Committed Cytomegalovirus-Specific CD8 T Cells to Eliminate Tumor Cells by Bifunctional Major Histocompatibility Class I Antibody Fusion Molecules

Martina Schmittnaegel1, Victor Levitsky2, Eike Hoffmann1, Guy Georges1, Olaf Mundigl1, Christian Klein2, and Hendrik Knoetgen1

Abstract

Tumor cells escape immune eradication through multiple mechanisms, including loss of antigenicity and local suppression of effector lymphocytes. To counteract these obstacles, we aimed to direct the unique cytomegalovirus (CMV)-specific immune surveillance against tumor cells. We developed a novel generation of fusion proteins composed of a tumor antigen-specific full immunoglobulin connected to a single major histocompatibility class I complex bearing a covalently linked virus-derived peptide (pMHCI-IgG). Here, we show that tumor antigen-expressing cancer cells, which are decorated with pMHCI-IgGs containing a HLA-A*0201 molecule associated with a CMV-derived peptide, are specifically eliminated through engagement of antigen-specific CD8+ T cells isolated from peripheral blood mononuclear cell preparations of CMV-infected humans. These CD8+ T cells act without additional expansion, preactivation, or provision of costimulatory signals. Elimination of tumor cells is induced at similar concentrations and with similar time kinetics as those seen with bispecific T-cell engagers (BiTE). However, while BiTE-like reagents indiscriminately activate T cells through binding to the T-cell receptor complex, pMHCI-IgGs selectively engage antigen-specific, constantly renewable, differentiated effector cytotoxic T lymphocytes to tumor cells, thereby representing a novel class of anticancer immunotherapeutics with potentially improved safety and efficacy profiles. Cancer Immunol Res; 3(7): 764–76. ©2015 AACR.

Introduction

Immunologic memory is a fundamental feature of the adaptive immune system. It enables a rapid and vigorous response to infectious pathogens that have been encountered previously. In particular, memory CD8+ T cells play a major role in host defense by rapid recognition and lysis of virus-infected cells (reviewed in ref. 1).

Human cytomegalovirus (CMV) is a widespread persistent β-herpesvirus that infects 60% to 90% of the population, with slightly lower seroprevalence of 40% to 60% in Western Europe and the United States (2). Primary infection in healthy subjects usually goes unnoticed but leads to a life-long persistence of the virus in a replicative dormant state known as “viral latency” (3, 4). During CMV latency, reactivation of the viral genome occurs periodically in a fraction of infected cells. This viral reactivation, however, is controlled by the immune system, preventing any clinical symptoms in immunocompetent individuals (4, 5).

In contrast, viral reactivation causes morbidity and mortality in immunocompromised patients and neonates and has a substantial influence on the number and the distribution of various T-cell subsets, especially CD8+ T cells, driving them toward “memory inflation,” which is characterized by increased numbers of CMV-specific T cells against certain peptide epitopes (5) similar to an infection with Epstein–Barr virus (EBV; ref. 6). About 10% of CD8+ T cells in the peripheral blood of healthy virus carriers, and up to 20% of CD8+ T cells in the peripheral blood of elderly individuals, can be specific for CMV-derived antigens (7).

CMV-specific CD8+ T cells are believed to play an essential role in the defense against CMV (8), and a correlation is observed between a higher percentage of CMV-specific CD8+ T cells and more effective control of the virus (9). Furthermore, adoptive T-cell therapies for CMV disease and CMV-associated cancer are remarkably successful (10, 11). CMV-specific CD8+ T cells exhibit the phenotype of late-differentiated effector T cells (CD45RA− or +, CCR7+, CD62L−, C27+, and CD28−), but do not appear to be exhausted, senescent, or dysfunctional (12, 13). It is still controversial how memory inflation of CMV-specific T cells is maintained and if these cells are mainly short-lived or long-lived effector cells, but the consensus holds that in CMV-infected individuals, a population of persisting long-lived memory-like cells is established, which serves as a source for constant or increasing effector cell numbers (reviewed in ref. 5). In summary,
Materials and Methods

Cell lines

H322M and H460M (both grown in RPMI-1640) express low levels of insulin-like growth factor 1 receptor (IGFIR), I24-M6 (grown in DMEM) are murine NIH 3T3 cells expressing high levels of human IGFIR (26). Colo38, MDA-MB435, MDA-MB_L1UC (all grown in RPMI-1640), and WM266-4 (grown in Eagle MEM with Earle BSS, 1.0 mmol/L sodium pyruvate, 0.1 mmol/L nonessential amino acids) express high levels of melanoma-associated chondroitin sulfate proteoglycan (MCSP). WM266-4 are HLA-A*0201 positive. All media were supplemented with 10% fetal bovine serum and L-glutamine (2 mmol/L). MDA-MB435_L1UC cells were transduced with replication incompetent lentivirus-based virus-like particles for stable firefly luciferase expression using Lent-X HT Packaging Mix (Clontech). Cells were cultured for multiple passages under selection at 1 μg/mL puromycin and tested for homogenous firefly luciferase expression. Cell lines were verified as pathogen-free and identity was verified by SNP-PCR or short tandem repeat (STR) analysis before use.

Cloning, transient transfection, and protein production

The recombinant pMHCI complexes have been described previously as single-chain trimers (27–29). Recombinant pMHCI-IgG fusions were cloned into standard mammalian expression vectors (pCDNA3-derived; Life Technologies) and consist of viral peptide, first linker (sequence: (G4S)3), β2-microglobulin, second linker (sequence: (G4S)3), α 1–3 domains of HLA-A*0201, third linker (sequence: GS) followed by the antibody heavy chain. Monoclonal antibodies, R1507 and M4-3-ML2 (30), directed against IGFIR (31) and MCSP (CSPG4; ref. 32), respectively, mediated tumor cell-specific targeting, nonbinding control antibody is based on antibody germline sequence DP47 (33) with modified CDR3 region. Unfused heavy chain and antibody light chain were coexpressed from a separate expression vector. Heterodimerization of heavy chains is driven by knob-into-hole mutations, stabilized by an artificial disulphide bridge (34, 35). The heavy chain construct bearing the pMHCI complex was of the “hole” type. The isoform IgG1 was engineered to be devoid of all effector functions (36). Detailed sequences are provided in the Supplementary Data (37). The FreeStyle 293 transient expression system was used for protein expression (Life Technologies).

PBMC preparations

Peripheral blood mononuclear cells (PBMC) were isolated by ficoll gradient centrifugation from heparinized blood of healthy volunteers. The effector-to-target-cell ratio (e.g., 10:1) was calculated for total PBMCs comprising 0.03% to 3.3% CMV–pp65-specific CD8+ T cells (see Supplementary Table S1).

Expansion of CMV–pp65-specific CD8+ T cells

PBMCs were peptide-pulsed (25 μg/mL), washed, and expanded. Media were supplemented with IL2 (20 U/mL; Roche). IL7, and IL15 (both 25 ng/mL; Peprotech). Cells were restimulated every 2 weeks with freshly prepared or thawed autologous peptide-pulsed PBMCs. These PBMCs were irradiated and washed before use. Interleukins as described above were added. Cells were expanded every 2 to 3 days with fresh medium supplemented with interleukins. The effector-to-target-cell ratio (e.g., 3:1)
was calculated for total expansion culture containing about 50% expanded CMV–pp65-specific CD8⁺ T cells and the remaining irradiated PBMCs.

**Cytokine release**

Cell-free supernatants from the cytotoxicity assays with PBMCs were analyzed for secreted cytokines using the Cytometric Bead Array Flex Sets (BD Biosciences) according to the manufacturer’s instructions and measured with FACS Array and analyzed using FCAP software.

**Time-lapse live-cell imaging by confocal microscopy**

Tumor cells were labeled with 5 μmol/L CMFDA (Life Technologies). Human CMV–pp65-specific CD8⁺ T cells were stained with 0.4–μL PKH26-GL in 100–μL Diluent C (Sigma-Aldrich) according to the manufacturer’s instructions. The final effector-to-target-cell ratio used was 3:1. pMHCI–IgGs were added at a concentration of 15 mmol/L. Time-lapse fluorescence imaging was performed on a LEICA SP5x confocal microscope (Leica Microsystems).

**Real-time cytotoxicity assays**

Cytotoxicity was measured in a real-time cell analyzer xCELLigence (Roche) using adherent tumor cell lines as target cells. Tumor cells were added and cultured for 24 hours. The test molecules were tested at concentrations ranging from 0.005 to 25 mmol/L and effecter cells were added. The ratio of effector-to-target cells was in the range of 20:1 to 10:1 for PBMCs and 1:1 to 2:1 for stimulated CTLs. Measurements were performed up to 48 hours in triplicates. Spontaneous release is defined as cell index from target cell + T cells, specific release from cell index of each specimen. Specific lysis in percentage (%) is calculated as [(cell index spontaneous release – cell index specimen)/(cell index spontaneous release)] × 100.

For measurement of lactate dehydrogenase (LDH) release, supernatants of cytotoxicity assays were incubated with Cytotoxicity Detection Kit (Roche) according to the manufacturer’s protocol. Absorption was detected at 490 nm using Tecan Sunrise Reader (Tecan). Specific lysis in percentage (%) is calculated as (specific release – spontaneous release)/(maximum release – spontaneous release) × 100. Maximum release was determined through addition of 1% Triton X-100 (Sigma-Aldrich) to target cells.

**In vivo tumor model**

All experiments were carried out according to the German Animal Welfare Act guidelines. Four- to 8-week-old CIEA NOG mice were obtained from Taconic Biosciences. The number of specific T cells was calculated for total culture comprising approximately 50% expanded CMV–pp65-specific CD8⁺ T cells and the remaining irradiated PBMCs. For measurement of bioluminescence, mice were injected intraperitoneally (i.p.) with 150 mg/kg ß-luciferin (Promega). Twelve minutes after luciferin application, mice were anesthetized with 2% isoflurane and bioluminescence was measured with IVIS Spectrum (PerkinElmer). Bioluminescence signals were read out with Living Image software (PerkinElmer) as total radiance (photons/second; p/s) of tumors. Statistical significance is determined using multiple t test with α = 5% and Holm–Sidak correction. Flow cytometry was performed from blood (d22) and spleen (d27) with anti-human CD8 PE-Cy7 (Becton Dickinson) and HLA-A*0201 Dextramers CMV (NLVPMVATV; Immudex). Blocking of Fc-receptors was performed with monoclonal anti-mouse CD16/32 (Becton Dickinson).

**Results**

**Recombinant expression and properties of heterodimeric pMHCI–IgG fusion proteins**

Extensive protein engineering and testing of various molecular designs finally led to identification of the two novel formats of pMHCI–IgG shown in Fig. 1. These new-format...
recombinant proteins could be expressed as fully human IgG1 antibody fusions to peptide–MHCI complexes in a stable and robust manner similar to standard, unmodified monoclonal antibodies (Schmittnaegel et al., in preparation). In our preferred design, the peptide MHCI complexes, described before as single-chain trimer (see Materials and Methods; ref. 38), are fused N-terminally to the variable domain of one antibody heavy chain and consist of a virus-derived antigenic peptide, human β-2-microglobulin, and MHCI class I heavy chain domains α 1 to 3 truncated at the transmembrane domain. Applying the “knob-into-hole” technology, a heterodimeric format with a second, unfused antibody heavy chain can be formed. This heterodimeric format enabled production of the pMHCI-IgG human antibody fusions in standard mammalian expression systems. Homodimeric formats with two pMHCI molecules, one on each of the heavy chains, can only be expressed at very low levels and were largely impaired by aggregation (Schmittnaegel et al., in preparation). Furthermore, the knob-into-hole technology allows the construction and evaluation of pMHCI–IgG fusion molecules being mono- or bivalent for the respective antigen (Fig. 1). Human IGFIR and MCSP were selected for targeting pMHCI–IgG fusion proteins to tumor cells. To exclusively evaluate pMHCI–derived effects, human IgG1 Fc-region was engineered to lack effector functions completely (see Materials and Methods).

To characterize the binding properties of the new fusion molecules, we first confirmed by flow cytometric analysis that the pMHCI–antibody fusion molecules retained their specific antibody-dependent target-binding capacity on cells and demonstrated that recombinant HLA-A2 was delivered to the surface of the target cells (data not shown). Antigen binding of the N-terminally modified fusion molecules was unaltered compared with the original anti-IGFIR and anti-MCSP antibodies in both the mono- and bivalent antibody forms (39). The HLA-A2 moiety was detected using the BB7.2 antibody, which recognizes a conformational HLA-A2 epitope, and thus confirmed the presence of properly folded HLA-A2 via pMHCI–IgG fusion molecules. Not surprisingly, due to their avidity, the bivalent pMHCI–IgGs showed superior antigen binding when compared with the monovalent antibody format at concentrations below 0.05 nmol/L (data not shown).

Next, single-dose blood plasma pharmacokinetic parameters of pMHCI–IgGs were determined in mice at three different dose levels (0.1, 1, and 2 mg/kg, n = 3 per group) over a period of 8 days. Here, the in vivo half-life was calculated to be between 4 and 6 days (see Supplementary Fig. S1). Finally, we were able to confirm that the pMHCI–IgG molecules detected in serum recovered from mice 16 hours after injection with the 2-mg/kg dose were active in a cytotoxicity assay (see Supplementary Fig. S2).

In vitro expanded CMV–pp65-specific CD8+ T cells kill pMHCI–IgG-targeted tumor cells

Next, we tested whether the pMHCI–IgG fusion molecules were able to activate CD8+ effector T cells and induce pMHCI-dependent and target-specific lysis of tumor cells. To this end, different tumor cell lines were cocultured with in vitro stimulated and expanded CMV–pp65-specific CD8+ T cells in the presence of a CMV (pp65-peptide: NLVPMVATV)—MHCI–IgG fusion molecule, a nonbinding CMV–pMHCI–IgG control molecule, or an EBV (BMLF1-peptide: GITCIVAML)–MHCI–IgG control molecule. As shown in Fig. 2, both CMV–pMHCI–IgG fusion molecules described here were able to redirect CMV-specific CD8+ effector T cells to lyse tumor cells that expressed the respective antigen. All tested IGFIR- or MCSP-expressing tumor cell lines were effectively eliminated after targeting with the corresponding CMV–pMHCI–IgG fusion molecule. Target-cell lysis was fast and virtually complete within 1 to 10 hours at an effector-to-target-cell ratio of 3:1 (effector cells are derived from donor 1 and about 50% of them were CMV–pp65-specific; see Supplementary Table S1). Importantly, lysis was strictly peptide specific and dependent on antibody binding to target-expressing cells. Accordingly, both control molecules either bearing the EBV-derived control peptide or the non–antigen-binding antibody moiety were completely inactive in the cytotoxicity assay. On the other
hand, the efficiency of target-cell lysis was dependent on the format and concentration of the fusion molecules, assay time, and effector-to-target-cell ratio. Thus, almost complete target cell lysis was observed at CMV–pMHCI–IgG concentrations between 0.05 and 0.5 nmol/L for the bivalent construct and at 5 nmol/L of the monovalent construct, depending on the cell line used as a target (Fig. 2). Most likely, the bivalent pMHCI–IgG constructs were more effective in inducing cytotoxicity especially at lower concentrations as compared with the monovalent constructs due to avidity. Specific target-cell killing was confirmed by LDH-release testing (data not shown), and time-lapse live-cell microscopy demonstrated that pp65-specific T cells induced serial killing of target cells (Fig. 3 and time-lapse microscopy movies in Supplementary Fig. S3).

Frequency and phenotype of CMV–pp65-specific CD8+ T cells in human donor PBMCs

We scrutinized PBMCs from 8 of 11 human donors who were positive for HLA-A*0201, carried CMV-specific CD8+ T cells against CMV pp65 peptide (495–503): NLVPMVATV, and were negative for EBV-specific CD8+ T cells against BMLF-1 peptide (260–268): GLCTLVAML (see Supplementary Table S1). The frequency of CMV-pp65-specific T cells of all CD8+ T cells was between 0.5% and 11.3%. More than 55% of these T cells were of the late-stage, peripheral effector-memory phenotype (TEM) followed in frequency by central-memory T cells (TCM; ~20%), and naïve T cells (Tnaïve; ~20%). One donor carried a larger proportion of TCM (Supplementary Table 1; donor 2), which could be due to a more recent CMV infection because TEM and TEMRA memory T cells were reported to develop from TCM cells (40, 41). Interleukin-7 receptor α (CD127) is upregulated in CMV–pp65-specific T-cell populations of all donors at frequencies between 34% and 58% and defines a phenotype of precursors of long-living memory T cells (Supplementary Table 1; ref. 42). Activation markers, such as CD25 and CD69, were not upregulated, indicating an absence of recent antigenic challenge (43). CXCR3 that enables T cells to migrate to inflamed tissues and is characteristic for a memory phenotype with the ability to produce IL2 and IFNγ (44) was widely expressed. Markers of activation and exhaustion, such as PD-1, and of replicative senescence, such as CD57, were expressed at low levels (PD-1) but with significant frequencies (both PD-1 and CD57), but did not correlate with functional impairment or replicative constraints, because CMV–pp65-specific CD8+ T cells of almost all donors could be stimulated ex vivo with CMV peptide in the presence of cytokines.

In vitro expansion of CMV–pp65-specific CD8+ T cells was successful for 10 of the 11 donors tested. In particular, 2 weeks after the initial stimulation, nine of the T-cell cultures contained between 10% and 80% of peptide-specific CD8+ T cells. After restimulation, an almost homogenous population of CMV–pp65-specific CD8+ T cells was obtained (data not shown).

PBMCs of CMV-positive donors mediate lysis of pMHCI–IgG-targeted tumor cells

Next, we evaluated the potency of CMV–pp65-specific CD8+ T cells to lyse target cells without prior in vitro stimulation after isolation from human PBMCs. To this end, we used freshly isolated human PBMCs as effector cells without any pretreatment or additional stimulation at an effector-to-target-cell ratio of 10:1 or 20:1 (Fig. 4). The frequency of CMV–pp65-specific
CD8⁺ T cells within the PBMC population ranged from 0.03% to 3.3%, depending on the individual donor (see Supplementary Table S1). CMV—pMHCI—IgGs were added at concentrations between 5 pmol/L and 5 nmol/L. Control molecules were non-binding CMV—pMHCI—IgG (black circles), EBV—pMHCI—IgG control (white circles), and CMV—pMHCI—IgG nonbinding control (white squares) were added at concentrations ranging from 50 to 0.0005 nmol/L or as indicated (x-axis). Specific tumor cell lysis (y-axis) is calculated as a percentage (%) for endpoint analysis after 30 to 48 hours. Error bars, SD of replicates (n = 3).

Figure 4. xCELLigence and LDH cytotoxicity analysis using pMHCI—IgG molecules with PBMCs from different donors. Lysis of MCSP⁺ tumor cell lines Colo38, WM266-4, and MDA-MB435 (indicated above columns) with unstimulated PBMCs from different donors at a PBMC-to-target-cell ratio of 10:1 or 20:1. Frequencies of CMV—pp65—specific CD8⁺ T cells in PBMCs are indicated on the left (3.3%–0.03%). Bivalent CMV—pMHCI—IgG (black circles), EBV—pMHCI—IgG control (white circles), and CMV—pMHCI—IgG nonbinding control (white squares) were added at concentrations ranging from 50 to 0.0005 nmol/L or as indicated (x-axis). Specific tumor cell lysis (y-axis) is calculated as a percentage (%) for endpoint analysis after 30 to 48 hours. Error bars, SD of replicates (n = 3).
to kill between three and eight target cells on an average to achieve complete killing. With lower effector-to-target ratios, we estimate that about 40 target cells or more were lysed per T cell. This implies that CMV–pp65–specific CD8+ T cells were able to kill multiple target cells and/or that a significant expansion of CD8+–pp65–memory T cells had taken place during the time of the assay, a phenomenon that has been previously shown for peptide-specific memory T cells (45). Again, no statistically significant killing was observed using control pMHCI–IgG molecules.

The monovalent nature of TCR interaction with pMHCI–IgG complexes prevents direct T-cell activation in the absence of target cells

To compare antibodies fused with one or two pMHCI molecules, we expressed and purified an antibody with two pMHCI molecule, one fused to each antibody heavy chain. These homodimeric fusions expressed at very low levels and only a small proportion was not aggregated and correctly folded (data not shown). We tested the activation of CMV–pp65–specific CD8+ T cells in PBMCs in the absence of target cells to follow the unspecific activation of T cells. We specifically looked at the induction of the activation markers CD25 and CD69, and at the downregulation of the TCR as a consequence of its activation at 0.5, 5, and 50 nmol/L of both pMHCI–antibody fusions either carrying one or two pMHCI complexes. CD25 and CD69 expression was not changed and a downregulation of the TCR was not observed at 0.5 and 5 nmol/L. The frequency of T cells expressing CD25 or CD69 was unchanged for the pMHCI–IgG with one pMHCI complex at 50 nmol/L concentration but increased when the pMHCI–IgG carrying two pMHCI molecules per IgG were analyzed (4.4-fold and 9.0-fold compared with the pMHCI–IgG with one pMHCI molecule, respectively). The downregulation of the TCR that correlates with T-cell activation was observed in 31.5% of the CMV–pp65–specific CD8+ T cells for the pMHCI–IgG with two pMHCI molecules compared with only 4.9% for the pMHCI–IgG with one pMHCI molecule.

Comparison of pMHCI and anti-CD3-mediated T-cell activation

We compared pMHCI–IgG fusion molecules to conventional bispecific T-cell engagers (BiTE), which interact with T cells by binding to CD3, the signal transduction component of the TCR complex, and thus potently activate all CD3-expressing cells (46). This, however, comes at the price of disadvantages such as binding to and activation of unwanted T-cell populations, which is known to cause a massive release of inflammatory cytokines. Accordingly, we assessed specific target cell killing both by enriched CMV–pp65–specific CD8+ T cells and by freshly isolated PBMCs (Figs. 5 and 6). The in vitro expanded CMV–pp65–specific CD8+ T cells mediated complete target cell killing after 10 hours at an effector-to-target-cell ratio of 3:1 following engagement by either the anti-MCSP-BiTE or the pMHCI–anti-MCSP–IgG. In contrast to pMHCI–IgG, BiTE molecules induced almost complete killing with little dose-dependency at a range of concentrations reaching as low as 1 pmol/L, while CMV–pMHCI–IgG-induced cell lysis was more concentration-dependent below 500 pmol/L. Half-maximal killing of target cells was achieved at 1 pmol/L of the BiTE and 35 pmol/L of the pMHCI–IgG (Fig. 5).

PBMCs from CMV-infected donors induce comparable tumor cell lysis in a time- and concentration-dependent manner with pMHCI–IgGs in comparison with BiTEs

Next, we tested the capacity of pMHCI–IgG fusion molecules to induce T cell–mediated cytolysis in comparison with BiTEs using freshly isolated PBMCs as effector cells. These PBMCs were tested in cytotoxicity assays using real-time xCELLigence cytotoxicity system. Cytotoxicity assays were performed to determine compound concentrations required for induction of half-maximal lysis (EC50) of MCSP+ tumor cells (Colo38). Prestimulated CMV–pp65–specific CD8+ T cells were added at an effector-to-target-cell ratio of 3:1; readout was after 10 hours. Compound concentrations for bivalent CMV–pMHCI–IgG (black circles) and BiTEs (white circles) range from 0.005 to 5,000 pmol/L (x-axis). The y-axis shows the percentage of specific tumor cell lysis. Error bars represent SD of replicates (n = 3).

Figure 5. EC50 analysis of CMV–pMHCI–IgG and BiTEs using xCELLigence cytotoxicity system. Cytotoxicity assays were performed to determine compound concentrations required for induction of half-maximal lysis (EC50) of MCSP+ tumor cells (Colo38). Prestimulated CMV–pp65–specific CD8+ T cells were added at an effector-to-target-cell ratio of 3:1; readout was after 10 hours. Compound concentrations for bivalent CMV–pMHCI–IgG (black circles) and BiTEs (white circles) range from 0.005 to 5,000 pmol/L (x-axis). The y-axis shows the percentage of specific tumor cell lysis. Error bars represent SD of replicates (n = 3).
comparable absolute potency of target cell killing, albeit at a later point in time, even at low frequencies of CMV–specific CD8⁺ T cells.

Cytokine release after CMV–pMHCI–IgG or BiTE stimulation

We expected the pMHCI–IgG-mediated T-cell targeting to tumor cells to be associated with less cytokine release as compared with BiTEs because only a small antigen-specific subpopulation of CD8⁺ T cells is engaged by these reagents, whereas CD8-specific triggering activates all T cells in a polyclonal manner. Accordingly, we measured the concentration of various cytokines secreted during the cytotoxicity assays using PBMCs containing 3.3% CMV–pp65–specific CD8⁺ T cells. All molecules (BiTEs, bivalent pMHCI–IgG, and pMHCI–IgG nonbinding control molecules) were tested on two different target cell lines, Colo38 and WM266-4, at a concentration of 5 nmol/L, and the amounts of cytokines secreted under different conditions were measured and compared (representative analysis for Colo38 cells is shown in Fig. 7).

Supernatants were tested after 48 hours in two independent experiments, when killing of target cells was completed. The concentrations of cytokines released were at least 20-fold up to 200-fold higher when BiTE molecules were tested as compared with pMHCI–IgGs. In particular, the main mediators of systemic inflammatory cytokine release and macrophage activation syndrome (47), such as TNFα, IL1α, IL1β, and IL6, were upregulated using BiTEs approximately 125-fold, 50-fold, 11-fold, and 16-fold, respectively. Secrecion of IL12p70 indicates a cytokine-mediated bystander activation of cells of the myeloid lineage and could not be detected after activation with pMHCI–IgGs but was upregulated to 175 pg/mL with BiTE molecules. Cytokines associated with CD4⁺ T cells of Th2 and regulatory T-cell lineage, such as IL4, IL5, and IL10, were not induced with pMHCI–IgGs but upregulated with BiTEs to 45, 200, and 210 pg/mL, respectively. Induction of IL13 was 8-fold increased by BiTEs compared with that by pMHCI–IgGs. IL13 is produced mostly by CD4⁺ T cells and some CD8⁺ subset (48). Th1 and cytotoxic T-cell–associated cytokines IFNy and IL2 were increased 7-fold and 3-fold, respectively, representing the activation of larger numbers of CD8⁺ T cells with BiTEs. In summary, a much lower and more selective cytokine production was induced by pMHCI–IgGs.

Expanded CMV–pp65–specific CD8⁺ T cells impair MCSP pMHCI–IgG-targeted tumor-cell outgrowth in vivo

SCID beige or RAG2⁻/⁻ge⁻/⁻ mice engrafted with human in vitro expanded pp65-specific CD8⁺ T cells showed insufficient survival and engraftment of these T cells, and T cells could not be recovered from blood or spleen 72 hours after transfer (data not shown). We tried to improve the engraftment of human pp65-specific CD8⁺ T cells by cotransfer of autologous PBMCs and changed the mouse strain to NOG mice. Those mice lack

Figure 6. Analysis of pMHCI–IgG– and BiTE-induced target cell lysis with PBMCs containing 3.3% CMV during the cytotoxicity assays using PBMCs containing 3.3% CMV–specific CD8⁺ T cells are indicated in the top left corner of the respective panels (3.3%, 1.3%, and 0.09%, respectively). A–C, left, real-time xCELLigence analysis of cytotoxicity; outlining CMV–pMHCI–IgGs (red triangles), BiTEs (violet squares) and control constructs EBV–pMHCI–IgG (blue circles) and nonbinding CMV–pMHCI–IgG (green diamonds) in the highest concentration of 5 nmol/L as well as spontaneous release (target cells + PBMCs; gray circles with black line) and target cells only (black stars). A–C, right, endpoint analysis of percentage of specific tumor cell lysis (%) for different constructs, including all measured concentrations (5, 0.5, and 0.05 nmol/L, as indicated). CMV–pMHCI–IgGs (red BiTEs; violet) and nonbinding CMV–pMHCI–IgG (green). Error bars represent SD of replicates (n = 3).
functional macrophages in addition to lymphocyte deficiency, thus allowing better engraftment of human T cells. These changes improved the overall survival and engraftment of human T cells. The best results were achieved when tumor cells, specific T cells, and PBMCs were cotransferred subcutaneously (s.c.) and when specific T cells and PBMCs were concomitantly cotransferred i.p. Intraperitoneal administration of 2 mg/kg (every 2 to 3 days) of MCSP-targeting pMHCI-IgGs led to a significantly reduced outgrowth of the MDA-MB435-LUC tumor cells with CMV–pMHCI–IgG treatment, which could be followed by noninvasive measurement of bioluminescence signal of tumor cells (Fig. 8, bottom left) until day 15, even before tumor volume could be measured. On days 17 and 21, tumor volume of CMV–pMHCI–IgG–treated group was significantly reduced in comparison with that of the control groups (not shown). After 27 days tumor weight was determined, showing significantly smaller tumors for CMV–pMHCI–IgG–treated mice compared with all control groups (see Fig. 8, bottom right). All mice showed engraftment of CMV–pp65-specific T cells, with percentages ranging from 2.2% ± 1.3% of all human CD3+ cells in blood (human CD3+ in blood: 5.5% ± 2.7% of viable cells) on day 22 and from 1.5% ± 0.9% of all human CD3+ cells in spleen (human CD3+ in spleen: 17.7% ± 7.0% of viable cells) on day 27 (data not shown).

Discussion

T-cell therapies are among the most potent approaches to cancer treatment and have received a lot of attention recently (49). The goal of T cell–based cancer therapies is to combine the extraordinary effector functions of tumor-specific T cells with minimal side effects. Cytotoxic T-cell responses can be initiated using vaccination, cytokines, adoptive T-cell transfer, and recombinant antibody-based molecules. However, the antitumor efficacy of such T cells is often thwarted by multiple inhibitory or escape mechanisms used by tumors. Interestingly, chronic viral infections pose a similar challenge for the human immune system, and here, too, multiple ways to evade immune defenses have evolved (reviewed in ref. 16). However, whereas cancers finally escape immune eradication, many chronic viral infections persist lifelong but are controlled by the immune system.

In general, the phenotypic characteristics of virus-specific T cells vary in different chronic infections. Both the number and functionality of CMV-specific CD8+ T cells recognizing immunodominant peptides such as peptides derived from viral pp65 protein seem to be unique as compared with other viral infections (14). These CMV–pp65-specific CD8+ T cells mainly contribute to the early control of CMV reactivation and constitute a large and persistent population with high proliferative and cytotoxic capacities (50). They are characterized as effector memory T cells, mostly TEM and TEMRA, with the capability to be rapidly activated and exhibit cytotoxicity. Because of the chronic nature of the CMV infection, which is associated with occasional viral reactivations, new effector cells are likely to be produced in a continuous and essentially infinite way. Here, we confirm that peripheral blood CMV-specific T cells derived from healthy donors are predominately effector T cells (TEM or TEMRA). They constitute more than 50% of the antigen-specific population while minor populations exhibit the phenotypes of TCM or naïve T cells. These CMV–pp65-specific CD8+ T cells are predominantly CD127+ long-lived memory T cells that are known to possess homeostatic proliferative capacity in the absence of antigen (42), and virtually all such T cells express chemokine receptor CCR3 that presumably enables them to migrate to inflamed tissues. Despite the expression of the inhibitory receptor PD-1 and CD57, markers generally associated with exhaustion and indicating replicative senescence, respectively, our data show that these CMV-specific CD8+ T cells are fully functional upon antigen-mediated reactivation. Indeed, almost all donor-derived CMV–pp65-specific CD8+ T cells could be stimulated with CMV peptide in vitro, and were capable of eliminating a large number of target cells without additional stimulation. Despite the fact that CMV has developed multiple ways to modulate and escape the immune response of the host, CMV is effectively controlled for the lifetime in healthy individuals and also in patients with cancer. Although cancer chemotherapy can

![Figure 7](image-url)

**Figure 7.** Cytokine release induced by pMHCI-IgGs in comparison with BiTE molecules with PBMCs using real-time xCELLigence cytotoxicity system. Supernatants of xCELLigence cytotoxicity assays with PBMCs (3.3% CMV–pp65-specific CD8+ T cells) and MCSP+ tumor cells (Colo38) were analyzed for cytokines released after 48 hours via flow cytometric bead-based measurement. CMV–pMHCI-IgGs (gray), BiTEs (black), and nonbinding CMV–pMHCI–IgG control (white) were administered at a concentration of 5 nmo/L. The respective cytokines are indicated and shown from left to right: IFNγ, IL2, TNF, IL12p70, IL1x, IL1β, IL6, IL4, IL5, IL13, and IL10. The y-axis shows cytokine release in pg/mL. Error bars represent SD of replicates (n = 3).
suppress immune cell function, CMV–pp65-specific CD8+ T cells are known to retain their antiviral capacity and even increase in number as they are the main defense against viral reactivation to this otherwise life-threatening infection (ref. 51 and references within). This is also supported by the recent finding that subsets of CMV-specific CD8+ T cells are functionally intact in patients with chronic lymphocytic leukemia (CLL) despite the global T-cell dysfunction characteristic of CLL (51).

Recently, latent CMV infection has been documented in a significant proportion of glioblastomas in which the expression of several viral genes, including pp65, was shown (52). Some of them may play a tumor-promoting role as they can suppress the immune response by downregulation of MHC class I on the tumor cell surface and by producing immunosuppressive cytokines (53). Although CMV-infected glioblastomas progress in the presence of endogenous pp65-specific CTLs, effective control over tumor development can be restored in this group of patients either by vaccination with dendritic cells pulsed with the relevant viral peptide or by adoptive transfer of pp65-specific in vitro expanded CTLs (11, 54).

Delivery of pMHCI complexes that display CMV-specific peptides may be a powerful way to mimic CMV infection in cancer cells. Fortunately, the immune response against CMV is largely focused on only a few immunodominant peptides. Importantly, a single virus-derived peptide is sufficient to attract a large number of T cells. The concept of mimicking a viral infection in cancer cells by targeting pMHCI complexes to their surface was proposed already several years ago (19, 28). To date, various fusion molecules were designed for this purpose; basically all of them were produced as antibody fragments in bacteria (combined with refolding), followed in most cases by in vitro peptide-loading, chemical-conjugation, or

Figure 8. In vivo xenograft model in NOG mice. Human MCSP+ tumor cells (2 × 10⁶; MDA-MB435, stably transduced with luciferase) were coengrafted s.c. with 6 × 10⁵ donor-derived, in vitro expanded T cells. Concomitantly, 3 × 10⁵ expanded T cells and 3 × 10⁵ autologous PBMCs were engrafted i.p. PBS (tumor and T-cell group) or pMHCI-IgG constructs were administered at 50 μg/mouse (~2 mg/kg) beginning 6 hours after engraftment (day 0), after 24 hours (day 1), and then every 2 to 3 days. Bioluminescence was measured 2 hours after tumor/T-cell engraftment, on day 1 (d1) and subsequently every 2 to 3 days. Tumor weight was determined on day 27. Bioluminescence is shown as mean values of all groups (n = 15) ± SEM.
streptavidin–biotin conjugation (19, 23–25, 55). However, complete human monoclonal IgG antibodies fused with pMHCI complexes have not been described previously. Here, we have established a new technology to produce full IgG antibodies appended with a single pMHCI complex per antibody molecule. These pMHCI–IgG molecules possess excellent expression efficiency and stability, allowing them to be produced using standard antibody manufacturing and purification processes. In addition, these molecules also possess pharmacokinetic properties comparable with those of conventional monoclonal IgG antibodies. Importantly, in comparison with fusion molecules carrying two or more pMHCI complexes, a single pMHCI complex per antibody molecule prevents non-specific TCR cross-linking and, consequently, unwanted activation of T cells in the absence of target cells. Finally, fusion of pMHCI to a full monoclonal antibody also confers higher potency of binding to tumor targets due to the avidity effect as compared with antibody fragment-based monovalent targeting moieties.

On the other hand, the concept of antibody-mediated delivery of pMHCI complexes to tumor cells as a therapeutic approach was never tested with unstimulated human PBMCs (19, 25, 28) although most likely these cells would constitute the primary source of CD8+ effector T cells. It was unclear, however, whether naturally observed frequencies of CMV-specific effector CD8+ T cells were sufficient to mediate significant tumor cell killing. Here, we provide evidence that freshly isolated CMV–pp65–specific CD8+ T cells, which were present at frequencies between 0.03% and 3.3% within the PBMCs from various donors, indeed were capable of lysing tumor cells in vitro. Clearly, serial killing of target cells was demonstrated by live-cell imaging and deduced from the rates of tumor cell lysis.

In vivo efficacy of CMV-specific T cells adoptively transferred into tumor-bearing NOG mice was significant but not as potent as expected, most likely due to a reduced viability and function of the transferred human effector cells. For successful survival of the CMV–pp65–specific CD8+ T cells, large numbers needed to be transferred and a cotransfer of autologous PBMCs was necessary to allow CMV-specific CD8+ T cells to survive for 27 days in NOG mice. In comparison with the in vitro results, the observed in vivo elimination of tumor cells was impaired, indicating that the T cells could not exert full killing capacity after transfer into a xenogeneic host.

Generally, using antigen-specific CD8+ cytotoxic T cells to eliminate tumor cells is expected to be advantageous both with regard to limiting side effects and improving specific tumor cell lysis. In comparison with pMHCI–IgGs, CD3-based molecules, such as BiTEs, recruited T cells in a polyclonal manner and were extremely potent in inducing rapid target cell lysis already at very low concentrations, whereas pMHCI–IgG–based molecules induced a more graded and perhaps more manageable, concentration-dependent lysis that is most likely due to the lower number of CD8+ T effector cells activated initially. In addition, engagement of only a subpopulation of T cells via the pMHCI/TCR interaction has the potential advantage of using a naturally occurring, highly effective and selective anti-viral subpopulation of CD8+ T cells that are functionally devoted to cell killing and do not need additional costimulation. Furthermore, because TCRs intrinsically possess a relatively low affinity for their cognate pMHCI complexes, activation through pMHCI–TCR interaction is only successful after multivalent interactions of several TCRs with pMHCI–IgG ligands (56). With respect to pharmacokinetics, we predict that there will be no T-cell sink effect due to the low intrinsic affinity of the pMHCI–TCR interaction and the low frequency of CMV-specific T cells that may allow for better tumor targeting and less drug deposition in organs of the immune system. Thus, non-specific T-cell activation is unlikely to occur even at high drug concentrations that would allow a broad safety window and, consequently, administration of higher doses and/or less frequent administration than possible for CD3-based T-cell recruiting molecules. Finally, the side effects caused by the non-specific cytokine release are expected to be significantly reduced. Indeed, we found a much reduced release of cytokines for the pMHCI–IgGs compared with CD3-based T-cell activation after killing of tumor cells in vitro. Importantly, we anticipate no impact of pMHCI–IgGs on effector CD8+ T cells and regulatory CD4+ T cells, in contrast to their effective engagement by CD3 targeting.

In comparison with the universal recruitment of all T cells via CD3-engagement, T-cell recruitment via pMHCI is limited to specific patient populations. In particular, the extensive polymorphism of class I HLA molecules limits the use of a single pMHCI class I complex to 35% to 40% of all patients for HLA A*0201 ([57] http://www.allelefrequencies.net). The second prerequisite is prior exposure to CMV infection. In most populations, this does not appear to be a major problem, because the vast majority of the human population tests CMV-seropositive. More specifically, the frequency of CMV infection in the human population increases with age, starting at 30% to 50% in individuals aged 6 to 11 years and eventually reaching 85% to 90% in individuals aged 75 to 80 years ([58, 59]). Because both HLA allotyping and determination of CMV infection status are clinically feasible ([60, 61]), this does not appear to be a major obstacle for clinical application of pMHCI–IgGs.

The data presented here demonstrate the feasibility of using pMHCI–IgG fusion proteins to redirect human CMV-specific CD8+ T cells for tumor therapy and shed new light on the functionality of this T-cell subset. We believe that further investigations are warranted to develop this concept towards clinical application.

Disclosure of Potential Conflicts of Interest
All of the authors are employees of F. Hoffman-LaRoche AG.

Authors’ Contributions
Conception and design: M. Schmittnaegel, V. Levitsky, G. Georges, C. Klein, H. Knoetgen
Development of methodology: M. Schmittnaegel, E. Hoffmann, O. Mundigl
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Schmittnaegel, E. Hoffmann, O. Mundigl
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Schmittnaegel, H. Knoetgen
Writing, review, and/or revision of the manuscript: M. Schmittnaegel, V. Levitsky, C. Klein, H. Knoetgen
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Hoffmann, G. Georges, O. Mundigl
Study supervision: H. Knoetgen

Acknowledgments
The authors thank Sylke Poehling, Pablo Ulmana, and John C. Reed for supporting the program, and Georg Tiefenthaler, Ulrich Brinkmann, John C. Reed, and William Pao for critical reading of the article. The authors also thank...
and her group, especially Margarete Galm, for assistance with in vivo imaging; Johannes Sam and Frank Herting and his group, especially Carsten Wolter, for supporting in new work; and Valeria Bunza and her group, especially Margarete Calm, for assistance with ex vivo analysis.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References
45. Rea IM, McNewlan SE, Alexander HD. CD69, CD25, and HLA-DR activation antigen expression on CD3(+) lymphocytes and relationship to serum TNF-alpha, IFN-gamma, and sIL-2R levels in aging. Exp Gerontol 1999;34:79–86.
Cancer Immunology Research

Committing Cytomegalovirus-Specific CD8 T Cells to Eliminate Tumor Cells by Bifunctional Major Histocompatibility Class I Antibody Fusion Molecules

Martina Schmittnaegel, Victor Levitsky, Eike Hoffmann, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/2326-6066.CIR-15-0037

Supplementary Material
Access the most recent supplemental material at:
http://cancerimmunolres.aacrjournals.org/content/suppl/2015/02/17/2326-6066.CIR-15-0037.DC1

Cited articles
This article cites 58 articles, 24 of which you can access for free at:
http://cancerimmunolres.aacrjournals.org/content/3/7/764.full.html#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
/content/3/7/764.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.