Persistent High Frequencies of Varicella-Zoster Virus ORF4 Protein-Specific CD4⁺ T Cells after Primary Infection

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Open reading frame 4 (ORF4) of varicella-zoster virus (VZV) encodes an immediate-early protein that is believed to be important for viral infectivity and establishing latency. Evidence suggests that VZV-specific T cells are crucial in the control of viral replication, but there are no data addressing the existence of potential ORF4 protein-specific CD4⁺ T cells. We tested the hypothesis that VZV ORF4 protein-specific CD4⁺ T cells could be identified and characterized within the peripheral blood of healthy immune donors following primary infection. Gamma interferon (IFN- γ) immunosorbent assays were used to screen peripheral blood mononuclear cells obtained from healthy seropositive donors for responses to overlapping ORF4 peptides, viral lysate, and live vaccine. High frequencies of ORF4 protein-specific T cells were detected *ex vivo* in individuals up to 52 years after primary infection. Several immunogenic regions of the ORF4 protein were identified, including a commonly recognized epitope which was restricted through HLA-DRB1*07. Total ORF4 protein-specific responses comprised 19.7% and 20.7% of the total lysate and vaccine responses, respectively, and were dominated by CD4⁺ T cells. Indeed, CD4⁺ T cells were found to dominate the overall virus-specific IFN- γ cellular immune response both *ex vivo* and after expansion *in vitro*. In summary, we have identified an ORF4 protein as a novel target antigen for persistent VZV-specific CD4⁺ T cells, with implications for disease pathogenesis and future vaccine development.

Varicella-zoster virus (VZV) is a cytopathic human alpha-herpesvirus. VZV-specific T lymphocytes are believed to be important in the control of viral replication both during primary infection, where the detection of T lymphocyte proliferation correlates with milder disease, and in the maintenance of latency (4). In addition, CD8⁺ T cells can mediate lysis of VZV-infected fibroblasts and lymphoblastoid cells (6, 21). Potential CD8⁺ T-cell epitopes have been documented within immediate-early protein 62 (IE62), but reactive cells have been detectable only after *in vitro* expansion (18). However, in a further study, VZV lysate-specific T cells circulating in healthy immune donors were found to be largely of the CD4⁺ T-cell subset, and CD8⁺ T cells were not detectable (7). VZV-specific CD4⁺ T cells synthesize Th1-like cytokines, such as interleukin-2 (IL-2) and gamma interferon (IFN- γ) (22, 31), and are capable of major histocompatibility complex class II-restricted cytotoxicity (6, 13, 16, 20). T-cell antigen specificity has been addressed in a number of studies which have documented reactivity to several VZV proteins, including the regulatory and structural proteins encoded by open reading frame 10 (ORF10), ORF62, and ORF63 (5, 28) and glycoproteins gB, gC, gE, and gI (3, 6, 19). One study documented the existence of ORF4 protein-specific cytotoxic T lymphocytes, which were again detectable only following expansion *in vitro* (5). Despite evidence supporting a role for T cells in the control of

VZV replication, there are no previous studies that have identified VZV-specific CD4⁺ T-cell epitopes.

The VZV genome comprises more than 70 unique open reading frames which encode proteins that are potential targets for the host immune system (2, 5, 14). ORF4 encodes a 51-kDa IE protein (15, 23, 26). The ORF4 protein is associated with the tegument in purified virions (23) and is found in the nuclei of infected cells during the initial stages of infection but later localizes to the cytoplasm (15). Functionally, the ORF4 protein is a transcriptional activator and influences several promoters from each of the three classes of genes. Furthermore, the ORF4 protein works cooperatively with ORF62 to function at both transcriptional and posttranscriptional levels. ORF4 RNA and protein have been detected in latently infected human ganglia by *in situ* hybridization (2). ORF4 has recently been shown to be important for infection and to have an important role in the establishment of latency (12, 29). Thus, the ORF4 protein is present during different phases of the viral life cycle and would be a reasonable candidate for a T-cell antigenic target for both control of viral replication and induction of responses through vaccination.

We tested the hypothesis that VZV ORF4 protein-specific CD4⁺ T cells could be identified and characterized in the peripheral blood of healthy immune donors. Immunosorbent assays with IFN- γ were used to screen healthy seropositive individuals with a history of primary infection for responses to overlapping peptides spanning the ORF4 protein. High frequencies of responses were observed both *ex vivo* and after *in vitro* restimulation. We have identified immunogenic regions of the ORF4 protein and have characterized one of the strongest responses in detail. The

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epitope was restricted by HLA-DRB1*07 and was naturally processed and presented after restimulation with infected cell lysate or the live VZV vaccine. The total ORF4 protein responses comprised approximately 20% of the total lysate or vaccine responses, suggesting that the ORF4 protein is an immunodominant target antigen. These data show that ORF4 protein-specific T cells comprise a large proportion of the overall VZV-specific response in healthy donors many years following primary infection and raise the possibility that such T cells have a role in the control of viral reactivation. As well as providing insight into the role of ORF4 protein-specific T cells in the control of viral replication, these findings will also inform future recombinant or subunit vaccine development.

MATERIALS AND METHODS

Study subjects. The study participants consisted of 19 healthy seropositive adult individuals with a history of primary VZV infection but no clinical reactivation and 12 seronegative individuals. The mean age of the donors was 30.7 years, with a mean age at primary infection of 5.6 years. Ethical approval was granted by the Oxfordshire ethics committee, and informed consent was obtained from all donors.

Peripheral blood mononuclear cells (PBMC) were isolated from fresh heparinized blood samples by Ficoll-Hypaque density gradient centrifugation. The cells were then resuspended in RPMI 1640 plus 10% fetal calf serum (R10) or 10% human AB serum (hR10) for ex vivo enzyme-linked immunospot (ELISPOT) assays, ex vivo intracellular cytokine assays, and cell cultures, as detailed below.

Peptide generation. A panel of 45 synthetic 20-mer peptides overlapping by 10 amino acids were synthesized using standard 9-fluorenylmethoxy carbonyl chemistry. These peptides span the entire protein encoded by open reading frame 4 of VZV (14). The purity was established by high-pressure liquid chromatography, and the individual peptides were dissolved at 10 mg/ml in dimethyl sulfoxide and stored at -20°C. They were diluted to 20 µg/ml in RPMI 1640 for use in ELISPOT assays.

IFN-γ ELISPOT and ELISPOT culture assays. Assays were performed using Immobilon-P-lined 96-well multiscreen plates (Millipore Ltd., United Kingdom). The wells were coated with 50 µl of IFN-γ catcher antibody (monoclonal antibody [MAB] 1.D1K; Mabtech) diluted to 15 µg/ml in sterile phosphate-buffered saline, pH 7.4, and incubated for 16 h at 4°C. The plates were washed six times and blocked with 200 µl R10 per well for 1 h at 37°C. PBMC were diluted to 1 × 10⁶/ml in hR10, and 100 µl was added to each well. The peptides were added to a final concentration of 5 µM, and the plates were incubated overnight (16 h) at 37°C. When the pool of 45 ORF4 peptides was used, each peptide was at a final concentration of 2 µM. Phytohemagglutinin was used as the positive control at 2 µg/ml per well, and RPMI 1640 alone was used as a negative control. An irrelevant peptide was used to assess nonspecific IFN-γ responses. The plates were developed by the addition of 1 µg/ml of biotin-linked anti-IFN-γ MAb (MAb 7-B6-1-biotin; Mabtech) as a detection antibody, which was subsequently conjugated to streptavidin alkaline phosphatase (Mabtech) and visualized using an AP conjugate substrate kit (Bio-Rad). Spots were enumerated using an automated AID ELISPOT reader. The background (cells plus irrelevant peptide) was subtracted, and the data were expressed as the number of spot-forming units per 10⁶ PBMC. Significant positive responses were defined as those greater than the mean + 2 standard deviations (SD) of the results for the irrelevant control peptide.

For ELISPOT culture assays, T-cell lines were set up as described below and incubated at 37°C and 5% CO₂ for 10 days. On day 10, the cells were washed twice in PBS and then returned to a 24-well culture plate in hR10. On day 11, the cells were counted and diluted to 0.4 × 10⁶/ml in hR10, and then ELISPOT assays were carried out as described above.

Partially matched antigen-presenting cells (B-cell lines and keratinocytes) were incubated with 100 µl of 0.1 mM peptide, lysate, or vaccine for 1 h at 37°C and then washed three times. One thousand to 5,000 antigen-presenting cells were added to wells with PBMC/T-cell-line concentrations as described above. ELISPOT assays were then carried out as described above.

Intracellular cytokine staining. Fresh PBMC or T-cell lines were stimulated with either culture medium alone, phorbol myristate acetate (50 ng/ml) and ionomycin (1 mM), peptide (5 µM), or pooled peptides (1 µM each peptide) for

16 h at 37°C and 5% CO₂ with the addition of GolgiStop (Becton Dickinson [BD] catalog no. 554715) after 2 h of incubation. On day 2, the cells were stained for the surface markers CD3, CD8, and CD4 (BD Pharmingen) and, after permeabilization using an intracellular cytokine staining kit (BD catalog no. 554715) for the cytokine IFN-γ, were analyzed by flow cytometry (8).

Serology and HLA typing. Serum was analyzed for VZV immunoglobulin G antibodies using the VIDAS varicella-zoster immunoglobulin G assay (bio-Mérieux). All PBMC, keratinocyte, and B-cell lines were HLA typed by PCR with sequence-specific primer phototyping (9).

Establishment of T-cell, B-cell, and keratinocyte lines. Freshly separated PBMC were diluted to 2 × 10⁶ cells/ml, plated onto a 24-well plate, and then incubated with peptides at a final concentration of 4 µM at 37°C and 5% CO₂. On day 3, IL-2 was added at a concentration of 100 units/ml. The cells were maintained in RPMI 1640 containing IL-2 and 10% human AB serum. VZV-infected cell extract (ABI catalog no. 10-514-001) was used at 5 µg/ml, and uninfected Vero cell extract (ABI catalog no. 10-508-001) was used at 5.4 µg/ml. A live, attenuated varicella virus vaccine (Varilrix; GlaxoSmithKline) was added to a final concentration of 10⁴ PFU/ml. B-cell lines were generated by pulsing PBMC with supernatant from an Epstein-Barr virus-producing marmoset cell line, B958 (in-house), for 1 hour at 37°C and 5% CO₂. The cells were cultured in RPMI 1640 containing 15% fetal calf serum and 1 µg/ml cyclosporine A (Sandemans) and then incubated at 37°C and 5% CO₂. Lines were maintained in RPMI 1640 containing 10% fetal calf serum and regularly screened for mycoplasmas.

Three established keratinocyte lines were used: HaCat cells (a gift from N. Fusenig) are spontaneously developed from adult epidermal keratinocytes, and NK and NFK cells are human papillomavirus type 16 immortalized keratinocytes (a gift from E. O'Toole). All three lines were HLA typed as described above.

CD4⁺ or CD8⁺ Dynal bead depletion. Dynabeads (either CD4 or CD8) were washed twice in RPMI 1640. PBMC were then incubated with 4 to 5 beads per target at 4°C for 30 min with gentle rotation. The cell-bead suspension was then placed on the magnet, and the supernatant was removed. The cells were then recounted and used at the concentrations stated above.

RESULTS

Responses to overlapping ORF4 peptides ex vivo. First, we determined the overall ex vivo T-cell responses to the VZV ORF4 protein using PBMC derived from healthy seropositive individuals with a history of primary infection. PBMC were tested by IFN-γ ELISPOT assays for responses to 45 overlapping 20-mer peptides spanning the ORF4 protein. The responses to the overlapping ORF4 peptides ranged in both breadth and magnitude, as seen in Fig. 1. One donor failed to respond to any peptide with a result above the positive cutoff (mean + 2 SD of the irrelevant peptide), but the remainder (95%) responded to at least one peptide. There was also a range in magnitude of responses to individual peptides, with the maximum reaching 1,800 IFN-γ-producing cells per million PBMC for an individual peptide. Such levels were comparable to the frequencies observed in individuals infected with persistently replicating viruses, including human immunodeficiency virus (17). In contrast, we did not observe responses to any peptides in the 12 seronegative controls (data not shown). There was no significant correlation between individual peptide-specific responses or total ORF4 peptide responses and age or time from primary infection. The average age of the donors was 32.5 years (range, 24 to 62 years), and the average age at primary infection was 6.4 years (range, 3 to 15 years). The median time since primary infection was 21.7 years (range, 17 to 52 years), and none of the donors had a history of reactivation. Overall, these data suggest that ORF4 protein-specific T cells circulate at persistently high levels in the blood of healthy immune donors with a history of primary infection.

We proceeded to further characterize the responding populations, and using CD4/CD8 depletion prior to ELISPOT assay

FIG. 1. Ex vivo IFN- γ ELISPOT responses to overlapping ORF4 peptides in healthy, immune donors with a history of primary VZV infection (each dot represents the mean response in one individual). The long horizontal bar shows the mean + 2 SD of the results for the irrelevant peptide control. For each peptide, the median is shown as a small horizontal bar.

and anti-CD4/CD8 staining with intracellular cytokine analysis, we observed that the majority of ex vivo and cultured ORF4 protein-specific responses were mediated by CD4⁺ T cells. Indeed, we could not identify ORF4 protein-specific CD8⁺ T cells even after culture. Although the system involving overlapping 20-mer peptides is widely used for the identification of both CD4⁺- and CD8⁺-restricted responses, we wanted to eliminate the possibility that our observed CD4⁺ T-cell dominance might be an artifact of the peptide-based stimulation system, with a bias away from CD8⁺ T cells. We therefore used a number of different VZV antigens, both ex vivo and also to stimulate VZV-specific T-cell expansion in vitro. Table 1 shows that when ORF4-based peptides, VZV-infected cell lysate, or the live viral vaccine was used to stimulate the PBMC and when cells

were tested for reactivity at day 10 of the culture, the majority of ORF4 protein-specific responses were mediated by CD4⁺ T cells. The only significant populations of CD8⁺ T cells that we could document occurred after the PBMC were stimulated with live viral vaccine and then tested at day 10 with the vaccine (Table 1). However, even under these circumstances, the proportion of CD8-positive cells to CD4-positive cells averaged 1:10.

Overall, these data show that ORF4 protein-specific T cells are detectable ex vivo and show rapid effector function in healthy immune donors with a history of primary infection. Furthermore, the responses were strongly dominated by CD4⁺ T cells. We observed responses to several of the 45 overlapping peptides ex vivo and after culture. We therefore proceeded to investigate these responses in more detail.

Characterization of the peptide 26 epitope. Peptide 26 comprised the sequence MLYGHELYRTFESYKMDSRI and was observed to induce strong responses in many individuals. To determine the optimal epitope and anchor residues, truncations were made by sequentially removing residues from each end. The responses to the resulting truncated peptides were assessed by IFN- γ ELISPOT culture assays. Figure 2 documents the responses observed and shows that residues 8 and 16 are critical for responses to this peptide. The optimal epitope was determined to comprise residues 6 to 18 (ELYRTFESYKMDS) (data not shown). In order to confirm whether IFN- γ responses to peptide 26 were mediated by CD4⁺ T cells, we used intracellular cytokine assays and CD4/CD8 T-cell depleting beads. As shown in Fig. 3, the IFN- γ responses to this peptide are mediated entirely by CD4⁺ T cells.

To determine the HLA restriction of the response to peptide 26, we used partially matched EBV-transformed B-cell lines or keratinocyte lines. These lines were pulsed for 1 h either with the peptide or the control, washed, and then used as antigen-presenting cells in IFN- γ ELISPOT assays. Figure 4 shows that

TABLE 1. ORF4 protein and peptide 26 are important target antigens^a

Initial stimulus	Second stimulus	Mean % response ^b	
		CD3 ⁺ CD4 ⁺ IFN- γ ⁺	CD3 ⁺ CD8 ⁺ IFN- γ ⁺
Peptide	Peptide	1.75*	0.06
Peptide	Lysate	0.07*	0.005
Peptide	Vaccine	0.04	0.01
Lysate	Peptide	0.1*	0.007
Lysate	ORF4, pooled	0.55*	0.02
Lysate	Lysate	2.81*	0.03
Lysate	Vaccine	4.92*	0.06
Vaccine	Peptide	0.23*	0.06
Vaccine	ORF4, pooled	0.36*	0.02
Vaccine	Lysate	0.81*	0.03
Vaccine	Vaccine	1.71*	0.17*

^a PBMC were incubated with an initial stimulus for 10 days and then tested for IFN- γ production in response to a second stimulus.

^b Mean responses for nine individuals. *, significantly above value for irrelevant control. $P < 0.05$.

FIG. 2. IFN- γ ELISPOT responses to peptide truncations of peptide 26 of ORF4. nil, no peptide; irr, irrelevant peptide.

the T-cell line responds to the lines that match HLA-DRB1*07 and not other partially matched antigen-presenting cells (all unpulsed antigen-presenting cells elicited no response). There-

fore, the response to peptide 26 is restricted via HLA-DRB1*07.

The ORF4 protein is an important target antigen. To determine the relative contributions that the responses to this peptide and to ORF4 make to the overall virus-specific response, we compared responses in HLA-DRB1*07-positive individuals to the epitope, to a pool of all 45 overlapping ORF4 peptides, to infected cell lysate, and to the vaccine. When lysate was used to stimulate PBMC, the total ORF4 pooled-peptide response comprised a mean of 19.7% of the day 10 response to the lysate (Table 1). Furthermore, the peptide 26 response was a mean of 18% of the total ORF4 pooled-peptide response. When vaccine was used to stimulate PBMC, the total ORF4 pooled-peptide response was a mean of 20.7% of the day 10 response to the lysate, and the peptide 26 response was a mean of 64% of the overall ORF4 pooled-peptide response. It is possible that there was competition between peptides within the pools, which we have tried to address using a number of different stimulation and testing forms of VZV antigens. Figure 5 shows an example of a day 10 T-cell line that was generated by incubating PBMC with live viral vaccine. In this example, the contributions of peptide 26 and overall ORF4 pooled peptides to the vaccine response were 10% and 31.5%, respectively. In order to confirm whether the peptide is naturally processed and presented, we generated peptide 26-specific T-cell lines and showed recognition of target cells (HLA-DRB1*07-positive transformed B lymphoblastoid cells and keratinocytes) sensitized by the use of viral lysate and live vaccine. Taken together, these data show that ORF4 protein is an important antigen and may comprise a significant proportion of the overall virus-specific T-cell response. In addition, we have shown that peptide 26 makes a large contribution to the overall ORF4 protein CD4⁺ T-cell response and that pep-

FIG. 3. Examples of results for PBMC incubated with peptide 26 and then assayed at 10 days by intracellular cytokine staining for IFN- γ production in response to either no stimulus (upper panels) or peptide 26 (lower panels). The responses to peptide 26 are mediated entirely by CD4⁺ T cells. PerCP, peridinin-chlorophyll-protein; APC, allophycocyanin.

FIG. 4. HLA-DRB1*07-mismatched EBV-transformed B-cell lines (BCL) pulsed with peptide 26 are recognized by specific CD4⁺ T cells. The overall data are shown on the left, and the corresponding ELISPOT wells are shown on the right.

tide 26-specific T cells can recognize naturally processed and presented antigen.

DISCUSSION

The ORF4 protein is believed to be important for viral infectivity and establishing latency, yet the existence of potential ORF4 protein-specific CD4⁺ T cells has not been previously examined. The ORF4 protein is expressed during both latency and viral replication and is therefore a reasonable candidate for understanding the immune control of viral replication and for potential future vaccination development. Using IFN- γ ELISPOT assays, we have shown high levels of ex vivo rapid effector functional responses to ORF4 peptides in healthy immune donors with a history of primary infection. We have shown that the responses to ORF4 peptides are dominated by CD4⁺ T cells and that such T cells recognize naturally processed and presented antigen.

We proceeded to characterize one of the strongest responses in detail and showed that residues 8 and 16 of the 20-mer (MLYGHELYRTFESYKMDSRI) were critical for presentation. By using partially matched antigen-presenting cells, we have shown that the responses seen to this peptide were restricted by HLA-DRB1*07. T-cell specificity for VZV antigens has been documented for several proteins, including the products of ORF10, ORF62, and ORF63 and glycoproteins gB, gC, gE, and gI, and one study demonstrated the presence of ORF4 protein-specific cytotoxic T lymphocytes which were detectable only after expansion in vitro (5). By using VZV lysate and live viral vaccine, we have been able to show that the overall ORF4 responses comprise a significant proportion of the ex vivo virus-specific T-cell response. Therefore, in addition to providing insight into the potential role of ORF4-specific T cells in the control of reactivation, the data also suggest that inclusion of the ORF4 gene or protein within future subunit or recombinant vaccine approaches might be beneficial. The existing vaccine is highly effective but carries a risk of systemic symptoms, particularly in those who are arguably most in need of vaccination, namely, individuals with immunodeficiency. The levels of ORF4 peptide-specific T cells were comparable to the frequencies observed in individuals infected with persistently replicating viruses, such as human immunodeficiency virus

FIG. 5. Examples of results for PBMC incubated with VZV live vaccine for 10 days and then analyzed for IFN- γ production in response to no stimulus (A), peptide 26 (B), all the ORF4 peptides (C), viral lysate (D), or vaccine (E).

(17). There was no correlation between the level of ORF4 responses and the age of the donor or the time from primary infection, which suggests that there is persistence of the ORF4-specific T cells. It is possible that exposure to primary varicella or periodic subclinical reactivation may boost the VZV-specific T-cell frequency, thus maintaining such high levels of reactive cells (24, 30). The presence of ORF4 protein-specific T cells in healthy immune donors with a history of primary infection suggests that such cells are involved in the control of viral reactivation. Clearly, it will now be important to build on these data by temporal analysis of different groups of individuals (including those with primary infection or reactivation, the elderly, and those recently vaccinated) in order to more closely define the potential role of these cells in the control of the disease. It will also be important to investigate how long ORF4-specific T cells are stable through larger cohort studies, particularly studies including elderly volunteers. The identification of an ORF4 protein-specific epitope will facilitate such investigations.

The existence of VZV-specific CD8⁺ T cells has been addressed in a number of studies, and indeed, IE62-specific CD8⁺ T-cell epitopes have been documented (18). However, in all studies, the CD8⁺ T cells were detectable only after *in vitro* expansion, suggesting that they circulate at relatively low frequencies (5, 18). Comparable findings were also documented by Asanuma et al., who showed that VZV-specific CD4⁺ T cells but not CD8⁺ T cells could be detected using stimulation with viral lysate but that both CMV-specific CD8⁺ and CD4⁺ T cells were readily detectable (7). Our data regarding ORF4 protein-specific CD4⁺ and CD8⁺ T cells would fit with such studies. However, this relative paucity of virus-specific CD8⁺ T cells was strikingly different from our and others' previous experience with other virus-specific responses, including those to human immunodeficiency virus and other herpesviruses such as Epstein-Barr virus and cytomegalovirus, in which strong CD8⁺ T-cell responses can be readily generated (10, 11, 27). We compared different antigen sources to help avoid potential approach-specific CD4⁺ T-cell bias, but despite using peptides, lysate, and live vaccine, we consistently observed proportionately low levels of VZV-specific CD8⁺ T cells. Such relatively low frequencies of circulating VZV-specific CD8⁺ T cells may contribute to viral persistence, possibly reflecting mechanisms of viral escape such as the known VZV-induced HLA class I down-regulation (1, 25).

Overall, we have shown that the ORF4 protein is an important T-cell antigen within VZV and that responses are dominated by CD4⁺ T cells. We have identified an ORF4 protein CD4⁺ T-cell epitope and documented that responding T cells recognize naturally processed and presented antigen. These data suggest that ORF4 protein-specific CD4⁺ T cells are important in the control of viral reactivation and will inform future studies of viral pathogenesis and novel vaccine development.

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