Antiviral activity of human γδ-T cells against enterovirus 71

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KEY MESSAGES

1. Vγ9Vδ2-T cells can directly kill enterovirus (EV) 71–infected cells.
2. The cytotoxicity of Vγ9Vδ2-T cells against EV71-infected cells is mediated by the Fas-FasL pathway.
3. Vγ9Vδ2-T cells have non-cytolytic antiviral activity against EV71 through their secreted IFN-γ.

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Introduction

Enterovirus 71 (EV71) is the most common cause of hand foot and mouth disease (HFMD).[1] Although the clinical manifestations of HFMD are usually mild and self-limiting, HFMD caused by EV71 is occasionally associated with severe complications such as encephalitis, aseptic meningitis, and brain stem encephalitis.[1] HFMD usually occurs in infants and young children and has become a major threat to public health in Southeast Asia (including Hong Kong) and mainland China.[1] Currently, there are no antiviral agents against EV71 infection. Therefore, there is a need for development of novel antiviral strategies for EV71 infection.

γδ-T cells play an important role in the defence against pathogens and tumours.[2] Phosphoantigens pamidronate (PAM) and isopentenyl pyrophosphate (IPP) can selectively activate and expand by up to 50-fold the Vγ9Vδ2-T cells in human peripheral blood.[3,4] PAM and IPP also trigger γδ-T cells to produce IFN-γ and other cytokines and chemokines, hence displaying their antiviral activities.[5] Vγ9Vδ2-T cells expanded by IPP or PAM can efficiently kill influenza virus–infected monocyte-derived macrophages (MDMs) thus reducing viral replication in vitro.[3,4] Vγ9Vδ2-T cells can also inhibit human influenza virus replication by releasing IFN-γ.[5] However, whether PAM-expanded γδ-T cells can efficiently kill EV71-infected cells and inhibit the virus replication (and their underlying mechanisms) remain unknown.

We aimed to investigate the antiviral cytokine responses of human Vγ9Vδ2-T cells and the cytotoxic and non-cytolytic antiviral activities of Vγ9Vδ2-T cells against EV71.

Methods

Human EV71 were propagated in Vero cells with DMEM and 5% FBS. The virus titre was also determined by Vero cell infection. The cytopathic effect of the infected cell was observed daily, and the TCID 50 was calculated according to the Reed-Muench formula.

PAM-expanded Vγ9Vδ2-T cells were generated from human peripheral blood mononuclear cells.[3] MDM were generated from monocytes as described previously.[4] Human peripheral blood mononuclear cells were infected with virus at multiplicity of infection of 2 or treated with mock; cells were harvested every 4 hours; brefeldin A was added at 4h before harvesting. Cells were stained for surface markers (Vδ2, CD3, Fas, and FasL from Biolegend) and then fixed and permeabilised and stained with intracellular cytokines (IFN-γ, TNF-α, perforin, granzyme A and B, or their respective isotype controls from Biolegend). All sample data were acquired by LSR II flow machine (BD Bioscience). The flow cytometry data was analysed by FlowJo software (8.8.6).

PAM-expanded Vγ9Vδ2-T cells (effector (E)) were co-cultured with EV71-infected MDM or mock-treated MDM (targets (T)) at different T:E ratios: 1:0, 1:10, 1:20, and 1:30 for 2 to 4 hours. The cell-free coculture medium was harvested for lactic dehydrogenase–based in vitro toxicology assay (Sigma-Aldrich) to determine Vγ9Vδ2-T cells cytotoxicity as described by supplier instructions. The percentage of dead MDM was calculated by sample optical density value divided by the optical density value of 100% lysed MDM. The blocking assay was performed by first incubated Vγ9Vδ2-T with neutralisation antibodies (mouse anti-human NKG2D, mouse anti TRAIL, Fas L, and standardised IgG1 isotype control antibodies at 10 μg/mL) or perforin inhibitors concanamycin A and granzyme blocker, Bcl2 for 30 mins before co-culture with vMDM at different T:E ratios. After co-culture for 4

hrs, the cell free supernatant was assayed with lactic dehydrogenase method as above.

VP1 gene copies in the cells and supernatant were quantified by SYBR green real-time RT-PCR. The supernatant and infected cells were processed for RNA using RNA extraction kit (TAKARA) and same known amount of RNA of each samples were converted to cDNA for Q-PCR by TAKARA reverse transcription kit. The expression of viral VP1 gene was accessed by ABI Prism 7900 detection system. Results were expressed as the number of target gene copies per 10^5 MDMs.

To evaluate cell-cell contact requirement for the direct killing of Vγ9Vδ2-T cells, the Transwell (24 wells, pore size 0.45 μm; Millipore) system was used. Target cells (T) in the bottom were infected by EV71 at multiplicity of infection of 2. Vγ9Vδ2-T cells (E) were added directly into the bottom wells or into Transwell inserts at a T: E ratio of 1: 10. After 4 h, the supernatant in the bottom wells were harvested and analysed for cell death by lactic dehydrogenase assay as above. To determine the non-cytolytic antiviral activity of Vγ9Vδ2-T cells, a 24-well Transwell culture system as described above with smaller pore size at 0.45 um was used. PAM-expanded Vγ9Vδ2-T cells were added in the upper wells, and 100 000 MDM were plated in lower wells with virus infection (multiplicity of infection of 2). The EV71 viral RNA copies in MDM and supernatants were quantified by Q PCR as described in the above. To investigate which soluble molecules were involved in the non-cytolytic antiviral activity of Vγ9Vδ2-T cells, the same Transwell system and blocking assay as described above were used.

Data were expressed as mean ± SEM. Statistical significance was determined by paired or nonpaired parametric-test using GraphPad Prism 5 software. $P < 0.05$ was considered to be significant.

Results

To determine the magnitude and kinetics of the antiviral cytokine responses of human Vγ9Vδ2-T cells in response to EV71, human peripheral blood mononuclear cells were infected with EV71 virus at multiplicity of infection of 2 or mock for 24 hours, and surface expressions of Fas and FasL, and intracellular expressions IFN-γ, TNF-α, perforin, granzyme A and B were analysed at different time points by FACS. Human Vγ9Vδ2-T cells had optimal responses to EV71 after 16 hours of infection. Vγ9Vδ2-T cells had very strong responses to EV71 in terms of the induction of IFN-γ and TNF-α. However, EV71- and mock-treated groups did not differ significantly in terms of Fas, FasL, granzyme A and B, and perforin expressions in Vγ9Vδ2-T cells.

To determine whether Vγ9Vδ2-T cells could kill EV71-infected MDM, we used lactic dehydrogenase assay to examine their cytolytic activities during the co-culture of PAM-expanded Vγ9Vδ2-T cells (effector, E) with EV71-infected autologous MDM (target, T). Vγ9Vδ2-T cells could kill EV71-infected MDM. Using the Transwell culture assay, we found that Vγ9Vδ2-T cells lost their cytotoxicity toward EV71-infected MDM when Vγ9Vδ2-T cells were physically separated from EV71-infected MDM. This result indicated that cell-cell contact is required for the direct killing of Vγ9Vδ2-T cells.

To understand mechanisms underlying the direct killing of PAM-expanded Vγ9Vδ2-T cells, neutralisation mAbs for NKG2D, FasL, TRAIL, perforin-specific inhibitor concanamycin A, and granzyme B inactivator Bcl-2 were used. Only the blockade of FasL significantly reduced the cytotoxicity of Vγ9Vδ2-T cells. However, the blockade of other pathways such as KGN2D, TRAIL, perforin, and granzyme B did not inhibit Vγ9Vδ2-T cells cytotoxicity. These results indicated that the direct killing of Vγ9Vδ2-T cells against EV71-infected cells was mediated through Fas/FasL pathway.

To evaluate whether PAM-expanded Vγ9Vδ2-T cells can inhibit EV71 replication through their released soluble molecules, the 24-well Transwell culture system was used. Even when Vγ9Vδ2-T cells were physically separated from EV71-infected MDM, the EV71 VP1 gene copies in EV71-infected MDM and culture supernatants were significantly reduced, indicating that the soluble molecules released from PAM-expanded Vγ9Vδ2-T cells can inhibit the EV71 replication. To understand which soluble molecules were involved in this inhibition, the IFN-γ neutralisation mAb, perforin-specific inhibitor concanamycin A, and granzyme B inactivator Bcl-2 were added into the Transwell culture system. Inhibition of EV71 viral replication was significantly abrogated only when IFN-γ neutralising mAb was added. These results indicated that the non-cytolytic antiviral activity of PAM-expanded Vγ9Vδ2 T cells against EV71 mainly depended on IFN-γ.

Discussion

We demonstrated that human Vγ9Vδ2-T cells have both cytotoxic and non-cytolytic antiviral activities against EV71. To the best of our knowledge, this is the first report to demonstrate that γδ-T cells have a potent antiviral activity against EV71.

γδ-T cells play an important role in the defence against pathogens and tumours.[2] They have broad antiviral activities against different viruses. In our previous studies, we demonstrated that phosphoantigen-expanded Vγ9Vδ2-T cells could efficiently kill influenza virus–infected MDM and thus reducing viral replication in vitro, and that this cytotoxicity against influenza virus–infected MDM depended on NKG2D activation and mediated by

Fas-FasL and perforin-granzyme B pathways.[3,4] In this study, we showed that PAM-expanded Vγ9Vδ2-T cells could directly kill EV71-infected MDM and reduce EV71 replication. However, we found only Fas-FasL pathway was involved in the killing of Vγ9Vδ2-T cells against EV71-infected MDM. These data suggest that Vγ9Vδ2-T cells may use different pathways to kill different virus-infected cells.

In our previous study, we showed that Vγ9Vδ2-T cells can produce IFN-γ to inhibit human influenza H1N1 viral replication.[5] In this study, we found that Vγ9Vδ2-T cells produced a large amount of IFN-γ in response to EV71 as compared to TNF-α. Using the Transwell culture system and IFN-γ neutralisation mAb, we demonstrated that Vγ9Vδ2-T cells may inhibit EV71 replication through their released IFN-γ. Therefore, Vγ9Vδ2-T cells have non-cytolytic antiviral activity against EV71.

Conclusion

Vγ9Vδ2-T cells have both cytotoxic and non-cytolytic antiviral activities against EV71. The cytotoxicity of Vγ9Vδ2-T cells against EV71-infected MDM is mediated by Fas-FasL pathway. IFN-γ released from Vγ9Vδ2-T cells can inhibit EV71 viral replication. Our study suggests a novel approach by using pamidronate to activate and expand Vγ9Vδ2-T cells against EV71 infection.

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