Identification of Varicella-Zoster Virus-Specific CD8 T Cells in Patients after T-Cell-Depleted Allogeneic Stem Cell Transplantation[▽]

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To study the role of CD8 T cells in the control of varicella-zoster virus (VZV) reactivation, we developed multimeric major histocompatibility complexes to identify VZV-specific CD8 T cells. Potential HLA-A2 binding peptides from the putative immediate-early 62 protein (IE62) of VZV were tested for binding, and peptides with sufficient binding capacity were used to generate pentamers. Patients with VZV reactivation following stem cell transplantation were screened with these pentamers, leading to the identification of the first validated class I-restricted epitope of VZV. In 42% of HLA-A2 patients following VZV reactivation, these IE62-ALW-A2 T cells could be detected ex vivo.

Varicella-zoster virus (VZV) infects about 95% of the population, persists throughout life, and may lead to herpes zoster when the virus reactivates. After T-cell-depleted allogeneic stem cell transplantation (TCD alloSCT), reactivation of the virus leads to considerable morbidity (10). Primary infection elicits both humoral and cellular responses, but cellular immunity is essential for preventing herpes zoster. The VZV genome comprises more than 70 unique open reading frames that encode proteins that are coordinately expressed during replication. The product of open reading frame 62, the immediate-early 62 (IE62) protein, is required for the initiation of VZV replication (9) and is expressed at high levels before viral replication has occurred (8). Previous research has demonstrated that IE62-specific T cells were detected after primary VZV infection and in immune subjects (2, 4). In addition, T cells recognizing various other IE proteins and glycoproteins of VZV, as demonstrated by gamma interferon (IFN- γ) production upon stimulation with peptides or lysate derived from these proteins, have been described (1, 6, 13). The VZV-specific memory T cells found in these studies were predominantly CD4 T cells, while no VZV-specific CD8 T cells were demonstrated without prior in vitro expansion, possibly due to the low frequency of VZV-specific CD8 T cells or to the low sensitivity of the screening methods used to detect CD8 T cells by IFN- γ production upon stimulation. Frey et al. described CD8 epitopes of IE62 detected following in vitro restimulation. However, the HLA restriction and specificity of these T cells were not confirmed (4). Due to the lack of validated VZV-derived immunodominant peptides for major histocompatibility complex (MHC) class I, the analysis of VZV-specific CD8 T-cell responses is hampered (14). To be able to analyze

the role of CD8 T cells in VZV reactivation, we therefore set out to identify epitopes for VZV by using VZV-IE62-specific MHC class I peptide complexes.

The predictive algorithms BIMAS (11) and SYFPEITHI (12) were used to select potential HLA-A2 binding peptides from the IE62 protein. Peptides with a score of ≥ 3 (BIMAS) or ≥ 20 (SYFPEITHI) were considered to have potentially significant binding affinity. The 81 resulting 9-mer peptides were synthesized and tested for binding affinity with the REVEAL MHC-peptide binding assay (ProImmune, Oxford, United Kingdom). HLA-A2 binding affinity was determined by the ability of the peptides to stabilize the HLA-peptide complex. Based on the binding affinity measurements, 34 high- to medium-affinity HLA-A2 binding peptides were selected and used to generate ProVE MHC pentamers (ProImmune, Oxford, United Kingdom). To enable screening of this large number of pentamers, the pentamers were divided into five pools, each containing six or seven pentamers. In the initial screening with pooled pentamers, four HLA-A2-positive patients were screened after a clinical diagnosis of VZV reactivation after TCD alloSCT. The presence of viral DNA in plasma at the time of clinical observations of VZV reactivation was confirmed by real-time PCR on plasma samples as previously described (7). After informed consent was obtained, peripheral blood mononuclear cells (PBMCs) were cryopreserved and thawed and 0.5×10^6 cells were incubated with pentamers at a concentration of 0.03 mg/ml for 10 min at room temperature in RPMI medium supplemented with 2% fetal bovine serum. After the cells were washed twice, 8 μ l of FluoroTag-phycoerythrin (PE) was added for 20 min of incubation at 4°C and the cells were counterstained with CD4, CD40, and CD19-fluorescein isothiocyanate (FITC). Flow cytometric analysis was performed on a FACScalibur fluorescence-activated cell sorter (FACS; Becton-Dickinson [BD], San Jose, CA). In one of four patients, pentamer pool 6, containing pentamers 61, 62, 64, 65, 66, and 67, was positive (0.06% of CD8 T cells); no

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FIG. 1. Screening with pentamers containing VZV-derived immunogenic epitopes. PBMCs of a patient after VZV reactivation following TCD alloSCT were incubated with pentamers and then stained with FluoroTag-PE to detect the pentamer-positive cells (A and B) and counterstained with CD4-, CD40-, and CD19-FITC. Pentamer staining of the CD4-, CD40-, and CD19-negative cells is shown. (A) PBMCs stained with pentamer 67 containing the epitope ALPHAAAV, showing no specific staining. (B) PBMCs stained with pentamer 66 containing the epitope ALWALPHAA, showing specific staining. IE62-ALW-A2-specific T-cell clones were sorted into a single cell per well and expanded nonspecifically. The clones were stained with an irrelevant tetramer (C) and the IE62-ALW-A2 tetramer (D) in combination with CD8-FITC. Clones 1 and 2 were stained with a V β kit (BD) to demonstrate that clone 1 (E) and clone 2 (F) express different T-cell receptors. The results demonstrate that we isolated different T-cell clones that specifically stain with the IE62-ALW-A2 tetramer.

other positive signals were observed. Staining with the individual pentamers revealed that pentamer 66, containing the epitope ALWALPHAA derived from the IE62 protein of VZV (IE62-ALW-A2) was responsible for the positive signal (0.06% of CD8 T cells, Fig. 1B).

To confirm the specificity of the IE62-ALW-A2-specific T cells, the pentamer-positive T cells were sorted into a single cell per well with a FACSDiva (BD) and expanded as previously described (5). The expanded T-cell clones were labeled specifically with the IE62-ALW-A2 PE-conjugated tetramer that was constructed as previously described (3) (Fig. 1D), and V β analysis with the T-cell receptor V β repertoire kit (BD) showed that at least two different T-cell clones were isolated, demonstrating the oligoclonal origin of IE62-ALW-A2-positive T cells (Fig. 1E and F). To assess the cytolytic capacity of IE62-ALW-A2 T cells, chromium release assays were performed as described earlier (5). ^{51}Cr -labeled Epstein-Barr virus (EBV) lymphoblastoid cell lines (LCLs) loaded with the IE62-ALW peptide were incubated with IE62-ALW-A2 T cells for 4 h. As demonstrated in Fig. 2A, HLA-A2-positive EBV LCLs loaded with the IE62-ALW-A2 peptide were lysed by both T-cell clones, whereas unloaded EBV LCLs were not lysed. To determine the avidity of the T-cell clones, the IE62-ALW-A2 peptide was titrated on EBV LCLs, and after 24 h of coculture, supernatants were harvested and used to determine the IFN- γ production of the stimulated T cells by standard enzyme-linked immunosorbent assay. Half-maximum IFN- γ production of the T-cell clones was observed when the stimu-

lator cells were loaded with 10 ng/ml peptide, indicative of high-avidity T-cell clones (Fig. 2B). To determine whether the T cells recognized cells endogenously expressing the IE-62-encoding gene, COS-A2 cells were transfected with Lipofectamine (Invitrogen, Carlsbad, CA) by using pcDNA vectors coding for different VZV genes, which were kindly provided by E. Wiertz (Department of Medical Microbiology, Leiden University Medical Center, Leiden, The Netherlands). The transfected COS-A2 cells were used 24 h after transfection as stimulator cells in this assay. After 24 h of coculture, supernatants were harvested and used to determine the IFN- γ production of the stimulated T cells. IE62-ALW-A2 T-cell clones produced IFN- γ in response to COS-A2 cells endogenously expressing the IE62 protein, as well as COS-A2 cells pulsed with the IE62-ALW-A2 peptide. No IFN- γ was produced when the COS-A2 cells were transfected with the IE63-encoding gene of VZV or pulsed with an irrelevant peptide (Fig. 2C).

To determine whether IE62-ALW-A2-specific T cells were present in healthy individuals, cryopreserved PBMCs from 18 healthy, VZV-seropositive, HLA-A2-positive individuals were screened with the PE-conjugated VZV tetramer. PBMCs were labeled with tetramers for 15 min at 37°C in RPMI medium without phenol supplemented with 2% fetal bovine serum, washed, and analyzed with a FACScalibur. In 3 of these 18 serologically VZV-positive individuals, IE62-ALW-A2 tetramer-positive T cells could be detected (range, 0.01 to 0.02% of CD8 T cells). These data demonstrate that IE62-ALW-A2-specific T cells can be observed and that the frequency of these

FIG. 2. IE62-ALW-A2 T cells recognize IE62-ALW-A2 peptide-loaded target cells and target cells endogenously expressing IE62. (A) The cytolytic activity of IE62-ALW-A2-positive T-cell clones 1 and 2 was analyzed with the ^{51}Cr release assay. T cells were incubated for 4 h with IE62-ALW-A2 peptide (pep)-loaded or unloaded, HLA-A2-positive EBV LCLs at an effector-to-target ratio of 10:1. (B) IE62-ALW-A2 T-cell clone 1 was stimulated with HLA-A2-positive EBV LCLs loaded with different concentrations of the IE62-ALW-A2 peptide. Release of IFN- γ (pg/ml) after 24 h of stimulation is shown. (C) IE62-ALW-A2 T-cell clones 1 and 2 were stimulated with HLA-A2-positive COS-A2 cells, left untreated, or loaded with the IE62-ALW-A2 peptide or with the IE4-ALR-B8 peptide as an irrelevant peptide or transfected with the IE63-encoding gene (COS-A2-IE63) or the IE62-encoding gene (COS-A2-IE62). Release of IFN- γ (picograms per milliliter) after 24 h of stimulation is shown.

T cells is low under steady-state conditions in immunocompetent persons.

To assess the frequency of IE62-ALW-A2-specific T cells in a cohort of patient who suffered from VZV reactivation following TCD alloSCT, 19 HLA-A2-positive patients after VZV reactivation following TCD alloSCT were screened by using the IE62-ALW-A2 tetramer. We screened these patients at a median of 47 days after the clinical diagnosis of VZV reactivation. In 8 of these 19 patients, IE62-ALW-A2-specific T cells could be directly detected ex vivo (mean, 0.04% [range, 0.01 to 0.11%] of CD8 T cells), indicating that this epitope is recognized in 42% of the HLA-A2-positive patients during VZV reactivation (Table 1). In VZV-seronegative patients (six screened), no IE62-ALW-A2 tetramer-positive cells could be detected.

To verify the presence of the IE62-ALW-A2-specific T cells in the patient and donor cohort and to investigate whether individuals negative for IE62-ALW-A2-specific T cells were

unable to mount a response against the epitope or whether the frequency of IE62-ALW-A2-specific T cells was too low to detect by FACS, the presence of these T cells was further measured after in vitro stimulation. PBMCs were cultured at a concentration of $1 \times 10^6/\text{ml}$ in 24-well plates in Iscove's modified Dulbecco's medium supplemented with 10% human serum in the presence of IE62-ALW peptide (1 $\mu\text{g}/\text{ml}$), interleukin-2 (IL-2; 50 IU/ml), and IL-15 (10 ng/ml). After stimulation for 7 days, the presence of IE62-ALW-A2-specific T cells was reassessed by tetramer labeling. These in vitro stimulations demonstrated that IE62-ALW-A2 CD8 T cells were detectable in another four patients and confirmed the presence of IE62-ALW-A2-specific T cells in eight patients and three healthy, VZV-seropositive individuals with ex vivo-detectable IE62-ALW-A2-specific T cells (Table 1; Fig. 3A to D). Thus, in 12 (63%) of 19 patients, IE62-ALW-A2 CD8 T cells could be detected either by direct tetramer labeling or after in vitro expansion, indicating that this HLA-A2-restricted epitope is commonly used in HLA-A2-positive individuals.

To study whether the immune response against the IE62-ALW-A2 epitope correlated with clinical reactivation, the percentage of IE62-ALW-A2-positive T cells was analyzed during the course of VZV reactivation in one patient. To determine the presence of viral DNA in plasma before and during the course of VZV reactivation, real-time PCR was performed on plasma samples derived at different time points. Six days prior to clinical signs of VZV reactivation, only 0.03% of the CD8 T cells were IE62-ALW-A2 specific. At 42 days after the onset of VZV reactivation, 0.23% of the CD8 T cells were IE62-ALW-A2 specific. After the VZV infection resolved, the per-

TABLE 1. Presence of IE62-ALW-A2-specific T cells in HLA-A2 patients after VZV reactivation following TCD alloSCT

Patient	No. of days after:		% IE62-ALW-A2 ⁺ T cells (SD)	
	TCD alloSCT	VZV reactivation	Before IVS ^a	After IVS ^b
1	180	46	Negative	0.22 (0.15)
2	190	38	0.03 (0.01)	0.51 (0.21)
3	545	31	Negative	Negative
4	294	52	Negative	0.12 (0.06)
5	82	38	Negative	Negative
6	183	16	Negative	0.01 (0.01)
7	176	81	0.02 (0.01)	0.44 (0.06)
8	99	35	0.11 (0.02)	0.22 (0.04)
9	601	88	Negative	0.01 (0.01)
10	95	63	Negative	Negative
11	90	83	Negative	Negative
12	179	48	Negative	Negative
13	1,224	62	Negative	Negative
14	173	20	0.03 (0.01)	0.22 (0.12)
15	514	21	0.03 (0.01)	ND ^c
16	635	40	0.02 (0.01)	ND
17	161	8	Negative	Negative
18	174	48	0.01 (0.00)	0.02 (0.01)
19	92	49	0.04 (0.01)	0.06 (0.02)

^a Mean percentages of IE62-ALW-A2 tetramer-positive cells of CD8 T cells of three tetramer stainings performed on different days are shown.

^b PBMCs were in vitro stimulated (IVS) for 7 days with IE62-ALW-A2 peptide, and the mean percentages of tetramer-positive cells of three to six stimulations are shown. A negative result was defined as <0.01% of CD8⁺ T cells.

^c ND, no PBMCs were available to do the analysis.

FIG. 3. Detection and kinetics of IE62-ALW-A2-specific T cells. PBMCs with detectable IE62-ALW-A2 T cells (A, left side), a low level of detectable tetramer-positive cells (B, left side), or no detectable tetramer-positive cells (C and D, left side) were in vitro stimulated for 7 days with IE62-ALW-A2 peptide (1 μ g/ml) in the presence of IL-2 and IL-15 (A to D, right side). Cells were stained with CD4-FITC, CD40-FITC, and IE62-ALW-A2 tetramer, and the percentages of CD8⁺ T cells that were IE62-ALW-A2 tetramer positive are indicated. CD8⁺ T cells are defined as CD4⁺ CD40⁺ lymphocytes. (E) PBMCs of a patient during the course of VZV reactivation following TCD alloSCT were stained with the IE62-ALW-A2 tetramer in combination with CD8-FITC. The percentages of IE62-ALW-A2-specific CD8 T cells before, during, and after VZV reactivation are shown. In the box, the presence of viral DNA in peripheral blood is shown as measured by real-time PCR at various time points. The bold line illustrates the use of valaciclovir to treat the VZV reactivation.

centage of IE62-ALW-A2-specific CD8 T cells declined to 0.09% at day 49 and 0.03% at day 145 after reactivation (Fig. 3D). The T cells present at the peak of the response were predominantly HLA-DR positive, CD45RA negative, CCR7

negative, CD28 negative, and CD27 positive, consistent with an activated effector memory phenotype.

In this study, we demonstrate that CD8 T cells specific for VZV are detectable without prior in vitro stimulation in patients with VZV reactivation following TCD alloSCT. We identified the ALWALPHA peptide derived from the IE62-encoding gene of VZV as the first validated VZV-specific HLA class I-restricted immunogenic epitope by a pentamer-based epitope discovery method. The detection of the IE62-ALW peptide as an immunogenic peptide for VZV-specific CD8 T cells demonstrates the usefulness of this procedure for discovering new immunogenic virus- or tumor-specific epitopes. We demonstrated that, despite the low frequency, it is possible to detect VZV-specific CD8 T cells, allowing ex vivo analysis of the immune response to VZV infection, reactivation, and possibly VZV vaccination.

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