Development of Virus-Specific CD4⁺ and CD8⁺ Regulatory T Cells Induced by Human Herpesvirus 6 Infection

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Human herpesvirus 6 (HHV-6) is an important immunosuppressive and immunomodulatory virus. The mechanisms by which HHV-6 establishes latency and immunosuppression in its host are not well understood. Here we characterized HHV-6-specific T cells in peripheral blood mononuclear cells (PBMCs) from HHV-6-infected donors. Our results showed that HHV-6 infection could induce both CD4⁺ and CD8⁺ HHV-6-specific regulatory T (Treg) cells. These HHV-6-specific Treg cells had potent suppressive activity and expressed high levels of Treg-associated molecules CD25, FoxP3, and GITR. Both CD4⁺ and CD8⁺ Treg cells secreted gamma interferon (IFN- γ) and interleukin-10 (IL-10) but little or no IL-2, IL-4, or transforming growth factor β (TGF- β). Furthermore, HHV-6-specific Treg cells not only could suppress naive and HHV-6-specific CD4⁺ effector T cell immune responses but also could impair dendritic cell (DC) maturation and functions. In addition, the suppressive effects mediated by HHV-6-specific Treg cells were mainly through a cell-to-cell contact-dependent mechanism but not through the identified cytokines. These results suggest that HHV-6 may utilize the induction of Treg cells as a strategy to escape antiviral immune responses and maintain the latency and immunosuppression in infected hosts.

Human herpesvirus 6 (HHV-6) was first discovered in patients with AIDS and lymphoma (1). Two variants, A and B, have been identified and have been implicated in a number of disorders, including multiple sclerosis, hematological malignancies, and complications following stem cell or organ transplantation (2–4). HHV-6 infection may be associated with the nodular sclerosing (NS)-type Hodgkin lymphoma and angioimmunoblastic T cell lymphoma (AITL). In addition, recent studies from our group and others demonstrated that HHV-6 may also be associated with glioma (5–8).

Modulation of host immune responses represents an important mechanism by which viruses create a favorable environment for their growth and persistence. HHV-6 is an important immunosuppressive and immunomodulatory virus which can induce immunomodulation through a variety of mechanisms, such as lytic infection of CD4⁺ and/or cytotoxic effector T cells, impairment of antigen-presenting cell functions, induction of inflammatory and immunosuppressive cytokines and/or chemokines, and downmodulation of the CD3/T cell receptor complex (9, 10). Our recent studies have demonstrated that HHV-6A infection induced cell cycle G₂/M arrest in infected T cells via various molecular regulatory processes, further suggesting that this potential mechanism involved in its immune suppression and modulation (11). However, the mechanisms responsible for the regulation and suppression mediated by HHV-6 infection are still under investigation.

Increasing evidence suggests that induction of regulatory T (Treg) cells is another important mechanism utilized by viruses to establish chronic infections or latency. Increased frequencies of Treg cells have been observed during chronic viral infections with hepatitis B virus (HBV), HCV, and HIV (12–14). We have also shown the existence of HHV-6-specific interleukin-10 (IL-10)-producing CD4⁺ T cells in HHV-6-infected individuals that pos-

sessed T regulatory type 1 (Tr1) cell activity (15). These Tr1 cells may contribute to the HHV-6-mediated chronic infection and latency. However, whether HHV-6 infection can directly induce virus-specific Treg cells affecting the magnitude of antiviral immunity is still unknown.

In our efforts to further explore the mechanisms responsible for the immunosuppression and latency of HHV-6 infection, we found that HHV-6 infection could induce both CD4⁺ and CD8⁺ HHV-6-specific Treg cells. These HHV-6-specific Treg cells had potent suppressive activity and exhibited Treg-associated phenotypes. Furthermore, HHV-6-specific Treg cells not only could suppress naive and HHV-6-specific CD4⁺ effector T cell immune responses but also could impair DC maturation and functions. These studies have important implications regarding the mechanisms utilized by HHV-6 to mediate virus immune evasion and latency.

MATERIALS AND METHODS

Ethics statement. This study was approved by the Committee of the Ethics of Treatment of Human Subjects at Nanjing Medical University, and written informed consent was provided by the study participants.

Preparation of virus antigens. The GS strain of HHV-6 variant A was propagated in HSB-2 cells and cell-free virus were prepared, as described previously (16). Mock-infected HSB-2 cells were included as a control.

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The YY5 strain of HHV-7 was propagated in SupT1 cells, and cell-free virus was prepared for the studies. Mock-infected SupT1 cells were included as a control. The Stoker strain of herpes simplex virus 1 (HSV-1) was propagated in Vero cells, and cell-free virus was prepared. Mock-infected Vero cells were included as a control.

Generation of HHV-6-specific T cell clones. Ten HHV-6-seropositive healthy volunteers were enrolled for the study (15). Peripheral blood mononuclear cells (PBMCs) from volunteers were seeded in 96-well round-bottom microtiter plates at the concentration of 1×10^5 cells per well in OpTmizer T-Cell Expansion SFM (GIBCO) medium supplemented with 10% human AB serum. HHV-6 lysates were then mixed with PBMCs. After 10 days of coculture, T cell clones were further obtained through a limiting-dilution method, as described previously (17).

To screen the HHV-6-specific T cell clones (17, 18), autologous irradiated PBMCs pulsed with HHV-6 lysates, or controls of HHV-7, HSV-1, and mock lysates, were cocultured with 5×10^4 cells of each T cell clone for 2 days. HHV-6-specific T cell clones were selected based on gamma interferon (IFN- γ) secretion in culture supernatants and expanded for the further experiments.

FACS analysis. T cell clones markers were determined by fluorescence-activated cell sorter (FACS) analysis after staining with specific antibodies conjugated with either phycoerythrin (PE) or fluorescein isothiocyanate (FITC). These antibodies included anti-CD4, anti-CD8, anti-CD25, anti-FoxP3, anti-GITR, anti-CTLA-4, anti-CD127, anti-transforming growth factor β (anti-TGF- β), anti-CD80, anti-CD86, anti-CD83, anti-CD14, anti-CD1a, and anti-HLA-DR (BD Bioscience). All stained cells were analyzed by FACSscan flow cytometry (BD Bioscience) and data analyzed with FlowJo software (Tree Star).

Ab blocking and cytokine release assays. T cell clones activity was measured in the presence or absence of specific neutralizing antibodies, as previously described (19). These antibodies included W6/32 (HLA-A, -B, and -C) and IVA12 (anti-human-HLA-DR, -DP, and -DQ) (Bio Legend). Autologous irradiated PBMCs pulsed with HHV-6 lysates were preincubated with antibody (Ab) (200 μ g/ml) for 30 min, and 5×10^4 T clone cells were then added and incubated overnight. The amount of IFN- γ released from T cell clones was measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Inc.).

To determine cytokine profiles of T cell clones, autologous irradiated PBMCs pulsed with HHV-6 lysates were cocultured with 5×10^4 cells of different T clones overnight. The amounts of IFN- γ , IL-2, IL-4, IL-10, and TGF- β released from the T cell clones were measured by ELISA.

For suppression of IL-2 secretion by effector T cell clones, HHV-6-specific Treg cell clones and effector T cell clones were cocultured at a 1:1 ratio in the presence of autologous irradiated PBMCs pulsed with HHV-6 lysates. IL-2 secretion was determined after 18 h of incubation. The CD4-W3 T cell clone was used as a control.

Proliferation assays. Proliferation assays were performed as previously described (20). Briefly, naive CD4⁺ T cells (1×10^5) were cocultured with autologous irradiated PBMCs (5×10^4) and different numbers of CD4⁺ or CD8⁺ Treg or effector T cell clone cells in the presence of 500 ng/ml anti-CD3 Ab (eBioscience) for 56 h. Proliferation of naive CD4⁺ T cells was determined by the incorporation of [³H]thymidine. For some experiments, neutralizing antibodies against IL-10 and TGF- β (Bio Legend) were added in the assay mixture at a final concentration of 10 μ g/ml.

Transwell experiments were performed in 24-well plates with a pore size of 0.4 μ m (Corning Costar). Naive CD4⁺ T cells (1×10^5) were cultured in the outer wells of 24-well plates in medium containing 500 ng/ml anti-CD3 Ab and 1×10^5 irradiated PBMCs. Equal numbers of Treg clone or effector T cell clone cells were added to the inner wells in the same medium. After 56 h of culture, the cells in the outer and inner wells were harvested separately and transferred to 96-well plates. [³H]thymidine was added, and the radioactivity was counted with a liquid scintillation counter.

Inhibition of DC maturation and function by HHV-6-specific Treg cells. Immature and mature dendritic cells (DCs) were derived from the monocytes of healthy donors in culture with IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) with or without TNF- α (PeproTech). Immature or mature DCs (1×10^6) were cocultured with preactivated HHV-6-specific Treg clone cells or naive CD4⁺ T cells (5×10^5) in 24-well plates in medium containing IL-4 and GM-CSF with or without TNF- α . HHV-6-specific T cells were preactivated with HHV-6 lysates. After 48 h of coculture, the treated and untreated DCs were separated from the cocultures using CD4- or CD8-positive immunomagnetic beads and divided into different groups. The surface markers CD83, CD80, CD86, and HLA-DR (BD Bioscience) were analyzed by FACSscan flow cytometry (BD Bioscience) and data analyzed with FlowJo software (Tree Star).

Treg-treated or untreated immature and mature DCs were cocultured with naive CD4⁺ T cells at different ratios in the presence of soluble OKT3 antibody (eBioscience), and the proliferation of naive CD4⁺ T cells was determined as described above.

Transwell experiments were performed in 24-well plates with a pore size of 0.4 μ m (Corning Costar). HHV-6-specific T cells were preactivated with HHV-6 lysates. Preactivated HHV-6-specific Treg clone cells or naive CD4⁺ T cells (5×10^5) were cultured in the inner wells of 24-well plates. Equal numbers of immature and mature DCs were added to the outer wells in medium containing IL-4 and GM-CSF with or without TNF- α (PeproTech). After 48 h of culture, the DCs in the outer wells were harvested and transferred to 96-well plates. The surface markers and the functions of DCs were analyzed by the same method described above.

Statistical analysis. Unless indicated otherwise, data were expressed as mean $\pm$ standard deviation (SD). All statistical analyses were carried out using SPSS 16.0 (SPSS Inc., USA). The significance of differences between groups was determined by one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) *post hoc* test. A *P* value of ≤ 0.05 was considered to be statistically significant.

RESULTS

Generation of HHV-6-specific effector and suppressive CD4⁺ and CD8⁺ T cells. To determine whether HHV-6 infection can induce virus-specific Treg cells, resulting in immunosuppression, we screened PBMCs from 10 HHV-6-infected donors after stimulation with HHV-6 lysates. PBMCs from donors C, P, and W were found to have higher levels of precursors for HHV-6-specific T cells, which were used for the generation of HHV-6-specific T cell lines and clones by further *in vitro* stimulation with HHV-6 lysates. After three cycles of stimulation with the HHV-6 lysates, T cells showing marked cell growth were tested for the recognition of HHV-6 and then used to generate HHV-6-specific T cell lines. As shown in Fig. 1A, three HHV-6-specific T cell lines generated 93 T cell clones, of which 36 T cell clones specifically recognized HHV-6 lysate-pulsed autologous PBMCs detected by IFN- γ secretion, compared with the response to HHV-6 mock lysate-pulsed autologous PBMCs (Fig. 1B). To further determine the specificity of these T cell clones, we analyzed the reactivities of those 16 HHV-6-specific T cell clones to HHV-7 or HSV-1 lysates. However, none of the T cell clones was reactive with HHV-7 or with HSV-1 (Fig. 1C).

In functional proliferation assays, we found that 11 (31%) of 36 T cell clones had little or no suppressive activity on the proliferation of naive CD4⁺ T cells in response to anti-CD3 Ab, while the remaining 25 T cell clones (69%) showed a strong ability to suppress naive CD4⁺ T cell proliferation (Fig. 1D).

FACS analysis of 36 T cell clones showed that 20 T cell clones (56%) were CD4⁺ T cells, while the remaining 16 T cell clones (44%) were CD8⁺ T cells (Fig. 2A). Moreover, 5 of 20 CD4⁺ T cell

FIG 1 Generation of HHV-6-specific T cell clones. (A) T cell recognition was evaluated by IFN- γ release from 93 T cell clones. Thirty-six T cell clones generated from T cell lines from donors C, P, and W specifically recognized HHV-6 lysate-pulsed autologous irradiated PBMCs but did not respond to HHV-6 mock lysate-pulsed autologous irradiated PBMCs. (B) Thirty-six T cell clones generated from T cell lines from donors C, P, and W did not respond to HHV-6 mock lysate-pulsed autologous irradiated PBMCs. IFN- γ secretion by 36 T cell clones was lower than 200 pg/ml. (C) Sixteen HHV-6-specific T cell clones generated from T cell lines from donors C, P, and W did not respond to HHV-7, HSV-1, or mock lysate-pulsed autologous irradiated PBMCs. (D) Generation of HHV-6-specific effector and suppressive CD4⁺ and CD8⁺ T cell clones. HHV-6-specific T cell clones were screened for their suppressive activity on the proliferation of naive CD4⁺ T cells in response to anti-CD3 Ab. Twenty-five T cell clones showed strong suppressive activity, whereas 11 T cell clones did not suppress or weakly suppressed naive CD4⁺ T cell proliferation in response to anti-CD3 Ab.

clones (25%) were effector T cells, which were HHV-6 specific but had no suppressive activity on the proliferation of naive CD4⁺ T cells in response to anti-CD3 Ab, while the remaining 15 CD4⁺ T cell clones (75%) showed potent ability to suppress naive CD4⁺

T cell proliferation. In addition, 6 of 16 CD8⁺ T cell clones (38%) did not produce a suppressive effect on naive CD4⁺ T cell proliferation, while the remaining 10 CD8⁺ T cell clones (62%) showed a potent ability to suppress naive CD4⁺ T cell proliferation.

FIG 2 Identification and characterization of HHV-6-specific T cell clones. (A) Two discrete T cell subsets were identified based on the expression of CD4⁺ and CD8⁺ molecules using FACS analyses. Data shown are representative results of FACS analysis of these T cell clones. (B) HLA restriction of HHV-6-specific T cell clones. HHV-6-specific T cell clones were cocultured with autologous irradiated PBMCs pulsed with HHV-6 lysates in the presence or absence of various anti-HLA neutralizing antibodies. IFN-γ release was determined with an ELISA kit after 18 h of incubation. CD4⁺ T cell clone (C4, P4, W4, and W1) recognition of HHV-6 antigen was specifically blocked by an anti-human-HLA-DR, -DP, -DQ antibody but not by anti-HLA class I antibody. CD8⁺ T cell clone (C15, P7, W6, and C7) recognition was specifically blocked by an anti-HLA class I antibody but not by an anti-HLA class II antibody. Similar results were obtained with other T cell clones. Data shown are means ± SDs from three independent experiments. Asterisks indicated a significant difference (**, *P* < 0.01) from the control group.

As expected, T cell recognition of HHV-6 by CD4⁺ T cell clones was specifically inhibited by the neutralizing Ab against major histocompatibility complex (MHC) class II but not by an antibody against MHC class I molecules. Furthermore, T cell recognition of HHV-6 by CD8⁺ T cell clones was specifically blocked by the neutralizing Ab against MHC class I but not by antibody against MHC class II molecules (Fig. 2B). These results suggested that HHV-6-specific recognition and reactivity mediated by both CD4⁺ and CD8⁺ T cell clones required MHC molecule involvement, no matter whether they were effector or suppressive T cell clones.

Phenotypic analyses of HHV-6-specific suppressive CD4⁺ and CD8⁺ T cells. Given that the majority of the HHV-6-specific T cell clones had suppressive activity, we hypothesized that these suppressive T cell clones may represent HHV-6-specific Treg cells.

To test this possibility, we first tested the phenotypes of HHV-6-specific CD4⁺ and CD8⁺ T cell clones. As shown in Fig. 3, both HHV-6-specific CD4⁺ (C4, P4, and W4) and CD8⁺ (C15, P7, and W6) T cell clones with suppressive activities expressed CD25, GITR, and Foxp3 molecules. However, nonsuppressive HHV-6-specific CD4⁺ or CD8⁺ T cells (W1 and C7) were negative for these Treg cell-associated markers. The expressions of CTLA-4 and CD127 were low, and there was no significant difference between suppressive and nonsuppressive HHV-6-specific T cells. These data suggested that HHV-6-specific T cell clones with suppressive function possessed the phenotypes of Treg cells.

Results of cytokine profiling showed that suppressive HHV-6-specific T cell clones (C4, P4, W4, C15, P7, and W6) secreted IFN-γ and IL-10, but no or little IL-2, IL-4, and TGF-β, when they were stimulated with HHV-6 lysates. However, HHV-6-specific

FIG 3 Phenotypic analysis of suppressive HHV-6-specific T cell clones. HHV-6-specific CD4⁺ and CD8⁺ T cell clones were stained with PE- or FITC-labeled monoclonal Abs to human CD25, GITR, Foxp3, CTLA-4, and CD127 molecules. The proportions of CD25⁺, GITR⁺, Foxp3⁺, CTLA-4⁺, and CD127⁺ T cells were quantified by flow cytometry in the CD4⁺/CD8⁺-gated cell populations. An isotype antibody served as the control (black graphs). HHV-6-specific suppressive T cell clones (C4, P4, W4, C15, P7, and W6) expressed high levels of CD25, GITR, and Foxp3 molecules, while the HHV-6-specific effector T cell clones (W1 and C7) expressed low levels of CD25, GITR, and Foxp3 molecules. The expression of CTLA-4 and CD127 was low, and there was no significant difference between suppressive and nonsuppressive HHV-6-specific T cells. After T cell expansion, all T cell clones were maintained under the same conditions and in growth medium containing IL-2 (30 IU/ml) for more than 1 week before the analyses.

effector T cell clones (W1 and C7) secreted IL-2 and IFN- γ , but little or no IL-4, IL-10, and TGF- β , after stimulation with HHV-6 lysates (Fig. 4). TGF- β was important for Treg cell suppressive activity. The membrane-bound TGF- β expression was very similar for suppressive and nonsuppressive HHV-6-specific T cells. We also examined TGF- β production by these HHV-6-specific T cells after stimulation with phorbol myristate acetate (PMA)-ionomycin and found no TGF- β secretion by those cells (data not shown). Taken together, the phenotypic and cytokine profile analyses further suggested that HHV-6-specific suppressive CD4⁺ and CD8⁺ T cell clones represented virus-specific CD4⁺ and CD8⁺ Treg cells.

HHV-6-specific CD4⁺ and CD8⁺ Treg cells inhibit both naive and HHV-6-specific CD4⁺ effector T cell functions. After analyzing Treg cell-associated phenotypes of HHV-6-specific suppressive CD4⁺ and CD8⁺ T cell clones, we next determined whether the HHV-6-specific CD4⁺ and CD8⁺ Treg cell clones had suppressive effects on naive and HHV-6-specific CD4⁺ effector T cells. As shown in Fig. 5A, we found that HHV-6-specific CD4⁺ and CD8⁺ Treg cell clones (C4, P4, W4, C15, P7, and W6) possessed only scant proliferative activity themselves. When cocultured with purified naive CD4⁺ T cells, these clones significantly suppressed naive CD4⁺ T cell proliferation induced by the anti-CD3 antibody stimulation in a dose-dependent manner. In

FIG 4 Cytokine profiles of suppressive HHV-6-specific T cell clones. Cytokine profiles of HHV-6-specific suppressive CD4⁺ (C4, P4, and W4) and CD8⁺ (C15, P7, and W6) T cell clones were analyzed by ELISA. IFN-γ, IL-2, IL-4, IL-10, and TGF-β were determined from T cell culture supernatants after coculture with HHV-6 lysate-pulsed autologous irradiated PBMCs. HHV-6-specific effector T cell clones W1 (CD4⁺) and C7 (CD8⁺) were used as controls and representatives of effector T cells.

contrast, HHV-6-specific effector T cell clones (W1 and C7) proliferated themselves and also enhanced the proliferative activity of naive CD4⁺ T cells. Our data clearly showed that HHV-6-specific Treg cell clones possessed potent nonspecific immunosuppressive activity.

We next tested whether these HHV-6-specific Treg cell clones could inhibit IL-2 secretion from HHV-6-specific CD4⁺ T effector cells in response to virus stimulation. We used the W1 clone as a representative of HHV-6-specific CD4⁺ effector T cells. As shown in Fig. 5B, coculture with HHV-6-specific Treg cell clones dramatically inhibited IL-2 secretion by the W1 clone activated by HHV-6 lysates, whereas treatment with another HHV-6-specific CD4⁺ effector T cell clone (W3, as a control) had no suppressive effect on IL-2 secretion by the HHV-6-specific CD4-W1 effector T cell clone. These results suggested that HHV-6-specific Treg cell clones had potent ability to suppress IL-2 production by HHV-6-specific CD4⁺ effector T cells. Collectively, HHV-6-specific CD4⁺ and CD8⁺ Treg cells not only suppressed the proliferation of naive CD4⁺ T cells but also inhibited the function of HHV-6-specific CD4⁺ effector T cells.

Cell-cell contact is required for the suppressive activity mediated by HHV-6-specific CD4⁺ and CD8⁺ Treg cells. Since HHV-6-specific Treg cell clones secreted high levels of IL-10, we reasoned that IL-10 may be responsible for the immunosuppressive activity of HHV-6-specific Treg cell clones. Naive CD4⁺ T cell proliferation was determined in the presence of neutralizing antibodies against IL-10 and TGF-β. However, neither anti-IL-10 nor anti-TGF-β neutralizing antibody could restore proliferation of

naive CD4⁺ T cells cocultured with HHV-6-specific Treg cell clones (Fig. 6A).

To determine whether cell-to-cell contact was required for HHV-6-specific CD4⁺ and CD8⁺ Treg cell clones to exert their suppressive activity, we used transwell experiments. HHV-6-specific Treg cell clones inhibited naive CD4⁺ T cell proliferation in the coculture assay system. In contrast, HHV-6-specific Treg cell clones, when separated by the transwell system, did not suppress the proliferative activity of naive CD4⁺ T cells (Fig. 6B). In addition, HHV-6-specific effector T cell clones did not have the ability to inhibit the proliferative response of the naive CD4⁺ T cells to anti-CD3 stimulation, regardless of the coculture or transwell conditions. These results suggested that cell-cell contact was required for immunosuppression mediated by HHV-6-specific Treg cell clones.

HHV-6-specific CD4⁺ and CD8⁺ Treg cells impair DC maturation and function. We also determined whether HHV-6 specific Treg cells suppressed innate immune cell functions. As shown in Fig. 7, DCs treated with or without effector CD4⁺ T cell clones expressed large amounts of maturation markers CD83, CD80, CD86, and HLA-DR in the presence of the DC maturation cytokines IL-4, GM-CSF, and TNF-α. In sharp contrast, coculture with HHV-6-specific Treg cell clones significantly blocked DC maturation, leading to the downregulation of the four maturation molecules in the treated DCs. However, in the transwell system, HHV-6-specific Treg cell clones could not block DC maturation, showing that maturation molecules in the treated DCs did not decrease compared to those in the untreated DCs (Fig. 8).

To test whether HHV-6-specific CD4⁺ and CD8⁺ Treg cell

FIG 5 HHV-6-specific CD4⁺ and CD8⁺ Treg cells suppress naive and HHV-6-specific CD4⁺ effector T cell functions. (A) HHV-6-specific CD4⁺ (C4, P4, and W4) or CD8⁺ (C15, P7, and W6) Treg cells significantly suppressed the proliferation of naive CD4⁺ T cells induced by anti-CD3 antibody stimulation in a dose-dependent manner. In contrast, no suppressive activity was mediated by HHV-6-specific effector T cell clones W1 (CD4⁺) and C7 (CD8⁺), and both of them enhanced the proliferative activity of naive CD4⁺ T cells. Proliferation of naive CD4⁺ T cells was determined by [³H]thymidine incorporation assays. The results shown are representative of three independent experiments. (B) HHV-6-specific Treg cells inhibit IL-2 secretion by HHV-6-specific CD4⁺ effector cells (W1) to recognize HHV-6 lysates, whereas treatment with or without another HHV-6-specific CD4⁺ effector T cell clone (W3, as a control) had no suppressive effect on IL-2 secretion by the W1 clone. HHV-6-specific cells (C4, P4, W4, and W3) and effector T cells (W1) were cocultured at a 1:1 ratio in the presence of autologous irradiated PBMCs pulsed with HHV-6 lysates. IL-2 secretion in the coculture supernatants was determined by ELISA after 18 h of coculture. IL-2 secretion by C4, P4, and W4 clone was significantly decreased compared to that by the W3 clone (**, $P < 0.01$). Data shown are means $\pm$ SDs from three independent experiments.

FIG 6 Cell-cell contact is required for T cell suppression mediated by HHV-6-specific Treg cells. (A) Anti-IL-10 and anti-TGF- β neutralizing antibodies fail to block the suppressive activity of HHV-6-specific CD4⁺ (C4) or CD8⁺ (C15) Treg cells. Purified naive CD4⁺ T cells were cocultured with HHV-6-specific CD4⁺ or CD8⁺ Treg cells and autologous irradiated PBMCs in the presence of anti-CD3 antibody, as well as anti-IL-10, anti-TGF- β , or control antibodies. The suppression of naive CD4⁺ T cell proliferative activity was determined by [³H]thymidine incorporation assays. There was no significant alteration of the proliferative activity of naive CD4⁺ T cells in the presence of anti-IL-10, anti-TGF- β , or control antibody compared to that of the medium group ($P > 0.05$). Data shown are means $\pm$ SDs from three independent experiments. (B) Cell-cell contact is required for HHV-6-specific CD4⁺ and CD8⁺ Treg cell-mediated suppression. In the transwell system, equal numbers of naive CD4⁺ T cells were cultured in the outer wells, and HHV-6-specific CD4⁺ (C4, P4, and W4) and CD8⁺ (C15, P7, and W6) Treg cells or effector T cells (W1 and C7) were cultured in the inner wells. Otherwise, culture conditions were identical for the inner and outer wells. To ensure that the HHV-6-specific Treg cells used had suppressive activity, we used the coculture system as our positive control for the suppressive activity of HHV-6-specific Treg cells. Once the Treg cells were separated with the responding naive CD4⁺ T cells in the transwell system, there was no detectable suppressive activity mediated by Treg cells on naive CD4⁺ T cell proliferation in the outer wells. There was a significant difference between HHV-6-specific Treg cells (C4, P4, W4, C15, P7, and W6) and effector T cells (W1 and C7) ($P < 0.01$). Data shown are means $\pm$ SDs from three independent experiments.

clones could also impair DC functions, we cultured DCs with HHV-6-specific Treg cell clones or control naive CD4⁺ T cells and then assessed DC ability to stimulate naive CD4⁺ T cell proliferation. Naive CD4⁺ T cells were cocultured with different numbers of immature or mature DCs pretreated with or without HHV-6-specific Treg cell clones or control CD4⁺ T cells, and then their proliferation was determined using [³H]thymidine incorporation assays. The untreated DCs or DCs treated with control CD4⁺ T cells strongly stimulated the proliferation of naive CD4⁺ T cells, regardless of their maturation status. However, neither immature nor mature DCs treated with HHV-6-specific Treg cell clones

stimulated naive CD4⁺ T cell proliferation (Fig. 9A). With separation with the transwell system, immature and mature DCs treated with HHV-6-specific Treg cell clones or control CD4⁺ T cells restored the stimulatory capacity and strongly stimulated the proliferation of naive CD4⁺ T cells (Fig. 9B). These results suggested that HHV-6-specific CD4⁺ and CD8⁺ Treg cell clones could not only block DC maturation but also suppress DC functions.

DISCUSSION

Like other herpesviruses, HHV-6 establishes latency after the initial productive infection and thus is hard to be cleared from its

FIG 7 Impairment of DC maturation by HHV-6-specific Treg cells. (A) Inhibition of DC maturation by HHV-6-specific CD4⁺ and CD8⁺ Treg cells. Treated and untreated DCs were stained with anti-CD83, -CD80, -CD86, and -HLA-DR antibodies and analyzed by FACS. DCs treated with or without HHV-6-specific effector CD4⁺ T cells (W1) expressed large amounts of CD83, CD80, CD86, and HLA-DR in the presence of the maturation cytokines. In contrast, expression of these four maturation molecules in DCs cocultured with HHV-6-specific CD4⁺ (C4) or CD8⁺ (P7) Treg cells was markedly inhibited. The percentage of cells expressing and mean fluorescence intensities (MFI) for CD83, CD80, CD86, and HLA-DR in the treated and untreated DCs are indicated. (B) Summary of the percent molecule expression and mean fluorescence intensities (MFI) in the DCs from different treatment groups. Boxes represent the upper and lower values of the data, and the dividing line in the box represents the median. There was a significant difference between HHV-6-specific Treg cells (C4 and P7) and effector T cells (W1) (*, $P < 0.01$; **, $P < 0.001$). The results shown are from five independent experiments.

host. To date, the immune evasion and regulation mechanisms deployed by HHV-6 have been poorly characterized. In this study, we demonstrated that HHV-6 infection could induce virus-specific Treg cells. HHV-6 may utilize the induction of Treg cells as a

strategy to escape antiviral immune responses and maintain the latency and immunosuppression in infected hosts.

Many pathogens have evolved strategies to manipulate the regulatory network of the host for their advantage, thereby generat-

FIG 8 The effect on DC maturation mediated by HHV-6-specific Treg cells is dependent on a cell-cell contact mechanism. There was no suppressive effect on DC maturation if HHV-6-specific CD4⁺ and CD8⁺ Treg cells were separated in the transwell system. (A) Treated and untreated DCs were stained with anti-CD83, -CD80, -CD86, and -HLA-DR antibodies and analyzed by FACS. DCs treated with or without HHV-6-specific CD4⁺ (C4) or HHV-6-specific CD8⁺ (P7) Treg cells or HHV-6-specific effector CD4⁺ T cells (W1) in a transwell system both expressed large amounts of CD83, CD80, CD86, and HLA-DR after supplementing with TNF- α . The percentage of cells expressing and mean fluorescence intensities (MFI) for CD83, CD80, CD86, and HLA-DR in the treated and untreated DCs are indicated. (B) Summary of the percent molecule expression and mean fluorescence intensities (MFI) in DCs from different treatment groups. Boxes represent the upper and lower values of the data, and the dividing line in the box represents the median. The results shown are from five independent experiments.

ing conditions that ensured their survival. These strategies included evasion of immunity by antigenic variation, interference with antigen processing or presentation, and subversion of phagocytosis or killing by cells of the innate immune system (10, 21).

Another important strategy used by pathogens to extend their survival involves the induction of regulatory responses that are normally associated with the inhibition of effector immune responses of the host. This could be achieved directly through the

FIG 9 Impairment of DC functions by HHV-6-specific Treg cells. (A) Significant inhibition of DC capacity to stimulate naive CD4⁺ T cell proliferation induced by treatment with HHV-6-specific CD4⁺ (C4) or CD8⁺ (P7) Treg cells ($P < 0.01$). Proliferation is plotted as mean $\pm$ SD. The results are representative data from three independent experiments. (B) There was no difference in naive CD4⁺ T cell proliferation stimulated by DCs after treatment with HHV-6-specific CD4⁺ (C4) or CD8⁺ (P7) Treg cells in the transwell system ($P > 0.05$). Proliferation is plotted as mean $\pm$ SD. Results are representative data from three independent experiments.

induction of suppressive cytokines, such as IL-10 (22) and TGF- β (23), which are produced by innate immune cells in response to pathogen-derived molecules, or through the generation of Treg cells. Increasing evidence has indicated that infection with some viruses, such as EBV, HBV, or HCV, could induce virus-specific Treg cells (12–14), which contribute to the increase of the viral load or the inhibition of antiviral T cell responses. In the current study, we further identified that HHV-6 infection can also induce HHV-6-specific Treg cells. These Treg cells had a broad immunosuppressive capacity that inhibited innate and adaptive immune cell functions, which may be critical for HHV-6-mediated latency and survival.

Several subsets of Treg cells, such as naturally occurring CD4⁺ CD25⁺ FoxP3⁺ Treg cells and adaptively induced Tr1, Th3, and

CD8⁺ Treg cells, have been identified in humans (24–27), the latter of which could also be provoked by specific pathogen-derived antigens. However, the majority of current studies have focused mainly on the roles and functional regulation of various CD4⁺ Treg cells during pathogen infections, and little is known about the functional role of CD8⁺ Treg cells in pathogen-mediated chronic infection, latency, and immunosuppression (28–31). One of the important findings in the present study was that HHV-6 infection not only induces HHV-6-specific CD4⁺ Treg cells but also induces virus-specific CD8⁺ Treg cells. These HHV-6-specific CD8⁺ Treg cells shared similar phenotypic features with CD4⁺ Treg cells exhibiting the CD4⁺ Treg-associated molecules FoxP3, CD25, and GITR. Cytokine profile analyses showed that suppressive HHV-6-specific Treg cell clones secreted IFN- γ and

IL-10 but no or little IL-2, IL-4, and TGF- β , which were similar to the finding by Nastke et al. (32). This study indicated that IFN- γ and IL-10 were both appropriate markers of the HHV-6-mediated cellular responses (32). However, our studies showed that HHV-6-specific effector T cell clones (W1 and C7) secreted IL-2 and IFN- γ but little or no IL-4, IL-10, and TGF- β after stimulation with HHV-6 lysates (Fig. 3B). It is true that mouse Treg cells express no or little IFN- γ and highly express FoxP3. However, IFN- γ secretion from tumor-specific Treg cells has been reported in melanoma patients (19). IFN- γ -induced Treg (iTreg) cells were also reported to be immunosuppressive and to play a key role in renal transplant recipients with stable long-term graft function and in patients with diabetes, as well as in mice with experimental colitis (33–35). There is no specific marker for human Treg cells, especially in inducible Treg cells. We could identify these human Treg cells only based on their suppressive function. Importantly, these HHV-6-specific Treg cells also have potent suppressive activity. HHV-6-specific Treg cells could strongly suppress naive and HHV-6-specific effector T cell immune responses and impair DC maturation and function. Innate immunity plays a critical role in the host response to HHV-6 infection. DCs are important components of the innate immune system that sense virus products and initiate immune responses against pathogens. Moreover, DCs are crucial for activating and conditioning HHV-6-specific T cells. In this process, DCs are largely influenced by the preceding innate immune response (36, 37). Impairment of DC maturation and function by HHV-6-specific Treg cells not only suppresses innate immunity but also influences the adaptive immune response to HHV-6 infection (38). Our studies clearly suggest that HHV-6 may utilize the induction of Treg cells as a strategy to escape anti-virus immune responses and maintain latency and immunosuppression in infected hosts.

Once Treg cells were activated, they suppressed CD4⁺ or CD8⁺ T cells in an antigen-nonspecific manner. Several mechanisms have been proposed to explain how CD4⁺ Treg cells inhibit CD4⁺ effector T cells (39–41). Some investigators have shown that IL-10 and/or TGF- β was directly involved in Treg cell-mediated suppression, while others argued that cell-cell contact was required for Treg suppression (42–45). In our study, although HHV-6-specific Treg cells secreted high levels of IL-10, their suppressor activities were mainly through a cell-cell contact-dependent mechanism but not through the identified cytokines. The mechanisms responsible for their suppression therefore require further study. Notably, recent studies have suggested that chronic inflammation may create a specific cytokine environment favoring the expansion of Treg cells (46, 47). Suppressive cytokines such as IL-10 and TGF- β not only recruited Treg cells to the infection sites but also favored the conversion of nonsuppressive T cells into Treg cells. Through the production of suppressive cytokines, HHV-6-specific Treg cells could possibly amplify the immunosuppression mediated by Treg cells during persistent and chronic virus infection. In addition to suppressive cytokines, multiple mechanisms have been identified for the induction of Treg cells in different experimental systems. Antigen-presenting cells, such as DCs, are essential for the expansion of Treg cells through costimulatory molecules B7 and ICOS and soluble factors, including TGF- β and prostaglandin E2 (PGE2). Recent studies have shown that Treg cells expand in response to *Mycobacterium tuberculosis* through mechanisms dependent on ManLAM, PGE2, PD-1, CISH, and PD-L1 (48–50).

Recent studies have suggested that some peptides from HHV-6 will allow HHV-6-specific CD4⁺ or CD8⁺ T cells to recognize HHV-6-infected target cells, thus overcoming the immunosuppressive properties of the virus (51, 52). Another challenging issue is the identification of pathogen-derived antigens which can specifically induce and activate virus-specific Treg cells. Our studies have shown that HHV-6-specific Treg cells could be specifically induced by HHV-6 lysates. These studies suggested that the chronic and persistent stimulation of some epitopes from the HHV-6 antigens might favor the generation of Treg cells. However, the peptides expressed in HHV-6 which are recognized by these HHV-6-specific Treg cells have not been identified in this study. Our future studies focusing on this issue may provide novel targets for the prevention of and/or therapy for HHV-6-associated diseases.

In summary, HHV-6 infection could induce both CD4⁺ and CD8⁺ HHV-6-specific Treg cells. They not only suppress activities of naive and HHV-6-specific CD4⁺ effector T cells but also impair DC maturation and function. Cell-cell contact was required for HHV-6-specific Treg cell-mediated immune suppression. Our findings not only will facilitate the understanding of mechanisms regarding HHV-6-mediated evasion, latency, and immunosuppression but also have important implications for the development of novel strategies to prevent infection and reverse immunosuppression mediated by HHV-6 and other types of viruses as well.

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