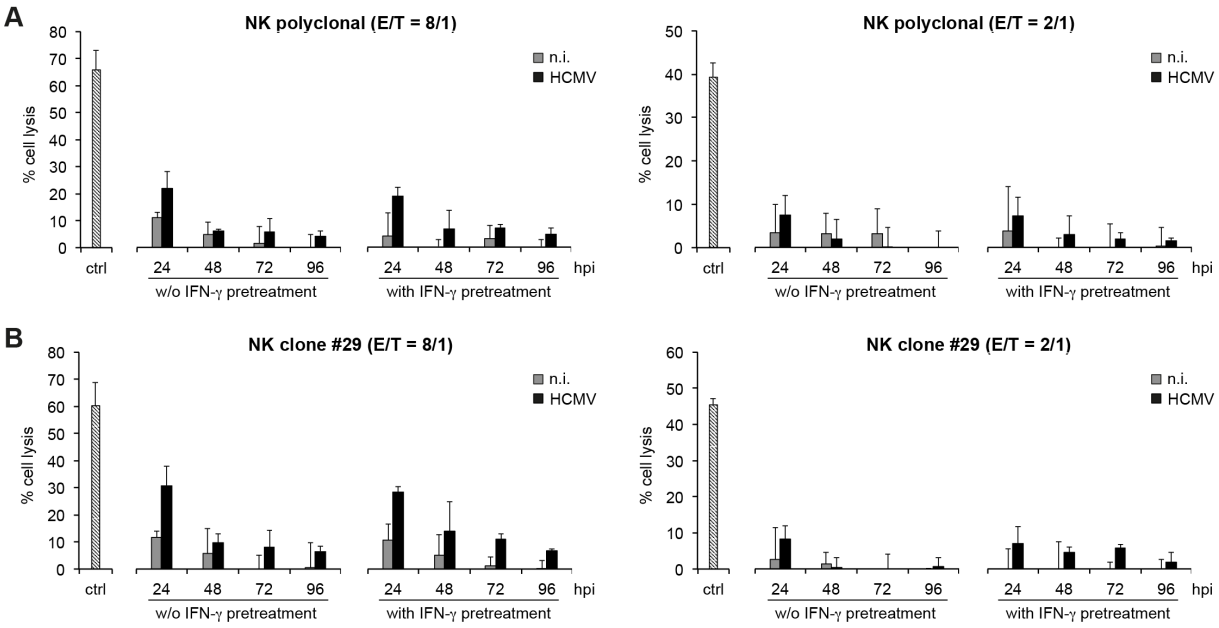


Fig. 4.46: Kinetics of NK cell killing of HCMV-infected fibroblasts. Polyclonal (**A**) and clonal (clone #29, **B**) NK cell lines of donor AJU were analyzed for killing of wild-type HCMV-infected MRC-5 fibroblasts (C*0702-positive), pretreated with IFN-γ for 72 hours or not pretreated prior to infection. NK cell killing of infected cells was measured at 24, 48, 72 and 96 hours post infection by calcein-release assay with effector/target ratios of 8/1 (left panel) and 2/1 (right panel). Killing of uninfected fibroblasts (n.i.) was detected in parallel at each time point as negative control. Killing of MHC class I-deficient cell line K562 served as positive control (ctrl) for HLA class I-dependent cell lysis and was detected at the 24 hours time point. Means and standard deviations of four replicates are shown.

The following calcein-release experiment (Fig. 4.47) was performed to assess the NK cell susceptibility of MRC-5 cells infected with wild-type HCMV or immunoevasion-defective HCMV strains deleted in all four immunoevasins (CMV-Δall) or expressing only US11 (CMV-US11) or US2 (CMV-US2). Again, MRC-5 cells were treated or not treated with IFN-γ for 72 hours before infection. MHC class I-deficient K562 cells served as positive control for HLA class I-dependent NK cell killing. In general, and in particular at the lower effector/target ratio, killing of wild-type HCMV-infected cells was low compared to killing of K562 cells. There was no lysis of CMV-Δall-infected or uninfected fibroblasts. Thus, wild-type HCMV was capable to partially downregulate HLA-C*0702, thereby inducing low levels of NK lysis. Infection with either mutant strain, CMV-US2 or CMV-US11, also rendered fibroblasts slightly susceptible to NK cell lysis, in accordance with the finding that US2 and US11 partially interfered with IE-1 recognition by HLA-C*0702-

specific T cell clones, especially in IFN-γ-untreated cells (Fig. 4.40). Lysis of CMV-US2 and -US11-infected cells tended to be lower than lysis of CMV-wt-infected cells, in particular at the higher effector/target ratio, indicating cumulative effects of US2, US11 and other immunoevasins (presumably US6, see Fig. 4.40) on HLA-C*0702 during wild-type HCMV infection. In this experiment, lysis of IFN-γ-pretreated cells was marginally lower than lysis of cells not pretreated with IFN-γ, reflecting the enhancement of overall HLA class I expression by this proinflammatory cytokine.

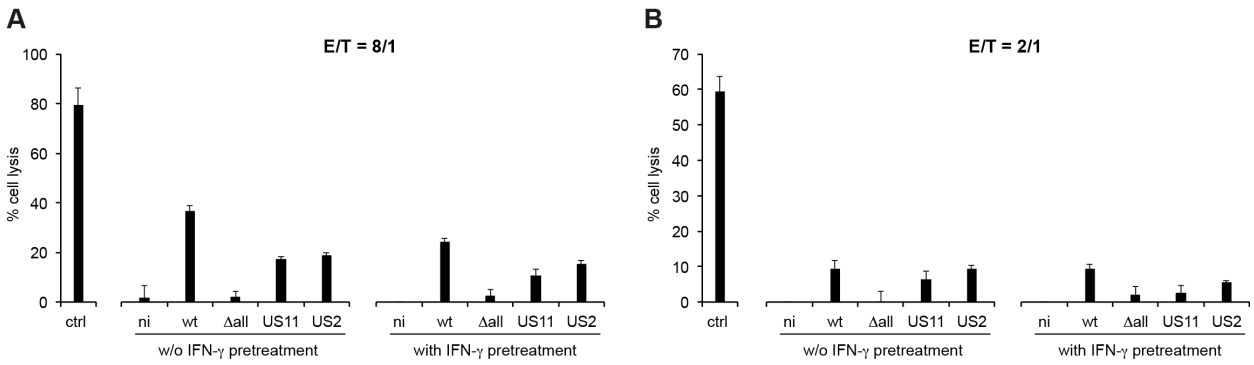


Fig. 4.47: Impact of HCMV immunoevasins on the susceptibility of infected fibroblasts to NK cell-mediated lysis. HLA-C*0702-sensitive polyclonal NK cells of donor AJU were analyzed for killing of IFN-γ pretreated or not pretreated MRC-5 fibroblasts (C*0702-positive) infected with wild-type HCMV (wt) or immunoevasion-defective HCMV strains deleted in all four immunoevasins (Δall) or only expressing US11 or US2. NK cell killing of infected cells was measured at 48 hours post infection by calcein-release assay with effector/target ratios of 8/1 (**A**) and 2/1 (**B**). Killing of uninfected fibroblasts (n.i.) was detected in parallel as negative control. Killing of MHC class I-deficient cell line K562 served as positive control (ctrl) for HLA class I-dependent cell lysis. Means and standard deviations of three replicates from one representative experiment out of two are shown.

4.6.3 Analysis of the capability of HLA-C*0702 to interfere with NK cell killing

The previous experiments showed that HCMV-infected cells were only poorly targeted by NK cells compared to MHC class I-deficient cells. To confirm a role for HLA-C*0702 and its specific KIR, KIR2DL3, in this inhibition of NK cell-mediated killing, HLA-A/B/C on wild-type HCMV-infected MRC-5 cells, or KIR2DL3 on NK cells, were blocked using specific antibodies (Fig. 4.48). Uninfected fibroblasts and L721.221 cells expressing HLA-C*0702 as their only HLA class I molecule were assessed in parallel.

For each NK cell line, polyclonal or clonal, blocking of HLA-C*0702 with anti-HLA-A/B/C antibody on L721.221 cells expressing only this HLA class I allotype induced NK cell-mediated lysis. A similar increase in NK cell cytotoxicity occurred when blocking total HLA-A/B/C on MRC-5 cells, infected with wild-type HCMV for 48 hours or not infected. As expected, an antibody against HLA-A2, which is not a KIR ligand, and isotype controls did not induce target cell lysis. Blocking of the KIR2DL3 receptor on NK cells led to a similar increase in NK cell lysis as HLA-A/B/C blocking for all three target cells tested, confirming that this lysis was due to an abolish-

ment of the C*0702/KIR2DL3 interaction. These results indicate that HLA-C*0702, as the only ligand of KIR2DL3 present in this system, prevented an attack on HCMV-infected fibroblasts by NK cells expressing this inhibitory receptor. To conclude, HCMV potentially preserves HLA-C*0702 on infected cells in order to evade killing by NK cells.

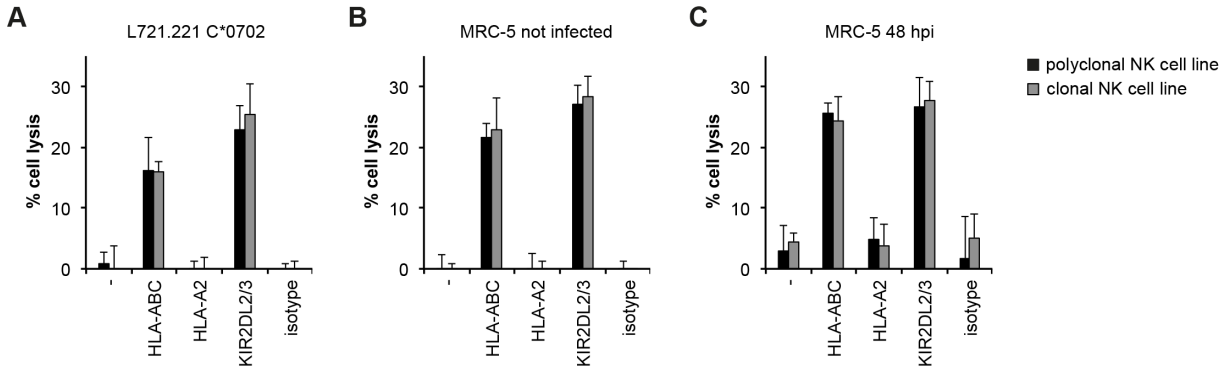


Fig. 4.48: HLA-mediated inhibition of NK cell recognition of HCMV infection. HLA-C*0702-sensitive polyclonal and clonal (clone #29) NK cells of donor AJU were analyzed for killing of C*0702-expressing L721.221 cells (**A**) and C*0702-positive MRC-5 fibroblasts, infected (**C**) or not infected (**B**) with CMV-wt for 48 hours (moi = 5), by calcein-release assay at an effector/target ratio of 2. Total HLA-A/B/C on target cells or KIR2DL2/3 on NK cells were blocked by preincubation with specific monoclonal antibodies for 1 hour prior to combining effectors and targets. Blockade of the non-KIR ligand HLA-A2, addition of isotype controls (shown for anti-HLA-A/B/C and -A2 antibodies) and samples without antibodies served as negative controls. Means and standard deviations of triplicate samples of one out of two independent experiments are shown.

5 Discussion

This study presents a refined analysis of the diversity and the effector functions of the T cell repertoire specific for the IE-1 antigen of HCMV. The IE-1 transcription activator is among the first endogenously expressed proteins in HCMV-infected cells (Stinski et al., 1983) and drives viral and cellular gene expression during the lytic replication cycle. Furthermore, reactivation of IE-1 expression in latently infected cells upon cell differentiation initiates productive HCMV replication with release of infectious virions (Sinclair and Sissons, 2006). Consequently, IE-1-specific T cells potentially control lytic HCMV infection but also viral reactivation from latency with particular efficiency, because they can act before progeny virus is released and spreads within the body (Simon et al., 2006; Besold and Plachter, 2008).

Both major arms of the IE-1-specific T cell response, CD4+ and CD8+ T cells, were readressed in this study, but with different focal points: key aspects of the CD4+ T cell part were the definition of IE-1 T cell epitopes and the analysis of T cell effector functions, in particular in the context of HCMV infection of DCs; the CD8+ T cell part mainly focused on the elucidation of the mechanism underlying the immunodominance of HLA-C-restricted T cells by analyzing IE-1 T cell recognition in context of HCMV immunoevasion. One intention behind these detailed analyses was the evaluation of the IE-1 protein as a target for HCMV immunotherapy to reduce the risk of HCMV disease in immunocompromised patients or of an intrauterine infection during pregnancy. For example, for the design of an effective peptide vaccine the identification of immunodominant regions within this HCMV T cell antigen is a prerequisite. The IE-1 protein is also considered as a particular attractive target for adoptive T cell transfer, because IE-1 specific T cells, especially CD8+ T cells, are found in the majority of healthy virus carriers (Sylwester et al., 2005). However, for patient studies it would be beneficial to know which of the various T cell specificities directed against IE-1 are particularly efficient in eradicating HCMV-infected cells, thus providing improved protection against HCMV disease.

In this work, IE-1-specific T cells were reactivated and expanded in bulk from PBMCs of healthy HCMV carriers by repeated stimulation with autologous mLCL expressing IE-1. IE-1-mLCLs were generated by infection of primary B cells with an IE-1-encoding mini-EBV vector, which provides the B cells with two constitutive properties, thus allowing specific T cell stimulation: endogenous expression of a foreign antigen of interest, here IE-1, and induction of costimulatory molecules (Wiesner et al., 2005). According to the classical picture of MHC presentation, cytosolic proteins are mainly fed into the MHC class I pathway of antigen processing and presentation, thereby stimulating CD8+ T cells. The MHC class II pathway was originally believed to encounter only exogenous antigens that have been endocytosed by the APC. However, it has been shown for EBV-immortalized B cells that cytosolic antigens like EBNA1 (Paludan et al., 2005) or a model antigen (Nimmerjahn et al., 2003) can also access the MHC class II

compartment via autophagy, resulting in their presentation on HLA class II to CD4+ T cells. The same is true for mLCLs, which is shown by their potential to present HCMV structural protein pp65 to CD4+ T cells and to expand both CD8+ and CD4+ T cells specific for pp65 (Moosmann et al., 2002; Wiesner et al., 2005). In this study, the same restimulation protocol has proven its worth for the enrichment of IE-1-specific T cells of both classes, CD8+ and CD4+. For all twelve donors studied except one, the resulting polyclonal T cell lines were dominated by CD8+ T cells. A likely reason for this are the much higher precursor frequencies of IE-1-specific CD8+ memory T cells in the donors' PBMCs compared to IE-1-specific CD4+ T cells.

Eight of the blood donors chosen for this study were known to be healthy carriers of both EBV and HCMV. Consequently, a coexpansion of T cells directed against EBV latent antigens coexpressed in the mLCLs (EBNA1, -2, -3A, -3B, -3C, -LP and LMP1, -2), most of them known to be recognized by more or less immunodominant CD8+ T cell populations in EBV-seropositive individuals (Rickinson and Moss, 1997), was expected for these donors. However, IE-1-specific T cells largely dominated the cultures from most EBV-seropositive donors. This went so far that T cells recognizing the ctrl-mLCL were completely absent in the cell lines of six donors. For one donor (ALT), it was confirmed by HLA/peptide multimer staining that T cells directed against epitopes from EBV latent antigens were expanded in the ctrl-mLCL-stimulated culture, but were lost in the IE-1- and pp65-stimulated cultures. As the ctrl-mLCL-stimulated T cell populations of all donors equally recognized both types of mLCLs, with and without IE-1, it can be assumed that EBV antigen expression and presentation by mLCLs was intact. Preferential expansion of HCMV-specific T cells was probably favored by their higher memory T cell frequencies in the peripheral blood. Expression of HCMV antigens in mLCLs is under control of the constitutive Simian Virus 40 (SV40) promoter; while this promoter is of lower activity in B cells than other heterologous viral promoters, its activity is similar to the one of the V-lambda promoter (Zarrin et al., 1999) and therefore should suffice for reliable antigen expression. Expression of IE-1 and pp65 might even be higher than expression of EBV latent antigens, contributing to a certain degree of competition for HLA molecules and the preferential expansion of T cells specific for the HCMV antigens. This hypothesis is supported by the observation that some EBV latent antigen-specific CD8+ T cells generated from seropositive individuals fail to lyse the autologous LCL unless antigen dose is artificially raised (Hill et al., 1995).

Detailed analyses of T cell attributes like minimal epitope, memory phenotype, TCR usage or cross-reactivity, were often only described for T cell specificities that are quite frequent in the peripheral blood of HCMV-seropositive donors, such as CD8+ pp65- and IE-1-specific T cells (Wills et al., 2002; Rist et al., 2009) or CD4+ T cells targeting structural proteins pp65 (Casazza et al., 2006; Crompton et al., 2008), and gB/gH (Elkington et al., 2004). Using the example of CD4+ IE-1-specific T cells, this study provides evidence that rare antigen-specific T cells can be expanded to significant numbers by mLCL-stimulation, thus being now available for further anal-

yses. Mini-EBV plasmids can be easily modified in *E. coli* to accommodate additional genetic information and their capacity for foreign DNA is extraordinary large: up to 80 kilobase pairs can theoretically be added without impairing the efficient packaging of mini-EBV DNA into virions (Bloss and Sugden, 1994). In this way, one could think about inserting HCMV genes encoding subdominant T cell antigens, such as the transcription factor IE-2 or immunoevasin US3, in order to characterize the less frequent T cell responses to these proteins (Sylwester et al., 2005).

5.1 The CD4+ T cell repertoire against the IE-1 antigen

There is growing interest in characterizing the CD4+ T cell response to HCMV. This is justified by HCMV's latency and lytic replication *in vivo* in cells of hematopoietic origin like macrophages and DCs (Sinzger et al., 2008) in which the HLA class II pathway of antigen presentation is constitutively active. Progenitor cells of the myeloid lineage are considered to be the main reservoir for latent HCMV and support virus reactivation upon differentiation into monocytes, macrophages and DCs (Reeves et al., 2005; Sinclair and Sissons, 2006). This makes it likely that virus-specific CD4+ T cells directly recognize lytically-infected or virus-reactivating cells, so that they can immediately provide T cell help for CD8+ T cells or, if they possess cytotoxic potential, can act as effectors in their own right. Adoptive T cell transfer studies have suggested a crucial helper role of HCMV-specific CD4+ T cells for the virus-specific CD8+ T cell response in control of HCMV or protection from viral disease (Einsele et al., 2008). Allogeneic SCT patients who failed to reconstitute endogenous CD4+ T cell responses to HCMV showed a progressive decline in the magnitude of adoptively transferred virus-specific CD8+ T cells (Walter et al., 1995), although it may be difficult to distinguish association from causation in such studies. Conversely, infusions of predominantly CD4+ T cell lines specific for HCMV into allogeneic SCT patients lacking a virus-specific CD4+ T cell response led to an efficient control of viremia by the preexisting endogenous HCMV-specific CD8+ T cell immunity (Einsele et al., 2002). Additional evidence for the pivotal role of HCMV-specific CD4+ T cells in virus control comes from studies on patients coinfected with HIV-1, which progressively depletes the host from CD4+ T cells. In such patients, diminished CD4+ T cell responses to HCMV were associated with an inability to sustain high levels of HCMV-specific CD8+ T cells and an increased risk of HCMV-associated end-organ disease (Komanduri et al., 2001). CD4+ T cells provide T cell help for cytotoxic CD8+ T cells by mechanisms that include secretion of cytokines, such as the T cell growth factor IL-2, and strengthening the antigen-presenting and costimulatory capacity of APCs through CD40-CD40L interaction (Schoenberger et al., 1998).

HCMV-specific CD4+ T cells have been described to target various functionally distinct viral antigens including virion proteins but also non-structural proteins such as IE-1 (Sylwester et al., 2005). However, dominant CD4+ T cell responses are largely directed against HCMV structural

proteins such as gB and pp65 (Beninga et al., 1995; Sylwester et al., 2005). Similar observations were also made for other herpesviruses, for example EBV-specific CD4+ T cells, which primarily target late-lytic structural proteins of the virus (Adhikary et al., 2007). These abundant virion proteins can efficiently access the MHC class II pathway of antigen presentation during direct infection of APCs involving endocytotic compartments or after engulfment and lysosomal degradation of material from infected cells by APCs. Efforts to isolate and characterize IE-1-specific CD4+ T cells started already more than two decades ago (Alp et al., 1991), quickly following the description of IE-1 as one of the first HCMV CD8+ T cell antigens (Rodgers et al., 1987; Borysiewicz et al., 1988). However, a more detailed analysis of IE-1-specific CD4+ T cells was apparently hampered by their low numbers in the peripheral blood of HCMV carriers. For example, Khan and collegues, who used IFN-γ ELISpot assays, detected IE-1-specific T cells only infrequently in young and elderly seropositive donors, and if they were detected, their proportion of the CD4+ T cell memory was low (<0.1%), in contrast to pp65-specific CD4+ T cell responses that were found to be quite strong (>1% of the CD4+ memory) (Khan et al., 2007). During evaluation of T cell reconstitution after solid organ transplantation by cytokine flow cytometry, Bunde and coworkers found IE-1-specific CD4+ T cells in 41% of the patients but not at all time points tested and again only at much lower numbers (0.05–0.19% of all CD4+ T cells) than pp65-specific CD4+ T cells (0.03–16.89% of all CD4+ T cells) (Bunde et al., 2005). Using the same technique, Slezak et al. could not detect IE-1-specific CD4+ T cells in any of 20 seropositive donors tested (Slezak et al., 2007). Controversially, Sylwester and collegues reported IE-1 peptide-specific CD4+ T cells in 11 out of 33 donors analyzed, with an average of approximately 0.7% of the total CD4+ memory T cell compartment, which was half of the frequency of pp65- and gB-specific CD4+ T cells (also analyzed by cytokine flow cytometry) (Sylwester et al., 2005). The data obtained in the present study for IE-1-specific CD4+ T cell frequencies in total PBMCs of HCMV carriers are in agreement with the majority of above mentioned studies, which report low numbers of these T cells. However, the experiments performed here were not primarily focused on the detection of IE-1-specific CD4+ T cells in PBMCs. To gain more exact information, it would be worth reanalyzing CD8+ T cell-depleted PBMCs from a variety of donors. Low numbers of IE-1-specific CD4+ T cells may be connected to the fact that IE-1, a nuclear protein, can reach the MHC class II pathway in infected cells only via autophagy and not, like abundant structural proteins, by the standard lysosomal pathway after uptake from extracellular material. Furthermore, as a regulatory protein expressed very early during the replicative cycle, IE-1 is less abundant in infected cells than late expressed structural proteins, whose amounts increase in molar ratios with the accumulation of newly synthesized viral DNA (Stinski, 1978). This together could result in smaller levels of IE-1 peptides presented on MHC class II and, consequently, less efficient stimulation of specific CD4+ T cells *in vivo*.

Despite their low precursor frequencies in PBMCs, IE-1-specific CD4+ T cells could be efficiently expanded by antigen-specific stimulation of PBMCs using individual peptides (as shown for EFF-specific T cells) or mLCLs expressing IE-1, making these T cells available for further analysis. Attempts to selectively enrich IE-1-specific CD4+ T cells have been made before by two groups: Alp and coworkers expanded IE-1-specific CD4+ T cell lines from five donors by stimulation of PBMCs for 12 days with recombinant baculovirus IE-1 protein or synthetic IE-1 peptides (Alp et al., 1991); Davignon et al. could enrich IE-1-specific CD4+ T cells from the PBMCs of 7 out of 23 seropositive donors by 7 day-stimulation with lysate of IE-1-transfected astrocytoma cells (U373MG) (Davignon et al., 1995). Both mentioned approaches have in common that they use exogenous antigen or peptides for specific stimulation of PBMCs. The professional APCs within PBMCs preferentially process these exogenously added antigens/peptides via endocytotic pathways thus favoring presentation on MHC class II over presentation on class I. In contrast to these approaches, this study used whole mLCLs endogenously expressing IE-1 for stimulation, which necessarily led to the coexpansion of IE-1-specific CD8+ and CD4+ T cells. Nevertheless, IE-1 epitope-specific CD4+ T cells could be enriched and isolated from all donors analyzed, indicating that MHC class II presentation of cytosolic proteins by APCs via autophagy is efficient enough to obtain antigen-specific CD4+ T cells. This process might also work *in vivo* for MHC class II presentation of IE-1-derived peptides by HCMV-infected APCs like DCs or macrophages, and might be of importance for early interference with reactivation of latent virus which takes place in these cell types.

In this study, IE-1-specific CD4+ polyclonal T cell lines and T cell clones were generated from eight healthy HCMV carriers with a broad panel of HLA class II types. IE-1 CD4+ epitopes were determined for each donor using a library of peptides covering the entire AD169 IE-1 sequence and also containing nine peptides with a selection of sequence variants found in HCMV strains TB40E, Toledo and other strains. A high diversity of 3 to 7 different T cell specificities was found per donor and was restricted through a manifold set of HLA-DR, -DQ and -DP allotypes suggesting that IE-1-specific CD4+ T cells can be generated for adoptive T cell therapy from most donors with divergent HLA types. With one exception, T cell clones recognized different strain-specific IE-1 sequence variants, allowing to target different HCMV strains with these T cells. Two CD4+ T cell epitopes, **EFFTKNSAFPKTT** (DRB5*0101 or DRB1*1501-restricted) and **VKSEPVSEIEEVAPE** (DQA1*0201/DQB1*0202-restricted), were found in different donors sharing the presenting HLA class II allotypes, thus being of particular interest for adoptive T cell therapy. Nevertheless, despite the identification of individual epitopes in more than one donor, the strongest CD4+ T cell responses in the donors analyzed did not consistently map to a particular subset of epitopes. This is in contrast to the IE-1-specific CD8+ T cell response, which targeted only 1 or 2 epitopes per donor and these were regularly found to elicit dominant responses in donors overlapping in the relevant HLA class I molecules (discussed in 5.2). This

discrepancy between the structures of virus-specific CD4+ and CD8+ T cell repertoires has been documented before. For example, for pp65-specific CD4+ T cells an average of 4 different epitopes (range 2–5) was recognized by each of the donors tested with responses to particular epitopes found in more than one donor but at variable intensity (Harcourt et al., 2006). By contrast, pp65-specific CD8+ T cell responses focused on few immunodominant epitopes that were recurrently identified in the donors tested (Khan et al., 2007). Similar reports came from the EBV field: CD4+ T cell responses to EBV latent-cycle antigens EBNA1, -2, -3A, and -3C, were diverse, and epitopes that were found in the highest number of donors were not necessarily immunodominant in terms of T cell numbers (Long et al., 2005). By contrast, the EBV-specific CD8+ T cell response to the same proteins is less diverse and focused preferentially on 1 or 2 immunodominant epitopes per donor (Khanna and Burrows, 2000).

Mapping of all identified IE-1 epitopes in the IE-1 sequence revealed that, with exception of the first 80 amino acids, the whole IE-1 sequence is well accessible for recognition by CD4+ T cells. Some epitopes were overlapping, thus creating hotspots of immunogenicity; for example, 6 different epitopes were covered by aa 85–107 and 8 different epitopes by aa 197–235. These regions might be particularly interesting for the generation of peptide vaccines. Besides the present work, the only major study attempting to map CD4+ T cell epitopes in IE-1 was published in 1991 (Alp et al., 1991). Alp and coworkers used polyclonal IE-1-specific CD4+ T cell lines of five donors to map IE-1 CD4+ T cell epitopes using 15–20-meric peptides with 6 amino acids overlap. They identified eight candidate epitopes, which were equally distributed over the IE-1 sequence thereby sparing the first 80 amino acids and overlapping in the first half of the protein as found in this study. One peptide recognized by clones of F63 and F65 in this study (RRKMMYMCYRNIEFFTKNS) was precisely matching a peptide defined as T cell epitope in the Alp study. Two other epitopes targeted in this study by clones from donors ARZ (ARAK**KDELRRKMMY**MCYRN) and F63 (CLQN**ALDILDKVHEP**FEEM) were partially overlapping with peptides of the older study. Unfortunately, Alp et al. did not establish T cell clones for more detailed analyses, such as minimal epitope definition. Putative HLA restrictions cannot be deduced for the candidate epitopes as only HLA-DR types are given and each epitope was identified only in one donor. Apart from the present work, IE-1-specific CD4+ T cell clones were only reported by Davrinche, Davignon and coworkers. They used clones of four different specificities, restricted through HLA-DR3, -DR7, -DR8 or -DR13, for studying T cell effector functions (see below) (Davrinche et al., 1993; Davignon et al., 1996; Le Roy et al., 2002). The HLA-DR8 epitope (DKREMWMACIKELH) (Gautier et al., 1996) may be identical to a T cell epitope identified in this work for DRB1*08-positive donor F63 (EDKR**EMWMACIKELH**DVSK).

A couple of IE-1 CD4+ T cell epitopes identified in this work were presented in different donors in the context of different HLA class II allotypes, suggesting some degree of promiscuity in peptide binding. For example, the HLA-DP-restricted epitope **SVMKRRIEEICMKVF**AQYI was

found in donors ARZ, AJU, and F61, who share only the DP α chain allele DPA1*0301, but no DPB allele encoding the DP β chain. It is tempting to speculate that class II molecules presenting the same peptides also recognize common peptide motifs. Unfortunately, the identification of binding motifs for MHC class II molecules is complicated by heterogeneity in peptide length due to an open-ended peptide binding cleft and a relatively degenerate amino acid usage at potential anchor positions in MHC class II peptide ligands (Rammensee, 1995). The specificity of peptide binding to MHC class II may be mediated by multiple weak interactions between residues inside the α and β chain pockets and residues of the bound peptide. Consequently, two or three obligatory anchor residues, as found in peptides binding to MHC class I, cannot be determined for MHC class II-presented peptides. The weak contacts leading to peptide binding might be mediated by different residues in different HLA class II molecules. The same peptide, when binding to different HLA class II allotypes, could adopt a different position on each HLA molecule. According to the literature, promiscuous peptide binding appears to be a common feature of HLA class II molecules. Using a pp65 peptide library, Li Pira and collegues screened 20 HCMV carriers for pp65 CD4+ epitopes and found six peptides that seemed to be recognized in different individuals in the context of more than one HLA-DR allele (Li Pira et al., 2004), although this is not definitive because donors were typed only for HLA-DRB1. HIV-1-derived peptides that were most frequently recognized by CD4+ T cells of the donors tested showed broad promiscuous binding capacity to a minimum of four out of eight HLA-DR alleles tested (Kaufmann et al., 2004). In addition to virus-derived CD4+ T cell epitopes, several MAGE-3 epitopes (Consogno et al., 2003) and one Her2/neu epitope (Kobayashi et al., 2000) were reported to promiscuously bind to a panel of different HLA-DR allotypes. The identification of broadly promiscuous epitopes can strongly increase the number of patients eligible for immunotherapy and offers the potential to induce CD4+ T cell responses in a genetically diverse population using only a limited number of peptides.

One aim of this work was to elucidate the role of IE-1-specific CD4+ T cells during HCMV infection by analyzing the effector functions of these cells. Only 5 out of 25 T cell clones tested showed significant cytolysis of IE-1 peptide-presenting target cells, suggesting that IE-1-specific CD4+ T cells tend to exert helper or indirect effector functions, by secretion of immunomodulatory cytokines, rather than mediating direct cytotoxicity. The cytokine profiles of all T cell clones tested contained the Th1 cytokines IL-2, IFN-γ and TNF-α. CD4+ T cells of the Th1 type appear to be the most abundant subtype of CD4+ T cells involved in antiviral immune responses and have been described for several herpesviruses including HCMV (Rentenaar et al., 2000), EBV (Bickham et al., 2001), HSV (Bettahi et al., 2006) and VZV (Zhang et al., 1994). Th1 cytokines stimulate the cellular arm of the immune response, which is crucial for elimination of virus-infected cells. The pleiotropic effects of IFN-γ on target cells include mechanisms that facilitate the recognition of infected cells by CD8+ cytotoxic T cells, for example upregulation of TAP

components and MHC class I molecules, as well as induction of the immunoproteasome (Schroder et al., 2004). The proliferation, differentiation and survival of antigen-selected CD8+ T cells are stimulated by the T cell growth factor IL-2 (Stern and Smith, 1986; Beadling and Smith, 2002). A helper cell function of IE-1-specific CD4+ T cells for CD8+ T cells would be in accordance with frequent reports about T helper cell dependence of HCMV-specific CD8+ T cell responses *in vitro* and *in vivo* (Walter et al., 1995; Li Pira et al., 2004; Salkowitz et al., 2004). IE-1-specific CD8+ T cells are found to be very abundant in HCMV carriers and might be particularly important for early control of HCMV infection or reactivation due to the immediate-early expression kinetics of their target antigen. However, during an early phase of infection, immune cells such as NK cells, macrophages and DCs may not suffice to create a supportive cytokine milieu for CD8+ T cells. Hence, IE-1-specific CD8+ T cells might especially rely on help by CD4+ T cells, which, for their own activation, have to see their target antigen simultaneously. Consequently, only IE-1-specific CD4+ T cells have the ability to fulfill this function during the early immune response. In addition to stimulation of CD8+ T cells, Th1 cytokines like IFN-γ exhibit direct antiviral activity (Gamadia et al., 2003) via induction of growth-inhibitory and pro-apoptotic signaling pathways in virus-infected cells (Schroder et al., 2004). Such growth-inhibitory effects were also detected in this study: cocultivation of IE-1/EFF-specific CD4+ T cells and mLCLs resulted in regression of mLCL outgrowth, which was largely dependent on specific stimulation by IE-1. Th1-like cytokine profiles have been reported by Davignon and collegues for HLA-DR3, -DR13 and -DR8-restricted IE-1-specific T cell clones (Davignon et al., 1996). These authors further showed that supernatants from these clones, activated with soluble antigen, inhibited HCMV replication in astrocytoma (U373MG) cells, and that this effect was mostly due to IFN-γ (Davignon et al., 1996). In line with the work of Davignon et al., the present study detected secretion of the Th2 cytokine IL-4 by some of the IE-1-specific CD4+ T cell clones in addition to the Th1 cytokines. Such T cells with broadened cytokine profiles have been designated Th0 cells, and have also been described for other viruses including HSV (Yasukawa et al., 1991) or Hepatitis B virus (Barnaba et al., 1994). IE-1-specific CD4+ T cells producing Th2 cytokines may contribute to anti-HCMV immunity by stimulating B cells to produce neutralizing antibodies, which are abundantly detected in the serum of HCMV carriers (Jackson et al., 2011), while the simultaneous production of Th1 cytokines may favor switching to appropriate antibody isotypes (Deenick et al., 2005).

A minority of the IE-1-specific CD4+ T cell clones tested displayed direct cytotoxic potential towards mLCLs expressing IE-1 or peptide-loaded mo-DCs. This emerging novel role of CD4+ T cells as direct antiviral effectors has been reported for several human viruses including influenza, poliovirus, measles virus, HIV, HSV, VZV, EBV and HCMV (Brown, 2010). Among these, CD4+ CTLs may be especially important for herpesviruses, which encode various mechanisms to suppress MHC class I antigen presentation in order to impede elimination of infected cells by

HLA class I-restricted CD8+ cytotoxic T lymphocytes (Hansen and Bouvier, 2009). CD4+ CTLs may remove infected cells that escape elimination through CD8+ T cells in an HLA class II-dependent fashion, thus expanding the capacity of the immune system to control such virus infections. The rare IE-1-specific CD4+ CTLs may be uniquely important in the control of HCMV reactivation and disease by directly targeting the reservoirs of latent virus, DCs and macrophages, which constitutively express MHC class II molecules. Evaluation of the cytotoxic potential of these T cells towards infected cells exceeded the scope of this study and remains to be done in the future. For HCMV, CD4+ T cells with cytolytic function are primarily described against structural proteins such as pp65 (Weekes et al., 2004), gB (Hopkins et al., 1996), and gH (Elkington et al., 2004), which are the quantitatively dominant target of HCMV-specific CD4+ T cells (Sylwester et al., 2005). This observation suggests a functional diversification of CD4+ T cell responses towards structural and non-structural proteins of HCMV, with the first ones being predominantly cytotoxic and the latter ones providing mainly T cell help or APC-activating function. This might reflect the biological need for different effector functions at different phases of the replication cycle: T cell help might be particularly important to initiate a successful immune response immediately after infection or reactivation from latency, when the lytic replication cycle is initiated by expression of regulatory proteins; later during infection, when HCMV virion proteins are abundantly expressed and presented, CD4+ CTLs contribute to the direct elimination of infected cells to restrict infection.

Assessment of the actual role of CD4+ IE-1-specific T cells in the HCMV immune response requires to study their ability to recognize IE-1 in context of an HCMV infection, because infection could dampen IE-1 presentation by virus-encoded immunomodulatory functions. Unfortunately, the *in vitro* analysis of CD4+ T cell recognition in context of HCMV infection is hampered by the limited availability of cell systems that are susceptible to HCMV infection and express MHC class II. MHC class II presentation can be triggered in many cell types of non-hematopoietic origin by IFN-γ, which induces the molecules HLA class II, invariant chain and HLA-DM via expression of the class II transactivator (CIITA) (Pieters, 1997). In this study, MHC class II expression was induced in primary fibroblast cell lines, a commonly used cell system to study T cell recognition of HCMV infection; however, the achievable MHC class II level was not high enough to demonstrate significant CD4+ T cell recognition of infected cells. This might be due to the capability of HCMV to repress IFN-γ-mediated MHC class II induction at the level of CIITA in order to escape CD4+ T cell recognition (Miller et al., 2001). To achieve permanent MHC class II expression, Le Roy et al. have transfected the HCMV-permissive astrocytoma cell line U373MG with CIITA (Le Roy et al., 2002). IE1-specific CD4+ T cell clones (HLA-DR3 and DR7-restricted) were efficiently activated by infected U373MG-CIITA cells through endogenous IE-1 presentation (Le Roy et al., 2002). By contrast, this study used mature mo-DCs with natural constitutive HLA class II expression to investigate IE-1-specific CD4+ T cell recognition of in-

fected cells. DCs were considered to be a more adequate cell system for this purpose as they are naturally targeted by HCMV *in vivo* and are known as cell reservoirs for reactivating virus (Sinclair and Sissons, 2006). Previous studies showed that mature mo-DCs permit low-level infection (≤20%) with fibroblast-adapted HCMV strain AD169 (Odeberg et al., 2003), and support all phases of viral gene expression, but do not release infectious progeny virus (Senechal et al., 2004). Strikingly, all CD4+ IE-1-specific T cell clones tested in the present study showed good recognition of HCMV-infected mo-DCs. This potent effector function of IE1-specific CD4+ T cells across various studied HLA class II allotypes is in contrast to IE1-specific CD8+ T cells, whose action *in vitro* is counteracted by viral functions that suppress most class I allotypes (discussed in 5.3). Interestingly, there is also some allotype-specific diversification among IE1-specific CD4+ T cells: only the HLA-DP-restricted T cell clones (SVM-specific) secreted similarly high IFN-γ amounts in response to infected cells and peptide-loaded cells; for HLA-DR and DQ-restricted T cells the response to infection was somewhat lower than to peptide-loaded cells. This could be due to intrinsic differences in the availability of the different target peptides or of HLA-DP versus DQ or DR, or due to differences in the avidities of the T cell clones. Furthermore, there is evidence that HCMV downregulates constitutive MHC class II expression in infected macrophages (endotheliotropic strains) (Miller et al., 2001; Sinzger et al., 2006) and DCs (strain AD169) (Odeberg et al., 2003). Inhibition of the MHC class II pathway may be particularly important during HCMV infection of APCs due to the accumulation of endogenously expressed structural proteins in the trans-Golgi network (TGN) for virion assembly (Tooze et al., 1993; Sanchez et al., 2000), thus delivering large quantities of viral antigens to endosomes and the MHC class II compartment (MIIC) (Hegde et al., 2002). To date, three HCMV-encoded proteins, pp65, US2 and US3, are described to interfere with the MHC class II pathway of antigen presentation. The tegument protein pp65 down-regulates cell surface expression of HLA-DR by accumulation of HLA-DR in lysosomes and destruction of the DR α chain (Odeberg et al., 2003). US2 causes rapid retro-translocation of DR α and DM α chains from the ER, followed by proteasomal degradation (Tomazin et al., 1999). US3 binds newly synthesized class II heterodimers and reduces their association with the invariant chain, thus preventing efficient sorting to MIIC (Hegde et al., 2002). However, while these studies described that such mechanisms inhibit the presentation of an irrelevant model antigen to CD4+ T cells (Tomazin et al., 1999; Hegde et al., 2002), it was shown later that genuine HCMV gB-specific CD4+ T cells recognize infected cells very well (Hegde et al., 2005). Potential immunoevasive effects of the virus on IE-1 presentation to CD4+ T cells remain to be investigated. To date, studies on MHC class II downregulation have focused on total HLA class II or HLA-DR, whereas HLA-DQ and -DP have not been investigated. As reported for class I immunoevasins, it is possible that the effects of class II immunoevasins are allele-specific. Experiments using defined HCMV immunoevasin

deletion mutants will clarify if HLA-DP-dependent presentation of IE-1 is less affected by viral immunoevasion than HLA-DR-dependent presentation.

In summary, the potent effector functions of IE-1-specific CD4+ T cells shown in this study suggest an important role of these cells during the immune response to HCMV. It is hypothesized that these T cells can efficiently control viral reactivation. To test this hypothesis it will be interesting to study the function of IE-1-specific CD4+ T cells in a cell culture model for HCMV reactivation from latency. The establishment of such a cell culture model should be feasible, because critical steps that promote the transition from HCMV latency to reactivation during myeloid progenitor differentiation have been described (Sinclair and Sissons, 2006; Reeves and Compton, 2011). On this basis, several experimental cell culture models for HCMV reactivation have been designed and evaluated. For example, CD14+ monocytes from peripheral blood of HCMV carriers were infected with clinical HCMV isolates and maintained in an undifferentiated state, in order to establish HCMV latency (Hargett and Shenk, 2010). When treated with cytokine mixtures to stimulate differentiation to macrophages or DCs, infected monocytes reactivated virus replication and produced infectious progeny. Such a cellular system will likely be a useful tool to study the function of IE-1-specific CD4+ T cells in greater detail.

5.2 The CD8+ T cell repertoire against the IE-1 antigen

Among HCMV-specific T cells, IE-1-specific CD8+ T cells might be of vital importance for preventing opportunistic HCMV disease in immunosuppressed patients, because these T cells could potentially eradicate cells at the onset of viral reactivation, before progeny virions are produced (Simon et al., 2006). In early studies in the mouse system, it was established that IE-1-specific CD8+ T cells protect against lethal MCMV infection (Reddehase et al., 1987; Jonjic et al., 1988). For HCMV, CD8+ T cells specific for IE-1, but not pp65, were associated with protection from viral disease in heart and lung transplant patients (Bunde et al., 2005) and with protection from early reactivation after stem cell transplantation (Sacre et al., 2008). In a comprehensive study covering all known 213 open reading frames of HCMV, IE-1 was one of only three HCMV proteins able to stimulate CD8+ T cells in PBMCs of more than half of healthy HCMV carriers tested; IE-1-specific CD8+ T cells were found in 55% (18/33) of donors (Sylwester et al., 2005). In contrast, the present study showed, by IFN-γ ELISpot assay of IE-1 mLCL-stimulated cultures, that IE-1-specific CD8+ T cells were present in all (11 of 11) seropositive donors tested (Fig. 4.23). Without prior stimulation, IE-1-specific T cells were detected in 75% of donors (Fig. 4.28). Furthermore, with up to 2% (donor LT12) of cells in the total CD8+ memory T cell compartment secreting IFN-γ in response to HCMV peptides in ELISpot, IE-1-specific CD8+ T cells appeared to be rather frequent. As only a fraction of antigen-specific T cells secrete sufficient amounts of IFN-γ within the time frame of an ELISpot assay to form a spot, the actual

proportion of IE-1-specific T cells is expected to be higher (see multimer staining for CRV-specific T cells, Fig. 4.27), presumably by a factor in the range of 4 (Tan et al., 1999). In their evaluation of HCMV ORFs for CD8+ T cell recognition, Sylwester and collegues detected IE-1 recognition by an average of ~2.9% of the total CD8+ T cell compartment (Sylwester et al., 2005), which largely agrees with the data of this study. Interestingly, the authors found that IE-1 stimulated the highest proportion of CD8+ T cells among the 213 ORFs tested (Sylwester et al., 2005). These extraordinary high numbers of CD8+ T cells specific for IE-1 could be explained with IE-1 being among the first proteins endogenously expressed in infected cells (Stinski et al., 1983). Consequently, IE-1-specific T cells are the first to be activated, and are therefore at an advantage in the competition with other T cells, for example for access to APCs (Kedl et al., 2003). This competition may be intensified by HCMV's progressive interference with MHC class I-dependent peptide presentation during the viral replication cycle. Furthermore, in the post-acute phase of infection, IE-1-specific CD8+ T cells may experience continuous stimulation by subclinical virus reactivation, leading to their sustained expansion (Khan et al., 2007). As IE-1-specific CTLs eliminate cells with reactivating virus at the early stage, they may limit the expansion of T cells recognizing antigens expressed later in the replication cycle such as pp65 (Khan et al., 2007).

In this study, 11 HCMV-seropositive blood donors were analyzed for IE-1 epitopes. Although these donors covered a broad panel of HLA class I types, only 1 or 2 epitopes restricted through HLA-A, -B or -C allotypes could be identified for each donor, and these epitopes were frequently found to be immunodominant in different donors sharing the respective class I alleles. For example, in HLA-A*0201 and/or C*0702-positive donors, the A*0201 epitope VLE and/or the C*0702-restricted epitope generally ranked first or second in frequency, irrespective of other HLA allotypes carried by the donor. This is in contrast to the IE-1-specific CD4+ T cell repertoire found to comprise 3–7 different specificities per donor with the same epitopes not always eliciting strongest responses in different donors. Such pronounced CD8+ T cell immunodominance hierarchies have previously been reported for other HCMV proteins like pp65 (Khan et al., 2007) and also other viruses such as EBV (Khanna and Burrows, 2000) and vaccinia (Kastenmuller et al., 2007). Several mechanisms which could lead to epitope selectivity and differential prominence of T cell specificities have been proposed. First of all, among the thousands of peptides that can be formed by degradation of a medium-sized protein like IE-1, few will bind with high affinity to a given HLA class I allotype, because there is a strict requirement for particular "anchor" residues in defined positions of the sequence, as well as a strict requirement of correct peptide length, often 9 amino acids (Yewdell and Bennink, 1999). The number of available peptides is further reduced by the substrate specificity of the proteasome: only a peptide carrying a preferred proteasomal cut site at its C-terminus, but not inside its sequence, will be produced with high efficiency. By contrast, HLA class II molecules appear to possess less stringently de-

fined peptide binding motifs, and there are much less stringent length requirements, which enables the engagement of a larger number of different peptides. Promiscuous binding of the same peptide to several different class II molecules may further enhance the spectrum of available HLA class II/peptide complexes (discussed in 5.1). If the restrictions mentioned above lead to unequal levels of different HLA class I/peptide complexes on the surface of infected cells or APCs, this might impact on the magnitude of epitope-specific T cell responses (Khan et al., 2007). Ultimately, the HLA/peptide complex density at the cell surface will depend on the processing efficiency of the target peptide and its MHC class I binding affinity (Yewdell and Bennink, 1999), but also on allele-specific effects of HCMV immunoevasins (discussed later in 5.3). Features of the T cells, the other side of the coin, participate in shaping the immunodominance structure, for example CD8+ T cell precursor frequencies (Thomas et al., 2007) and TCR affinity (Kedl et al., 2003). Selection of high affinity T cell clones from an initially more diverse T cell pool available during early primary response to virus infection was observed at the level of single epitopes such as pp65 NLV and TPR (Day et al., 2007). The rapid expansion of dominant T cell populations further restricts the diversity of the immune response through competition-based suppression of subdominant responses, a process called ‘immunodomination’ (Stock et al., 2006). In this process, dominant T cell specificities limit the access of low-frequency T cells to APCs and cytokines (Kedl et al., 2003; Oh et al., 2004) and deprive them of their target antigens by direct killing of infected cells or APCs (Stock et al., 2006). For example, during symptomatic primary infection, Khan and collegues detected a number of weaker subdominant T cell responses in addition to the dominant responses to pp65 and IE-1, however, these were undetectable after transition to the latent phase of virus carriage (Khan et al., 2007). When an immunodominant T cell epitope is lost from the antigen, an expansion of T cells targeting alternate epitopes may take place as shown for HSV (Stock et al., 2006) and influenza (Andreansky et al., 2005), demonstrating the importance of immunodomination.

Due to the restriction of the IE-1-specific CD8+ T cell repertoire to a limited number of targeted epitopes, the characterization of a large panel of 11 blood donors with various HLA class I types was necessary to allow the identification of novel T cell epitopes within the present study. Three new epitopes were characterized in more detail. The subdominant T cell epitope identified in donor F46 was restricted through HLA-A*03(01), which is, after A*0201 (~29% gene frequency), the second most common HLA allele in the German population (~16%) (Schmidt et al., 2009), thus making it an interesting candidate for immunotherapy. These A*0301-restricted T cell clones showed HLA-A*29(02)-dependent alloreactivity, which should be considered if these T cells are to be used for adoptive transfer in HLA-mismatched transplant patients (Amir et al., 2011). Based on common amino acid residues of well-described HLA-A*03(01)-restricted epitopes (Hill et al., 1995; Pepperl et al., 1998), the 10-mer RHRIKEHMLK was postulated as the minimal epitope of these T cells, but this remains to be confirmed in the future by peptide

titration studies. The other two new IE-1 epitopes evaluated in more detail in this study were identified as the first IE-1 epitopes restricted through HLA-C allotypes: an epitope uniquely found in donor ARZ was presented on HLA-C*0602, and an epitope found in donors ALT, AJJ, AJU, F46, and F60 was HLA-C*0702-restricted. The minimal epitope sequence was determined for the HLA-C*0702-restricted epitope. The sequence of this nonameric peptide was CRV-LCCYVL (IE-1, aa 309-317). A variant of this sequence, CRVLCCY**I**L, that is found in some HCMV strains such as Toledo, Davis and TB40E, was equally well recognized by specific T cell clones. The high prevalence of HLA-C*0702 in the population, e.g. in 28% of Germans (Schmidt et al., 2009) and 37% of southern Chinese (Trachtenberg et al., 2007), renders this T cell epitope a particularly attractive candidate for immunotherapy. Analyses of the frequency of such responses across donors showed that, strikingly, CRV-reactive T cells were present in all 15 HLA-C*0702-positive donors analyzed, and sometimes reached very high numbers (up to 4.5% of total lymphocytes, detected by multimer staining). In the majority (~73%) of HLA-C*0702-positive donors analyzed, the response to the CRV epitope dominated the response to the total IE-1 protein, even in HLA-A*0201 carriers that also had T cells specific for the known, strongly immunogenic epitope VLE (Khan et al., 2002). Furthermore, HLA-C*0702-positive donors were generally found to harbor higher numbers of total IE-1-specific T cells compared to HLA-C*0702-negative donors, thus confirming the importance of this particular epitope. A possible mechanism underlying the immunodominance of CRV-specific T cells was identified in this work and involves selective preservation of HLA-C surface expression despite HCMV immunoevasion, leading to escape from NK cell recognition (discussed in 5.4).

Interestingly, dominant CD8+ T cell responses to the CRV peptide have been reported earlier (Kern et al., 1999). Since that time, such responses were described to be restricted through HLA-B7, and this notion has been accepted in the literature (Wills et al., 2002; Khan et al., 2007), but was not experimentally verified. Data of the present work clearly demonstrate that this view is erroneous: experiments using recombinant HLA-B*0702 and -C*0702 in transfection studies showed that the CRV peptide-presenting HLA molecule was undoubtedly HLA-C*0702; moreover, the CRV peptide could be incorporated into MHC-peptide multimers of HLA-C*0702 (this study), but not of HLA-B*0702 (Khan et al., 2004), and CRV-specific T cells were well stained with C*0702/peptide multimers. It can easily be reconstructed how the original study mistakenly defined the CRV epitope as HLA-B7-restricted: Kern and collegues investigated *ex vivo* CD8+ T cell responses in donors of presumable European descent who were HLA-typed at low resolution, and responses to the CRV peptide were only found in HLA-B7+ Cw7+ donors, whereas B7– Cw7+ donors never responded, thus leading to the conclusion that CRV was B7-restricted (Kern et al., 1999). However, HLA-B and -C alleles are in strong linkage disequilibrium, their genes being positioned close to each other in the MHC locus. In European populations, HLA-C*0702 is almost always linked with HLA-B*07, whereas the equally frequent HLA-

C*0701 is usually linked with HLA-B*08 (Schmidt et al., 2009). Of note, such linkages may differ between worldwide populations; for example, in Southern Chinese HLA-C*0702 most frequently segregates with B*4001 and B*3802 (Trachtenberg et al., 2007).

Compared to HLA-A and -B molecules, the role of HLA-C in antigen presentation was long neglected. One historical reason for this is a technical limitation, as HLA-C alleles of blood donors are difficult to determine by human alloantisera that were routinely used for HLA typing before PCR-based techniques arose (McCutcheon et al., 1995). In addition, HLA-C was considered to be less effective in antigen presentation owing to its observed 10-fold lower surface expression compared to HLA-A and -B allotypes in some cell types (Snary et al., 1977; McCutcheon et al., 1995). Several mechanisms of HLA-C downregulation are discussed in the literature: rapid turnover of heavy chain mRNA due to miRNA targeting; inefficient association of HLA-C heavy chains with β_2-microglobulin; higher restricted peptide binding properties of HLA-C compared to HLA-A and -B molecules leading to accumulation of immature HLA-C molecules in the ER and eventually to subsequent degradation; promotion of internalization and lysosomal degradation by a di-hydrophobic signal in the HLA-C cytoplasmic tail (reviewed in (Kulpa and Collins, 2011)). However, low cell surface expression of HLA allotypes does not necessarily prevent T cell recognition, as it was shown that fewer than 10 MHC-peptide complexes per target cell can be sufficient to trigger T cell activation (Kageyama et al., 1995). Furthermore, phosphorylation of a serine residue in the cytoplasmic tail of HLA-C was reported to upregulate HLA-C surface expression upon macrophage differentiation suggesting a unique role for HLA-C in APCs (Schaefer et al., 2008). Today, the importance of HLA-C for antigen presentation to T cells is beyond question, since epitopes restricted through HLA-C allotypes have been identified in several viruses including HCMV (mainly for pp65) (Kondo et al., 2004; Slezak et al., 2007), EBV (Khanna and Burrows, 2000) and HIV (Adnan et al., 2006). As shown for an HCMV HLA-C*0602-restricted pp65 epitope (Rist et al., 2009) and for the CRV epitope from IE-1 (this study), HLA-C-restricted T cells can show similar or even higher memory T cell frequencies than dominant HLA-A and B-restricted epitopes. Precise information on conserved T cell epitope motifs for HLA binding would be useful to identify additional targets for monitoring and immunotherapy of HCMV and other viruses, but is still not available for several HLA-C allotypes. Accordingly, it is still difficult to predict HLA-C*0702-restricted epitopes. Although tyrosine (Y) and proline were defined to be anchors in position two, arginine (R) was similarly enriched in this position in pool-sequenced endogenous C*0702-bound peptides (Falk et al., 1993). Well-characterized C*0702-restricted T cell epitopes such as VRIGHLYIL from MAGE-A12 (Bettinotti et al., 2003) and FRCPRRFCF from HCMV UL28 (Kim et al., 2011) share with the CRV peptide an arginine at position two and a bulky hydrophobic residue at the C-terminus. Therefore, it can be hypothesized that these might be preferred anchors of HLA-C*0702-restricted T cell epitopes.

5.3 Differential resistance of CD8+ T cells to HCMV immunoevasion

Ultimately, the control of HCMV infection is achieved by CD8+ T cells (Riddell et al., 1992). Hence, it is not surprising that a substantial number of viral genes is devoted to counteracting T cell recognition of infected cells by interfering with the MHC class I pathway of antigen presentation (Manley et al., 2004). Immunoevasins encoded in the US2-11 region of the HCMV genome downmodulate MHC class I surface expression by preventing peptide transport (US6), retaining MHC molecules in the ER (US3), or targeting them for cytoplasmic degradation (US2, US11) (Jones et al., 1996; Wiertz et al., 1996a; Wiertz et al., 1996b; Ahn et al., 1997). The broad range of immunoevasins with overlapping function is astonishing, however, it may be necessary for cell-type-specific adaptation (Rehm et al., 2002) and, particularly, to cope with the high number of existing class I allotypes due to MHC polymorphism (Reddehase, 2002). A large body of research has been dedicated to allele-specific effects of HCMV immunoevasins (Miller-Kittrell and Sparer, 2009). However, most of these studies have been limited to molecular or phenotypic investigations of transfected or transduced cells, and the impact of the observed effects on genuine CD8+ T cell recognition of HCMV-infected cells has remained largely unexplored. Such studies are warranted because CD8+ T cells might well be sensitive enough to recognize their antigen even when the presenting peptide/MHC class I complex is partially downregulated by the virus. For developing T cell therapies and vaccines, the identification of HCMV peptide/MHC complexes that are functionally recognized in spite of viral immunoevasion would be important.

The IE-1 protein is a prime example for strong impact of HCMV immunoevasion. However, the studies investigating recognition of IE-1 in context of HCMV infection did not produce consistent results. The majority of groups have observed that the recognition of HCMV-infected cells by IE-1-specific CD8+ T cells is severely suppressed (Gilbert et al., 1993; Mutimer et al., 2002; Manley et al., 2004; Khan et al., 2005; Besold et al., 2007). By contrast, a minority of reports described that IE-1-specific CD8+ T cells recognize and control HCMV infection *in vitro* (Borysiewicz et al., 1988). This controversy in the literature has remained unresolved until today. In the previous studies, T cell recognition of IE-1 in the context of HCMV infection was investigated using T cell clones directed against A2 epitopes VLE (Khan et al., 2005) and TMY (Besold et al., 2007) or with T cells of undefined HLA restriction (Borysiewicz et al., 1988; Mutimer et al., 2002; Manley et al., 2004). Hence, the conflicting data might reflect the analysis of T cell clones that were restricted through HLA allotypes differentially affected by viral immunoevasion. This study used an extended set of CD8+ T cell clones restricted through a variety of different HLA-A, -B and -C allotypes to analyze the recognition of IE-1-derived peptides in context of HCMV infection. Strikingly, only T cell clones directed against the IE-1 epitope CRV (HLA-C*0702-restricted) identified in this study recognized wild-type HCMV-infected cells in spite of the presence of viral immunoevasins, whereas T cell clones restricted through HLA-A and -B allotypes did not. As shown by peptide titration studies, this differential T cell recognition was not due to different

avidities of the T cell clones. By contrast, in the absence of class I immunoevasins US2, 3, 6, and 11, recognition of infected cells by HLA-A and B-restricted T cell clones was restored. As expected, pp65 recognition by specific T cell clones used for comparison was less affected by viral immunoevasion. As structural protein, pp65 is introduced into host cells with the virion and therefore processed and presented before the onset of full viral immunoevasive activity (Riddell et al., 1991; McLaughlin-Taylor et al., 1994). Cells infected with the US2/3/6/11-deleted virus stimulated every CD8+ T cell clone similarly strongly as uninfected cells that had been exogenously loaded with the antigenic peptide, indicating that HCMV antigens were very efficiently processed and presented in the absence of US2, 3, 6, and 11, and that other immunoevasins such as US10 (Park et al., 2010) did not interfere with antigen presentation by the HLA-A, -B or -C allotypes tested. To conclude, HLA-C*0702-restricted T cells largely resist HCMV class I imunoevasion. This may provide an explanation for the extraordinarily high frequency of these T cells in healthy carriers due to better stimulation by infected cells.

Employing a broad panel of IE-1- and pp65-specific T cell clones, this study provides, for the first time, a systematic dissection of HLA class I allotype-specific effects of individual HCMV immunoevasins on CD8+ T cell recognition of infected cells. The major focus was on IE-1: CD8+ T cell clones restricted through seven different HLA class I allotypes of all major classes were available (three different HLA-A allotypes, three HLA-B allotypes, and one HLA-C allotype), all of them generated in this study, and the interaction of these clones with HCMV-infected cells was studied side by side at different stages of infection (days 1, 2, 3, and 4). CD8+ cell clones specific for pp65 epitopes with three different HLA-A/B restrictions were studied in parallel. T cell reactivity to wild-type HCMV and to a set of HCMV gene deletion mutants that carried no or only one of the immunoevasin genes US2, US3, US6, and US11 were compared under all these conditions. Therefore, the results comprehensively inform on the following aspects: (1) allotype specificity of HCMV immunevasion for epitopes from the same HCMV antigen; (2) temporal pattern of immunoevasion after infection; (3) differential regulation of immunoevasion for representative antigens of different functional classes; (4) the individual role of each HCMV immunoevasin. The results of these analyses show that there is an unexpectedly large degree of diversification of HCMV immunoevasion on all these investigated levels: allotype, antigen, infection phase, and immunoevasin.

An influence of US6 was detected for all specificities tested as expected. The US6 protein prevents the translocation step of peptide ligands for HLA class I across the ER membrane and is found associated with the peptide loading complex including TAP1, TAP2, and tapasin (Hengel et al., 1997). Effects of US6 were largely revertable by IFN-γ, due to this cytokine's ability to induce TAP1, TAP2, tapasin and subunits of the immunoproteasome, which will increase the generation and supply of viral peptides for MHC class I assembly (Schroder et al., 2004). Furthermore, IFN-γ strongly impairs gpUS6 synthesis in HCMV-infected cells (Benz and

Hengel, 2000), which may have further contributed to the observed reversion of US6 effects by IFN-γ.

Analysis of US3-mediated impact on T cell recognition revealed that only IE-1 presentation in context of HLA-C*0702 and A*0301 was completely unimpaired by this immunoevasin. As US3 inhibits the ER-resident chaperone tapasin, the susceptibility of HLA class I allotypes to US3 is related to their grade of tapasin-dependence for peptide loading and surface expression (Park et al., 2004). The amino acid at position 114 of class I heavy chains was described to be decisive for the degree of tapasin-dependence: high for acidic residues (D, E), moderate for neutral residues (N, Q), and low for basic residues (H, R) (Park et al., 2003). This rule was only partially confirmed by the data of this study. Concordant with Park et al., peptide presentation on HLA-A*0301 (114R) was completely unaffected by US3, whereas presentation on HLA-B*0801 (114N), B*1801 (114D), B*0702 (114D) and B*3501 (114D) was affected to a greater or lesser extent. However, in discordance with the rule of Park et al., HLA-A*0201 (114H) and A*6801 (114R)-dependent T cell recognition was affected by US3, and HLA-C*0702 (114D)-dependent peptide presentation was fully resistant to US3, even under IE conditions (after accumulation of US3 mRNA). Hence, other residues of the MHC class I heavy chain, in addition to position 114, must be involved in interaction with tapasin. This hypothesis is supported by more recent studies on HLA allotypes which differ from each other only in one amino acid (position 116 or 156) that is determining for tapasin dependence (Sieker et al., 2007; Badrinath et al., 2012). In the present study, effects of US3 were fully compensated by IFN-γ, probably because the capability of US3 to retain HLA molecules is exhausted under these conditions.

For US2 and US11, more complex T cell recognition patterns arose: US2 and US11, both strongly and equally suppressed recognition of IE-1 peptides presented by HLA-A*0201, B*1801 and B*3501, weakly affected C*0702-dependent (only without IFN-γ pretreatment) and largely spared B*0801- and B*0702-dependent presentation; among the allotypes with different susceptibility to US2 and US11, HLA-A*6801 was more strongly affected by US2 and A*0301 by US11; HLA-B*4001-mediated presentation was only influenced by US2. These data partly confirm and partly challenge the conclusions drawn from earlier studies. Using CTL clones generated from HLA-A2 transgenic mice, strong effects of either immunoevasin, US2 and US11, were observed for HLA-A*0201-dependent presentation of IE-1_{TMY} (Besold et al., 2009) and weaker effects for $pp65_{NLV}$ (Besold et al., 2009) which is in good accordance with this work detecting generally less impact of immunoevasins on pp65 presentation. The binding of US2 to recombinant MHC/peptide complexes containing HLA-A2 and -A68 (Gewurz et al., 2001) is precisely mirrored by the observation of this study that CD8+ T cell recognition of IE-1 peptides presented by HLA-A2 and -A68 was fully inhibited after infection with a HCMV recombinant expressing only US2. Discordant with the HLA staining data of this study and of Besold et al. (2009), others reported that gpUS2 failed to interact with HLA-B7 in the ER and was insufficient to downregu-

late HLA-B7 on the cell surface (Gewurz et al., 2001; Llano et al., 2003; Barel et al., 2006). A potential reason for this discrepancy could be a higher baseline HLA class I expression in the lymphoid cells studied by these authors, which is more difficult to overcome by immunoevasins that have only a limited affinity for the relevant HLA allele, such as US2 for HLA-B7. By contrast, downregulation of HLA-B*0702 surface expression by US11 detected in this study, was also found by other groups (Llano et al., 2003; Barel et al., 2006). Interestingly, in the present work, partial downregulation of HLA-B*0702 permitted unimpaired peptide presentation to T cells, which is a good example that biochemical studies of immunoevasins' effects cannot reliably predict their impact on T cell recognition, because (I) they only study total levels of HLA but not levels of particular HLA/peptide complexes, and (II) they neglect that reductions in HLA levels will not be relevant for T cell recognition if they occur in dimensions that are far above the sensitivity thresholds of T cells. After coinfection of J26 cells with VV-US2 and VV-HLA-B8, cell surface expression of HLA-B8 was unaffected (Thilo et al., 2006), thus confirming the unimpared HLA-B*0801-dependent T cell recognition in presence of US2 observed in this study. In accordance with this study, HLA-C alleles generally do not seem to be retained by US2 (Schust et al., 1998; Gewurz et al., 2001; Barel et al., 2003a). By contrast, US11 appears to affect some HLA-C allotypes (Llano et al., 2003; Barel et al., 2006) but not others (Schust et al., 1998). Of note, previous studies reported partial downregulation of endogenous Cw7 in a B lymphoma cell line (RPMI 8866) by overexpression of either immunoevasin, US2 or US11 (Llano et al., 2003), which conforms the weak effects of both immunoevasins on HLA-C*0702-dependent presentation observed in this study, however, only without IFN-γ pretreatment of the infected cells. IFN-γ could fully compensate only for modest influences of US2 and US11 as detected for HLA-C*0702, but not for strong effects as in case of HLA-A*0201 or A*6801.

Studies that showed reduced surface expression of HLA-A2 and -G, but not of HLA-B7, -Cw3, or -E, by retrovirally expressed US2 in astrocytoma cells and murine fibroblasts (Barel et al., 2003b; Barel et al., 2006) indicate that amino acids 180–183, positioned at the junction of the α_2/α_3 domains of the MHC class I heavy chain, have a dominant role for US2-mediated downregulation. This agrees well with the observation of this study that US2 sensitivity of T cell recognition (but not MHC–T cell interaction) is conferred by amino acids that are C-terminal to residue 176. However, the variations in this region are complex and do currently not allow to predict the degree of US2-mediated downregulation for every allotype (Barel et al., 2006). With regard to US11, the data of this work confirm those of Barel et al. (2006) who described that HLA-A2 was the allotype most strongly affected by US11. These authors further showed that the presence of a lysine-valine (KV) motif at the C-terminus of the MHC class I cytoplasmic tail was associated with US11-mediated downregulation (Barel et al., 2003a), but their data were not fully consistent with this rule for every allotype and chimeric MHC class I chain tested (Barel et al., 2006). This is also the case for the functional data obtained in the present study: T cell

recognition in context of all HLA-A alleles (which carry the KV motif) was partially or completely sensitive to US11; the situation for the tested HLA-B and -C alleles (which all lack this motif) was more complex: most allotypes were resistant, but the allotypes B*1801 and B*3501 were not. This may indicate that other residues than KV may also sensitize to US11. In case of HLA-C*0702 and A*0201, US11 sensitivity was fully reverted by exchange of amino acids C-terminal of position 176, suggesting that residues in the α_1/α_2 domain might not be decisive for US11 sensitivity as supposed in a previous study (Barel et al., 2003a).

For most class I allotypes, a single immunoevasin was not able to reduce antigen presentation to levels detected for wild-type HCMV. Thus, gpUS2, 3, 6, and 11 cooperate to mediate efficient protection of infected cells from T cell recognition through most allotypes. This may be especially important after enhancement of total HLA class I expression by IFN-γ, which is abundantly released by NK cells and T cells during HCMV infection. Such cooperation has been studied by others in biochemical analyses showing that the turnover of class I molecules is increased in cells expressing both US2 and US3 than either immunoevasin alone (Noriega and Tortorella, 2009). Possibly, the ability of US3 to retain class I molecules in the ER creates a target-rich environment for US2 to mediate the destruction of class I heavy chains (Noriega and Tortorella, 2009). A recent report by the same group described that there is also some cooperation between US11 and US3 (Noriega et al., 2012). A prerequisite for an interplay amongst US2, 3, and 11 is a temporal overlap in their expression kinetics. However, so far US3 was the only immunoevasin of the US2/3/6/11 group shown to be expressed at immediate-early times post infection (Weston, 1988). Data of the present work and Hesse et al. (unpublished data) now clearly demonstrate that US2 and US11 are already active in the IE phase in which exogenously introduced structural proteins and regulatory IE proteins are present and available for presentation to CD8+ T cells. To attack the vast majority of HLA allotypes already at this critical phase, it appears to be required that several immunoevasins, US2, US3 and US11, are already present. In accordance with its reported expression at early/late times post infection (Jones and Muzithras, 1991), US6 effects could not be detected under IE conditions. TAP inhibition by US6 may be particularly important at later time points post infection to cope with the additional supply of antigenic peptides derived from abundantly expressed structural proteins.

Efficient recognition of wild-type HCMV infection by IE-1-specific T cells was only detected for HLA-C-restricted T cells, whereas HLA-A and B-dependent IE-1 presentation was severely suppressed. In spite of this suppression, a broad IE-1-specific CD8+ CTL response including T cells of HLA-A and -B restrictions is maintained in healthy carriers, often at high frequencies (Manley et al., 2004). How is this possible? Supposedly, HCMV immunoevasins targeting HLA class I presentation are not equally effective in inhibiting T cell activation *in vivo* due to the action of proinflammatory cytokines such as type I interferons (IFN-α/β), IFN-γ and TNF-α,

which might partially restore antigen presentation in HCMV-infected cells (Benz and Hengel, 2000). Second, reactivation from latency may differ from *de novo* infection/replication (as studied here) in its degree of immunoevasion. Third, besides direct priming through infected cells, T cell proliferation can also be induced by cross-priming via DCs that process exogenously acquired antigen but are not subjected to HCMV immunoevasion as they are not infected themselves. However, many of the so primed T cells may be biologically ineffective, since their cognate epitope is not presented by HCMV-infected cells when immunoevasion is active. Such a mechanism could be seen as a viral tactic of misleading the immune system to sustain an ineffective response (Khan et al., 2005) and has already be demonstrated vor MCMV (Holtappels et al., 2004). Hence, the critical question is: which of the various specificities have a protective role *in vivo*? Based on this work, it can be assumed that HLA-C*0702-restricted CTLs might be more effective in eliminating infected cells than HLA-A and B-restricted T cells and will therefore be particularly important for adoptive T cell therapy.

5.4 The relationship between HLA-C, CD8+ T cells and NK cells

Besides antigen presentation to CD8+ T cells, a subset of HLA class I molecules, including the HLA-C allotypes, functions in regulating NK cell activity through interaction with NK cell inhibitory receptors like NKG2A and KIRs (Lanier, 2005). The lack of such inhibitory signals ("missing self") due to downregulation of HLA expression, in combination with activating signals, would stimulate NK cells to attack infected cells (Lanier, 2005). These circumstances motivated the hypothesis, presented here, that HCMV may selectively preserve HLA-C at the cell surface in order to protect infected cells from NK cell lysis. HLA-C allotypes provide inhibitory signals either through KIR2DL1 (C2 group allotypes) or KIR2DL2/3 (C1 group allotypes), and every human KIR haplotype encodes KIRs specific for either group of HLA-C (Uhrberg et al., 1997). An individual NK cell typically expresses a subset of activating and inhibitory KIRs and other NK cell receptors like NKG2A, and in aggregate these receptors guide its reactivity (Valiante et al., 1997). To be able to analyze the role of HLA-C*0702 during NK cell recognition of HCMV-infected cells in primary NK cells (to obtain biologically relevant results) while minimizing the complexity of the system (to obtain mechanistic information), particular donors and techniques were chosen. Primary NK cells were isolated from a donor homozygous for KIR haplotype A, which is devoid of several activating KIR genes that might diversify the patterns of NK cell reactivity. Furthermore, the NK cell donor was carrier of two C1 group alleles, as it has recently become clear that the presence of class I allotypes matched to NK inhibitory receptors is required to license KIR-expressing NK cells for recognition of cells that lack those MHC class I allotypes (Orr and Lanier, 2010). As a result of such education, both the functional competence and the frequency of NK cells with self-specific KIRs is enhanced (Yu et al., 2007; Kim et al., 2008). However, such a licensing state of NK cells may change upon transfer into an environment with

altered KIR ligands, for example after stem cell transplantation (Yu et al., 2009). In an effort to minimize any potential functional "re-education" in culture, isolated KIR2DL3-positive NK cells were cultivated in the continuous presence of feeder cells that were biallelic for HLA-C group 1.

During HCMV infection, NK cell reactivity is modulated by interactions with additional inhibitory and activating NK receptors (Leong et al., 1998; Carr et al., 2002), and a multitude of HCMV proteins target such interactions (Wilkinson et al., 2008). Nontheless, using HLA-C*0702-specific NK cell lines, the present experiments indicate that the classical mechanism of inhibitory signaling through HLA-C and an inhibitory KIR (KIR2DL3) largely protects infected fibroblasts from NK cell recognition: compared to lysis of MHC-deficient cell lines, lysis of wild-type HCMV-infected cells was low during the whole replicative cycle (analyzed during 96 hours). The NK cell lines were even sensitive enough to confirm partial downregulation of HLA-C*0702 by wild-type HCMV compared to immunoevasion-defective HCMV strains, as detected in the T cell assays in absence of IFN-γ. Interestingly, in the NK cell assays, IFN-γ treatment of fibroblasts prior to infection to enhance total MHC expression reduced NK cell cytotoxicity only marginally, suggesting that lower numbers of HLA-C*0702 molecules might be already sufficient to inhibit NK cells. By contrast, complete downregulation of HLA-C*0702, as simulated in the antibody-blocking studies, would render infected cells much more susceptible to NK cell lysis.

Surprisingly, the idea that the downregulation of classical MHC class I molecules is causally connected to NK cell recognition of HCMV-infected cells is controversial, being supported by some (Huard and Fruh, 2000; Falk et al., 2002; Llano et al., 2003) but contested by other studies (Leong et al., 1998; Cerboni et al., 2000; Carr et al., 2002). Allotype-specific downregulation of MHC class I could explain some of these contradictions. For example, Falk and collegues demonstrated that NK cell lysis was induced after fibroblast infection with wild-type but not US2–11-deleted HCMV (Falk et al., 2002). Because the relevant receptor-ligand pair in this study was KIR2DL2 and (likely) HLA-C*0701, this rises the interesting possibility that the two major HLA-C*07 allotypes are differentially regulated by HCMV infection. Others suggested that KIRs and MHC class I do not play a role in NK recognition of HCMV-infected fibroblasts (Leong et al., 1998), but because the role of individual KIR-HLA pairings in the context of infection was not addressed, their data are consistent with the results of this work. The absence of a differential reactivity to non-infected and infected autologous fibroblasts observed in a majority of NK cell clones from two HLA-C*0702 carriers (Carr et al., 2002) is perfectly compatible with an HLA-C*0702-mediated inhibition via KIR2DL3, as demonstrated in this study.

The NK cell experiments of this study are restricted to the role of HLA-C*0702/KIR2DL3 interaction in NK cell defense of HCMV. Hence, it is unknown whether other HLA/KIR combinations similarly protect infected cells from NK cell recognition, but it is entirely possible that particular HLA/KIR pairings play uniquely favourable or unfavourable roles. For example, such a unique

role has been shown for resolution of hepatitis C virus infection, where superior protection is mediated by KIR2DL3 and group 1 HLA-C (Khakoo et al., 2004). In this line, experimental data of this work predict that NK cells whose specificity is determined by KIR3DL2 should be less inhibited from recognizing HCMV-infected cells, because HLA-A3, a ligand of this KIR, is fully downregulated after HCMV infection.

Taken together, the immunodominance of HLA-C*0702-restricted T cells observed in this study may be the consequence of a viral strategy that strives to minimize NK cell recognition of HCMV infection by selective preservation of NK cell inhibitory ligands on the surface of infected cells. NK cells are part of the innate immune response, the earliest host defense mechanism capable to immediately interfere with virus infection, before adaptive immunity takes effect. Therefore, NK cell evasion might be essential for HCMV to establish infection in the host. Later on, T cells (especially CRV-specific ones) may efficiently control productive HCMV replication but are not able to completely eliminate the virus due to its persistence in a latent state. This mechanism to escape NK cell attack may also be operative for other viruses. Indeed it has already been reported that the HIV Nef protein downregulates HLA-A and -B molecules on the cell surface but spares HLA-C (Cohen et al., 1999), implying that HLA-C-restricted T cells could potentially control HIV infection with particular efficiency (Kulpa and Collins, 2011).

According to the previous hypothesis, HLA-C*0702 carriers might have less efficient NK cells but very efficient T cells for HCMV control. Further studies will be required to assess if these persons are especially protected from HCMV disease and, conversely, if HLA-C*0702-negative individuals are more susceptible to HCMV or to a more severe manifestation of viral disease. Notably, in carriers of other C1 group HLA alleles such as C*0701, inhibition of KIR2DL2/3-positive NK cells would be operative as well, but might not be complemented by an IE-1-specific CD8+ T cell response that is as abundant and efficient as CRV-specific T cells. (It is worth noting that the CRV epitope is not recognized on C*0701 by T cells, although the differences by the two C*07 allotyes are rather minor.) Potentially, HLA-C*0702-negative donors carry unidentified specific T cells directed against other HCMV proteins than IE-1 and restricted through other HLA-C allotypes, that are similarly resistant to HCMV-mediated suppression. Such investigations might be particularly interesting in context of congenital HCMV infection as HLA-C, but not HLA-A and -B, is expressed on trophoblast cells (Apps et al., 2009) and might therefore be involved in protection of the fetus from HCMV infection. To think one step further, it is obvious that certain HLA alleles including HLA-C*0702 appear to be particularly frequent in the human population. It might well be that such alleles or also certain HLA/KIR combinations have been selected for during human evolution due to superior control of ubiquitous pathogens including HCMV and other human herpesviruses which have accompanied the human species since its existence.

References

Adhikary, D., U. Behrends, H. Boerschmann, A. Pfunder, S. Burdach, A. Moosmann, K. Witter, G.W. Bornkamm, and J. Mautner. 2007. Immunodominance of lytic cycle antigens in Epstein-Barr virus-specific CD4+ T cell preparations for therapy. *PLoS One*. 2:e583

Adler, S.P., S.E. Starr, S.A. Plotkin, S.H. Hempfling, J. Buis, M.L. Manning, and A.M. Best. 1995. Immunity induced by primary human cytomegalovirus infection protects against secondary infection among women of childbearing age. *J Infect Dis*. 171:26-32

Adnan, S., A. Balamurugan, A. Trocha, M.S. Bennett, H.L. Ng, A. Ali, C. Brander, and O.O. Yang. 2006. Nef interference with HIV-1-specific CTL antiviral activity is epitope specific. *Blood*. 108:3414-3419

Ahn, K., A. Angulo, P. Ghazal, P.A. Peterson, Y. Yang, and K. Fruh. 1996. Human cytomegalovirus inhibits antigen presentation by a sequential multistep process. *Proc Natl Acad Sci U S A*. 93:10990-10995

Ahn, K., A. Gruhler, B. Galocha, T.R. Jones, E.J. Wiertz, H.L. Ploegh, P.A. Peterson, Y. Yang, and K. Fruh. 1997. The ER-luminal domain of the HCMV glycoprotein US6 inhibits peptide translocation by TAP. *Immunity*. 6:613-621

Alp, N.J., T.D. Allport, J. Van Zanten, B. Rodgers, J.G. Sissons, and L.K. Borysiewicz. 1991. Fine specificity of cellular immune responses in humans to human cytomegalovirus immediate-early 1 protein. *J Virol*. 65:4812-4820

Amir, A.L., D.M. van der Steen, R.S. Hagedoorn, M.G. Kester, C.A. van Bergen, J.W. Drijfhout, A.H. de Ru, J.H. Falkenburg, P.A. van Veelen, and M.H. Heemskerk. 2011. Allo-HLA-reactive T cells inducing graft-versus-host disease are single peptide specific. *Blood*. 118:6733-6742

Andreansky, S.S., J. Stambas, P.G. Thomas, W. Xie, R.J. Webby, and P.C. Doherty. 2005. Consequences of immunodominant epitope deletion for minor influenza virus-specific CD8+-T-cell responses. *J Virol*. 79:4329-4339

Apps, R., S.P. Murphy, R. Fernando, L. Gardner, T. Ahad, and A. Moffett. 2009. Human leucocyte antigen (HLA) expression of primary trophoblast cells and placental cell lines, determined using single antigen beads to characterize allotype specificities of anti-HLA antibodies. *Immunology*. 127:26-39

Arase, H., E.S. Mocarski, A.E. Campbell, A.B. Hill, and L.L. Lanier. 2002. Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors. *Science*. 296:1323-1326

Arnon, T.I., H. Achdout, O. Levi, G. Markel, N. Saleh, G. Katz, R. Gazit, T. Gonen-Gross, J. Hanna, E. Nahari, A. Porgador, A. Honigman, B. Plachter, D. Mevorach, D.G. Wolf, and O. Mandelboim. 2005. Inhibition of the NKp30 activating receptor by pp65 of human cytomegalovirus. *Nat Immunol*. 6:515-523

Badrinath, S., P. Saunders, T. Huyton, S. Aufderbeck, O. Hiller, R. Blasczyk, and C. Bade-Doeding. 2012. Position 156 influences the peptide repertoire and tapasin dependency of human leukocyte antigen B*44 allotypes. *Haematologica*. 97:98-106

Barel, M.T., N. Pizzato, P. Le Bouteiller, E.J. Wiertz, and F. Lenfant. 2006. Subtle sequence variation among MHC class I locus products greatly influences sensitivity to HCMV US2- and US11-mediated degradation. *Int Immunol*. 18:173-182

Barel, M.T., N. Pizzato, D. van Leeuwen, P.L. Bouteiller, E.J. Wiertz, and F. Lenfant. 2003a. Amino acid composition of alpha1/alpha2 domains and cytoplasmic tail of MHC class I molecules determine their susceptibility to human cytomegalovirus US11-mediated down-regulation. *Eur J Immunol*. 33:1707-1716

Barel, M.T., M. Ressing, N. Pizzato, D. van Leeuwen, P. Le Bouteiller, F. Lenfant, and E.J. Wiertz. 2003b. Human cytomegalovirus-encoded US2 differentially affects surface expression of MHC class I locus products and targets membrane-bound, but not soluble HLA-G1 for degradation. *J Immunol*. 171:6757-6765

Barnaba, V., A. Franco, M. Paroli, R. Benvenuto, G. De Petrillo, V.L. Burgio, I. Santilio, C. Balsano, M.S. Bonavita, G. Cappelli, and a. et. 1994. Selective expansion of cytotoxic T lymphocytes with a CD4+CD56+ surface phenotype and a T helper type 1 profile of cytokine secretion in the liver of patients chronically infected with Hepatitis B virus. *J Immunol*. 152:3074-3087

Barnes, P.D., and J.E. Grundy. 1992. Down-regulation of the class I HLA heterodimer and beta 2-microglobulin on the surface of cells infected with cytomegalovirus. *J Gen Virol*. 73:2395-2403

Beadling, C., and K.A. Smith. 2002. DNA array analysis of interleukin-2-regulated immediate/early genes. *Med Immunol*. 1:2

Beninga, J., B. Kropff, and M. Mach. 1995. Comparative analysis of fourteen individual human cytomegalovirus proteins for helper T cell response. *J Gen Virol*. 76:153-160

Benz, C., and H. Hengel. 2000. MHC class I-subversive gene functions of cytomegalovirus and their regulation by interferons-an intricate balance. *Virus Genes*. 21:39-47

Besold, K., N. Frankenberg, S. Pepperl-Klindworth, J. Kuball, M. Theobald, G. Hahn, and B. Plachter. 2007. Processing and MHC class I presentation of human cytomegalovirus pp65-derived peptides persist despite gpUS2-11-mediated immune evasion. *J Gen Virol*. 88:1429-1439

Besold, K., and B. Plachter. 2008. Recombinant viruses as tools to study human cytomegalovirus immune modulation. *Med Microbiol Immunol*. 197:215-222

Besold, K., M. Wills, and B. Plachter. 2009. Immune evasion proteins gpUS2 and gpUS11 of human cytomegalovirus incompletely protect infected cells from CD8 T cell recognition. *Virology*. 391:5-19

Bettahi, I., X. Zhang, R.E. Afifi, and L. BenMohamed. 2006. Protective immunity to genital herpes simplex virus type 1 and type 2 provided by self-adjuvanting lipopeptides that drive dendritic cell maturation and elicit a polarized Th1 immune response. *Viral Immunol*. 19:220-236

Bettelli, E., T. Korn, and V.K. Kuchroo. 2007. Th17: the third member of the effector T cell trilogy. *Curr Opin Immunol*. 19:652-657

Bettinotti, M.P., M.C. Panelli, E. Ruppe, S. Mocellin, G.Q. Phan, D.E. White, and F.M. Marincola. 2003. Clinical and immunological evaluation of patients with metastatic melanoma undergoing immunization with the HLA-Cw*0702-associated epitope MAGE-A12:170-178. *Int J Cancer*. 105:210-216

Bickham, K., C. Munz, M.L. Tsang, M. Larsson, J.F. Fonteneau, N. Bhardwaj, and R. Steinman. 2001. EBNA1-specific CD4+ T cells in healthy carriers of Epstein-Barr virus are primarily Th1 in function. *J Clin Invest*. 107:121-130

Biegalke, B.J. 1995. Regulation of human cytomegalovirus US3 gene transcription by a cis-repressive sequence. *J Virol*. 69:5362-5367

Biron, C.A., K.S. Byron, and J.L. Sullivan. 1989. Severe herpesvirus infections in an adolescent without natural killer cells. *N Engl J Med*. 320:1731-1735

Bloss, T.A., and B. Sugden. 1994. Optimal lengths for DNAs encapsidated by Epstein-Barr virus. *J Virol*. 68:8217-8222

Boeckh, M., and A.P. Geballe. 2011. Cytomegalovirus: pathogen, paradigm, and puzzle. *J Clin Invest*. 121:1673-1680

Boehme, K.W., J. Singh, S.T. Perry, and T. Compton. 2004. Human cytomegalovirus elicits a coordinated cellular antiviral response via envelope glycoprotein B. *J Virol*. 78:1202-1211

Boppana, S.B., and W.J. Britt. 1995. Antiviral antibody responses and intrauterine transmission after primary maternal cytomegalovirus infection. *J Infect Dis*. 171:1115-1121

Boppana, S.B., and K.B. Fowler. 2007. Persistence in the population: epidemiology and transmisson. In "Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis". *Cambridge University Press*

Boppana, S.B., R.F. Pass, W.J. Britt, S. Stagno, and C.A. Alford. 1992. Symptomatic congenital cytomegalovirus infection: neonatal morbidity and mortality. *Pediatr Infect Dis J*. 11:93-99

Borrego, F., M. Ulbrecht, E.H. Weiss, J.E. Coligan, and A.G. Brooks. 1998. Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. *J Exp Med*. 187:813-818

Borysiewicz, L.K., J.K. Hickling, S. Graham, J. Sinclair, M.P. Cranage, G.L. Smith, and J.G. Sissons. 1988. Human cytomegalovirus-specific cytotoxic T cells. Relative frequency of stage-specific CTL recognizing the 72-kD immediate early protein and glycoprotein B expressed by recombinant vaccinia viruses. *J Exp Med*. 168:919-931

Bradley, A.J., N.S. Lurain, P. Ghazal, U. Trivedi, C. Cunningham, K. Baluchova, D. Gatherer, G.W. Wilkinson, D.J. Dargan, and A.J. Davison. 2009. High-throughput sequence analysis of variants of human cytomegalovirus strains Towne and AD169. *J Gen Virol*. 90:2375-2380

Bresnahan, W.A., and T. Shenk. 2000. A subset of viral transcripts packaged within human cytomegalovirus particles. *Science*. 288:2373-2376

Britt, B. 2007a. Maturation and egress. In "Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis". *Cambridge University Press*

Britt, W. 2007b. Virus entry into host, establishment of infection, spread in host, mechanisms of tissue damage. In "Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis". *Cambridge University Press*

Britt, W.J., L. Vugler, E.J. Butfiloski, and E.B. Stephens. 1990. Cell surface expression of human cytomegalovirus (HCMV) gp55-116 (gB): use of HCMV-recombinant vaccinia virus-infected cells in analysis of the human neutralizing antibody response. *J Virol*. 64:1079-1085

Brown, D.M. 2010. Cytolytic CD4 cells: Direct mediators in infectious disease and malignancy. *Cell Immunol*. 262:89-95

Bunde, T., A. Kirchner, B. Hoffmeister, D. Habedank, R. Hetzer, G. Cherepnev, S. Proesch, P. Reinke, H.D. Volk, H. Lehmkuhl, and F. Kern. 2005. Protection from cytomegalovirus after transplantation is correlated with immediate early 1-specific CD8 T cells. *J Exp Med*. 201:1031-1036

Caligiuri, M.A. 2008. Human natural killer cells. *Blood*. 112:461-469

Cannon, M.J., D.S. Schmid, and T.B. Hyde. 2010. Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection. *Rev Med Virol*. 20:202-213

Carr, W.H., A.M. Little, E. Mocarski, and P. Parham. 2002. NK cell-mediated lysis of autologous HCMV-infected skin fibroblasts is highly variable among NK cell clones and polyclonal NK cell lines. *Clin Immunol*. 105:126-140

Casazza, J.P., M.R. Betts, D.A. Price, M.L. Precopio, L.E. Ruff, J.M. Brenchley, B.J. Hill, M. Roederer, D.C. Douek, and R.A. Koup. 2006. Acquisition of direct antiviral effector functions by CMV-specific CD4+ T lymphocytes with cellular maturation. *J Exp Med*. 203:2865-2877

Cerboni, C., M. Mousavi-Jazi, A. Linde, K. Soderstrom, M. Brytting, B. Wahren, K. Karre, and E. Carbone. 2000. Human cytomegalovirus strain-dependent changes in NK cell recognition of infected fibroblasts. *J Immunol*. 164:4775-4782

Cha, T.A., E. Tom, G.W. Kemble, G.M. Duke, E.S. Mocarski, and R.R. Spaete. 1996. Human cytomegalovirus clinical isolates carry at least 19 genes not found in laboratory strains. *J Virol*. 70:78-83

Chalupny, N.J., A. Rein-Weston, S. Dosch, and D. Cosman. 2006. Down-regulation of the NKG2D ligand MICA by the human cytomegalovirus glycoprotein UL142. *Biochem Biophys Res Commun*. 346:175-181

Champagne, E. 2011. gammadelta T cell receptor ligands and modes of antigen recognition. *Arch Immunol Ther Exp (Warsz)*. 59:117-137

Chan, H.W., Z.B. Kurago, C.A. Stewart, M.J. Wilson, M.P. Martin, B.E. Mace, M. Carrington, J. Trowsdale, and C.T. Lutz. 2003. DNA methylation maintains allele-specific KIR gene expression in human natural killer cells. *J Exp Med*. 197:245-255

Chapman, T.L., A.P. Heikeman, and P.J. Bjorkman. 1999a. The inhibitory receptor LIR-1 uses a common binding interaction to recognize class I MHC molecules and the viral homolog UL18. *Immunity*. 11:603-613

Chapman, T.L., A.P. Heikeman, and P.J. Bjorkman. 1999b. The inhibitory receptor LIR-1 uses a common binding interaction to recognize class I MHC molecules and the viral homolog UL18. *Immunity*. 11:603-613

Chiang, C.L., A.R. Hagemann, R. Leskowitz, R. Mick, T. Garrabrant, B.J. Czerniecki, L.E. Kandalaft, D.J.J. Powell, and G. Coukos. 2011. Day-4 myeloid dendritic cells pulsed with whole tumor lysate are highly immunogenic and elicit potent anti-tumor responses. *PLoS One*. 6:e28732

Cobbold, M., N. Khan, B. Pourgheysari, S. Tauro, D. McDonald, H. Osman, M. Assenmacher, L. Billingham, C. Steward, C. Crawley, E. Olavarria, J. Goldman, R. Chakraverty, P. Mahendra, C. Craddock, and P.A. Moss. 2005. Adoptive transfer of cytomegalovirus-specific CTL to stem cell transplant patients after selection by HLA-peptide tetramers. *J Exp Med*. 202:379-386

Cohen, G.B., R.T. Gandhi, D.M. Davis, O. Mandelboim, B.K. Chen, J.L. Strominger, and D. Baltimore. 1999. The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. *Immunity*. 10:661-671

Compton, T., and A. Feire. 2007. Early events in human cytomegalovirus infection. In "Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis". *Cambridge University Press*

Compton, T., E.A. Kurt-Jones, K.W. Boehme, J. Belko, E. Latz, D.T. Golenbock, and R.W. Finberg. 2003. Human cytomegalovirus activates inflammatory cytokine responses via CD14 and Toll-like receptor 2. *J Virol*. 77:4588-4596

Compton, T., R.R. Nepomuceno, and D.M. Nowlin. 1992. Human cytomegalovirus penetrates host cells by pH-independent fusion at the cell surface. *Virology*. 191:387-395

Consogno, G., S. Manici, V. Facchinetti, A. Bachi, J. Hammer, B.M. Conti-Fine, C. Rugarli, C. Traversari, and M.P. Protti. 2003. Identification of immunodominant regions among promiscuous HLA-DR-restricted CD4+ T-cell epitopes on the tumor antigen MAGE-3. *Blood*. 101:1038-1044

Cooper, M.A., T.A. Fehniger, S.C. Turner, K.S. Chen, B.A. Ghaheri, T. Ghayur, W.E. Carson, and M.A. Caligiuri. 2001. Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset. *Blood*. 97:3146-3151

Cousins, D.J., T.H. Lee, and D.Z. Staynov. 2002. Cytokine coexpression during human Th1/Th2 cell differentiation: direct evidence for coordinated expression of Th2 cytokines. *J Immunol*. 169:2498-2506

Cresswell, P., A.L. Ackerman, A. Giodini, D.R. Peaper, and P.A. Wearsch. 2005. Mechanisms of MHC class I-restricted antigen processing and cross-presentation. *Immunol Rev*. 207:145-157

Crompton, L., N. Khan, R. Khanna, L. Nayak, and P.A. Moss. 2008. CD4+ T cells specific for glycoprotein B from cytomegalovirus exhibit extreme conservation of T-cell receptor usage between different individuals. *Blood*. 111:2053-2061

Crotzer, V.L., and J.S. Blum. 2010. Autophagy and adaptive immunity. *Immunology*. 131:9-17

Crough, T., and R. Khanna. 2009. Immunobiology of human cytomegalovirus: from bench to bedside. *Clin Microbiol Rev*. 22:76-98

D'Asaro, M., F. Dieli, N. Caccamo, M. Musso, F. Porretto, and A. Salerno. 2006. Increase of CCR7- CD45RA+ CD8 T cells (T(EMRA)) in chronic graft-versus-host disease. *Leukemia*. 20(3):545-547

Davignon, J.L., P. Castanie, J.A. Yorke, N. Gautier, D. Clement, and C. Davrinche. 1996. Anti-human cytomegalovirus activity of cytokines produced by CD4+ T-cell clones specifically activated by IE1 peptides in vitro. *J Virol*. 70:2162-2169

Davignon, J.L., D. Clement, J. Alriquet, S. Michelson, and C. Davrinche. 1995. Analysis of the proliferative T cell response to human cytomegalovirus major immediate-early protein (IE1): phenotype, frequency and variability. *Scand J Immunol*. 41:247-255

Davison, A.J. 2007a. Comparative analysis of the genomes. In "Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis". *Cambridge University Press*

Davison, A.J. 2007b. Overview of classification. In "Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis". *Cambridge University Press*

Davrinche, C., C. Pasquier, M. Cerutti, L. Serradell, D. Clement, G. Devauchelle, S. Michelson, and J.L. Davignon. 1993. Expression of human cytomegalovirus immediate early protein IE1 in insect cells: splicing of RNA and recognition by CD4+ T-cell clones. *Biochem Biophys Res Commun*. 195:469-477

Day, E.K., A.J. Carmichael, I.J. ten Berge, E.C. Waller, J.G. Sissons, and M.R. Wills. 2007. Rapid CD8+ T cell repertoire focusing and selection of high-affinity clones into memory following primary infection with a persistent human virus: human cytomegalovirus. *J Immunol*. 179:3203-3213

Deenick, E.K., J. Hasbold, and P.D. Hodgkin. 2005. Decision criteria for resolving isotype switching conflicts by B cells. *Eur J Immunol*. 35:2949-2955

del Val, M., H. Hengel, H. Hacker, U. Hartlaub, T. Ruppert, P. Lucin, and U.H. Koszinowski. 1992. Cytomegalovirus prevents antigen presentation by blocking the transport of peptide-loaded major histocompatibility complex class I molecules into the medial-Golgi compartment. *J Exp Med*. 176:729-738

Delecluse, H.J., D. Pich, T. Hilsendegen, C. Baum, and W. Hammerschmidt. 1999. A first-generation packaging cell line for Epstein-Barr virus-derived vectors. *Proc Natl Acad Sci U S A*. 96:5188-5193

Diamond, D.J., J. York, J.Y. Sun, C.L. Wright, and S.J. Forman. 1997. Development of a candidate HLA A*0201 restricted peptide-based vaccine against human cytomegalovirus infection. *Blood*. 90:1751-1767

Dunn, C., N.J. Chalupny, C.L. Sutherland, S. Dosch, P.V. Sivakumar, D.C. Johnson, and D. Cosman. 2003. Human cytomegalovirus glycoprotein UL16 causes intracellular sequestration of NKG2D ligands, protecting against natural killer cell cytotoxicity. *J Exp Med*. 197:1427-1439

Einsele, H., M. Kapp, and G.U. Grigoleit. 2008. CMV-specific T cell therapy. *Blood Cells Mol Dis*. 40:71-75

Einsele, H., E. Roosnek, N. Rufer, C. Sinzger, S. Riegler, J. Loffler, U. Grigoleit, A. Moris, H.G. Rammensee, L. Kanz, A. Kleihauer, F. Frank, G. Jahn, and H. Hebart. 2002. Infusion of cytomegalovirus (CMV)-specific T cells for the treatment of CMV infection not responding to antiviral chemotherapy. *Blood*. 99:3916-3922

Elkington, R., N.H. Shoukry, S. Walker, T. Crough, C. Fazou, A. Kaur, C.M. Walker, and R. Khanna. 2004. Cross-reactive recognition of human and primate cytomegalovirus sequences by human CD4 cytotoxic T lymphocytes specific for glycoprotein B and H. *Eur J Immunol*. 34:3216-3226

Elkington, R., S. Walker, T. Crough, M. Menzies, J. Tellam, M. Bharadwaj, and R. Khanna. 2003. Ex vivo profiling of CD8+-T-cell responses to human cytomegalovirus reveals broad and multispecific reactivities in healthy virus carriers. *J Virol*. 77:5226-5240

Evans, P.C., A. Soin, T.G. Wreghitt, C.J. Taylor, D.G. Wight, and G.J. Alexander. 2000. An association between cytomegalovirus infection and chronic rejection after liver transplantation. *Transplantation*. 69:30-35

Falagas, M.E., D.R. Snydman, R. Ruthazer, J. Griffith, B.G. Werner, R. Freeman, and R. Rohrer. 1997. Cytomegalovirus immune globulin (CMVIG) prophylaxis is associated with increased survival after orthotopic liver transplantation. The Boston Center for Liver Transplantation CMVIG Study Group. *Clin Transplant*. 11:432-437

Falk, C.S., M. Mach, D.J. Schendel, E.H. Weiss, I. Hilgert, and G. Hahn. 2002. NK cell activity during human cytomegalovirus infection is dominated by US2-11-mediated HLA class I down-regulation. *J Immunol*. 169:3257-3266

Falk, C.S., A. Steinle, and D.J. Schendel. 1995. Expression of HLA-C molecules confers target cell resistance to some non-major histocompatibility complex-restricted T cells in a manner analogous to allospecific natural killer cells. *J Exp Med*. 182:1005-1018

Falk, K., O. Rotzschke, B. Grahovac, D. Schendel, S. Stevanovic, V. Gnau, G. Jung, J.L. Strominger, and H.G. Rammensee. 1993. Allele-specific peptide ligand motifs of HLA-C molecules. *Proc Natl Acad Sci U S A*. 90:12005-12009

Feuchtinger, T., K. Opherk, W.A. Bethge, M.S. Topp, F.R. Schuster, E.M. Weissinger, M. Mohty, R. Or, M. Maschan, M. Schumm, K. Hamprecht, R. Handgretinger, P. Lang, and H. Einsele. 2010. Adoptive transfer of pp65-specific T cells for the treatment of chemorefractory cytomegalovirus disease or reactivation after haploidentical and matched unrelated stem cell transplantation. *Blood*. 116:4360-4367

Fietze, E., S. Prosch, P. Reinke, J. Stein, W.D. Docke, G. Staffa, S. Loning, S. Devaux, F. Emmrich, R. von Baehr, and a. et. 1994. Cytomegalovirus infection in transplant recipients. The role of tumor necrosis factor. *Transplantation*. 58:675-680

Gallina, A., L. Simoncini, S. Garbelli, E. Percivalle, G. Pedrali-Noy, K.S. Lee, R.L. Erikson, B. Plachter, G. Gerna, and G. Milanesi. 1999. Polo-like kinase 1 as a target for human cytomegalovirus pp65 lower matrix protein. *J Virol*. 73:1468-1478

Gamadia, L.E., E.B. Remmerswaal, J.F. Weel, F. Bemelman, R.A. van Lier, and I.J. Ten Berge. 2003. Primary immune responses to human CMV: a critical role for IFN-gamma-producing CD4+ T cells in protection against CMV disease. *Blood*. 101:2686-2692

Gattinoni, L., E. Lugli, Y. Ji, Z. Pos, C.M. Paulos, M.F. Quigley, J.R. Almeida, E. Gostick, Z. Yu, C. Carpenito, E. Wang, D.C. Douek, D.A. Price, C.H. June, F.M. Marincola, M. Roederer, and N.P. Restifo. 2011. A human memory T cell subset with stem cell-like properties. *Nat Med*. 17:1290-1297

Gautier, N., E. Chavant, E. Prieur, B. Monsarrat, H. Mazarguil, C. Davrinche, J.E. Gairin, and J.L. Davignon. 1996. Characterization of an epitope of the human cytomegalovirus protein IE1 recognized by a CD4+ T cell clone. *Eur J Immunol*. 26:1110-1117

Gavin, M.A., M.J. Gilbert, S.R. Riddell, P.D. Greenberg, and M.J. Bevan. 1993. Alkali hydrolysis of recombinant proteins allows for the rapid identification of class I MHC-restricted CTL epitopes. *J Immunol*. 151:3971-3980

Gazit, R., B.Z. Garty, Y. Monselise, V. Hoffer, Y. Finkelstein, G. Markel, G. Katz, J. Hanna, H. Achdout, R. Gruda, T. Gonen-Gross, and O. Mandelboim. 2004. Expression of KIR2DL1 on the entire NK cell population: a possible novel immunodeficiency syndrome. *Blood*. 103:1965-1966

Geginat, G., T. Ruppert, H. Hengel, R. Holtappels, and U.H. Koszinowski. 1997. IFN-gamma is a prerequisite for optimal antigen processing of viral peptides in vivo. *J Immunol*. 158:3303-3310

Germain, R.N. 1994. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. *Cell*. 76:287-299

Gewurz, B.E., E.W. Wang, D. Tortorella, D.J. Schust, and H.L. Ploegh. 2001. Human cytomegalovirus US2 endoplasmic reticulum-lumenal domain dictates association with major histocompatibility complex class I in a locus-specific manner. *J Virol*. 75:5197-5204

Gilbert, M.J., S.R. Riddell, C.R. Li, and P.D. Greenberg. 1993. Selective interference with class I major histocompatibility complex presentation of the major immediate-early protein following infection with human cytomegalovirus. *J Virol*. 67:3461-3469

Gilbert, M.J., S.R. Riddell, B. Plachter, and P.D. Greenberg. 1996. Cytomegalovirus selectively blocks antigen processing and presentation of its immediate-early gene product. *Nature*. 383:720-722

Groettrup, M., S. Khan, K. Schwarz, and G. Schmidtke. 2001. Interferon-gamma inducible exchanges of 20S proteasome active site subunits: why? *Biochimie*. 83:367-372

Halary, F., V. Pitard, D. Dlubek, R. Krzysiek, H. de la Salle, P. Merville, C. Dromer, D. Emilie, J.F. Moreau, and J. Dechanet-Merville. 2005. Shared reactivity of V{delta}2(neg) {gamma}{delta} T cells against cytomegalovirus-infected cells and tumor intestinal epithelial cells. *J Exp Med*. 201:1567-1578

Hamprecht, K., R. Goelz, and J. Maschmann. 2005. Breast milk and cytomegalovirus infection in preterm infants. *Early Hum Dev*. 81:989-996

Hansasuta, P., T. Dong, H. Thananchai, M. Weekes, C. Willberg, H. Aldemir, S. Rowland-Jones, and V.M. Braud. 2004. Recognition of HLA-A3 and HLA-A11 by KIR3DL2 is peptide-specific. *Eur J Immunol*. 34:1673-1679

Hansen, T.H., and M. Bouvier. 2009. MHC class I antigen presentation: learning from viral evasion strategies. *Nat Rev Immunol*. 9:503-513

Harcourt, G.C., T.J. Scriba, N. Semmo, S. Bounds, E. Taylor, and P. Klenerman. 2006. Identification of key peptide-specific CD4+ T cell responses to human cytomegalovirus: implications for tracking antiviral populations. *Clin Exp Immunol*. 146:203-210

Hargett, D., and T.E. Shenk. 2010. Experimental human cytomegalovirus latency in CD14+ monocytes. *Proc Natl Acad Sci U S A*. 107:20039-20044

Hebart, H., S. Daginik, S. Stevanovic, U. Grigoleit, A. Dobler, M. Baur, G. Rauser, C. Sinzger, G. Jahn, J. Loeffler, L. Kanz, H.G. Rammensee, and H. Einsele. 2002. Sensitive detection of human cytomegalovirus peptide-specific cytotoxic T-lymphocyte responses by interferon-gamma-enzyme-linked immunospot assay and flow cytometry in healthy individuals and in patients after allogeneic stem cell transplantation. *Blood*. 99:3830-3837

Hegde, N.R., C. Dunn, D.M. Lewinsohn, M.A. Jarvis, J.A. Nelson, and D.C. Johnson. 2005. Endogenous human cytomegalovirus gB is presented efficiently by MHC class II molecules to CD4+ CTL. *J Exp Med*. 202:1109-1119

Hegde, N.R., R.A. Tomazin, T.W. Wisner, C. Dunn, J.M. Boname, D.M. Lewinsohn, and D.C. Johnson. 2002. Inhibition of HLA-DR assembly, transport, and loading by human cytomegalovirus glycoprotein US3: a novel mechanism for evading major histocompatibility complex class II antigen presentation. *J Virol*. 76:10929-10941

Hengel, H., T. Flohr, G.J. Hammerling, U.H. Koszinowski, and F. Momburg. 1996. Human cytomegalovirus inhibits peptide translocation into the endoplasmic reticulum for MHC class I assembly. *J Gen Virol*. 77:2287-2296

Hengel, H., J.O. Koopmann, T. Flohr, W. Muranyi, E. Goulmy, G.J. Hammerling, U.H. Koszinowski, and F. Momburg. 1997. A viral ER-resident glycoprotein inactivates the MHC-encoded peptide transporter. *Immunity*. 6:623-632

Hengel, H., P. Lucin, S. Jonjic, T. Ruppert, and U.H. Koszinowski. 1994. Restoration of cytomegalovirus antigen presentation by gamma interferon combats viral escape. *J Virol*. 68:289-297

Hill, A.B., S.P. Lee, J.S. Haurum, N. Murray, Q.Y. Yao, M. Rowe, N. Signoret, A.B. Rickinson, and A.J. McMichael. 1995. Class I major histocompatibility complex-restricted cytotoxic T lymphocytes specific for Epstein-Barr virus (EBV)-transformed B lymphoblastoid cell lines against which they were raised. *J Exp Med*. 181:2221-2228

Holtappels, R., J. Podlech, M.F. Pahl-Seibert, M. Julch, D. Thomas, C.O. Simon, M. Wagner, and M.J. Reddehase. 2004. Cytomegalovirus misleads its host by priming of CD8 T cells specific for an epitope not presented in infected tissues. *J Exp Med*. 199:131-136

Hopkins, J.I., A.N. Fiander, A.S. Evans, M. Delchambre, D. Gheysen, and L.K. Borysiewicz. 1996. Cytotoxic T cell immunity to human cytomegalovirus glycoprotein B. *J Med Virol*. 49:124-131

Huard, B., and K. Fruh. 2000. A role for MHC class I down-regulation in NK cell lysis of herpes virus-infected cells. *Eur J Immunol*. 30:509-515

Ito, M., T. Maruyama, N. Saito, S. Koganei, K. Yamamoto, and N. Matsumoto. 2006. Killer cell lectin-like receptor G1 binds three members of the classical cadherin family to inhibit NK cell cytotoxicity. *J Exp Med*. 203:289-295

Jackson, S.E., G.M. Mason, and M.R. Wills. 2011. Human cytomegalovirus immunity and immune evasion. *Virus Res*. 157:151-160

Janeway, C., P. Travers, M. Walport, and M. Shlomchik. 2006. Immunobiology: The Immune System in Health and Disease. Garland Science

Jensen, P.E. 2007. Recent advances in antigen processing and presentation. *Nat Immunol*. 8:1041-1048

Jones, T.R., L.K. Hanson, L. Sun, J.S. Slater, R.M. Stenberg, and A.E. Campbell. 1995. Multiple independent loci within the human cytomegalovirus unique short region down-regulate expression of major histocompatibility complex class I heavy chains. *J Virol*. 69:4830-4841

Jones, T.R., and V.P. Muzithras. 1991. Fine mapping of transcripts expressed from the US6 gene family of human cytomegalovirus strain AD169. *J Virol*. 65:2024-2036

Jones, T.R., and L. Sun. 1997. Human cytomegalovirus US2 destabilizes major histocompatibility complex class I heavy chains. *J Virol*. 71:2970-2979

Jones, T.R., E.J. Wiertz, L. Sun, K.N. Fish, J.A. Nelson, and H.L. Ploegh. 1996. Human cytomegalovirus US3 impairs transport and maturation of major histocompatibility complex class I heavy chains. *Proc Natl Acad Sci U S A*. 93:11327-11333

Jonjic, S., M. del Val, G.M. Keil, M.J. Reddehase, and U.H. Koszinowski. 1988. A nonstructural viral protein expressed by a recombinant vaccinia virus protects against lethal cytomegalovirus infection. *J Virol*. 62:1653-1658

Jonjic, S., I. Pavic, B. Polic, I. Crnkovic, P. Lucin, and U.H. Koszinowski. 1994. Antibodies are not essential for the resolution of primary cytomegalovirus infection but limit dissemination of recurrent virus. *J Exp Med*. 179:1713-1717

Jonuleit, H., A. Giesecke, A. Kandemir, L. Paragnik, J. Knop, and A.H. Enk. 2000. Induction of tumor peptide-specific cytotoxic T cells under serum-free conditions by mature human dendritic cells. *Arch Dermatol Res*. 292:325-332

Kageyama, S., T.J. Tsomides, Y. Sykulev, and H.N. Eisen. 1995. Variations in the number of peptide-MHC class I complexes required to activate cytotoxic T cell responses. *J Immunol*. 154:567-576

Kalejta, R.F. 2008. Tegument proteins of human cytomegalovirus. *Microbiol Mol Biol Rev*. 72:249-65

Kastenmuller, W., G. Gasteiger, J.H. Gronau, R. Baier, R. Ljapoci, D.H. Busch, and I. Drexler. 2007. Cross-competition of CD8+ T cells shapes the immunodominance hierarchy during boost vaccination. *J Exp Med*. 204:2187-2198

Kaufmann, D.E., P.M. Bailey, J. Sidney, B. Wagner, P.J. Norris, M.N. Johnston, L.A. Cosimi, M.M. Addo, M. Lichterfeld, M. Altfeld, N. Frahm, C. Brander, A. Sette, B.D. Walker, and E.S. Rosenberg. 2004. Comprehensive analysis of human immunodeficiency virus type 1-specific CD4 responses reveals marked immunodominance of gag and nef and the presence of broadly recognized peptides. *J Virol*. 78:4463-4477

Kedl, R.M., J.W. Kappler, and P. Marrack. 2003. Epitope dominance, competition and T cell affinity maturation. *Curr Opin Immunol*. 15:120-127

Kempkes, B., D. Pich, R. Zeidler, and W. Hammerschmidt. 1995a. Immortalization of human primary B lymphocytes in vitro with DNA. *Proc Natl Acad Sci U S A*. 92:5875-5879

Kempkes, B., D. Pich, R. Zeidler, B. Sugden, and W. Hammerschmidt. 1995b. Immortalization of human B lymphocytes by a plasmid containing 71 kilobase pairs of Epstein-Barr virus DNA. *J Virol*. 69:231-238

Kern, F., T. Bunde, N. Faulhaber, F. Kiecker, E. Khatamzas, I.M. Rudawski, A. Pruss, J.W. Gratama, R. Volkmer-Engert, R. Ewert, P. Reinke, H.D. Volk, and L.J. Picker. 2002. Cytomegalovirus (CMV) phosphoprotein 65 makes a large contribution to shaping the T cell repertoire in CMV-exposed individuals. *J Infect Dis*. 185:1709-1716

Kern, F., I.P. Surel, N. Faulhaber, C. Frommel, J. Schneider-Mergener, C. Schonemann, P. Reinke, and H.D. Volk. 1999. Target structures of the CD8(+)-T-cell response to human cytomegalovirus: the 72-kilodalton major immediate-early protein revisited. *J Virol*. 73:8179-8184

Khakoo, S.I., C.L. Thio, M.P. Martin, C.R. Brooks, X. Gao, J. Astemborski, J. Cheng, J.J. Goedert, D. Vlahov, M. Hilgartner, S. Cox, A.M. Little, G.J. Alexander, M.E. Cramp, S.J. O'Brien, W.M. Rosenberg, D.L. Thomas, and M. Carrington. 2004. HLA and NK cell inhibitory receptor genes in resolving hepatitis C virus infection. *Science*. 305:872-874

Khan, N., D. Best, R. Bruton, L. Nayak, A.B. Rickinson, and P.A. Moss. 2007. T cell recognition patterns of immunodominant cytomegalovirus antigens in primary and persistent infection. *J Immunol*. 178:4455-4465

Khan, N., R. Bruton, G.S. Taylor, M. Cobbold, T.R. Jones, A.B. Rickinson, and P.A. Moss. 2005. Identification of cytomegalovirus-specific cytotoxic T lymphocytes in vitro is greatly enhanced by the use of recombinant virus lacking the US2 to US11 region or modified vaccinia virus Ankara expressing individual viral genes. *J Virol*. 79:2869-2879

Khan, N., M. Cobbold, R. Keenan, and P.A. Moss. 2002. Comparative analysis of CD8+ T cell responses against human cytomegalovirus proteins pp65 and immediate early 1 shows similarities in precursor frequency, oligoclonality, and phenotype. *J Infect Dis*. 185:1025-1034

Khan, N., A. Hislop, N. Gudgeon, M. Cobbold, R. Khanna, L. Nayak, A.B. Rickinson, and P.A. Moss. 2004. Herpesvirus-specific CD8 T cell immunity in old age: cytomegalovirus impairs the response to a coresident EBV infection. *J Immunol*. 173:7481-7489

Khanna, R., and S.R. Burrows. 2000. Role of cytotoxic T lymphocytes in Epstein-Barr virus-associated diseases. *Annu Rev Microbiol*. 54:19-48

Kilger, E., G. Pecher, A. Schwenk, and W. Hammerschmidt. 1999. Expression of mucin (MUC-1) from a mini-Epstein-Barr virus in immortalized B-cells to generate tumor antigen specific cytotoxic T cells. *J Gene Med*. 1:84-92

Kim, S., S. Lee, J. Shin, Y. Kim, I. Evnouchidou, D. Kim, Y.K. Kim, Y.E. Kim, J.H. Ahn, S.R. Riddell, E. Stratikos, V.N. Kim, and K. Ahn. 2011. Human cytomegalovirus microRNA miR-US4-1 inhibits CD8(+) T cell responses by targeting the aminopeptidase ERAP1. *Nat Immunol*. 12:984-991

Kim, S., J. Poursine-Laurent, S.M. Truscott, L. Lybarger, Y.J. Song, L. Yang, A.R. French, J.B. Sunwoo, S. Lemieux, T.H. Hansen, and W.M. Yokoyama. 2005. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature*. 436:709-713

Kim, S., J.B. Sunwoo, L. Yang, T. Choi, Y.J. Song, A.R. French, A. Vlahiotis, J.F. Piccirillo, M. Cella, M. Colonna, T. Mohanakumar, K.C. Hsu, B. Dupont, and W.M. Yokoyama. 2008. HLA alleles determine differences in human natural killer cell responsiveness and potency. *Proc Natl Acad Sci U S A*. 105:3053-3058

Kleihauer, A., U. Grigoleit, H. Hebart, A. Moris, P. Brossart, A. Muhm, S. Stevanovic, H.G. Rammensee, C. Sinzger, S. Riegler, G. Jahn, L. Kanz, and H. Einsele. 2001. Ex vivo generation of human cytomegalovirus-specific cytotoxic T cells by peptide-pulsed dendritic cells. *Br J Haematol*. 113:231-239

Klein, E., G. Klein, J.S. Nadkarni, J.J. Nadkarni, H. Wigzell, and P. Clifford. 1968. Surface IgM-kappa specificity on a Burkitt lymphoma cell in vivo and in derived culture lines. *Cancer Res*. 28:1300-1310

Kline, J.N., G.M. Hunninghake, B. He, M.M. Monick, and G.W. Hunninghake. 1998. Synergistic activation of the human cytomegalovirus major immediate early promoter by prostaglandin E2 and cytokines. *Exp Lung Res*. 24:3-14

Knabel, M., T.J. Franz, M. Schiemann, A. Wulf, B. Villmow, B. Schmidt, H. Bernhard, H. Wagner, and D.H. Busch. 2002. Reversible MHC multimer staining for functional isolation of T-cell populations and effective adoptive transfer. *Nat Med*. 8:631-637

Knight, A., A.J. Madrigal, S. Grace, J. Sivakumaran, P. Kottaridis, S. Mackinnon, P.J. Travers, and M.W. Lowdell. 2010. The role of Vdelta2-negative gammadelta T cells during cytomegalovirus reactivation in recipients of allogeneic stem cell transplantation. *Blood*. 116:2164-2172

Kobayashi, H., M. Wood, Y. Song, E. Appella, and E. Celis. 2000. Defining promiscuous MHC class II helper T-cell epitopes for the HER2/neu tumor antigen. *Cancer Res*. 60:5228-5236

Komanduri, K.V., S.M. Donahoe, W.J. Moretto, D.K. Schmidt, G. Gillespie, G.S. Ogg, M. Roederer, D.F. Nixon, and J.M. McCune. 2001. Direct measurement of CD4+ and CD8+ T-cell responses to CMV in HIV-1-infected subjects. *Virology*. 279:459-470

Kondo, E., Y. Akatsuka, K. Kuzushima, K. Tsujimura, S. Asakura, K. Tajima, Y. Kagami, Y. Kodera, M. Tanimoto, Y. Morishima, and T. Takahashi. 2004. Identification of novel CTL epitopes of CMV-pp65 presented by a variety of HLA alleles. *Blood*. 103:630-638

Kulpa, D.A., and K.L. Collins. 2011. The emerging role of HLA-C in HIV-1 infection. *Immunology*. 134:116-122

Lanier, L.L. 2005. NK cell recognition. *Annu Rev Immunol*. 23:225-274

Lautscham, G., A. Rickinson, and N. Blake. 2003. TAP-independent antigen presentation on MHC class I molecules: lessons from Epstein-Barr virus. *Microbes Infect*. 5:291-299

Le Roy, E., M. Baron, W. Faigle, D. Clement, D.M. Lewinsohn, D.N. Streblow, J.A. Nelson, S. Amigorena, and J.L. Davignon. 2002. Infection of APC by human cytomegalovirus controlled through recognition of endogenous nuclear immediate early protein 1 by specific CD4(+) T lymphocytes. *J Immunol*. 169:1293-1301

Leong, C.C., T.L. Chapman, P.J. Bjorkman, D. Formankova, E.S. Mocarski, J.H. Phillips, and L.L. Lanier. 1998. Modulation of natural killer cell cytotoxicity in human cytomegalovirus infection: the role of endogenous class I major histocompatibility complex and a viral class I homolog. *J Exp Med*. 187:1681-1687

Li Pira, G., L. Bottone, F. Ivaldi, R. Pelizzoli, F. Del Galdo, L. Lozzi, L. Bracci, A. Loregian, G. Palu, R. De Palma, H. Einsele, and F. Manca. 2004. Identification of new Th peptides from the cytomegalovirus protein pp65 to design a peptide library for generation of CD4 T cell lines for cellular immunoreconstitution. *Int Immunol*. 16:635-642

Li, C.R., P.D. Greenberg, M.J. Gilbert, J.M. Goodrich, and S.R. Riddell. 1994. Recovery of HLA-restricted cytomegalovirus (CMV)-specific T-cell responses after allogeneic bone marrow transplant: correlation with CMV disease and effect of ganciclovir prophylaxis. *Blood*. 83:1971-1979

Liu, F., and Z. Hong Zhou. 2007. Comparative virion structures of human herpesviruses. In "Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis". *Cambridge University Press*

Liu, J., and G.F. Gao. 2011. Major Histocompatibility Complex: Interaction with Peptides. *eLS*

Liu, W., Y. Zhao, and B. Biegalke. 2002. Analysis of human cytomegalovirus US3 gene products. *Virology*. 301:32-42

Llano, M., M. Guma, M. Ortega, A. Angulo, and M. Lopez-Botet. 2003. Differential effects of US2, US6 and US11 human cytomegalovirus proteins on HLA class Ia and HLA-E expression: impact on target susceptibility to NK cell subsets. *Eur J Immunol*. 33:2744-2754

Loewendorf, A., and C.A. Benedict. 2010. Modulation of host innate and adaptive immune defenses by cytomegalovirus: timing is everything. *J Intern Med*. 267:483-501

Long, H.M., T.A. Haigh, N.H. Gudgeon, A.M. Leen, C.W. Tsang, J. Brooks, E. Landais, E. Houssaint, S.P. Lee, A.B. Rickinson, and G.S. Taylor. 2005. CD4+ T-cell responses to Epstein-Barr virus (EBV) latent-cycle antigens and the recognition of EBV-transformed lymphoblastoid cell lines. *J Virol*. 79:4896-4907

Lozzio, C.B., and B.B. Lozzio. 1975. Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. *Blood*. 45:321-334

Luckheeram, R.V., R. Zhou, A.D. Verma, and B. Xia. 2012. CD4(+)T cells: differentiation and functions. *Clin Dev Immunol*. 2012:925135

Lurain, N.S., and S. Chou. 2010. Antiviral drug resistance of human cytomegalovirus. *Clin Microbiol Rev*. 23:689-712

MACPHERSON, I., and M. STOKER. 1962. Polyoma transformation of hamster cell clones--an investigation of genetic factors affecting cell competence. *Virology*. 16:147-151

Maecker, H.T., H.S. Dunn, M.A. Suni, E. Khatamzas, C.J. Pitcher, T. Bunde, N. Persaud, W. Trigona, T.M. Fu, E. Sinclair, B.M. Bredt, J.M. McCune, V.C. Maino, F. Kern, and L.J. Picker. 2001. Use of overlapping peptide mixtures as antigens for cytokine flow cytometry. *J Immunol Methods*. 255:27-40

Mandelboim, O., N. Lieberman, M. Lev, L. Paul, T.I. Arnon, Y. Bushkin, D.M. Davis, J.L. Strominger, J.W. Yewdell, and A. Porgador. 2001. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature*. 409:1055-1060

Manley, T.J., L. Luy, T. Jones, M. Boeckh, H. Mutimer, and S.R. Riddell. 2004. Immune evasion proteins of human cytomegalovirus do not prevent a diverse CD8+ cytotoxic T-cell response in natural infection. *Blood*. 104:1075-1082

Marsh, S.G.E., P. Parham, and L.D. Barber. 2000. The HLA FactsBook (1st Ed.) Academic Press. ISBN 0-12-545025-7

Marshall, N.B., and S.L. Swain. 2011. Cytotoxic CD4 T cells in antiviral immunity. *J Biomed Biotechnol*. 2011:954602

Matzinger, P. 1994. Tolerance, danger, and the extended family. *Annu Rev Immunol*. 12:991-1045

McCutcheon, J.A., J. Gumperz, K.D. Smith, C.T. Lutz, and P. Parham. 1995. Low HLA-C expression at cell surfaces correlates with increased turnover of heavy chain mRNA. *J Exp Med*. 181:2085-2095

McLaughlin-Taylor, E., H. Pande, S.J. Forman, B. Tanamachi, C.R. Li, J.A. Zaia, P.D. Greenberg, and S.R. Riddell. 1994. Identification of the major late human cytomegalovirus matrix protein pp65 as a target antigen for CD8+ virus-specific cytotoxic T lymphocytes. *J Med Virol*. 43:103-110

Miller-Kittrell, M., and T.E. Sparer. 2009. Feeling manipulated: cytomegalovirus immune manipulation. *Virol J*. 6:4

Miller, D.M., C.M. Cebulla, B.M. Rahill, and D.D. Sedmak. 2001. Cytomegalovirus and transcriptional down-regulation of major histocompatibility complex class II expression. *Semin Immunol*. 13:11-18

Miyashita, E.M., B. Yang, K.M. Lam, D.H. Crawford, and D.A. Thorley-Lawson. 1995. A novel form of Epstein-Barr virus latency in normal B cells in vivo. *Cell*. 80:593-601

Moore, K.W., R. de Waal Malefyt, R.L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol*. 19:683-765

Moosmann, A., I. Bigalke, J. Tischer, L. Schirrmann, J. Kasten, S. Tippmer, M. Leeping, D. Prevalsek, G. Jaeger, G. Ledderose, J. Mautner, W. Hammerschmidt, D.J. Schendel, and H.J. Kolb. 2010. Effective and long-term control of EBV PTLD after transfer of peptide-selected T cells. *Blood*. 115:2960-2970

Moosmann, A., N. Khan, M. Cobbold, C. Zentz, H.J. Delecluse, G. Hollweck, A.D. Hislop, N.W. Blake, D. Croom-Carter, B. Wollenberg, P.A. Moss, R. Zeidler, A.B. Rickinson, and W. Hammerschmidt. 2002. B cells immortalized by a mini-Epstein-Barr virus encoding a foreign antigen efficiently reactivate specific cytotoxic T cells. *Blood*. 100:1755-1764

Morales, O., S. Depil, D. Mrizak, N. Martin, P.A. Ndour, F. Dufosse, C. Miroux, J. Coll, Y. Launoit, C. Auriault, V. Pancre, and N. Delhem. 2012. EBV Latency II-derived peptides induce a specific CD4+ cytotoxic t-cell activity and not a CD4+ regulatory T-cell response. *J Immunother*. 35:254-266

Moretta, A., C. Bottino, M. Vitale, D. Pende, C. Cantoni, M.C. Mingari, R. Biassoni, and L. Moretta. 2001. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu Rev Immunol*. 19:197-223

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*. 65:55-63

Moss, P. 2010. The emerging role of cytomegalovirus in driving immune senescence: a novel therapeutic opportunity for improving health in the elderly. *Curr Opin Immunol*. 22:529-534

Munoz, I., A. Gutierrez, C. Gimeno, A. Farga, J. Alberola, C. Solano, F. Prosper, J. Garcia-Conde, and D. Navarro. 2001. Lack of association between the kinetics of human cytomegalovirus (HCMV) glycoprotein B (gB)-specific and neutralizing serum antibodies and development or recovery from HCMV active infection in patients undergoing allogeneic stem cell transplant. *J Med Virol*. 65:77-84

Murphy, E., D. Yu, J. Grimwood, J. Schmutz, M. Dickson, M.A. Jarvis, G. Hahn, J.A. Nelson, R.M. Myers, and T.E. Shenk. 2003. Coding potential of laboratory and clinical strains of human cytomegalovirus. *Proc Natl Acad Sci U S A*. 100:14976-14981

Mutimer, H.P., Y. Akatsuka, T. Manley, E.L. Chuang, M. Boeckh, R. Harrington, T. Jones, and S.R. Riddell. 2002. Association between immune recovery uveitis and a diverse intraocular cytomegalovirus-specific cytotoxic T cell response. *J Infect Dis*. 186:701-705

Nakajima, H., F. Toyoshima-Morimoto, E. Taniguchi, and E. Nishida. 2003. Identification of a consensus motif for Plk (Polo-like kinase) phosphorylation reveals Myt1 as a Plk1 substrate. *J Biol Chem*. 278:25277-25280

Neefjes, J., M.L. Jongsma, P. Paul, and O. Bakke. 2011. Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat Rev Immunol*. 11:823-836

Neuhierl, B., R. Feederle, W. Hammerschmidt, and H.J. Delecluse. 2002. Glycoprotein gp110 of Epstein-Barr virus determines viral tropism and efficiency of infection. *Proc Natl Acad Sci U S A*. 99:15036-15041

Nigro, G., S.P. Adler, R. La Torre, and A.M. Best. 2005. Passive immunization during pregnancy for congenital cytomegalovirus infection. *N Engl J Med*. 353:1350-1362

Nimmerjahn, F., S. Milosevic, U. Behrends, E.M. Jaffee, D.M. Pardoll, G.W. Bornkamm, and J. Mautner. 2003. Major histocompatibility complex class II-restricted presentation of a cytosolic antigen by autophagy. *Eur J Immunol*. 33:1250-1259

Ning, R.J., X.Q. Xu, K.H. Chan, and A.K. Chiang. 2011. Long-term carriers generate Epstein-Barr virus (EBV)-specific CD4(+) and CD8(+) polyfunctional T-cell responses which show immunodominance hierarchies of EBV proteins. *Immunology*. 134:161-171

Noriega, V., V. Redmann, T. Gardner, and D. Tortorella. 2012. Diverse immune evasion strategies by human cytomegalovirus. *Immunol Res*. 10.1007/s12026-012-8304-8.

Noriega, V.M., and D. Tortorella. 2009. Human cytomegalovirus-encoded immune modulators partner to downregulate major histocompatibility complex class I molecules. *J Virol*. 83:1359-1367

Odeberg, J., B. Plachter, L. Branden, and C. Soderberg-Naucler. 2003. Human cytomegalovirus protein pp65 mediates accumulation of HLA-DR in lysosomes and destruction of the HLA-DR alpha-chain. *Blood*. 101:4870-4877

Oh, S., L.P. Perera, D.S. Burke, T.A. Waldmann, and J.A. Berzofsky. 2004. IL-15/IL-15Ralpha-mediated avidity maturation of memory CD8+ T cells. *Proc Natl Acad Sci U S A*. 101:15154-15159

Orr, M.T., and L.L. Lanier. 2010. Natural killer cell education and tolerance. *Cell*. 142:847-856

Paludan, C., D. Schmid, M. Landthaler, M. Vockerodt, D. Kube, T. Tuschl, and C. Munz. 2005. Endogenous MHC class II processing of a viral nuclear antigen after autophagy. *Science*. 307:593-596

Parham, P. 2005. MHC class I molecules and KIRs in human history, health and survival. *Nat Rev Immunol*. 5:201-214

Park, B., Y. Kim, J. Shin, S. Lee, K. Cho, K. Fruh, S. Lee, and K. Ahn. 2004. Human cytomegalovirus inhibits tapasin-dependent peptide loading and optimization of the MHC class I peptide cargo for immune evasion. *Immunity*. 20:71-85

Park, B., S. Lee, E. Kim, and K. Ahn. 2003. A single polymorphic residue within the peptide-binding cleft of MHC class I molecules determines spectrum of tapasin dependence. *J Immunol*. 170:961-968

Park, B., S. Lee, E. Kim, K. Cho, S.R. Riddell, S. Cho, and K. Ahn. 2006. Redox regulation facilitates optimal peptide selection by MHC class I during antigen processing. *Cell*. 127:369-382

Park, B., E. Spooner, B.L. Houser, J.L. Strominger, and H.L. Ploegh. 2010. The HCMV membrane glycoprotein US10 selectively targets HLA-G for degradation. *J Exp Med*. 207:2033-2041

Pawelec, G., and E. Derhovanessian. 2011. Role of CMV in immune senescence. *Virus Res*. 157:175-179

Peggs, K., S. Verfuerth, and S. Mackinnon. 2001. Induction of cytomegalovirus (CMV)-specific T-cell responses using dendritic cells pulsed with CMV antigen: a novel culture system free of live CMV virions. *Blood*. 97:994-1000

Peggs, K.S., S. Verfuerth, A. Pizzey, N. Khan, M. Guiver, P.A. Moss, and S. Mackinnon. 2003. Adoptive cellular therapy for early cytomegalovirus infection after allogeneic stem-cell transplantation with virus-specific T-cell lines. *Lancet*. 362:1375-1377

Pegram, H.J., D.M. Andrews, M.J. Smyth, P.K. Darcy, and M.H. Kershaw. 2011. Activating and inhibitory receptors of natural killer cells. *Immunol Cell Biol*. 89:216-224

Pepperl, S., G. Benninger-Doring, S. Modrow, H. Wolf, and W. Jilg. 1998. Immediate-early transactivator Rta of Epstein-Barr virus (EBV) shows multiple epitopes recognized by EBV-specific cytotoxic T lymphocytes. *J Virol*. 72:8644-8649

Pieters, J. 1997. MHC class II restricted antigen presentation. *Curr Opin Immunol*. 9:89-96

Plotkin, S.A., S.E. Starr, H.M. Friedman, K. Brayman, S. Harris, S. Jackson, N.B. Tustin, R. Grossman, D. Dafoe, and C. Barker. 1991. Effect of Towne live virus vaccine on cytomegalovirus disease after renal transplant. A controlled trial. *Ann Intern Med*. 114:525-531

Prichard, M.N., M.E. Penfold, G.M. Duke, R.R. Spaete, and G.W. Kemble. 2001. A review of genetic differences between limited and extensively passaged human cytomegalovirus strains. *Rev Med Virol*. 11:191-200

Prod'homme, V., C. Griffin, R.J. Aicheler, E.C. Wang, B.P. McSharry, C.R. Rickards, R.J. Stanton, L.K. Borysiewicz, M. Lopez-Botet, G.W. Wilkinson, and P. Tomasec. 2007. The human cytomegalovirus MHC class I homolog UL18 inhibits LIR-1+ but activates LIR-1- NK cells. *J Immunol*. 178:4473-4481

Prod'homme, V., D.M. Sugrue, R.J. Stanton, A. Nomoto, J. Davies, C.R. Rickards, D. Cochrane, M. Moore, G.W. Wilkinson, and P. Tomasec. 2010. Human cytomegalovirus UL141 promotes efficient downregulation of the natural killer cell activating ligand CD112. *J Gen Virol*. 91:2034-2039

Prosch, S., K. Staak, J. Stein, C. Liebenthal, T. Stamminger, H.D. Volk, and D.H. Kruger. 1995. Stimulation of the human cytomegalovirus IE enhancer/promoter in HL-60 cells by TNFalpha is mediated via induction of NF-kappaB. *Virology*. 208:197-206

Quinnan, G.V.J., N. Kirmani, A.H. Rook, J.F. Manischewitz, L. Jackson, G. Moreschi, G.W. Santos, R. Saral, and W.H. Burns. 1982a. Cytotoxic t cells in cytomegalovirus infection: HLA-restricted T-lymphocyte and non-T-lymphocyte cytotoxic responses correlate with recovery from cytomegalovirus infection in bone-marrow-transplant recipients. *N Engl J Med*. 307:7-13

Quinnan, G.V.J., N. Kirmani, A.H. Rook, J.F. Manischewitz, L. Jackson, G. Moreschi, G.W. Santos, R. Saral, and W.H. Burns. 1982b. Cytotoxic t cells in cytomegalovirus infection: HLA-restricted T-lymphocyte and non-T-lymphocyte cytotoxic responses correlate with recovery from cytomegalovirus infection in bone-marrow-transplant recipients. *N Engl J Med*. 307:7-13

Rammensee, H., J. Bachmann, N.P. Emmerich, O.A. Bachor, and S. Stevanovic. 1999. SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics*. 50:213-219

Rammensee, H.G. 1995. Chemistry of peptides associated with MHC class I and class II molecules. *Curr Opin Immunol*. 7:85-96

Rauser, G., H. Einsele, C. Sinzger, D. Wernet, G. Kuntz, M. Assenmacher, J.D. Campbell, and M.S. Topp. 2004. Rapid generation of combined CMV-specific CD4+ and CD8+ T-cell lines for adoptive transfer into recipients of allogeneic stem cell transplants. *Blood*. 103:3565-3572

Reddehase, M.J. 2002. Antigens and immunoevasins: opponents in cytomegalovirus immune surveillance. *Nat Rev Immunol*. 2:831-844

Reddehase, M.J., W. Mutter, K. Munch, H.J. Buhring, and U.H. Koszinowski. 1987. CD8-positive T lymphocytes specific for murine cytomegalovirus immediate-early antigens mediate protective immunity. *J Virol*. 61:3102-3108

Reeves, M.B., and T. Compton. 2011. Inhibition of inflammatory interleukin-6 activity via extracellular signal-regulated kinase-mitogen-activated protein kinase signaling antagonizes human cytomegalovirus reactivation from dendritic cells. *J Virol*. 85:12750-12758

Reeves, M.B., P.A. MacAry, P.J. Lehner, J.G. Sissons, and J.H. Sinclair. 2005. Latency, chromatin remodeling, and reactivation of human cytomegalovirus in the dendritic cells of healthy carriers. *Proc Natl Acad Sci U S A*. 102:4140-4145

Rehm, A., A. Engelsberg, D. Tortorella, I.J. Korner, I. Lehmann, H.L. Ploegh, and U.E. Hopken. 2002. Human cytomegalovirus gene products US2 and US11 differ in their ability to attack major histocompatibility class I heavy chains in dendritic cells. *J Virol*. 76:5043-5050

Rentenaar, R.J., L.E. Gamadia, N. van DerHoek, F.N. van Diepen, R. Boom, J.F. Weel, P.M. Wertheim-van Dillen, R.A. van Lier, and I.J. ten Berge. 2000. Development of virus-specific CD4(+) T cells during primary cytomegalovirus infection. *J Clin Invest*. 105:541-548

Retiere, C., V. Prod'homme, B.M. Imbert-Marcille, M. Bonneville, H. Vie, and M.M. Hallet. 2000. Generation of cytomegalovirus-specific human T-lymphocyte clones by using autologous B-lymphoblastoid cells with stable expression of pp65 or IE1 proteins: a tool to study the fine specificity of the antiviral response. *J Virol*. 74:3948-3952

Reusser, P., S.R. Riddell, J.D. Meyers, and P.D. Greenberg. 1991. Cytotoxic T-lymphocyte response to cytomegalovirus after human allogeneic bone marrow transplantation: pattern of recovery and correlation with cytomegalovirus infection and disease. *Blood*. 78:1373-1380

Revello, M.G., and G. Gerna. 2002. Diagnosis and management of human cytomegalovirus infection in the mother, fetus, and newborn infant. *Clin Microbiol Rev*. 15:680-715

Revello, M.G., and G. Gerna. 2010. Human cytomegalovirus tropism for endothelial/epithelial cells: scientific background and clinical implications. *Rev Med Virol*. 20:136-155

Rickinson, A.B., and D.J. Moss. 1997. Human cytotoxic T lymphocyte responses to Epstein-Barr virus infection. *Annu Rev Immunol*. 15:405-431

Riddell, S.R., M. Rabin, A.P. Geballe, W.J. Britt, and P.D. Greenberg. 1991. Class I MHC-restricted cytotoxic T lymphocyte recognition of cells infected with human cytomegalovirus does not require endogenous viral gene expression. *J Immunol*. 146:2795-2804

Riddell, S.R., K.S. Watanabe, J.M. Goodrich, C.R. Li, M.E. Agha, and P.D. Greenberg. 1992. Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. *Science*. 257:238-241

Ringden, O., P. Pihlstedt, L. Volin, J. Nikoskelainen, B. Lonnqvist, P. Ruutu, T. Ruutu, A. Toivanen, and B. Wahren. 1987. Failure to prevent cytomegalovirus infection by cytomegalovirus hyperimmune plasma: a randomized trial by the Nordic Bone Marrow Transplantation Group. *Bone Marrow Transplant*. 2:299-305

Rist, M., C. Smith, M.J. Bell, S.R. Burrows, and R. Khanna. 2009. Cross-recognition of HLA DR4 alloantigen by virus-specific CD8+ T cells: a new paradigm for self-/nonself-recognition. *Blood*. 114:2244-2253

Rodgers, B., L. Borysiewicz, J. Mundin, S. Graham, and P. Sissons. 1987. Immunoaffinity purification of a 72K early antigen of human cytomegalovirus: analysis of humoral and cell-mediated immunity to the purified polypeptide. *J Gen Virol*. 68:2371-2378

Romagnani, S. 1994. Lymphokine production by human T cells in disease states. *Annu Rev Immunol*. 12:227-257

Rudolph, M.G., R.L. Stanfield, and I.A. Wilson. 2006. How TCRs bind MHCs, peptides, and coreceptors. *Annu Rev Immunol*. 24:419-466

Ryckman, B.J., M.A. Jarvis, D.D. Drummond, J.A. Nelson, and D.C. Johnson. 2006. Human cytomegalovirus entry into epithelial and endothelial cells depends on genes UL128 to UL150 and occurs by endocytosis and low-pH fusion. *J Virol*. 80:710-722

Sacre, K., S. Nguyen, C. Deback, G. Carcelain, J.P. Vernant, V. Leblond, B. Autran, and N. Dhedin. 2008. Expansion of human cytomegalovirus (HCMV) immediate-early 1-specific CD8+ T cells and control of HCMV replication after allogeneic stem cell transplantation. *J Virol*. 82:10143-10152

Salkowitz, J.R., S.F. Sieg, C.V. Harding, and M.M. Lederman. 2004. In vitro human memory CD8 T cell expansion in response to cytomegalovirus requires CD4+ T cell help. *J Infect Dis*. 189:971-983

Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature*. 401:708-712

Sanchez, V., K.D. Greis, E. Sztul, and W.J. Britt. 2000. Accumulation of virion tegument and envelope proteins in a stable cytoplasmic compartment during human cytomegalovirus replication: characterization of a potential site of virus assembly. *J Virol*. 74:975-986

Saric, T., S.C. Chang, A. Hattori, I.A. York, S. Markant, K.L. Rock, M. Tsujimoto, and A.L. Goldberg. 2002. An IFN-gamma-induced aminopeptidase in the ER, ERAP1, trims precursors to MHC class I-presented peptides. *Nat Immunol*. 3:1169-1176

Saulquin, X., C. Ibisch, M.A. Peyrat, E. Scotet, M. Hourmant, H. Vie, M. Bonneville, and E. Houssaint. 2000. A global appraisal of immunodominant CD8 T cell responses to Epstein-Barr virus and cytomegalovirus by bulk screening. *Eur J Immunol*. 30:2531-2539

Scalzo, A.A., and W.M. Yokoyama. 2008. Cmv1 and natural killer cell responses to murine cytomegalovirus infection. *Curr Top Microbiol Immunol*. 321:101-122

Schaefer, M.R., M. Williams, D.A. Kulpa, P.K. Blakely, A.Q. Yaffee, and K.L. Collins. 2008. A novel trafficking signal within the HLA-C cytoplasmic tail allows regulated expression upon differentiation of macrophages. *J Immunol*. 180:7804-7817

Schleiss, M.R. 2008. Cytomegalovirus vaccine development. *Curr Top Microbiol Immunol*. 325:361-382

Schmidt, A.H., D. Baier, U.V. Solloch, A. Stahr, N. Cereb, R. Wassmuth, G. Ehninger, and C. Rutt. 2009. Estimation of high-resolution HLA-A, -B, -C, -DRB1 allele and haplotype frequencies based on 8862 German stem cell donors and implications for strategic donor registry planning. *Hum Immunol*. 70:895-902

Schmitt, A., T. Tonn, D.H. Busch, G.U. Grigoleit, H. Einsele, M. Odendahl, L. Germeroth, M. Ringhoffer, S. Ringhoffer, M. Wiesneth, J. Greiner, D. Michel, T. Mertens, M. Rojewski, M. Marx, S. von Harsdorf, H. Dohner, E. Seifried, D. Bunjes, and M. Schmitt. 2011. Adoptive transfer and selective reconstitution of streptamer-selected cytomegalovirus-specific CD8+ T cells leads to virus clearance in patients after allogeneic peripheral blood stem cell transplantation. *Transfusion*. 51:591-599

Schoenberger, S.P., R.E. Toes, E.I. van der Voort, R. Offringa, and C.J. Melief. 1998. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature*. 393:480-483

Schonberg, K., M. Sribar, J. Enczmann, J.C. Fischer, and M. Uhrberg. 2011. Analyses of HLA-C-specific KIR repertoires in donors with group A and B haplotypes suggest a ligand-instructed model of NK cell receptor acquisition. *Blood*. 117:98-107

Schroder, K., P.J. Hertzog, T. Ravasi, and D.A. Hume. 2004. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol*. 75:163-189

Schultze, J.L., S. Michalak, M.J. Seamon, G. Dranoff, K. Jung, J. Daley, J.C. Delgado, J.G. Gribben, and L.M. Nadler. 1997. CD40-activated human B cells: an alternative source of highly efficient antigen presenting cells to generate autologous antigen-specific T cells for adoptive immunotherapy. *J Clin Invest*. 100:2757-2765

Schust, D.J., D. Tortorella, J. Seebach, C. Phan, and H.L. Ploegh. 1998. Trophoblast class I major histocompatibility complex (MHC) products are resistant to rapid degradation imposed by the human cytomegalovirus (HCMV) gene products US2 and US11. *J Exp Med*. 188:497-503

Senechal, B., A.M. Boruchov, J.L. Reagan, D.N. Hart, and J.W. Young. 2004. Infection of mature monocyte-derived dendritic cells with human cytomegalovirus inhibits stimulation of T-cell proliferation via the release of soluble CD83. *Blood*. 103:4207-4215

Serwold, T., F. Gonzalez, J. Kim, R. Jacob, and N. Shastri. 2002. ERAAP customizes peptides for MHC class I molecules in the endoplasmic reticulum. *Nature*. 419:480-483

Shimizu, Y., and R. DeMars. 1989. Production of human cells expressing individual transferred HLA-A,-B,-C genes using an HLA-A,-B,-C null human cell line. *J Immunol*. 142:3320-3328

Sieker, F., S. Springer, and M. Zacharias. 2007. Comparative molecular dynamics analysis of tapasin-dependent and -independent MHC class I alleles. *Protein Sci*. 16:299-308

Simon, C.O., R. Holtappels, H.M. Tervo, V. Bohm, T. Daubner, S.A. Oehrlein-Karpi, B. Kuhnapfel, A. Renzaho, D. Strand, J. Podlech, M.J. Reddehase, and N.K. Grzimek. 2006. CD8 T cells control cytomegalovirus latency by epitope-specific sensing of transcriptional reactivation. *J Virol*. 80:10436-10456

Sinclair, J., and P. Sissons. 2006. Latency and reactivation of human cytomegalovirus. *J Gen Virol*. 87:1763-1779

Sinzger, C., M. Digel, and G. Jahn. 2008. Cytomegalovirus cell tropism. *Curr Top Microbiol Immunol*. 325:63-83

Sinzger, C., K. Eberhardt, Y. Cavignac, C. Weinstock, T. Kessler, G. Jahn, and J.L. Davignon. 2006. Macrophage cultures are susceptible to lytic productive infection by endothelial-cell-propagated human cytomegalovirus strains and present viral IE1 protein to CD4+ T cells despite late downregulation of MHC class II molecules. *J Gen Virol*. 87:1853-1862

Sissons, J.G., and A.J. Carmichael. 2002. Clinical aspects and management of cytomegalovirus infection. *J Infect*. 44:78-83

Slezak, S.L., M. Bettinotti, S. Selleri, S. Adams, F.M. Marincola, and D.F. Stroncek. 2007. CMV pp65 and IE-1 T cell epitopes recognized by healthy subjects. *J Transl Med*. 5:17

Slobedman, B., P.A. Barry, J.V. Spencer, S. Avdic, and A. Abendroth. 2009. Virus-encoded homologs of cellular interleukin-10 and their control of host immune function. *J Virol*. 83:9618-9629

Snary, D., C.J. Barnstable, W.F. Bodmer, and M.J. Crumpton. 1977. Molecular structure of human histocompatibility antigens: the HLA-C series. *Eur J Immunol*. 7:580-585

Starr, T.K., S.C. Jameson, and K.A. Hogquist. 2003. Positive and negative selection of T cells. *Annu Rev Immunol*. 21:139-176

Stern-Ginossar, N., N. Elefant, A. Zimmermann, D.G. Wolf, N. Saleh, M. Biton, E. Horwitz, Z. Prokocimer, M. Prichard, G. Hahn, D. Goldman-Wohl, C. Greenfield, S. Yagel, H. Hengel, Y. Altuvia, H. Margalit, and O. Mandelboim. 2007. Host immune system gene targeting by a viral miRNA. *Science*. 317:376-381

Stern, J.B., and K.A. Smith. 1986. Interleukin-2 induction of T-cell G1 progression and c-myb expression. *Science*. 233:203-206

Stinski, M.F. 1978. Sequence of protein synthesis in cells infected by human cytomegalovirus: early and late virus-induced polypeptides. *J Virol*. 26:686-701

Stinski, M.F., and H. Isomura. 2008. Role of the cytomegalovirus major immediate early enhancer in acute infection and reactivation from latency. *Med Microbiol Immunol*. 197:223-231

Stinski, M.F., D.R. Thomsen, R.M. Stenberg, and L.C. Goldstein. 1983. Organization and expression of the immediate early genes of human cytomegalovirus. *J Virol*. 46:1-14

Stock, A.T., C.M. Jones, W.R. Heath, and F.R. Carbone. 2006. CTL response compensation for the loss of an immunodominant class I-restricted HSV-1 determinant. *Immunol Cell Biol*. 84:543-550

Sutherland, C.L., N.J. Chalupny, and D. Cosman. 2001. The UL16-binding proteins, a novel family of MHC class I-related ligands for NKG2D, activate natural killer cell functions. *Immunol Rev*. 181:185-192

Sylwester, A.W., B.L. Mitchell, J.B. Edgar, C. Taormina, C. Pelte, F. Ruchti, P.R. Sleath, K.H. Grabstein, N.A. Hosken, F. Kern, J.A. Nelson, and L.J. Picker. 2005. Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. *J Exp Med*. 202:673-685

Tan, L.C., N. Gudgeon, N.E. Annels, P. Hansasuta, C.A. O'Callaghan, S. Rowland-Jones, A.J. McMichael, A.B. Rickinson, and M.F. Callan. 1999. A re-evaluation of the frequency of CD8+ T cells specific for EBV in healthy virus carriers. *J Immunol*. 162:1827-1835

Tenney, D.J., and A.M. Colberg-Poley. 1991. Human cytomegalovirus UL36-38 and US3 immediate-early genes: temporally regulated expression of nuclear, cytoplasmic, and polysome-associated transcripts during infection. *J Virol*. 65:6724-6734

Thilo, C., P. Berglund, S.E. Applequist, J.W. Yewdell, H.G. Ljunggren, and A. Achour. 2006. Dissection of the interaction of the human cytomegalovirus-derived US2 protein with major histocompatibility complex class I molecules: prominent role of a single arginine residue in human leukocyte antigen-A2. *J Biol Chem*. 281:8950-8957

Thomas, P.G., S.A. Brown, R. Keating, W. Yue, M.Y. Morris, J. So, R.J. Webby, and P.C. Doherty. 2007. Hidden epitopes emerge in secondary influenza virus-specific CD8+ T cell responses. *J Immunol*. 178:3091-3098

Tomasec, P., V.M. Braud, C. Rickards, M.B. Powell, B.P. McSharry, S. Gadola, V. Cerundolo, L.K. Borysiewicz, A.J. McMichael, and G.W. Wilkinson. 2000. Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. *Science*. 287:1031

Tomasec, P., E.C. Wang, A.J. Davison, B. Vojtesek, M. Armstrong, C. Griffin, B.P. McSharry, R.J. Morris, S. Llewellyn-Lacey, C. Rickards, A. Nomoto, C. Sinzger, and G.W. Wilkinson. 2005. Downregulation of natural killer cell-activating ligand CD155 by human cytomegalovirus UL141. *Nat Immunol*. 6:181-188

Tomazin, R., J. Boname, N.R. Hegde, D.M. Lewinsohn, Y. Altschuler, T.R. Jones, P. Cresswell, J.A. Nelson, S.R. Riddell, and D.C. Johnson. 1999. Cytomegalovirus US2 destroys two components of the MHC class II pathway, preventing recognition by CD4+ T cells. *Nat Med*. 5:1039-1043

Tooze, J., M. Hollinshead, B. Reis, K. Radsak, and H. Kern. 1993. Progeny vaccinia and human cytomegalovirus particles utilize early endosomal cisternae for their envelopes. *Eur J Cell Biol*. 60:163-178

Trachtenberg, E., M. Vinson, E. Hayes, Y.M. Hsu, K. Houtchens, H. Erlich, W. Klitz, Y. Hsia, and J. Hollenbach. 2007. HLA class I (A, B, C) and class II (DRB1, DQA1, DQB1, DPB1) alleles and haplotypes in the Han from southern China. *Tissue Antigens*. 70:455-463

Trapani, J.A., and M.J. Smyth. 2002. Functional significance of the perforin/granzyme cell death pathway. *Nat Rev Immunol*. 2:735-747

Trowsdale, J. 2002. The gentle art of gene arrangement: the meaning of gene clusters. *Genome Biol*. 3:COMMENT2002

Uhrberg, M. 2005. The KIR gene family: life in the fast lane of evolution. *Eur J Immunol*. 35:10-15

Uhrberg, M., N.M. Valiante, B.P. Shum, H.G. Shilling, K. Lienert-Weidenbach, B. Corliss, D. Tyan, L.L. Lanier, and P. Parham. 1997. Human diversity in killer cell inhibitory receptor genes. *Immunity*. 7:753-763

Valiante, N.M., M. Uhrberg, H.G. Shilling, K. Lienert-Weidenbach, K.L. Arnett, A. D'Andrea, J.H. Phillips, L.L. Lanier, and P. Parham. 1997. Functionally and structurally distinct NK cell receptor repertoires in the peripheral blood of two human donors. *Immunity*. 7:739-751

van Leeuwen, E.M., E.B. Remmerswaal, M.H. Heemskerk, I.J. ten Berge, and R.A. van Lier. 2006. Strong selection of virus-specific cytotoxic CD4+ T-cell clones during primary human cytomegalovirus infection. *Blood*. 108:3121-3127

van Leeuwen, E.M., E.B. Remmerswaal, M.T. Vossen, A.T. Rowshani, P.M. Wertheim-van Dillen, R.A. van Lier, and I.J. ten Berge. 2004. Emergence of a CD4+CD28- granzyme B+, cytomegalovirus-specific T cell subset after recovery of primary cytomegalovirus infection. *J Immunol*. 173:1834-1841

Vancha, A.R., S. Govindaraju, K.V. Parsa, M. Jasti, M. Gonzalez-Garcia, and R.P. Ballestero. 2004. Use of polyethyleneimine polymer in cell culture as attachment factor and lipofection enhancer. *BMC Biotechnol*. 4:23

Vancikova, Z., and P. Dvorak. 2001. Cytomegalovirus infection in immunocompetent and immunocompromised individuals--a review. *Curr Drug Targets Immune Endocr Metabol Disord*. 1:179-187

Varnum, S.M., D.N. Streblow, M.E. Monroe, P. Smith, K.J. Auberry, L. Pasa-Tolic, D. Wang, D.G.n. Camp, K. Rodland, S. Wiley, W. Britt, T. Shenk, R.D. Smith, and J.A. Nelson. 2004. Identification of proteins in human cytomegalovirus (HCMV) particles: the HCMV proteome. *J Virol*. 78:10960-10966

Vivier, E., J.A. Nunes, and F. Vely. 2004. Natural killer cell signaling pathways. *Science*. 306:1517-1519

Vivier, E., D.H. Raulet, A. Moretta, M.A. Caligiuri, L. Zitvogel, L.L. Lanier, W.M. Yokoyama, and S. Ugolini. 2011. Innate or adaptive immunity? The example of natural killer cells. *Science*. 331:44-49

von Bergwelt-Baildon, M.S., R.H. Vonderheide, B. Maecker, N. Hirano, K.S. Anderson, M.O. Butler, Z. Xia, W.Y. Zeng, K.W. Wucherpfennig, L.M. Nadler, and J.L. Schultze. 2002. Human primary and memory cytotoxic T lymphocyte responses are efficiently induced by means of CD40-activated B cells as antigen-presenting cells: potential for clinical application. *Blood*. 99:3319-3325

Wallace, L.E., M. Rowe, J.S. Gaston, A.B. Rickinson, and M.A. Epstein. 1982. Cytotoxic T cell recognition of Epstein-Barr virus-infected B cells. III. Establishment of HLA-restricted cytotoxic T cell lines using interleukin 2. *Eur J Immunol*. 12:1012-1018

Walter, E.A., P.D. Greenberg, M.J. Gilbert, R.J. Finch, K.S. Watanabe, E.D. Thomas, and S.R. Riddell. 1995. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *N Engl J Med*. 333:1038-1044

Wang, E.C., B. McSharry, C. Retiere, P. Tomasec, S. Williams, L.K. Borysiewicz, V.M. Braud, and G.W. Wilkinson. 2002a. UL40-mediated NK evasion during productive infection with human cytomegalovirus. *Proc Natl Acad Sci U S A*. 99:7570-7575

Wang, E.C., B. McSharry, C. Retiere, P. Tomasec, S. Williams, L.K. Borysiewicz, V.M. Braud, and G.W. Wilkinson. 2002b. UL40-mediated NK evasion during productive infection with human cytomegalovirus. *Proc Natl Acad Sci U S A*. 99:7570-7575

Weekes, M.P., M.R. Wills, K. Mynard, A.J. Carmichael, and J.G. Sissons. 1999. The memory cytotoxic T-lymphocyte (CTL) response to human cytomegalovirus infection contains individual peptide-specific CTL clones that have undergone extensive expansion in vivo. *J Virol*. 73:2099-2108

Weekes, M.P., M.R. Wills, J.G. Sissons, and A.J. Carmichael. 2004. Long-term stable expanded human CD4+ T cell clones specific for human cytomegalovirus are distributed in both CD45RAhigh and CD45ROhigh populations. *J Immunol*. 173:5843-5851

Weston, K. 1988. An enhancer element in the short unique region of human cytomegalovirus regulates the production of a group of abundant immediate early transcripts. *Virology*. 162:406-416

Wiertz, E.J., T.R. Jones, L. Sun, M. Bogyo, H.J. Geuze, and H.L. Ploegh. 1996a. The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. *Cell*. 84:769-779

Wiertz, E.J., D. Tortorella, M. Bogyo, J. Yu, W. Mothes, T.R. Jones, T.A. Rapoport, and H.L. Ploegh. 1996b. Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. *Nature*. 384:432-438

Wiesner, M., 2005. Die Diversität Cytomegalovirus-spezifischer T-Zellen nach Selektion mittels B-Zell-Vektoren. Fakultät für Chemie und Pharmazie, Ludwig-Maximilians-Universität München.

Wiesner, M., C. Zentz, M.H. Hammer, M. Cobbold, F. Kern, H.J. Kolb, W. Hammerschmidt, R. Zeidler, and A. Moosmann. 2005. Selection of CMV-specific CD8+ and CD4+ T cells by mini-EBV-transformed B cell lines. *Eur J Immunol*. 35:2110-2121

Wiesner, M., C. Zentz, C. Mayr, R. Wimmer, W. Hammerschmidt, R. Zeidler, and A. Moosmann. 2008. Conditional immortalization of human B cells by CD40 ligation. *PLoS One*. 3:e1464

Wilkinson, G.W., P. Tomasec, R.J. Stanton, M. Armstrong, V. Prod'homme, R. Aicheler, B.P. McSharry, C.R. Rickards, D. Cochrane, S. Llewellyn-Lacey, E.C. Wang, C.A. Griffin, and A.J. Davison. 2008. Modulation of natural killer cells by human cytomegalovirus. *J Clin Virol*. 41:206-212

Willinger, T., T. Freeman, H. Hasegawa, A.J. McMichael, and M.F. Callan. 2005. Molecular signatures distinguish human central memory from effector memory CD8 T cell subsets. *J Immunol*. 175:5895-5903

Wills, M.R., A.J. Carmichael, K. Mynard, X. Jin, M.P. Weekes, B. Plachter, and J.G. Sissons. 1996. The human cytotoxic T-lymphocyte (CTL) response to cytomegalovirus is dominated by structural protein pp65: frequency, specificity, and T-cell receptor usage of pp65-specific CTL. *J Virol*. 70:7569-7579

Wills, M.R., A.J. Carmichael, J. Sinclair, and J.P. Sissons. 2007. Immunobiology and host response. In "Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis". *Cambridge University Press*

Wills, M.R., G. Okecha, M.P. Weekes, M.K. Gandhi, P.J. Sissons, and A.J. Carmichael. 2002. Identification of naive or antigen-experienced human CD8(+) T cells by expression of costimulation and chemokine receptors: analysis of the human cytomegalovirus-specific CD8(+) T cell response. *J Immunol*. 168:5455-5464

Wright, C.A., P. Kozik, M. Zacharias, and S. Springer. 2004. Tapasin and other chaperones: models of the MHC class I loading complex. *Biol Chem*. 385:763-778

Yasukawa, M., A. Inatsuki, T. Horiuchi, and Y. Kobayashi. 1991. Functional heterogeneity among herpes simplex virus-specific human CD4+ T cells. *J Immunol*. 146:1341-1347

Yewdell, J.W., and J.R. Bennink. 1999. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. *Annu Rev Immunol*. 17:51-88

Yu, J., G. Heller, J. Chewning, S. Kim, W.M. Yokoyama, and K.C. Hsu. 2007. Hierarchy of the human natural killer cell response is determined by class and quantity of inhibitory receptors for self-HLA-B and HLA-C ligands. *J Immunol*. 179:5977-5989

Yu, J., J.M. Venstrom, X.R. Liu, J. Pring, R.S. Hasan, R.J. O'Reilly, and K.C. Hsu. 2009. Breaking tolerance to self, circulating natural killer cells expressing inhibitory KIR for non-self HLA exhibit effector function after T cell-depleted allogeneic hematopoietic cell transplantation. *Blood*. 113:3875-3884.

Zarrin, A.A., L. Malkin, I. Fong, K.D. Luk, A. Ghose, and N.L. Berinstein. 1999. Comparison of CMV, RSV, SV40 viral and Vlambda1 cellular promoters in B and T lymphoid and non-lymphoid cell lines. *Biochim Biophys Acta*. 1446:135-139

Zhang, Y., M. Cosyns, M.J. Levin, and A.R. Hayward. 1994. Cytokine production in varicella zoster virus-stimulated limiting dilution lymphocyte cultures. *Clin Exp Immunol*. 98:128-133

Zhao, Y., and B.J. Biegalke. 2003. Functional analysis of the human cytomegalovirus immune evasion protein, pUS3 (22kDa). *Virology*. 315:353-361

Abbreviations

β_2m	beta 2-microglobulin
ActD	actinomycin D
AIDS	acquired immunodeficiency syndrome
ALP	alkaline phosphatase
APC	antigen-presenting cell
APC	Allophycocyanin
bp	base pairs
BMT	bone marrow transplantation
cam	chloramphenicol acetyltransferase
cAMP	cyclic AMP
CD	cluster of differentiation (cell surface marker)
CD40L	CD40 ligand
CHX	cycloheximide
CMV	cytomegalovirus
C-terminal	carboxy terminal
ctrl	control
DC	dendritic cell
(D)MEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
e.g.	exempli gratia (lat., for example)
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
ELISA	Enzyme-Linked Immunosorbent Assay
ELISpot	Enzyme-Linked ImmunoSpot
ER	endoplasmic reticulum
ERAP	ER-aminopeptidase
et al.	et alii
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
Fig.	figure
FITC	fluoresceinisothiocyanate
GM-CSF	granulocyte-macrophage colony-stimulating factor
gB/H	glycoprotein B/H
gp	glycoprotein
GVHD	graft-versus-host disease
hpi	hours post infection
HAART	highly active anti-retroviral therapy
HCMV	human cytomegalovirus
HHV	human herpesvirus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HSCT	hematopoietic stem cell transplantation
HSV	herpes simplex virus
i.e.	id est
IE	immediate-early
IE-1	immediate-early 1 protein
IFN	interferon
Ig	immune globuline

IL	Interleukin
ISG	interferon-stimulated gene
kb	kilobases/kilo base pairs
kDa	kilodalton
KIR	killer-cell immunoglobulin-like receptor
KSHV	Kaposi's sarcoma-associated herpesvirus
LB	Luria Bertani (bacteria culture medium)
MIIC	MHC class II compartment
MACS	magnetic cell sorting
Mb	megabases
MCMV	murine cytomegalovirus
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
miRNA	microRNA
mRNA	messenger RNA
mLCL	mini-lymphoblastoid cell line
mo-DCs	monocyte-derived dendritic cells
moi	multiplicity of infection
MVA	modified vaccinia virus Ankara
NK cell	natural killer cell
n.d.	not determined
N-terminal	amino terminal
ORF	open reading frame
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PE-Cy5	phycoerythrin-cyanine 5
PEI	polyethyleneimine
p-NPP	*para*-nitrophenyl-phosphate
pp65	phosphoprotein 65
P/S	penicillin/streptomycin
rhu	recombinant human
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SOT	solid organ transplantation
SV40	simian virus 40
rpm	revolutions per minute
RPMI	"Roswell Park Memorial Institute" (cell culture medium)
TAP	transporter associated with antigen processing
$TCID_{50}$	50% infectious tissue culture dose
TCR	T cell receptor
TLR	Toll-like receptor
TNF-α	Tumor Necrosis Factor alpha
TPA	tetradecanoylphorbol acetate
U	units
US	unique short
UL	unique long
vol/vol	volume per volume
VZV	varicella-zoster virus
w/o	without
wt	wild-type

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