Varicella-zoster virus glycoproteins B and E are major targets of CD4+ and CD8+ T cells reconstituting during zoster after allogeneic transplantation

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ABSTRACT

Background

After allogeneic hematopoietic stem-cell transplantation patients are at increased risk for herpes zoster as long as varicella-zoster virus specific T-cell reconstitution is impaired. This study aimed to identify immunodominant varicella-zoster virus antigens that drive recovery of virus-specific T cells after transplantation.

Design and Methods

Antigens were purified from a varicella-zoster virus infected cell lysate by high-performance liquid chromatography and were identified by quantitative mass spectrometric analysis. To approximate *in vivo* immunogenicity for memory T cells, antigen preparations were consistently screened with *ex vivo* PBMC of varicella-zoster virus immune healthy individuals in sensitive interferon-γ ELISpot assays. Candidate virus antigens identified by the approach were genetically expressed in PBMC using electroporation of *in vitro* transcribed RNA encoding full-length proteins and were then analyzed for recognition by CD4+ and CD8+ memory T cells.

Results

Varicella-zoster virus encoded glycoproteins B and E, and immediate early protein 62 were identified in immunoreactive lysate material. Predominant CD4+ T-cell reactivity to these proteins was observed in healthy virus carriers. Furthermore, longitudinal screening in allogeneic stem-cell transplantation patients showed strong expansions of memory T cells recognizing glycoproteins B and E after onset of herpes zoster, while immediate early protein 62 reactivity remained moderate. Reactivity to viral glycoproteins boosted by acute zoster was mediated by both CD4+ and CD8+ T cells.

Conclusions

Our data demonstrate that glycoproteins B and E are major targets of varicella-zoster virus specific CD4+ and CD8+ T-cell reconstitution occurring during herpes zoster after allogeneic stem-cell transplantation. Varicella-zoster virus glycoproteins B and E might form the basis for novel non-hazardous zoster subunit vaccines suitable for immunocompromised transplant patients.

Key words: varicella zoster virus, T cells, allogeneic transplantation, zoster vaccine, glycoprotein.

Introduction

Herpes zoster represents the clinical manifestation of reactivated varicella-zoster virus (VZV) infection and occurs with an incidence of 25-40% after allogeneic hematopoietic stem cell transplantation (HSCT).[1-3] Although long-term antiviral drug prophylaxis with acyclovir and its derivatives is very effective in preventing zoster following transplantation, it does not provide complete protection and can postpone the disease to the late post-HSCT period.[4-8] In addition, the success of antiviral prophylaxis can be compromised by lack of patient compliance or by renal dysfunction. In fact, renal dysfunction is quite common after transplantation and may require the discontinuation of prophylactic medication due to nephrotoxic side effects. Long-term application of antiviral agents may also favor the selection of drug-resistant virus mutants.[9,10] A more causal approach for zoster prevention is to boost VZV-specific cellular immunity by vaccination to levels that are sufficient to protect transplant recipients from the disease. However, live attenuated vaccines that are successfully used for varicella and zoster prophylaxis in immunocompetent individuals are not approved in immunocompromised HSCT patients due to safety concerns.[11-15]

VZV vaccines suitable for HSCT recipients[16] should be completely devoid of infectious virus. A candidate vaccine based on a heat-inactivated whole virus preparation of VZV has already demonstrated safety along with clinical and immunological activity in 2 pilot trials in the setting of HSCT.[17,18] Since effective antiviral control upon transplantation mainly depends on the successful reconstitution of virus-specific T cells,[1,19,20-22] VZV vaccines should contain immunodominant T-cell targets of VZV. However, there is a paucity of data on the nature of VZV antigens that drive posttransplant T-cell immunity and that are of sufficient immunostimulatory activity to protect HSCT recipients from the disease. A considerable number of glycoproteins and immediate early (IE) proteins of VZV have been identified as a source of CD4+ and CD8+ T-cell antigens.[23] Amongst those, glycoprotein E (gE), IE62, and IE63 were defined as being immunodominant.[24-28] The screening approaches that led to these findings mainly used memory T-cell populations which were expanded from PBMC of latently infected healthy individuals by antigen-specific stimulation over a culture period of several days to weeks. However, prolonged culturing may favor the proliferation of certain T-cell specificities over others and, consequently, the observed profile may not necessarily reflect the hierarchy of immunogenicity of different VZV antigens *in vivo*.

In order to match the *in vivo* situation as closely as possible and avoid *in vitro* bias, we established a novel screening approach. For this, VZV proteins derived from virus-infected cells were fractionated by reverse-phase high performance liquid chromatography (RP-HPLC). Individual fractions containing VZV proteins were subsequently incubated with PBMC from latently infected healthy donors in sensitive interferon (IFN)-γ ELISpot assays to stimulate antiviral memory CD4+ and CD8+ T lymphocytes directly *ex vivo*. Reactive fractions were further analyzed to identify individual VZV proteins by using concerted chromatography and mass spectrometry procedures. For verification, identified candidate antigens were tested as full-length recombinant proteins for recognition by T cells. T-cell stimulation assays were performed both with cells from latently infected healthy individuals and from patients with zoster after allogeneic HSCT, again restricting responder cell populations to *ex vivo* PBMC. Together these analyses confirmed VZV gE and IE62 were immunodominant T-cell antigens. Interestingly, the VZV glycoprotein B (gB) was identified as an additional major T-cell target. We further demonstrated that allogeneic HSCT patients develop strong *in vivo* expansion of CD4+ and CD8+ T cells targeting glycoproteins B and E during the onset of herpes zoster. Therefore, our data suggest that both glycoproteins are top candidates for the design of subunit VZV vaccines in the setting of HSCT.

Design and Methods

Donors and patients

The study was approved by the local ethics committee and was performed according to the Declaration of Helsinki. Informed consent was obtained from all participants. Healthy donors (HD) were VZV-immune volunteers (n=11). They provided whole blood donations used to isolate PBMC by buffy coat separation and subsequent Ficoll centrifugation. PBMC were stored frozen in liquid nitrogen until use. VZV-seropositive study patients (n=7) were treated with reduced-intensity allogeneic HSCT for acute or chronic leukemia. During conditioning therapy, 4 of them had received the lymphocyte-depleting antibody alemtuzumab, while T/B cell depletion agents had not been given to the other 3 patients. Anti-zoster drug prophylaxis with famciclovir was performed until day +365 after HSCT, unless toxic renal dysfunction prevented treatment. Patients developed localized herpes zoster at a median of +337 (range 90-935) days after HSCT. The disease was diagnosed clinically and was confirmed by VZV-specific PCR. Patients with acute zoster were treated with intravenous acyclovir over 10-14 days, followed by secondary prophylaxis with famciclovir. Patient PBMC were collected and cryopreserved before zoster, at zoster onset and at indicated time points thereafter. Serum samples of patients were analyzed for VZV IgG/IgM by ELISA (Virion-Serion, Würzburg, Germany). Stem cell donors were HLA-matched sibling (n=1), HLA-matched unrelated (n=4), or HLA-mismatched unrelated individuals (n=2). PBMC of stem cell donors were isolated from excess donor lymphocyte infusion material, if available. Mature dendritic cells (DC) were generated *in vitro* from peripheral blood monocytes as described.[29]

Biochemical purification of VZV-infected Vero cell lysate by reverse-phase HPLC

A lysate (0.4-0.7 mg/mL) prepared from VZV-infected Vero cells (Advanced Biotechnologies, Columbia, MD, USA) was used as antigen source. According to information provided by the manufacturer, cells had been infected with the VZV ROD strain for 7-9 days. A lysate prepared from uninfected Vero cells (Advanced Biotechnologies) by the same procedure was included as negative control. After 0.2 μm filtration, lysate filtrate was separated by RP-HPLC. RP-HPLC was performed with an Ettan LC system (GE Healthcare, Waukesha, WI, USA) equipped with a Jupiter 4 μm, C12, Proteo 90 Å, (250 x 2.0 mm) column (Phemomenex Inc, Aschaffenburg, Germany). Then 50 μL of 0.2 μm filtrate were injected and separated with an acetonitrile gradient (5-25% over 10 min, 25-65% over 40 min, 65-90% over 10 min). The aqueous and organic mobile phases contained 0.1% trifluoroacetic acid in H_2O and in acetonitrile, respectively. Fractions (each 150 μL) were collected at a flow rate of 150 μL/min and were split for subsequent use in bioassays and mass spectrometry (MS) analyses.

IFN-γ enzyme-linked immunosorbent spot (ELISpot) assay

ELISpot assays were performed as previously described[29] with minor modifications. PBMC, either loaded with VZV-infected cell lysate or HPLC fractions, or electroporated with RNA coding for VZV proteins, were incubated in antibody-coated ELISpot plates at 0.5-2×10⁶ cells/well over 40 h to allow for processing, presentation, and immune recognition of VZV proteins. In experiments analyzing HLA restriction of T-cell reactivity, the following murine mAb were added to ELISpot wells at saturating concentrations: W6/32, an anti-HLA class I IgG2a;[30] L243, an anti-HLA-DR IgG2a;[31] SPV-L3, an anti-HLA-DQ IgG2a;[32] and B7.21, an anti-HLA-DP IgG3.[33]

Protein identification by electrospray ionization mass spectrometry (ESI-MS)

RP-HPLC fractions (each 100 μL) were dried and re-solubilized in 25 mM ammonium bicarbonate containing 0.1% Rapigest (Waters, Eschborn, Germany). Solubilized proteins were subjected to reduction, alkylation and tryptic digestion as previously described.[34] After removal of detergent by acid hydrolysis and centrifugation,[34] the supernatant was transferred into an autosampler vial. Capillary liquid chromatography of tryptic peptides (2.6 μL injection) was performed with a Waters NanoAcquity UPLC system online coupled to a Waters Q-TOF Premier system as described.[34] Raw data processing and database searching were performed as detailed[35] with the IDENTITYᴱ Algorithm of ProteinLynx Global Server (version 2.3), using an in-house compiled database containing Uni-ProtKB/Swiss-Prot Protein Knowledgebase *(*http://expasy.org/sprot/*)* entries for macaca mulatta (349 entries), cercopithecus aethiops (181 entries), pongo pygmaeus (239 entries), homo sapiens (20,405 entries), pan troglodytes (688 entries), pan paniscus (122 entries), gorilla gorilla (279 entries), papio papio (190 entries), varicella-zoster virus (Dumas strain, 69 entries), supplemented with known possible contaminants (porcine trypsin). Maximum mass deviation was set to 15 ppm for precursor ions and 30 ppm for fragment ions. For valid protein identification at least two peptides had to be detected with altogether at least seven fragments. The false-positive rate for protein identification was set to 1% based on a search of a 5x-randomized database.

Production and electroporation of IVT-RNA

For *in vitro* transcription (IVT) of RNA coding for single VZV proteins, pcDNA™3.1-gE, pcDNA™3.1-gB, and pcDNA™3.1-IE62 vectors were used as DNA templates. The pcDNA™3.1 vectors encoding VZV proteins were kindly provided by Dr AM Arvin and Dr M Sommer (Stanford University, Stanford, CA, USA). IVT was performed with T7 RNA polymerase using the mMESSAGE mMACHINE T7 Ultra kit (Ambion/Applied Biosystems, Darmstadt, Germany). After enzymatic DNaseI digestion to remove the template DNA and subsequent enzymatic polyadenylation, the IVT-RNA was purified by the RNeasy Mini Kit (Qiagen, Hilden, Germany). PBMC or mature DC were then adjusted in OptiMEM medium (Gibco/Invitrogen, Darmstadt, Germany) to 2.5-10×10⁶ per 200 μL and were transferred with 20 μg IVT-RNA in 4 mm electroporation cuvettes (Peqlab, Erlangen, Germany). Electroporation was performed with the GenePulser Xcell system (Bio-Rad, Munich, Germany) applying a square wave pulse of 350V/12 ms. Electroporated cells were cultured for 4 h at 37°C and were thereafter used in ELISpot assays or cLSM analysis.

Confocal laser scanning microscopy (cLSM)

Intracellular expression of VZV proteins was determined with a confocal Zeiss LSM 510-UV device equipped with LSM Image Examiner software (Zeiss, Jena, Germany). Cells were intracellularly stained (Cytofix/Cytoperm™, BD Biosciences, Heidelberg Germany) for 30 min at 4°C with VZV protein-specific murine IgG1 mAb (Novus Biologicals, Littleton, USA) and FITC-conjugated goat-anti-mouse IgG (Immunotech, Marseille, France). Nuclear co-staining was performed by 200 nM Hoechst 33342 (Invitrogen). Subsequently, 10⁵ cells were transferred into Lab-Tek 8-well chamber slides at 300 μL medium/well for imaging.

## Results

VZV-infected cell lysate contains T-cell antigens of VZV

In a first set of experiments, a VZV-infected Vero cell lysate was used as source of VZV antigens. After 0.2 μm filtration of the lysate, filtrate and retentate were analyzed for immune reactivity in PBMC from healthy individuals with latent VZV infection in IFN-γ ELISpot assay. Cytokine secretion was significantly increased when the filtrate compared to the retentate was incubated with PBMC (*Online Supplementary Figure S1*). Recognition was specific, as IFN-γ secretion was not detected using the uninfected Vero cell lysate preparation as control. The immunogenic filtrate material was subsequently separated on a C12 column by RP-HPLC. Aliquots of all fractions were analyzed for immune recognition, again by using PBMC of VZV-immune healthy donors as screening populations in IFN-γ ELISpot assay. Distinct reactivity to several RP-HPLC fractions was found, indicating that they contained immunoreactive VZV antigens (Figure 1). The pattern of reactive and non-reactive fractions was consistent in 3 of 3 analyzed individuals. Subsequent ELISpot assays with purified T-cell subsets (i.e. CD4, CD8) and autologous dendritic cells showed that reactivity to fractions was predominantly mediated by CD4⁺ T cells (*data not shown*).

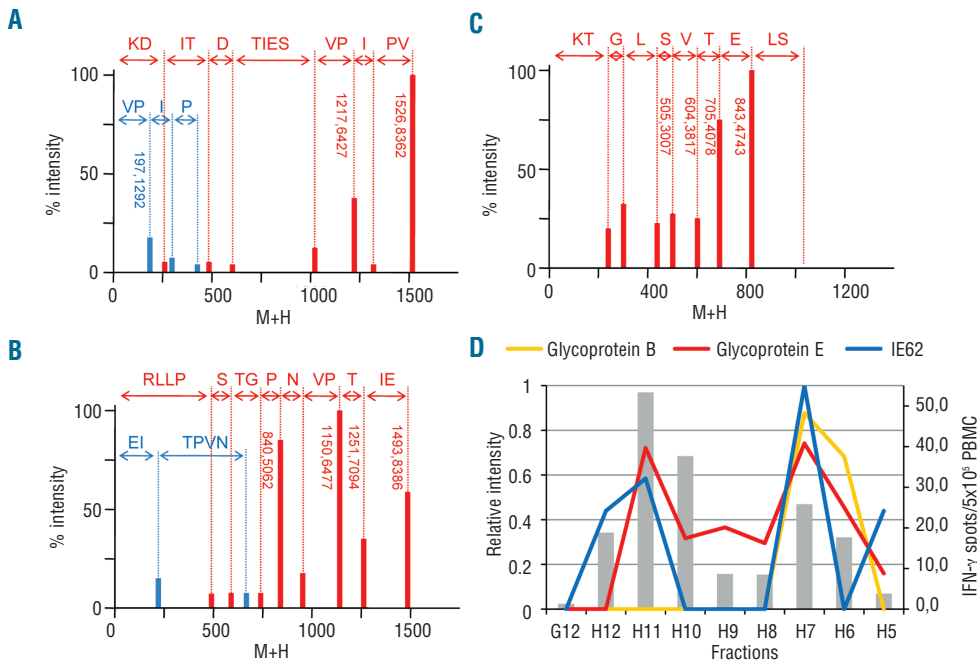### Mass spectrometry identification of VZV-encoded proteins

To identify immunoreactive VZV antigens, we performed quantitative mass spectrometric analysis (qMS) of RP-HPLC fractions. Proteins contained in those fractions that stimulated T-cell activation were first subjected to tryptic digestion. Resulting peptides were then analyzed by nanoUPLC-ESI-Q-TOF mass spectrometry. Acquired spectra were processed with ProteinLynx Global Server (PLGS 2.3), followed by a search in a composite database containing VZV and primate protein sequences. With the use of this approach, the VZV-encoded glycoproteins gB and gE as well as the immediate early protein 62 (IE62) were detected in immunoreactive HPLC fractions (Figures 2A-C). These viral proteins were not found in non-reactive fractions. The latter contained several other proteins, including VZV glycoprotein I (*data not shown*). Additionally, the relative abundance of gB, gE, and IE62 protein fragments in individual HPLC fractions as determined by qMS correlated well with the magnitude of reactivity of virus-immune donors in IFN-γ ELISpot assay (Figure 2D). Taken together, these data suggested viral gB, gE, and IE62 as major target proteins of the natural human T-cell response during the latency stage of VZV infection.

VZV gB, gE and IE62 are major targets of CD4⁺ T cells in latently infected individuals

To confirm our initial findings and to define T-cell pop-
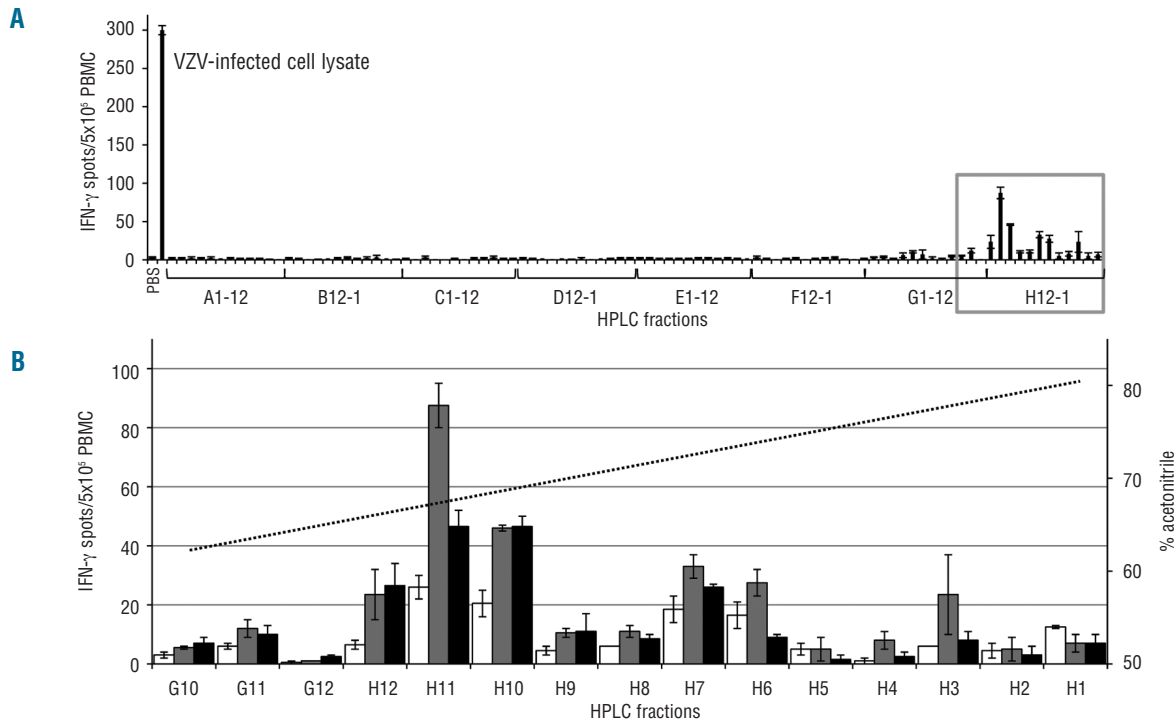
Figure 1. IFN-γ ELISpot screening of HPLC-fractionated cell lysate. The 0.2 μm filtrate (50 μL) of the VZV-infected Vero cell lysate was separated on a C12 column by RP-HPLC. Fractions A1-H1 (each 10 μL) were screened in duplicates for IFN-γ ELISpot reactivity with PBMC (5x10⁵/well) of VZV-seropositive healthy donors HD-1, HD-2, and HD-3. Unseparated VZV-infected cell lysate filtrate and PBS served as positive and negative controls, respectively. Reactivity was defined as significant if IFN-γ spot number per well exceeded 3-fold that of the background level (<5 spots/well). (A) Data shown are means ± SD of spot numbers obtained from all 3 donors. In fractions A1-G9 no significant IFN-γ ELISpot reactivity was detected. Reactivity clustered from fractions G10-H1, for which reason this region was scaled up in (B). (B) Individual data from healthy donors HD-1, HD-2, and HD-3 are shown as means of duplicates in white, gray, and black bars, respectively. ELISpot screening data were confirmed in a second independent assay for each donor and HPLC fraction, respectively. Dilution of fraction material with PBS led to concentration-dependent decrease of reactivity, with complete loss at 1:100 or lower (*data not shown*). The acetonitrile gradient (%) is added in (B) to estimate the hydrophobicity of eluted fraction material.

Figure 2. Mass spectra of VZV protein fragments and overlay data of VZV protein abundance and IFN-γ ELISpot reactivity in HPLC fractions. NanoUPLC-ESI-Q-TOF mass spectrometry identified protonated molecular ions (M+H) of peptides derived from VZV gB (A), gE (B), and IE62 (C) proteins. Data are presented for highest scoring peptides in single letter amino acid code. Scores calculated by ProteinLynx Global Server were 7427 for gB peptide (R)VPIPVSEITDTIDK(F) in HPLC fraction H7 (A), 3037 for gE peptide (K)EIT-PVNPGTSPLLR(Y) in HPLC fraction H11 (B), and 4074 for IE62 peptide (R)SLETVSLGTK(L) in fraction H7 (C), respectively. Scores over 200 were considered as results with less than 0.1% probability of being false positive. The molecular mass (Da) of identified tryptic peptides including % relative intensity

is shown. Apart from these VZV proteins, more than 350 Vero-cell derived proteins were also identified by the approach (*data not shown*). (D) Overlay data from mass spectrometry and IFN-γ ELISpot assay. Shown are the abundance of identified VZV protein fragments in relative intensity and the magnitude of immune reactivity for each individual HPLC fraction. ELISpot data are means of spot numbers per 5x10⁵ PBMC from healthy donors HD-1, HD-2, and HD-3, as shown in Figure 1.

ulations directed against individual VZV proteins, cDNA clones encoding full-length viral gB, gE, and IE62 were transcribed *in vitro* (IVT) into RNAs. The latter were subsequently transfected into PBMC by electroporation. Confocal LSM on electroporated DC (Figure 3) and PBMC verified expression of the viral proteins, with gB and gE being located in the cytoplasm and on the cell membrane and IE62 being mainly detectable in the nucleus of transfected cells (*data not shown*). Transfected PBMC were then analyzed for IFN-γ secretion by T cells in 10 healthy virus carriers. Clear recognition of 2 to 3 virus proteins was observed in every single donor tested (Figure 4A and B). The reactivity was detectable over a broad RNA dose range (1-30 μg) and reached peak intensities at 20 μg (*data not shown*). Median spot numbers per 10^6 PBMC, induced by glycoproteins B (87, 21-495) and E (83, 17-511) exceeded that induced by IE62 (49, 1-237), respectively (Figure 4C). By using mAb that block interaction of the T-cell receptor with either HLA class I or II molecules, VZV protein reactivity of healthy individuals was found to be mainly mediated by HLA class II restricted T cells. In contrast, T cells recognizing these three VZV proteins in association with HLA class I were detected at much lower numbers (Figure 4D). These data suggested that immune reactivity to VZV proteins in healthy virus carriers is predominantly mediated by virus-specific CD4+ T cells, which was confirmed in additional assays using purified T-cell subsets and DC (*Online Supplementary Figure S2*).

Posttransplant zoster triggers strong CD4+ and CD8+ T-cell responses to VZV gB and gE

The specificity of VZV-specific T cells was subsequently analyzed during the course of localized herpes zoster after allogeneic HSCT. Serial PBMC samples from zoster patients (n=5) were transfected with individual IVT-RNAs and tested in IFN-γ ELISpot assays. Vigorous expansions of T cells recognizing VZV gB and gE were observed after zoster onset, while IE62 reactivity was negative or at a very low level (Figure 5). In contrast, T-cell responses to any of these 3 VZV proteins were not detected in PBMC obtained from these patients before zoster occurred. However, T cells with reactivity to VZV proteins were clearly found in 3 of 4 stem cell donors prior to transplantation (Figure 5). In patients (n=7) analyzed during the first four weeks after zoster onset, median IFN-γ spot numbers per 10^6 PBMC to glycoproteins B (36, range 0-177) and E (47, range 8-323) exceeded that to IE62 (0, range 0-5), respectively (Figure 6A). Anti-HLA blocking assays suggested that this early glycoprotein reactivity after zoster was mediated by both CD4+ and CD8+ T cells (Figure 6C). Additionally, we observed that the frequency of circulating gE-specific T cells exceeded that of gB during the second month after zoster (Figure 6B) and that the level of VZV cell lysate reactivity was either below, similar or above that of total reactivity to all 3 VZV proteins (Figures 5 and 6). In contrast to mixed CD4+ and CD8+ T-cell responses to electroporated viral glycoproteins, reactivity to the VZV cell lysate was mediated only by CD4+ T cells (Figures 6D). Clinical follow up of the 7 analyzed patients showed that none of them developed a second episode of zoster during a median period of 25 (range 9-36) months after first occurrence. Together these experiments indicated that herpes zoster occurring after allogeneic HSCT triggers marked CD4+ and CD8+ T-cell responses to VZV gE and gB.

Discussion

Here we combined RP-HPLC fractionation of a virus-infected cell lysate with sensitive IFN-γ ELISpot assay and quantitative mass spectrometry to define T-cell antigens of VZV for subsequent immunogenicity analyses in latently infected donors and in allogeneic HSCT patients with zoster. Besides viral glycoprotein E and immediate early protein IE62, which had been reported before as major T-cell targets,[23,26,27] glycoprotein B was identified as an additional immunodominant VZV protein inducing both CD4+ and CD8+ T-cell reactivity. Our approach has considerable advantages over many other methods in identifying viral T-cell antigens. It allows for the rapid detection of naturally expressed candidate antigens from a relatively low (<1 mg) initial amount of virus-infected cell lysate material. Furthermore, the use of PBMC from VZV-seropositive donors as the preferred screening population in a sensitive IFN-γ ELISpot assay avoids *in vitro* culturing of T cells. The latter is both time-consuming and prone to bias due to possible selective expansion of distinct T-cell specificities under the chosen culture conditions. In addition, our approach is extremely flexible. First, screening with PBMC from patients with active herpes zoster may be suitable to help us unravel additional T-cell antigens of VZV that could play an important role during the acute phase of the disease. Moreover, the virus antigen source used in this study was prepared from Vero cells after one week of VZV infection. Modification of the infection period with regard to time and addition of drugs interfering with virus metabolism could most likely change the composition and con-
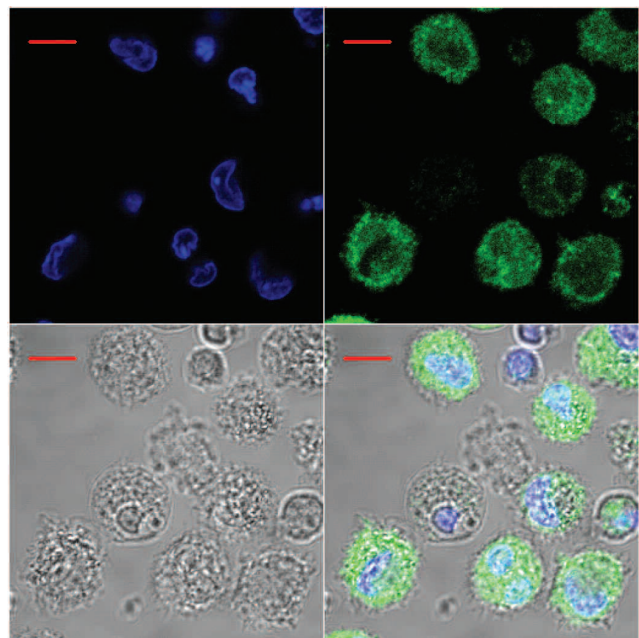
Figure 3. Expression of full-length VZV proteins in dendritic cells after RNA electroporation. Proteins were intracellularly stained in mature DC at 4 h after IVT-RNA electroporation by using VZV protein-specific primary antibodies and gam-FITC-labeled secondary antibody (green). Hoechst 33342 served as nuclear staining dye (blue). Visualization was performed by confocal LSM (oil immersion 63X, total magnification 1260X). The red size bar represents a distance of 10 μm. Pictures obtained for VZV glycoprotein E are shown and include the merged image at lower right quadrant.
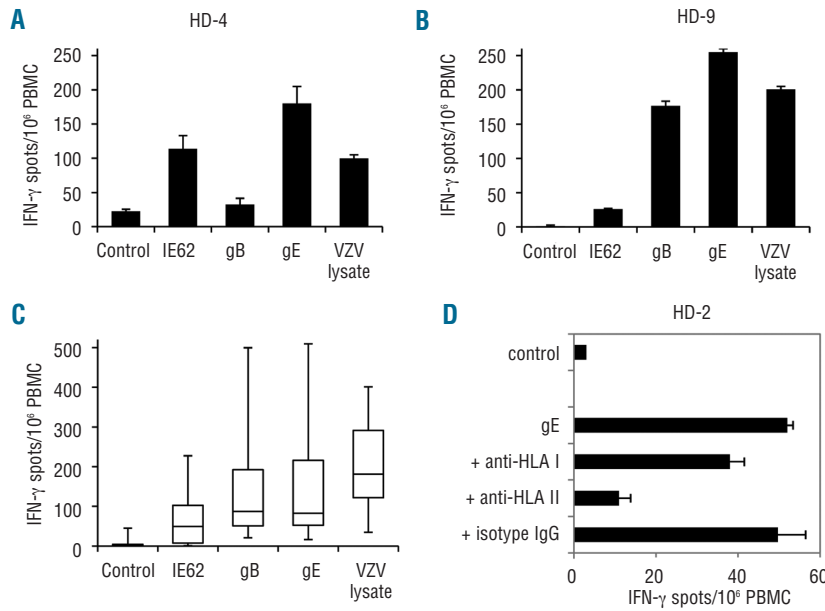
Figure 4. Frequency of VZV protein-reactive T cells in healthy virus carriers. PBMC of 10 VZV-seropositive donors with a median age of 28 (22-52) years were transfected in separate batches with IVT-RNA, encoding IE62, gB, and gE, respectively. PBMC transfected without adding RNA and transfected with an irrelevant RNA from green fluorescent protein (GFP) served as negative controls. After 4 h, PBMC were seeded at indicated numbers in IFN-γ ELISpot plates and were incubated for 40 h before spot development. Non-electroporated PBMC incubated with VZV-infected Vero cell lysate was included as positive control. Data from 2 individual donors (A and B) as well as from all 10 donors, combined in a box-and-whisker diagram with medians and ranges (C) are shown. (D) HLA restriction of T-cell reactivity to gE in donor HD-2. The assay used mAb for blocking HLA-A/B/C and HLA-DR/DQ/DP, as well as irrelevant isotype IgG mAb, respectively. In Figure 4 A-D, control represents data from wells that contained PBMC transfected without RNA and with GFP RNA.
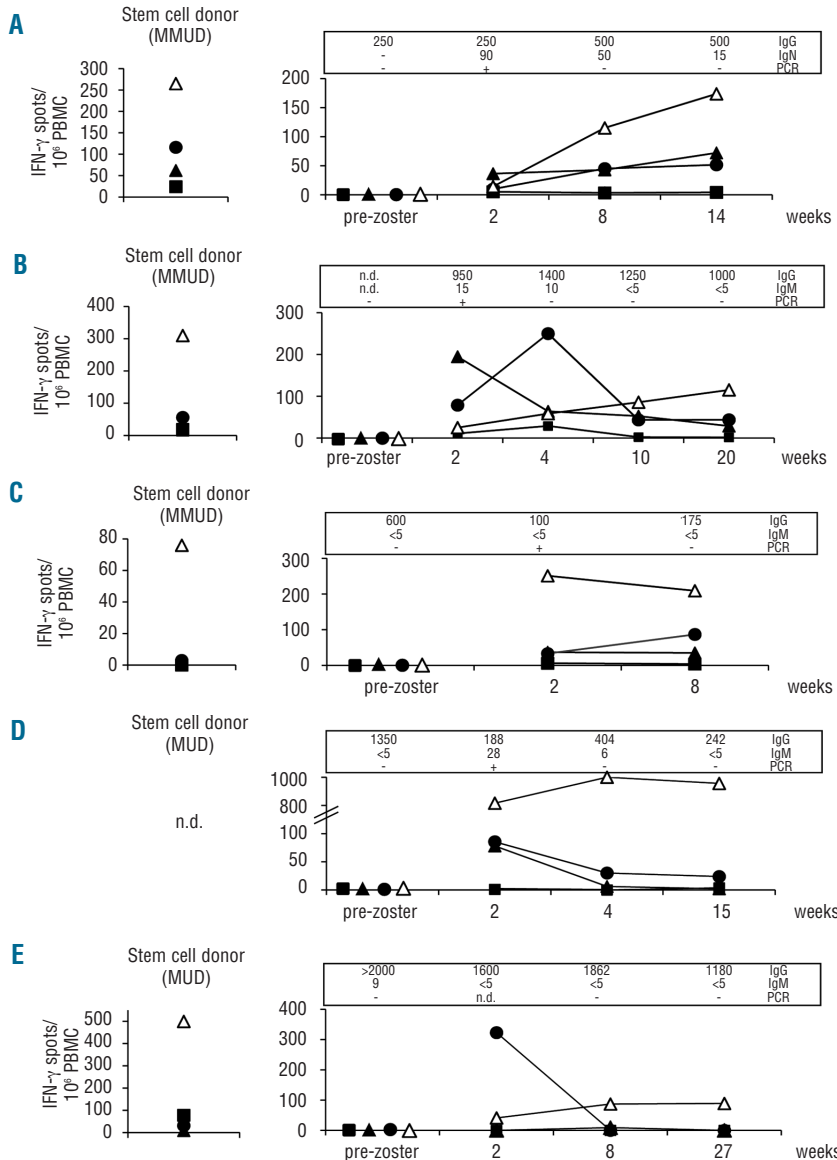
Figure 5. Expansion of VZV glycoprotein B and E specific T cells during posttransplant herpes zoster. Patients 1-5 (A-E) developed zoster at 225, 614, 935, 225, and 90 days after reduced-intensity allogeneic HSCT. Conditioning therapy included lympho-depleting antibody alemtuzumab in patients 1, 3, and 4, but not in patients 2 and 5. Diagrams show the frequencies of VZV-reactive T cells in leukapheresis-derived PBMC of stem cell donors before transplantation, and in PBMC of corresponding patients prior to zoster and at indicated time points after zoster. Results were obtained by IFN-γ ELISPOT assay using IVT-RNA of VZV proteins IE62 (■), gB (▲), and gE (●), as well as VZV-infected cell lysate (△), as described in Figure 4. Pre-zoster samples were also analyzed for existing T-cell reactivity to an inactivated whole influenza virus preparation and to phytohemagglutinin if sufficient PBMC numbers were available (*data not shown*). PBMC of stem cell donor 4 were not available. Diagrams include data on VZV-specific IgM (U/mL) and IgG (mIU/mL), as well as on VZV-PCR. MMUD: HLA-mismatched unrelated donor; MUD: HLA-matched unrelated donor; n.d.: not determined.
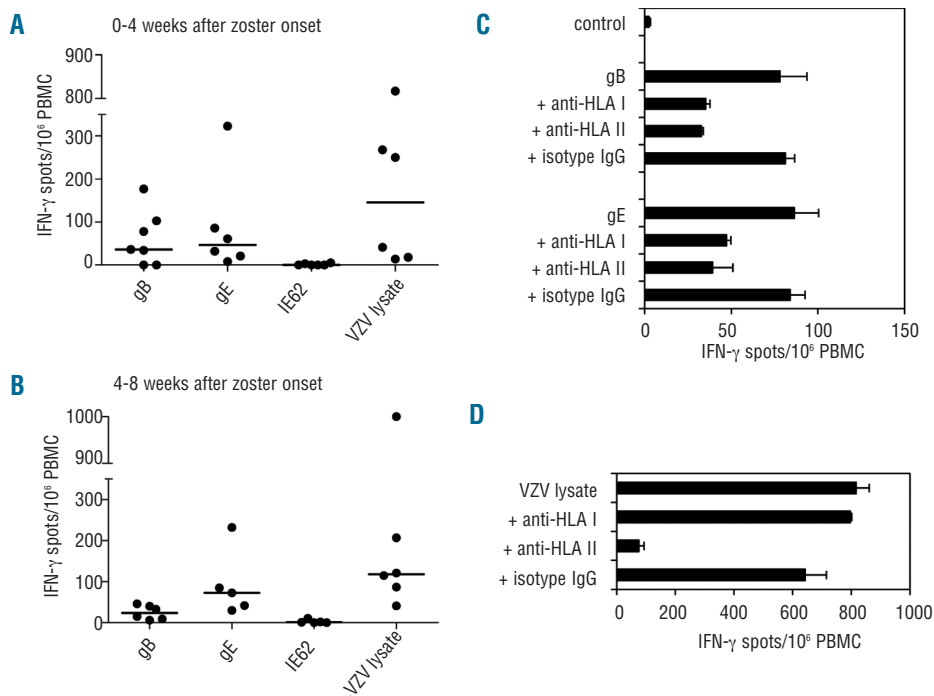
Figure 6. Post-zoster immune responses to glycoproteins B and E are mediated by CD4+ and CD8+ T cells. Shown is the IFN-γ ELISPOT reactivity to single VZV proteins (gB, gE, IE62) and to entire VZV-infected cell lysate in PBMC of allo-geneic HSCT patients during the first (A) and second (B) month after zoster onset. Medians are indicated as bars. Patients (n=7) had a median age of 61 (54-70) years. In some PBMC samples, reactivity could not be determined for all VZV proteins because of limited cell material. Also shown is the HLA restriction of T-cell reactivity to gB and gE (C), and to VZV cell lysate (D) in patient 4 during the first month after the onset of zoster. These assays were performed as described in Figure 4. Results are representative of 2 analyzed patients with sufficient PBMC numbers available.

centration of expressed virus proteins and might lead to the identification of additional known (e.g. glycoprotein I, IE63)[23] or even novel T-cell antigens of VZV. It is well conceivable that the system can be extended towards other viruses in which T-cell antigens are completely or partly undefined.

For verification, VZV candidate proteins identified by qMS were expressed in PBMC by electroporation of full-length IVT-RNAs. This procedure enabled the *in vitro* stimulation and detection of a broad repertoire of VZV-specific T cells, bypassing the requirement for prior analysis of HLA type and HLA allele-specific peptide epitopes of the test person. In contrast to loading target cells with purified proteins or cell lysates, protein expression after RNA transfection not only allows peptide presentation by MHC class II, but also efficiently generates epitopes presented by the MHC class I pathway.[36] Using this approach in combination with HLA-A/B/C- and HLA-DR/DQ/DP-blocking antibodies we demonstrated that virtually every latently infected healthy donor carried significant numbers of circulating CD4+ T cells directed against at least 2 of the 3 VZV proteins. In contrast, CD8+ T cells recognizing the same target proteins were detected at lower numbers in virus-immune healthy individuals. Considering the fact that in every assay the entire VZV lysate or protein reactivity could be completely blocked by HLA antibodies (of either class I or II specificity) indicated recognition by MHC restricted T cells and not by non-MHC restricted effector cells. Our observation is in accordance with previous reports which had shown a predominance of VZV-specific CD4+ memory T cells during latent infection.[23,26] We further found that the precursor frequencies of circulating VZV IE62-reactive T cells were much higher than previously reported.[27,37] In contrast, frequencies of gE-specific T cells were consistent with previous studies, using overlapping peptides and the IFN-γ ELISpot assay for monitoring.[38] Surprisingly, however, the highest median numbers of peripheral blood T cells were found to be targeting gB, a protein that was previously much less appre-

ciated as a source of antigenic peptides. This clearly demonstrates that gB is an important target of the T-cell response during VZV latency.

We monitored the frequencies of VZV protein-reactive T cells in seropositive patients who developed localized herpes zoster after reduced-intensity allogeneic HSCT. Before onset of zoster symptoms, the numbers of circulating protein-reactive T cells were below the detection limit of the assay (<5 per 10[6] PBMC), even in those patients who did not receive T-cell depleting agents during conditioning therapy. These findings matched the lack of IFN-γ ELISpot responses to VZV-infected cell lysate which we had reported for pre-zoster PBMC samples of 12 VZV-seropositive patients undergoing T-cell depleted allogeneic HSCT.[21] The frequencies of T cells specifically recognizing VZV glycoproteins B and E, however, increased considerably during the first four weeks after zoster onset, whereas that to IE62 remained negative or reached only a very low level. The reasons for the lack of detectable IE62-specific T cells in our zoster patients is still unclear. Interestingly, we could show that substantial numbers of HLA class I restricted gB and gE reactive CD8+ T cells are present during the first weeks after zoster onset, which most likely assisted antiviral CD4+ T cells[23,26,27] in the total cytolytic T-cell response. Supporting evidence for this hypothesis would require the detection of cytolysis-associated molecules (e.g. perforin, granzyme B) in PBMC, which was not possible in the current study due to limited sample material. The levels of IFN-γ ELISpot reactivity to gB and gE were followed for several months and reached almost those found in healthy virus-immune donors.

The described assay system can be implemented in similar monitoring studies[39] in patients in other situations of immunodeficiency (e.g. autologous HSCT, cancer, HIV, the elderly), where the incidence of herpes zoster is also significantly increased. These analyses might confirm the observed predominant role of gB and gE for T-cell mediated immunity to VZV and/or define other important T-cell antigens during zoster. Interestingly, we found in several

PBMC samples that the sum of reactivity to single VZV proteins was higher compared to that to the entire VZV cell lysate (e.g. patients 2 and 5). Thus, the use of viral cell lysates as the antigen source in monitoring assays may underestimate total anti-VZV reactivity, because lysates usually contain relatively low concentrations of VZV proteins and favor the stimulation of antiviral CD4 (and not CD8) responses. In other PBMC samples, however, the level of VZV cell lysate reactivity was clearly above that of total reactivity to all 3 VZV proteins (e.g. patients 3 and 4). This observation indicated that alternate targets beyond those studied here can dominate the T-cell response to VZV.

Live attenuated VZV vaccines are suitable to effectively enhance antiviral T-cell immunity in immunocompetent individuals.[40,41] It has also been shown in 2 randomized studies that a heat-inactivated formulation of a live attenuated VZV vaccine can boost VZV-reactive CD4+ T cells in patients undergoing autologous and allogeneic HSCT, and that this specific reconstitution correlates with protection from zoster.[17,18] We show here that VZV gB and gE are major targets of virus-specific CD4+ and CD8+ T-cell reconstitution occurring during zoster after allogeneic HSCT. None of the patients analyzed developed a second episode of zoster during a median period of 25 months after first occurrence. Although this observation does not prove a direct causative role of anti-glycoprotein reactivity for zoster prevention, VZV gB and gE may form the basis for a safe zoster subunit vaccine, suitable for vaccinating both donors and recipients of HSCT. Studies with gE subunit vaccines already demonstrated the induction of specific B and T-cell responses in animals.[42,43] Interestingly, preliminary results from an open randomized study in adults exploring a recombinant gE vaccine with adjuvant for zoster prevention demonstrated more robust cellular (i.e. CD4) and humoral immune responses in older adults compared to the application of a live varicella vaccine.[44] Clinical activity and adverse effect profile in ongoing trials as well as those to be performed in HSCT patients will show if VZV glycoprotein subunit vaccines are an attractive alternative compared to whole virus vaccines. Considering that B-cell immunity is not suitable for preventing zoster after allogeneic HSCT,[1] stimulation of VZV-specific T cells will be a major goal in developing an efficient vaccine for HSCT patients. Monitoring such T-cell responses on the basis of individual VZV proteins in vaccinated subjects could be an important application for the described assay system. In prospective trials, it may help to optimize vaccine strategies and to define a threshold frequency of circulating VZV antigen-specific T cells that correlates with protection from zoster and may allow for safe discontinuation of antiviral drug prophylaxis.

Authorship and Disclosures

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