Phosphoantigen-Expanded Human γδ T Cells Display Potent Cytotoxicity against Monocyte-Derived Macrophages Infected with Human and Avian Influenza Viruses

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Background. Influenza virus is a cause of substantial annual morbidity and mortality worldwide. The potential emergence of a new pandemic strain (eg, avian influenza virus) is a major concern. Currently available vaccines and anti-influenza drugs have limited effectiveness for influenza virus infections, especially for new pandemic strains. Therefore, there is an acute need to develop alternative strategies for influenza therapy. γδ T cells have potent antiviral activities against different viruses, but no data are available concerning their antiviral activity against influenza viruses.

Methods. In this study, we used virus-infected primary human monocyte-derived macrophages (MDMs) to examine the antiviral activity of phosphoantigen isopentenyl pyrophosphate (IPP)–expanded human Vγ9Vδ2 T cells against influenza viruses.

Results. Vγ9Vδ2 T cells were selectively activated and expanded by IPP from peripheral blood mononuclear cells. IPP-expanded Vγ9Vδ2 T cells efficiently killed MDMs infected with human (H1N1) or avian (H9N2 or H5N1) influenza virus and significantly inhibited viral replication. The cytotoxicity of Vγ9Vδ2 T cells against influenza virus–infected MDMs was dependent on NKG2D activation and was mediated by Fas–Fas ligand and perforin–granzyme B pathways.

Conclusion. Our findings suggest a potentially novel therapeutic approach to seasonal, zoonotic avian, and pandemic influenza—the use of phosphoantigens to activate γδ T cells against influenza virus infections.

Influenza A (fluA) virus is a cause of substantial annual morbidity and mortality worldwide. The potential emergence of a new pandemic strain through natural reassortment (eg, avian influenza virus) is a major concern [1–4]. Both the innate and adaptive immune systems play critical roles against fluA viruses, and direct manipulation of the immune system can protect individuals from fluA virus infections [5]. Because the next pandemic influenza virus subtype cannot be predicted with certainty, vaccines are unlikely to be available in sufficient supply to confront the next pandemic [4]. Therefore, alternative strategies to enhance innate immune responses—the first line of defense—have obvious benefits as an early therapeutic intervention for an influenza pandemic.

Unlike αβ T cells, γδ T cells constitute only a small

proportion (2%–10%) of T lymphocytes in the blood and peripheral organs of most adult animals and humans [6–8]. In humans, Vγ9Vδ2 T cells make up the majority of peripheral blood and lymphoid organ γδ T cells [9]. Human γδ T cells share characteristics of T cells, natural killer (NK) cells, and antigen-presenting cells [6–8]. Therefore, γδ T cells are thought to represent one of the first lines of the host immune defense.

The antiviral activities of γδ T cells have been demonstrated in different models [10–12]. In the mouse model, γδ T cells were shown to contribute to recovery from influenza pneumonia [13, 14], but no data are available on the contribution of γδ T cells at early stages of influenza virus infections. Activated mouse γδ T cells showed profound cytotoxicity against hemagglutinin (H1 or H3) expressing target cells in a non–major histocompatibility complex–restricted manner [15]. However, it is still unknown whether human γδ T cells have antiviral activities against human or avian fluA viruses or what their underlying mechanisms are.

Vγ9Vδ2 T cells are specifically activated in an HLA-unrestricted manner by small nonpeptidic phosphoantigens, which are metabolites of isoprenoid biosynthesis pathways in all organisms [16]. The most potent phosphoantigen is hydroxy-dimethylallyl-pyrophosphate, produced through a nonmevalonate pathway in microorganisms such as mycobacteria. Natural isopentenyl pyrophosphate (IPP), an intermediate produced through the mevalonate pathway that also leads to cholesterol synthesis in mammalian cells, was found to selectively activate and expand human Vγ9Vδ2 T cells in vitro or in vivo [17, 18]. Vγ9Vδ2 T cells expanded by synthetic phosphoantigens were demonstrated to have antiviral potential against other viruses [19, 20], but no data are available concerning their antiviral activities against human and avian fluA viruses.

One characteristic of the host immune response to fluA virus infection is the influx of both macrophages and T lymphocytes into the lungs [21]. We recently demonstrated that the macrophage is one of the major target cells for avian H5N1 virus in human lungs apart from alveolar epithelial cells [22]. Moreover, H1N1 and H5N1 viruses have been shown to replicate efficiently in both human primary lung epithelial cell cultures and macrophages in vitro [23]. Using the model of fluA virus–infected monocyte-derived macrophages (MDMs), we have found that macrophages infected with human and avian fluA viruses express differential proinflammatory cytokines and chemokines [23, 24] and exhibit differential apoptosis-inducing capability in T cells through the tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) pathway [25].

In the present study, we used the similar virus-infected MDMs model to examine the cytotoxicity of phosphoantigen-expanded Vγ9Vδ2 T cells against fluA viruses. Specifically, we demonstrated for the first time that IPP-expanded Vγ9Vδ2 T cells could efficiently kill MDMs infected with human (H1N1)

and avian (H9N2 and H5N1) fluA viruses and inhibit virus replication. The cytotoxicity of IPP-expanded Vγ9Vδ2 T cells was dependent on NKG2D activation and mediated by perforin–granzyme B and Fas–Fas ligand (FasL) pathways. Our findings suggest that phosphoantigen could be used to activate Vγ9Vδ2 T cells against fluA infections, in particular for avian fluA virus infections.

METHODS

Generation of IPP-expanded Vγ9Vδ2 T cells. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy-coat preparations of blood from healthy donors from the Hong Kong Red Cross by Ficoll-Paque (Pharmacia) gradient centrifugation, as described elsewhere [26]. The research protocol was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster. PBMCs were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum. IPP (Sigma) was added on days 0 and 3, to a final concentration of 6 μg/mL. Recombinant human interleukin 2 (IL-2) (Invitrogen) was added every 3 days beginning on day 3, to a final concentration of 500 IU/mL. After being cultured for 14 days, the cells were purified by negative selection with a TCRγ/δ+ T cell isolation kit (Miltenyi Biotec), in accordance with the manufacturer's instructions.

Culture of MDMs. Human MDMs were generated from mononuclear cells, as described elsewhere [25]. Briefly, adherent monocytes were seeded in 96-well flat-bottomed plates at 1×10^5 cells/well or in 24-well plates at 5×10^5 cells/well. They were then re-fed by RPMI 1640 supplemented with 5% autologous serum and allowed to differentiate to macrophages for 14 days. The purity of monocytes, as determined by flow cytometry with anti-CD14 monoclonal antibody, was consistently >90%.

Virus preparation, titration, and infection. Human fluA H1N1 (A/Hong Kong/54/98) and avian fluA H9N2 (A/Quail/Hong Kong/G1/97) and H5N1 (A/Hong Kong/483/97) viruses were used. All the viruses were cultured in Madin-Darby canine kidney cells (American Type Culture Collection), as described elsewhere [25]. The virus titer was determined by daily observation of cytopathic effect, and the median tissue culture infective dose was calculated according to the Reed-Muench formula. Differentiated MDMs on day 14 were infected by fluA viruses at a multiplicity of infection (MOI) of 2. After 1 h of viral adsorption, the cells were washed with phosphate-buffered saline to remove unadsorbed virus.

Cytotoxicity assay. Vγ9Vδ2 T cells (effector) were cocultured with H1N1-, H9N2-, or H5N1-infected MDMs (target) at different effector-to-target (E:T) ratios for 4–6 h. Afterward, nonadherent cells were harvested directly. Adherent cells were detached with 0.25% trypsin–ethylenediaminetetraacetic acid.
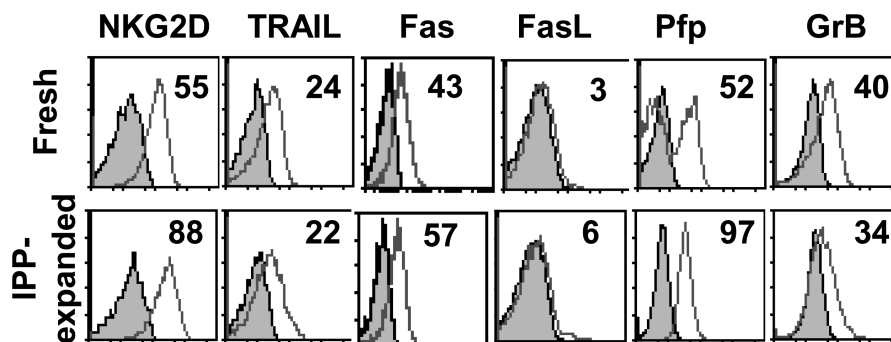
Figure 1. Fresh and isopentenyl pyrophosphate (IPP)–expanded Vγ9Vδ2 T cell phenotypes. Vγ9Vδ2 T cells in fresh peripheral blood mononuclear cells were analyzed by flow cytometry on day 0, and IPP-expanded cells were analyzed after being cultured for 14 days. White histograms depict surface expression of NKG2D, Fas, Fas ligand (FasL), tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), intracellular perforin (Pfp), and granzyme B (GrB), and gray histograms depict isotype controls. Data are representative of 4 separate experiments.

All of the adherent and nonadherent cells were then stained with anti-CD3 to identify Vγ9Vδ2 T cells and ethidium homodimer 2 (EthD-2) to identify dead cells [27]. The cytotoxicity of Vγ9Vδ2 T cells against virus-infected MDMs was assessed by flow cytometry as the percentage of EthD-2$^+$ cells in the CD3$^-$ population.

Transwell culture of Vγ9Vδ2 T cells and fluA virus–infected MDMs. To evaluate cell-cell contact requirement for Vγ9Vδ2 T cell cytotoxicity, a transwell system was used (24 wells; pore size, 0.4 μm; Millipore). MDMs (target) in the bottom well were infected with H1N1 or H9N2 virus at an MOI of 2. Vγ9Vδ2 T cells (effector) were added directly into the bottom wells or into transwell inserts at an E:T ratio of 10:1. In some inserts, the same amount of virus-infected MDMs was added to activate Vγ9Vδ2 T cells. After 6 h, the MDMs in the bottom wells were harvested and analyzed for cell death, as described above.

Blocking assay. Vγ9Vδ2 T cells (effector) were cocultured with H1N1 virus–infected or H9N2 virus–infected MDMs (target) at an E:T ratio of 10:1 for 6 h. The neutralization antibodies anti-NKG2D (10 μg/mL; 1D11, BD Biosciences), anti-FasL (10 μg/mL; 100419, R&D Systems), anti-TRAIL (10 μg/mL; RIK-2, R&D Systems) and their respective isotype controls were added in the coculture for blocking NKG2D-, FasL-, and TRAIL-mediated pathways, respectively [28]. For blocking perforin and granzyme B, the perforin inhibitor concanamycin A (CMA) (1 μg/mL; Sigma) and the granzyme B inactivator Bcl-2 (1 μg/mL; R&D Systems) were used, as described elsewhere [29]. Cytotoxicity was analyzed by flow cytometry, as described below, and calculated as the percentage of inhibition relative to that in controls.

Flow cytometry. Cells were stained for surface markers with the following antibodies: anti-CD3 (HIT3a), anti–T cell receptor (TCR)–γ9 (B3), anti–TCR-δ2 (B6), anti-NKG2D (1D11), anti-TRAIL (RIK-2), anti-CD107a (H4A3) (BD Biosciences),

anti-TCR-γδ (5A6.E9), anti-CD14 (TUK4), and anti-FasL (Alf-2.1) (Invitrogen). For intracellular staining, cells were fixed, permeabilized, and then stained with anti-perforin (Pfp, δG9) and anti–granzyme B (GrB, GB11) antibodies (BD Biosciences) or their respective isotype controls. All samples were acquired using a BD FACSAria cell sorter (BD Biosciences) and were analyzed by means of FlowJo software (version 8.8.3; Tree Star).

Quantification of fluA viral copies by reverse-transcription polymerase chain reaction. MDMs (1×10^5) were infected with H1N1, H9N2, or H5N1 virus at an MOI of 2. One hour later, unadsorbed virus was washed away carefully and the MDMs were cultured alone or with 1×10^6 Vγ9Vδ2 T cells for 48 h. The cells and supernatant were then harvested, and total RNA was extracted by means of TRIzol LS reagent (Invitrogen), in accordance with the manufacturer's instructions. Complementary DNA was synthesized with oligo(dT)$_{12-18}$ primer and Superscript II reverse transcriptase (Invitrogen). Viral matrix gene copies were quantified on the basis of a SYBR green fluorescence signal after polymerase chain reaction (forward primer, 5′-CTTCTAACCGAGGTCGAAACG-3′; reverse primer, 5′-GGCATTTTGGACAAAGCGTCTA-3′) by means of the ABI PRISM 7700 sequence detection system (Applied Biosystems). Results were expressed as the number of target gene copies per 1×10^5 MDMs.

Statistical analysis. Data were expressed as means ± standard errors of the mean. Statistical significance was determined by the Student t test or nonparametric tests using GraphPad Prism software (version 5). Differences were considered significant at $P < .05$.

RESULTS

Selective activation and expansion of Vγ9Vδ2 T cells by IPP. Consistent with previous findings [18, 19], we found that IPP and IL-2 could selectively expand Vγ9Vδ2 T cells. Freshly iso-
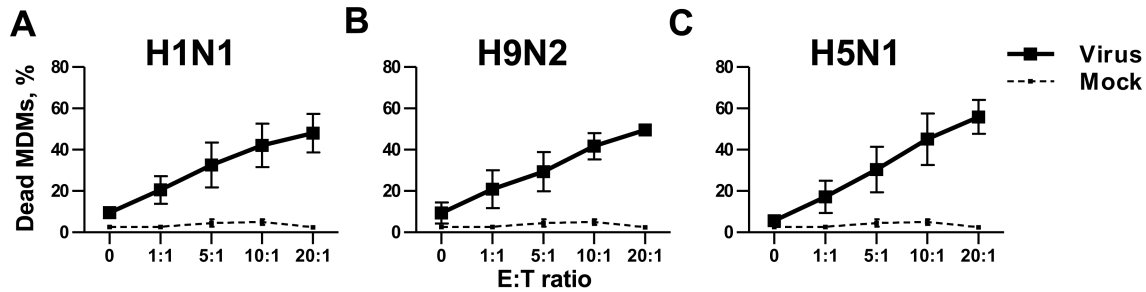
Figure 2. Potent cytotoxicity of isopentenyl pyrophosphate (IPP)–expanded Vγ9Vδ2 T cells against human monocyte-derived macrophages (MDMs) infected with human and avian influenza A viruses. MDMs (target) were either mock infected or infected with influenza H1N1 (*A*), H9N2 (*B*), or H5N1 (*C*) virus (multiplicity of infection, 2) and then cocultured with purified IPP-expanded Vγ9Vδ2 T cells (effector) at various effector-to-target (E:T) ratios (range, 0:1 to 20:1) for 4–6 h. The mean percentages of dead MDMs among whole target cells (CD3⁻ population) identified as CD3⁻ and ethidium homodimer 2⁺ for 4 separate experiments are shown; error bars indicate standard errors of the mean.

lated PBMCs contained 1%–5% of Vγ9Vδ2 T cells from 12 randomly selected healthy adult donors; after 14 days of in vitro culture in the presence of IPP and IL-2, the Vγ9Vδ2 T cells were expanded by 36-fold (range, 19–55-fold), and the percentage of Vγ9Vδ2 T cells within PBMCs increased to 60%–80%. IL-2 alone did not increase the absolute number of Vγ9Vδ2 T cells. The purity of Vγ9Vδ2 T cells after magnetic sorting was consistently >97%.

To determine the phenotypes of the Vγ9Vδ2 T cells, we examined cell surface markers (NKG2D, Fas, FasL, and TRAIL) and intracellular cytolytic granules (perforin and granzyme B) in both fresh and IPP-expanded Vγ9Vδ2 T cells. As shown in figure 1, fresh Vγ9Vδ2 T cells expressed medium levels of NKG2D, Fas, TRAIL, perforin, and granzyme B, whereas the expanded Vγ9Vδ2 T cells had much higher levels of NKG2D and perforin and similar levels of Fas, TRAIL, and granzyme B compared with fresh Vγ9Vδ2 T cells. In contrast, both fresh and expanded Vγ9Vδ2 T cells expressed little or no FasL (figure 1).

Efficient killing of human and avian fluA virus–infected MDMs by Vγ9Vδ2 T cells. To determine whether Vγ9Vδ2 T cells could kill fluA virus–infected MDMs, we examined their cytolytic activities during the coculture of IPP-expanded Vγ9Vδ2 T cells with virus-infected autologous MDMs. Vγ9Vδ2 T cells did not show any cytolytic activity against mock-infected MDMs, but they displayed potent cytotoxicity against both human fluA virus–infected and avian fluA virus–infected MDMs in a dose-dependent manner (figure 2). For human fluA H1N1–infected MDMs (target), Vγ9Vδ2 T cells (effector) killed ~50% of virus-infected MDMs at an E:T ratio of 20:1 after 6 h of coculture (figure 2*A*). Importantly, similar results were also found in avian fluA H9N2–infected and avian fluA H5N1–infected MDMs (figure 2*B* and 2*C*). There were no significant differences in the cytotoxicity of Vγ9Vδ2 T cells between these 3 fluA viruses (figure 2).

Inhibition of fluA virus replication in MDMs by Vγ9Vδ2 T cells. To determine whether there was a decreased viral load in virus-infected MDMs after coculture with Vγ9Vδ2 T cells, H1N1-, H9N2-, or H5N1-infected MDMs (target) were cultured alone or with IPP-expanded Vγ9Vδ2 T cells (effector) at an E:T ratio of 10:1. After 2 days of coculture, H1N1, H9N2, and H5N1 fluA virus matrix gene copies from whole virus–infected MDMs and culture supernatants were significantly re-
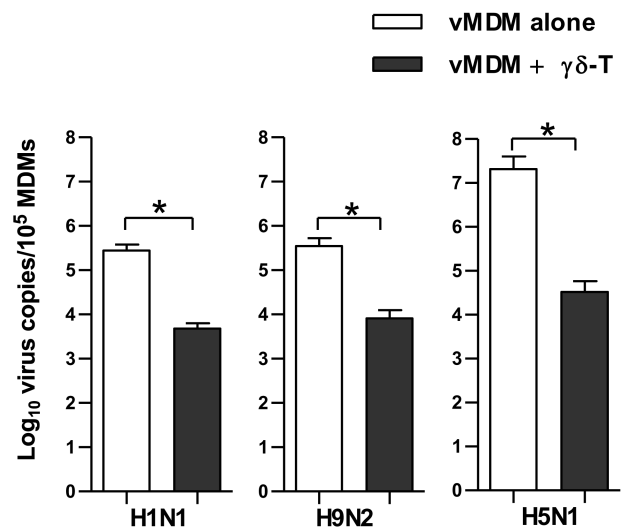
Figure 3. Inhibition of influenza virus replication in human monocyte-derived macrophages (MDMs) by Vγ9Vδ2 T cells. MDMs were infected with H1N1 (*A*), H9N2 (*B*), or H5N1 (*C*) virus (multiplicity of infection, 2). Virus-infected MDMs (vMDM) were cultured alone or with Vγ9Vδ2 T cells at an effector-to-target ratio of 10:1 for 48 h. Total RNA was extracted from both cells and supernatant, and viral matrix gene copies were quantified by reverse-transcription polymerase chain reaction, as described in Methods. Data are mean viral matrix gene copies per 1×10^5 MDMs for 4 separate experiments; error bars indicate standard errors of the mean. *P < .05.
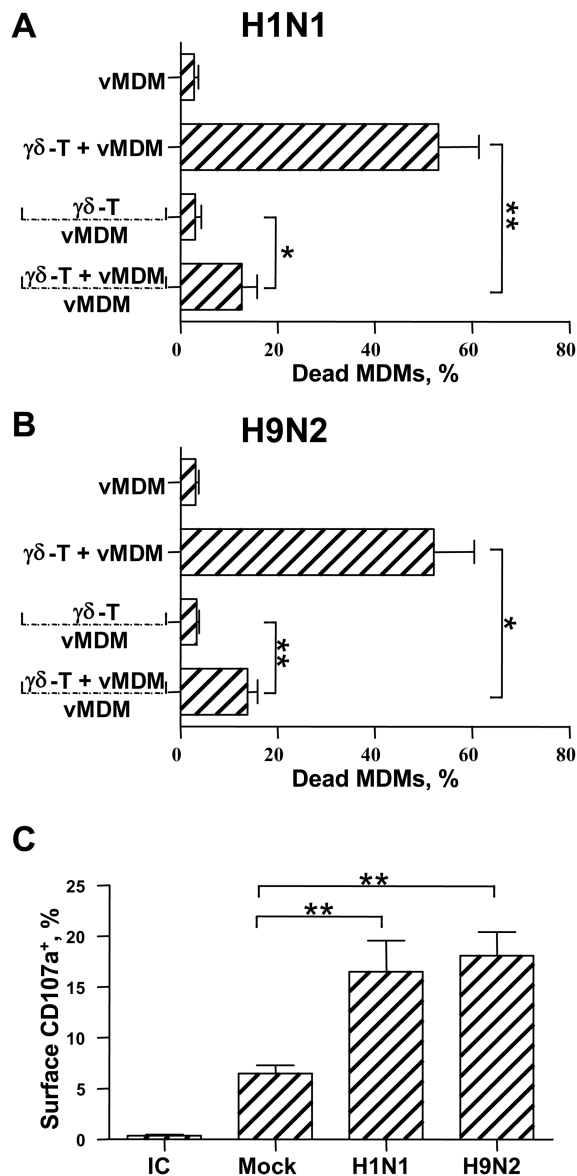
Figure 4. Dependency on cell-cell contact and requirement of virus-infected human monocyte-derived macrophage (MDM) activation for Vγ9Vδ2 T cell cytotoxicity. Vγ9Vδ2 T cells (effector) were directly cocultured with or physically separated from H1N1 virus–infected (*A*) or H9N2 virus–infected (*B*) MDMs (vMDM) (multiplicity of infection [MOI], 2) by a transwell system for 6 h at an effector-to-target (E:T) ratio of 10:1. The percentages of dead virus-infected MDMs in the direct coculture system or in the bottom wells of the transwell system were determined as described in Methods. In some experiments with the transwell system, Vγ9Vδ2 T cells were stimulated with same amount of virus-infected MDMs. Data are means for 4 separate experiments; error bars show standard errors of the mean. *C,* Up-regulation of surface CD107a expression on Vγ9Vδ2 T cells after coculture with influenza A virus–infected MDMs. Vγ9Vδ2 T cells were cocultured with mock-, H1N1-, or H9N2-infected MDMs (MOI, 2) at an E:T ratio of 10:1 for 4 h. Cells were stained with anti-CD107a monoclonal antibody and its isotype control (IC), along with staining for T cell receptor γδ and CD3. *$P < .05$; **$P < .01$.

duced in the coculture of virus-infected MDMs with Vγ9Vδ2 T cells, compared with virus-infected MDMs alone (figure 3).

Cytotoxicity of Vγ9Vδ2 T cells: dependence on cell-cell contact and requirement of virus-infected MDM activation. To determine whether efficient killing of virus-infected MDMs by Vγ9Vδ2 T cells is dependent on cell-cell contact, we used a transwell culture system. As shown in figure 4*A* and 4*B*, Vγ9Vδ2 T cells lost their cytotoxicity during H1N1 or H9N2 virus infections when Vγ9Vδ2 T cells were physically separated from virus-infected MDMs. However, when virus-infected MDMs were put with Vγ9Vδ2 T cells, cytotoxicity toward targets in the bottom wells was also observed, although their cytolytic activities were much lower than those in the direct cell-cell contact coculture. These data suggest that the cytotoxicity of Vγ9Vδ2 T cells is dependent on cell-cell contact and requires activation of virus-infected MDMs; diffusible soluble factors, such as granules released from activated Vγ9Vδ2 T cells, may also be involved.

We then sought to confirm granule release during the killing of fluA virus–infected MDMs by Vγ9Vδ2 T cells. The expressions of CD107a (lysosome-associated membrane protein 1), a marker associated with the degranulation of NK cells and cytotoxic T lymphocytes (CTLs) [30], were significantly upregulated in Vγ9Vδ2 T cells after coculture with H1N1- and H9N2-infected MDMs for 4 h, compared with mock-infected MDMs (figure 4*C*). These results suggest that virus-infected MDMs trigger more intensive granule release from Vγ9Vδ2 T cells.

Cytotoxicity of Vγ9Vδ2 T cells: dependence on NKG2D activation and mediation by Fas-FasL and perforin–granzyme B pathways. Because IPP-expanded Vγ9Vδ2 T cells also expressed high or medium levels of NKG2D, Fas, and TRAIL, we further determined whether NKG2D, Fas-FasL, and TRAIL pathways were involved in their cytotoxicity. Using neutralizing antibodies for NKG2D and FasL, we found that blockades of NKG2D and FasL significantly inhibited the cytolytic activities of Vγ9Vδ2 T cells against H1N1-infected MDMs (64.08% ± 9.15% inhibition by NKG2D blocking; 41.34% ± 9.51% by FasL blocking). In contrast, there was no significant change in their cytolytic activity against H1N1-infected MDMs after treatment with TRAIL-blocking antibody (figure 5*A*). Similar results were also found during the killing of H9N2-infected MDMs by Vγ9Vδ2 T cells (figure 5*B*). These results demonstrate that both the NKG2D and the Fas-FasL pathway are also involved in the killing of MDMs infected with human and avian fluA viruses by Vγ9Vδ2 T cells.

To further confirm the involvement of cytolytic granule release in the killing of virus-infected MDMs by Vγ9Vδ2 T cells, the perforin-specific inhibitor CMA and granzyme B inactivator Bcl-2 were used. As shown in figure 5*A*, the cytolytic activities of Vγ9Vδ2 T cells against H1N1-infected MDMs were sig-
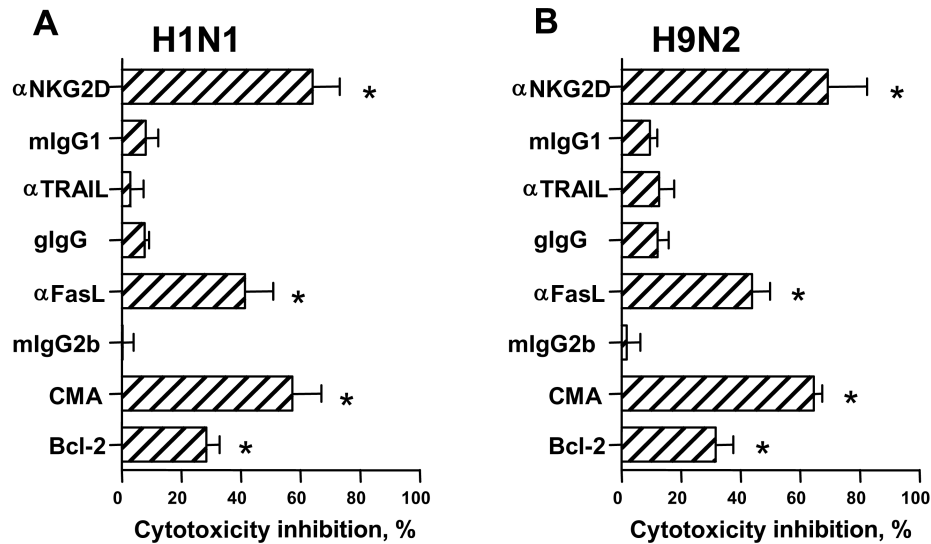
Figure 5. Dependency on NKG2D activation and mediation by Fas–Fas ligand (FasL) and perforin–granzyme B pathways for Vγ9Vδ2 T cell cytotoxicity. Vγ9Vδ2 T cells (effector) were cocultured with H1N1 virus–infected (*A*) or H9N2 virus–infected (*B*) human monocyte-derived macrophages (MDMs) (target) at an effector-to-target ratio of 10:1 for 6 h. The perforin inhibitor concanamycin A (CMA), the granzyme B inactivator Bcl-2, anti-NKG2D (αNKG2D), anti–tumor necrosis factor–related apoptosis-inducing ligand (αTRAIL), and anti-FasL (αFasL) blocking antibodies or their respective isotype controls were used as described in Methods. Cytotoxicity was analyzed by flow cytometry and calculated as the percentage of inhibition relative to that of controls. Data are means from 4 experiments; error bars represent standard errors of the mean. *$P < .05$ for the comparison with isotype control. gIgG, goat immunoglobulin G; mIgG1, mouse immunoglobulin G1; mIgG2b, mouse immunoglobulin G2b.

nificantly abrogated after CMA treatment (57.25% ± 9.77% inhibition) or Bcl-2 treatment (28.42% ± 4.41% inhibition). Similar results were also observed during the killing of H9N2-infected MDMs by Vγ9Vδ2 T cells (figure 5*B*). These results indicate that the perforin–granzyme B pathway is involved in the cytotoxicity of Vγ9Vδ2 T cells against MDMs infected with human and avian fluA viruses.

DISCUSSION

γδ T cells have been reported to play an important role in the defense against pathogens and tumors, and they have broad antiviral activities against different viruses [10, 12, 31, 32]. In human in vitro systems, γδ T cells have been shown to have potent cytolytic activities against virus-infected cells, suppressing the replication of human immunodeficiency virus, hepatitis B virus, herpes simplex virus, vaccinia virus, human cytomegalovirus, and severe acute respiratory syndrome coronavirus [20, 33–35]. In the present study, we focused on the cytotoxic properties of γδ T cells in influenza virus infection, because cell-mediated cytotoxicity is the major mechanism to eliminate virus-infected cells and thus to eliminate potential sources of new virus. In particular, as a component of innate immunity, γδ T cells may act as early responders in viral control and clearance, compared with specific CTL activity. We showed that IPP-expanded Vγ9Vδ2 T cells could kill ∼50% of human H1N1 virus–infected MDMs after 6 h of coculture. Most importantly, similar potent cytotoxic activities of these cells against MDMs

infected with the newly emerged avian fluA H5N1 virus and its precursor H9N2 virus were also found after 4–6 h of coculture. Furthermore, we found that IPP-expanded Vγ9Vδ2 T cells significantly inhibited H1N1, H9N2, and H5N1 viral replication by eliminating virus-infected MDMs. To the best of our knowledge, ours is the first study to demonstrate that IPP-expanded γδ T cells have rapid and potent antiviral activity against both human and avian fluA viruses.

Consistent with findings of other in vitro studies [17, 18], we found that the phosphoantigen IPP could selectively activate and expand human Vγ9Vδ2 T cells from PBMCs in the presence of IL-2. During 2 weeks of stimulation by IPP and IL-2, Vγ9Vδ2 T cells were expanded by ∼36-fold. In fact, phosphoantigens have demonstrated the potential to facilitate large-scale in vitro expansion of functional γδ T cells for use in adoptive immunotherapy for tumors [36] and infectious diseases in different models [19, 20]. Although the lack of murine counterparts of Vγ9Vδ2 T cells has dramatically hampered efforts to understand the in vivo roles of phosphoantigens in Vγ9Vδ2 T cells, the selective activation and expansion of these cells by phosphoantigens were also confirmed in some in vivo models, such as cynomolgus monkeys and severe combined immunodeficient mice engrafted with human peripheral blood leukocytes (human PBL-SCID mice) [17, 37]. Therefore, phosphoantigens such as IPP could be an alternative for treating human and avian fluA infections via targeting Vγ9Vδ2 T cells. Indeed, synthetic phosphoantigens named *therapeutic amino-*

bisphosphonates (pamidronate and zoledronate) have been commonly used clinically to treat osteoporosis through Vγ9Vδ2 T cell–mediated lysis of osteoclasts [38, 39].

One concern regarding γδ T cell–based immunotherapy as induced by phosphoantigens is whether these cells can traffic to infected sites, such as the lungs, during the fluA virus infection. Because γδ T cells express chemokine (C-C motif) receptor 5 and are capable of diapedesis [40], these cells should be able to migrate to inflammatory sites. A more recent study in a macaque model of *Mycobacterium tuberculosis* infection demonstrated that phosphoantigen-specific γδ T cells could accumulate at all inflammatory sites in lymphoid and nonlymphoid tissues (including the lungs) [41], suggesting that the strategy of targeting γδ T cells by phosphoantigens may be feasible for the treatment of human and avian fluA virus infections. Use of nonhuman primate models or human PBL-SCID mice to further evaluate γδ T cell–based immunotherapy for influenza virus infections in vivo may accelerate its future clinical application.

Although NKG2D was originally described as an activating receptor for NK cells, it has also been recognized as a potent costimulatory receptor of the cytotoxic functions of human Vγ9Vδ2 T cells [42, 43]. It has recently been demonstrated that NKG2D can directly activate Vγ9Vδ2 T cells and trigger their release of cytolytic granules through recognition of the NKG2D ligand [44]. In humans, it has been identified that stress-inducible major histocompatibility complex class I–related proteins A and B and members of the UL16-binding protein family (ULBP1–4 and RAET1G) are the ligands for NKG2D [45]. In different tumor models, NKG2D-mediated cytotoxicity was reported to be involved in the lysis of tumor cells by γδ T cells [18, 46]. In the present study, we showed that most IPP-expanded Vγ9Vδ2 T cells expressed NKG2D and that the cytotoxicity of Vγ9Vδ2 T cells against H1N1- and H9N2-infected MDMs was significantly blocked by NKG2D neutralizing antibody, suggesting that the killing of influenza virus–infected cells by Vγ9Vδ2 T cells requires NKG2D activation and recognition. That only influenza virus–infected MDMs expressed up-regulated major histocompatibility complex class I–related protein B, an NKG2D ligand [47], could also explain why IPP-expanded Vγ9Vδ2 T cells killed only H1N1-, H9N2-, and H5N1-infected MDMs but not mock-infected MDMs in the present study.

The Fas-FasL–mediated pathway was also shown to be involved in the killing of *Listeria monocytogenes*–infected macrophages by murine γδ T cells in vivo [48]. We found that IPP-expanded Vγ9Vδ2 T cells expressed high levels of Fas and that blockade of the Fas-FasL pathway significantly inhibited the cytotoxicity of Vγ9Vδ2 T cells against H1N1- and H9N2-infected MDMs, indicating that the Fas-FasL–mediated pathway

is also involved in the killing of influenza virus–infected cells by Vγ9Vδ2 T cells.

We recently demonstrated that TRAIL was significantly up-regulated by both H9N2 and H5N1 avian fluA viruses in human MDMs, compared with H1N1, and that avian fluA virus–infected MDMs could induce T cell apoptosis through the TRAIL pathway [25]. The present study, however, did not show involvement of the TRAIL pathway during the killing of H1N1- or H9N2-infected MDMs by Vγ9Vδ2 T cells; blockade of the TRAIL pathway did not reduce the cytotoxicity of Vγ9Vδ2 T cells against either H1N1- or H9N2-infected MDMs.

Using the transwell system, we demonstrated that the killing of fluA virus–infected MDMs by Vγ9Vδ2 T cells was dependent on cell-cell contact and required the activation of virus-infected cells. In addition, CD107a expression was increased only in Vγ9Vδ2 T cells cocultured with fluA virus–infected MDMs and not in those cocultured with mock-infected MDMs, supporting the hypothesis that granule release from Vγ9Vδ2 T cells requires virus-infected cell activation. Using the perforin inhibitor CMA and the granzyme B inactivator Bcl-2, we confirmed that perforin and granzyme B facilitated the cytolytic responses of Vγ9Vδ2 T cells to fluA virus–infected MDMs, which is consistent with the findings of other studies in tumor and other virus-infected cells [18, 20, 33–35]. Therefore, human Vγ9Vδ2 T cells closely resemble NK and CD8+ T cells in their cytotoxic function, using a predominantly granule-exocytosis mechanism to kill virus-infected cells.

In summary, we have demonstrated for the first time that Vγ9Vδ2 T cells can recognize and efficiently kill MDMs infected with human and avian fluA viruses and thus contribute to virus clearance. The cytotoxicity of Vγ9Vδ2 T cells against fluA virus–infected MDMs is dependent on NKG2D activation and is mediated by the Fas-FasL and perforin–granzyme B pathways. Our study suggests a novel approach of using phosphoantigens to activate Vγ9Vδ2 T cells against fluA virus infections—newly emerged avian fluA virus infections in particular.

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