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Chapter 6

Identification of varicella zoster virus-specific CD8⁺ T cells in patients after T cell depleted allogeneic stem cell transplantation

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Abstract

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 morbidity¹⁰. 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 replication⁹ and is expressed at high levels before viral replication has occurred⁸. 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 confirmed⁴. 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 hampered¹⁴. 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 BIMAS¹¹ and SYFPEITHI¹² 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 described⁷. 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 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, **Figure 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 described⁵. The expanded T cell clones were labeled specifically with the IE62-ALW-A2 PE-conjugated tetramer that was constructed as previously described³ (**Figure 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 (**Figure 1E and F**).

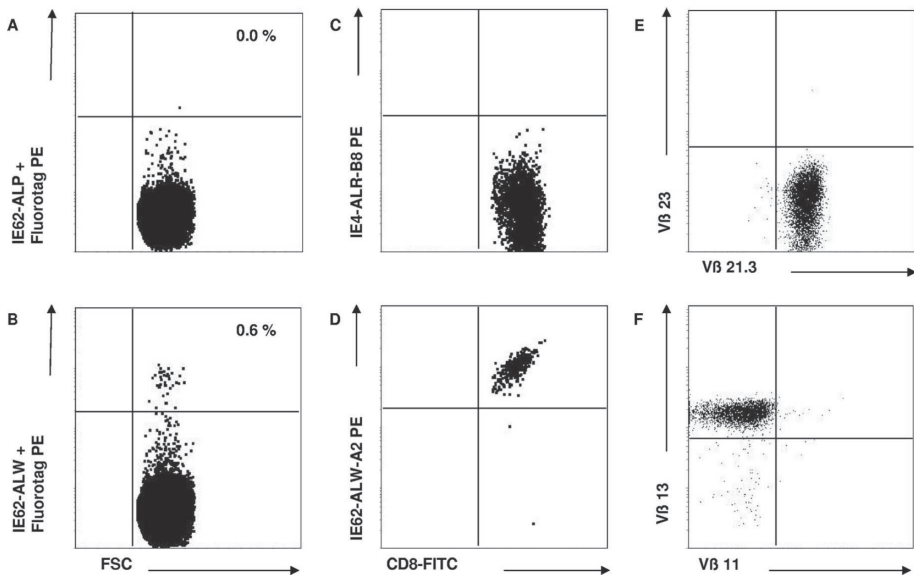


Figure 1. Screening with Pentamers containing VZV derived immunogenic epitopes.

PBMCs of a patient after VZV reactivation following a 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 CD19FITC. Pentamer staining is shown of the CD4, CD40, CD19 negative cells. (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 single cell per well and expanded non-specifically. 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 Vbeta kit (BD) to demonstrate that clone 1 (E) and clone 2 (F) express different TCR. The results demonstrate that we have isolated different T cell clones that specifically stain with the IE62-ALW-A2 tetramer.

To assess the cytolytic capacity of IE62-ALW-A2 T cells, chromium release assays were performed as described earlier⁵. ⁵¹Cr-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 Figure 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 stimulator cells were loaded with 10 ng/ml peptide, indicative of high-avidity T cell clones (**Figure 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 (**Figure 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 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.

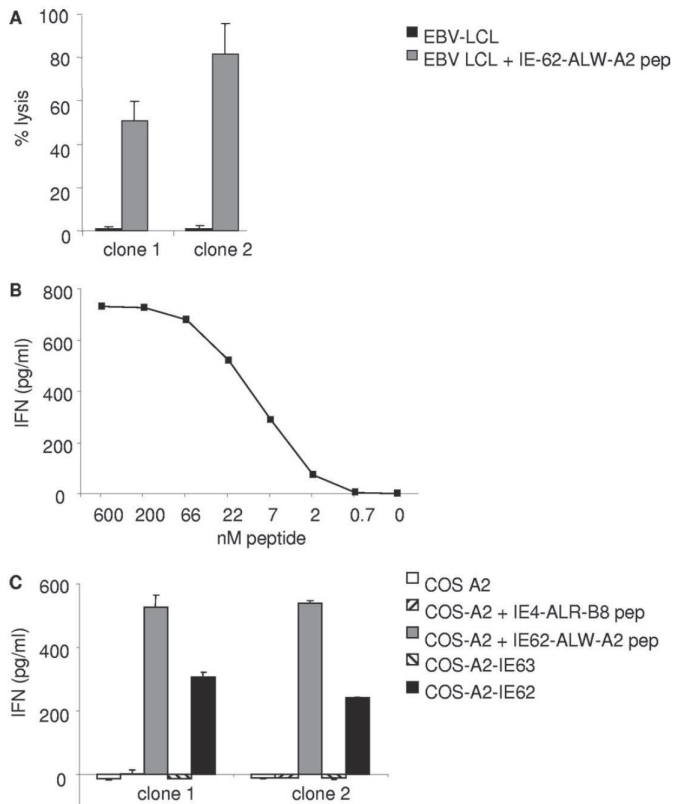


Figure 2. IE62-ALW-A2 T cells recognize IE62-ALW-A2 peptide loaded target cells and target cells endogenously expressing IE62.

(A) Cytolytic activity of the IE62-ALW-A2 positive T cell clones 1 and 2 was analyzed using the ^{51}Cr release assay. T cells were incubated for 4 h with IE62-ALW-A2 peptide 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 hours of stimulation is shown. (C) IE62-ALW-A2 T cell clones 1 and 2 were stimulated with HLA-A2 positive COS-A2 cells, 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 gene (COS-A2-IE63) or the IE62 gene (COS-A2-IE62). Release of IFN- γ (pg/ml) after 24 hours of stimulation is shown.

Table 1. The presence of IE62-ALW-A2-specific T cells in HLA-A2 patients after VZV reactivation following TCD alloSCT

Patient	days after alloSCT	days after VZV	IE62-ALW-A2+ T cells %* (SD)	after IVS# % (SD)
1	180	46	neg	0.22 (0.15)
2	190	38	0.03 (0.01)	0.51 (0.21)
3	545	31	neg	neg
4	294	52	neg	0.12 (0.06)
5	82	38	neg	neg
6	183	16	neg	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	neg	0.01 (0.01)
10	95	63	neg	neg
11	90	83	neg	neg
12	179	48	neg	neg
13	1224	62	neg	neg
14	173	20	0.03 (0.01)	0.22 (0.12)
15	514	21	0.03 (0.01)	nd [§]
16	635	40	0.02 (0.01)	nd
17	161	8	neg	neg
18	174	48	0.01 (0.00)	0.02 (0.01)
19	92	49	0.04 (0.01)	0.06 (0.02)

*Mean percentages of IE62-ALW-A2 tetramer positive cells of CD8⁺ T cells of 3 tetramer stainings performed on different days are indicated. #PBMCs were in vitro stimulated (IVS) for 7 days with IE62-ALW-A2 peptide, the mean percentages of tetramer positive cells of 3 to 6 stimulations are indicated. A negative result was determined as < 0.01% of CD8⁺ T cells. §No PBMCs were available to do the analysis.

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 1×10^6 /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 µg/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; Figure 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.

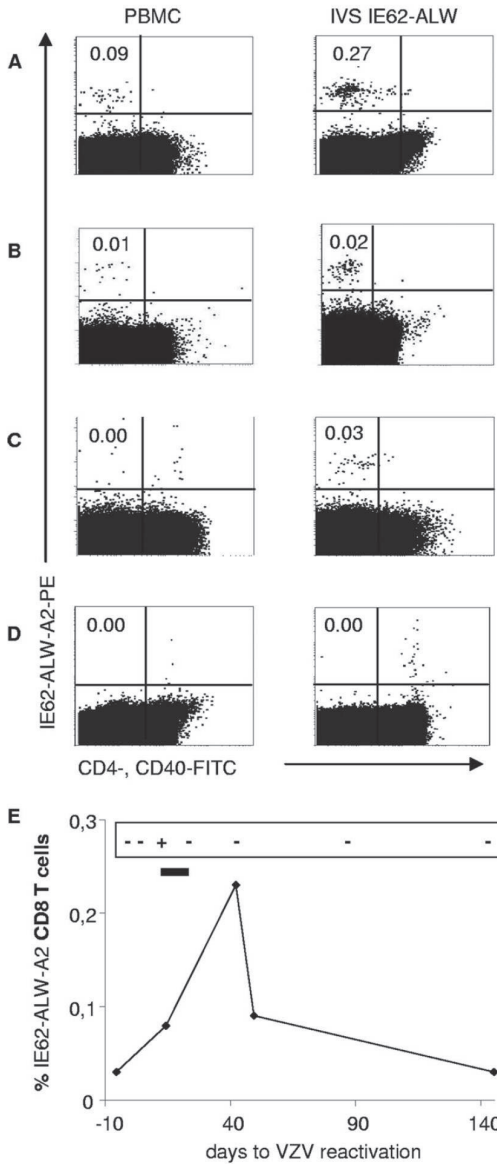


Figure 3. Detection and kinetics of IE62-ALW-A2-specific T cells.

PBMCs with detectable IE62-ALW-A2 T cells (A, left figure), or without detectable tetramer positive cells (C and D, left figures) were in-vitro stimulated (IVS) for 7 days with IE62-ALW-A2 peptide (1 µg/ml), in the presence of IL-2 and IL-15 (A, B, C, D right figures). Cells were stained with CD4 FITC, CD40 FITC and IE62-ALW-A2 tetramer, and percentages IE62-ALW-A2 tetramer positive cells of CD8⁺ T cells are indicated. CD8⁺ T cells are defined as CD4⁻ CD40⁻ lymphocytes. E) PBMCs of a patient during the course of a VZV reactivation following TCD alloSCT were stained with the IE62-ALW-A2 tetramer in combination with CD8⁺ FITC. The percentages 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 for treatment of the VZV reactivation.

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 percentage of IE62-ALW-A2-specific CD8⁺ T cells declined to 0.09% at day 49 and 0.03% at day 145 after reactivation (**Figure 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 ALWALPHAA 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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