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The aminobisphosphonate pamidronate controls influenza pathogenesis by expanding a γδ T cell population in humanized mice

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Influenza is an acute respiratory virus infection associated with significant morbidity and mortality that continues to threaten global public health (Guan et al., 2004; Palese, 2004; Tumpey et al., 2005; Dawood et al., 2009; Fraser et al., 2009; Garten et al., 2009). Both innate and adaptive immune systems play critical roles in protecting against influenza A viruses, and direct manipulation of host immunity may help protect individuals against these viruses (Peiris et al., 2009). Inactivated vaccines against seasonal influenza viruses are less effective for a novel pandemic strain, e.g., pandemic H1N1 virus (Hancock et al., 2009), although they may provide partial cross-protection (Medina et al., 2010; Tu et al., 2010). The commercially available antiviral agents, the adamantanes and neuraminidase inhibitors, target specific viral proteins, blocking virus uncoating and inhibiting virus release from infected cells, respectively. Viral mutation allows influenza viruses to evade the action of these antiviral drugs, leading to the emergence of antiviral resistance (Lackenby et al., 2008; Dharan et al., 2009; Layne et al., 2009; Moscona, 2009). The development of alternative strategies to contain virus infection by boosting innate immunity has obvious advantages for the treatment of influenza without the risk of encouraging antiviral resistance. One way to enhance innate immunity is to stimulate NK cell activity, but our recent studies demonstrated that influenza virus can use a novel strategy to evade NK cell immunity by directly killing
NK cells and inhibiting NK cell cytotoxicity (Mao et al., 2009, 2010). We therefore looked for an alternative way of augmenting innate immunity.

Another cell type that shares the characteristics of NK cells and antigen-presenting cells, playing a critical role in innate and adaptive immune responses to infectious agents and tumors, is γδ-T cells (Carding and Egan, 2002; Brandes et al., 2005; Poccia et al., 2005a; Born et al., 2006; Konigshofer and Chien, 2006; Eberl and Moser, 2009; Bonneville et al., 2010). These cells constitute only 1–5% of T lymphocytes in blood and peripheral organs of adult humans and other animals (Carding and Egan, 2002; Shen et al., 2002; Poccia et al., 2005a). In humans, the majority of peripheral blood and lymphoid organ γδ-T cells are Vγ9Vδ2 T cells, a major “innate-like” peripheral T cell subset (Carding and Egan, 2002; Beetz et al., 2008).

Vγ9Vδ2 T cells are specifically activated in an HLA-unrestricted manner by small nonpeptidic phosphoantigens, which are metabolites of isoprenoid biosynthesis pathways (Beetz et al., 2008). Isopentenyl pyrophosphate (IPP), an intermediate produced through the mevalonate pathway, was found to selectively activate and expand human Vγ9Vδ2 T cells in vitro and in vivo (Bonneville and Scotet, 2006; Puan et al., 2007; Qin et al., 2009). Pharmacological compounds, such as the aminobisphosphonates pamidronate (PAM) and zoledronic acid (ZOL), were shown to have antiviral activity (Poccia et al., 2005a; Agrati et al., 2006; Urban et al., 2010).

Phosphoantigen-expanded Vγ9Vδ2 T cells were shown to have antiviral activity (Poccia et al., 2005a; Agrati et al., 2006; Poccia et al., 2006). Recently, we demonstrated that IPP-expanded Vγ9Vδ2 T cells have potent cytotoxic activity against human and avian influenza virus–infected cells (Qin et al., 2009), but it is still not known whether PAM-expanded Vγ9Vδ2 T cells have similar effects on virus-infected cells. We wished to investigate the role of these cells in combating influenza virus infection in vivo; however, it was not possible to use the conventional mouse or ferret model for these studies because murine T cells do not express a homologue of the Vγ9Vδ2-TCR. (Sicard et al., 2005; Bonneville and Scotet, 2006; Born et al., 2006), and whether Vγ9Vδ2 T cells exist in ferret remains unknown. Studies on nonhuman primates are constrained by high cost, limited availability, and lack of genetically inbred strains suitable for adoptive cell transplantation (Pearson et al., 2008). In this study, we successfully established a humanized mouse model using C57BL/10SgAiRag2−/−γc−/− (Rag2−/−γc−/−) mice, with human peripheral blood mononuclear cells (huPBMCs). We demonstrated that PAM effectively controlled human and avian influenza virus infections and protected mice from death by boosting Vγ9Vδ2 T cell immunity. Our study suggests a novel strategy for treatment of seasonal, zoonotic, and pandemic influenza by using PAM to enhance host Vγ9Vδ2 T cell immunity.

RESULTS
Cytotoxic activity of Vγ9Vδ2 T cells against influenza virus in vitro
We recently demonstrated that IPP-expanded Vγ9Vδ2 T cells could kill human seasonal influenza H1N1/A/Hong Kong/54/98 (human H1N1) and avian influenza H9N2 and H5N1 virus–infected, monocyte-derived macrophages (MDMs; Qin et al., 2009). To determine whether PAM-expanded Vγ9Vδ2 T cells have similar effects on virus-infected cells, we examined their cytolytic activities during the co-culture of PAM-expanded Vγ9Vδ2 T cells with influenza H1N1/A/PR/8/34 (PR/8) virus–infected, autologous MDMs. PAM selectively expanded Vγ9Vδ2 T cells in vitro (Fig. S1). Similar to in our previous study (Qin et al., 2009), Vγ9Vδ2 T cells did not show any cytolytic activity against mock–infected MDMs (not depicted), but these cells displayed potent cytotoxicity against PR/8 H1N1 virus–infected MDMs (Fig. 1 A). Vγ9Vδ2 T cells significantly inhibited PR/8 H1N1 virus replication in MDMs (Fig. 1 A). In addition, PAM-expanded Vγ9Vδ2 T cells also displayed similar cytolytic activities against other influenza virus strains, such as H3N2, B, and H5N1 viruses (Fig. S2).

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 PR/8 virus–infected MDMs (Fig. 1 B). In contrast, there was no significant change on their cytolytic activity against H1N1–infected

![Figure 1. Cytotoxicity of PAM-expanded Vγ9Vδ2 T cells against virus-infected MDMs.](image-url)
The lymphocyte population comprised CD45+ lymphocytes, which had stably engrafted in Rag2-/-γc-/- mice with human peripheral mononuclear cells (huPBMCs). Human lymphocytes had stably engrafted in Rag2-/-γc-/- mice by 4 wk after transplantation, and this stable engraftment was maintained for up to 60 wk (Fig. S3). On day 28 after transplantation, ~80% of nucleated cells in the peripheral blood were human CD45+ cells and CD3+ T cells, respectively (Fig. 2 A). The lymphocyte population comprised CD3+ T cells (69.7%), CD4+ T cells (29.1%), CD8+ T cells (37.3%), CD19+ B cells (6.5%), CD56+ NK cells (22.7%), Vγ9Vδ2+ T cells (2.9%), CD25highCD4+ T cells (2.0%), and others (1.1%; Fig. 2 B). The percentages of the various human lymphocyte populations in mouse peripheral blood were comparable to that in huPBMCs before transplantation except the CD4/CD8 ratio was reversed (Table S1). A booster vaccination of tetanus toxoid (TT) in these mice induced human T and B cell proliferation, TT-specific IFN-γ secretion in human CD4 and CD8 T cells, and serum anti-TT–specific human antibody in vivo (Fig. 2 C). Therefore, these chimeric Rag2-/-γc-/- mice with functional human T and B cells, including a similar percentage of Vγ9Vδ2+ T cells, after 4 wk of huPBMC transplantation are used for following experiments and referred to as “humanized” mice.

Antiviral activity of human Vγ9Vδ2 T cells against influenza virus infection in vivo

Wild-type, Rag2-/-γc-/-, and humanized mice were infected with human seasonal influenza human H1N1 virus intranasally (i.n.). As this human H1N1 virus is not a mouse-adapted strain, it does not cause obvious disease in wild-type and Rag2-/-γc-/- mice (Fig. 3 A and Fig. S4). However, it did induce mild disease in mice reconstituted with huPBMCs, with evidence of 5–10% weight loss from day 6 after infection (Fig. 3 B). Mice reconstituted with Vγ9Vδ2 T cell–depleted huPBMCs (Fig. S5) showed comparable weight loss (Fig. 3 B).

We used adoptive transfer of human Vγ9Vδ2 T cells to investigate their antiviral activity in vivo. Highly purified (>97%) PAM-expanded Vγ9Vδ2 T cells (10⁶ cells/mouse) were adoptively transferred into influenza PR/8 virus–infected mice i.v. at day 2, 4, and 6 after infection. Consequently, the frequency of Vγ9Vδ2 T cells in peripheral blood lymphocytes in humanized mice increased to a peak level at day 5, and then gradually reduced to a basal level at day 15 after infection (Table S2). Compared with PBS-treated mice, mice receiving PAM–expanded Vγ9Vδ2 T cells showed reduced weight loss and less virus replication in the lungs (Fig. 3, C and D). Importantly, more survived (Fig. 3 E). These results indicate that the cytotoxicity of Vγ9Vδ2 T cells is dependent on NKG2D activation, and mediated by Fas–FasL and perforin–granzyme B pathways.

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indicated that PAM-expanded Vγ9Vδ2 T cells can help control influenza infection in vivo.

Humanized mice contain human NK cells, αβ-T, and B cells (Fig. 2 A). Because Vγ9Vδ2 T cells can enhance NK cell activity and mediate adaptive immune responses (Brandes et al., 2005, 2009; Maniar et al., 2010), the antiviral activity of adoptively transferred Vγ9Vδ2 T cells may be associated with NK cells and adaptive immunity. To exclude this possibility, we transferred highly purified (>97%) Vγ9Vδ2 T cells into PR/8 virus–infected Rag2−/−γc−/− mice, which lack both human and murine T, B, and NK cells. As in humanized mice, the frequency of Vγ9Vδ2 T cells in peripheral blood lymphocytes in Rag2−/−γc−/− mice increased to a peak level at day 5, and then gradually reduced to a basal level at day 15 after infection (Table S2). Mice with adaptively transferred PAM-expanded Vγ9Vδ2 T cells also had significantly reduced weight loss and mortality, and reduced virus replication in the lung, compared with PBS-treated mice (Fig. 3, F–H). These data demonstrated that PAM-expanded Vγ9Vδ2 T cells by themselves can effectively control influenza infection in vivo.

PAM selectively expands human Vγ9Vδ2 T cells in humanized mice

Consistent with previous studies that PAM could selectively expand Vγ9Vδ2 T cells in humans (Kunzmann et al., 1999; Das et al., 2001), we also found that PAM specifically expanded Vγ9Vδ2 T cells, but not non-Vγ9Vδ2 γδ T cells, in vitro (Fig. S1). To determine whether this occurs in humanized mice, the mice were injected with PAM (10 mg/kg body weight) i.p. (Wang et al., 2001). A significant increase in human Vγ9Vδ2 T cells was seen in peripheral blood, spleen, and liver, but not in lung (Fig. 4, A and B). Importantly, consistent with its effect in humans (Kunzmann et al., 1999), PAM expanded only Vγ9Vδ2 T cells, with no such effect on other cells, such as CD4, CD8, B, or NK cells (Fig. 4 C). In addition, in the absence of influenza infection, there were no significant weight changes in PAM-treated Rag2−/−γc−/− or humanized mice, compared with their controls (Fig. 4 D). These results indicated that PAM can selectively expand Vγ9Vδ2 T cells in humanized mice, but by itself has no effect on weight in mice.

PAM reduces influenza disease severity and mortality

We next investigated whether PAM could control influenza infection and disease in humanized mice by expanding Vγ9Vδ2 T cells in vivo. Humanized mice were infected with PR/8 virus i.n. and injected with PAM or PBS i.p from day 3 after infection. As observed in adoptive transfer experiments (Fig. 3), treatment with PAM significantly decreased weight loss and mortality in humanized mice (Fig. 5 A). Importantly, PAM had no effect in PR/8 virus–infected Rag2−/−γc−/− mice, which do not have Vγ9Vδ2 T cells (Fig. 5 B).

In humanized mice infected with human seasonal H1N1, PAM treatment that began on day 3 after infection completely prevented weight loss (Fig. 5 C). As human seasonal H1N1 virus infection does not kill mice, the effect of PAM...
on mortality could not be investigated. Whereas no Vγ9Vδ2 T cells were seen in lungs of PAM-treated uninfected humanized mice (Fig. 4 B), the accumulation of these cells in the lungs of H1N1 virus–infected humanized mice treated with PAM on day 6, 10, and 14 after infection was significantly higher than those in PBS-treated mice (Fig. 5 D and Fig. S6). These results indicated that in vivo PAM-expanded Vγ9Vδ2 T cells can move to infected lungs to exert their antiviral activity.

Importantly, treatment with PAM from day 1 after infection significantly reduced weight loss and mortality caused by avian influenza H5N1/A/Hong Kong/486/97 (avian H5N1) virus (Fig. 5 E). Significantly more Vγ9Vδ2 T cells accumulated in avian H5N1 virus–infected lung after treatment with PAM than after treatment with PBS (Fig. 5 F and Fig. S6), indicating that PAM-expanded Vγ9Vδ2 T cells can also migrate to infected lungs to exert their antiviral activity after avian H5N1 infection. As Rag2−/−/γc−/− mice have no Vγ9Vδ2 T cells, PAM treatment had no beneficial effect in these mice after avian H5N1 infection (Fig. 5 G). These observations exclude the possibility that PAM has direct antiviral activity. Without PAM treatment, H5N1 disease progressed faster in nonhumanized Rag2−/−/γc−/− than in humanized Rag2−/−/γc−/− mice (Fig. 5 E vs. Fig. 5 G), suggesting that reconstituted huPBMCs may provide some protection during the early stage of infection.

Collectively, these results demonstrated that PAM can effectively reduce influenza disease severity and mortality caused by human H1N1, PR/8, and avian H5N1 viruses.

**PAM inhibits influenza virus replication in lung**

In humanized mice, influenza virus replicated effectively in the lung, and the viral titers of human H1N1 and avian H5N1 viruses peaked by day 6 and 10 after infection, respectively (Fig. 6). Importantly, PAM treatment significantly suppressed replication of human seasonal H1N1 virus in the lung from day 6 onwards (Fig. 6). PAM treatment similarly reduced virus titers in the lung of humanized mice infected with avian H5N1 or PR/8 by day 10 after infection (Fig. 6). These results indicated that PAM treatment leads to the control of virus replication in the lung.

**PAM prevents inflammation in infected lung**

The lungs of humanized mice infected with human seasonal H1N1 and avian H5N1 viruses differed in levels of human and/or murine proinflammatory cytokines and chemokines, such as IL-6, IL-10, TNF, MCP-1, IP-10, IL-8, or RANTES (Fig. 7, A and B; and Fig. S7). Consistent with the findings in our previous in vitro studies in primary human cells (Cheung et al., 2002), avian H5N1 virus induced higher levels of human IL-6, TNF, and IP-10 and murine IL-6 and RANTES in the lungs of humanized mice compared with H1N1 virus–infected mice (Fig. 7, A and B; and Fig. S7). PAM treatment significantly reduced levels of human IL-10, TNF, and MCP-1 (Fig. 7 A) and murine MCP-1 in the lungs of human H1N1 virus–infected mice at day 10 after infection (Fig. S7). Similarly, PAM significantly decreased levels of human IL-6, IL-10, TNF, IL-8, IP-10, and MCP-1 (Fig. 7 B) and murine IL-6, TNF, and RANTES (Fig. S7) in the lungs of avian H5N1 virus–infected humanized mice on day 10 after infection. Furthermore, there were significantly fewer inflammatory human lymphocyte (i.e., CD3) infiltrates and less pathology in the lungs of human H1N1 or avian H5N1 virus–infected, PAM-treated humanized mice on day 6, 10, and 14 after infection, compared with those in PBS-treated mice that were infected with the same viruses (Fig. 7, C and D; and Fig. S8). Collectively, these results demonstrated that PAM can attenuate the lung inflammation and pathology induced by these viruses, possibly via reduction of viral replication.
PAM cannot control influenza diseases in humanized mice without Vγ9Vδ2 T cells

To further determine whether antiviral role of PAM against influenza virus is mediated by Vγ9Vδ2 T cells in humanized mice, humanized mice reconstituted with Vγ9Vδ2 T cell–depleted huPBMCs (Fig. S5) were used. Mice reconstituted with whole huPBMCs or Vγ9Vδ2 T cell–depleted huPBMCs were infected with PR/8 virus i.n. and injected with PAM or PBS i.p. from day 3 after infection. As shown in Fig. 8, treatment with PAM significantly decreased weight loss and mortality in humanized mice with whole huPBMCs (Fig. 8). In contrast, PAM had no such effects in PR/8 virus–infected humanized mice with Vγ9Vδ2 T cell–depleted huPBMCs (Fig. 8). These results demonstrated that the antiviral effect of PAM against influenza virus is mainly mediated by a Vγ9Vδ2 T cell–dependent mechanism.

DISCUSSION

In this study, we have used humanized mice to demonstrate that PAM treatment can effectively control influenza virus–induced disease and that this beneficial effect is mediated by boosting human Vγ9Vδ2 T cell immunity. Humanized mice are a widely used, powerful, and cost-effective animal model for the study of human innate and adaptive immunity to infectious diseases (Ohashi et al., 2000; Feuerer et al., 2001; Stoddart et al., 2001; Traggiai et al., 2004; Jiang et al., 2008; Pearson et al., 2008). For example, chimeric human PBMC-SCID mice have been used to study the antibacterial and antitumor effects of human Vγ9Vδ2 T cells (Wang et al., 2001; Kabelitz et al., 2004; Beck et al., 2010). The humanized mice established here contain functional human T and B cells, including a similar percentage of Vγ9Vδ2 T cells in peripheral blood as seen in humans (Fig. 2 and Table S1). Most importantly, we found that influenza viruses could efficiently replicate and cause pathology in these mice with...
that IPP-expanded Vγ9Vδ2 T cells can efficiently kill human seasonal H1N1- and avian influenza virus H9N2- and H5N1-infected MDMs, and inhibit virus replications in vitro (Qin et al., 2009). In this study, we further showed that PAM-expanded Vγ9Vδ2 T cells have a similar antiviral activity against influenza PR/8 H1N1 virus (Fig. 1) and other strains, such as H3N2, B, and H5N1 viruses (Fig. S2). Importantly, by the use of adoptive transfer of in vitro-expanded Vγ9Vδ2 T cells into influenza virus–infected immunodeficient Rag2−/− γc−/− and humanized mice, here we demonstrate for the first time that Vγ9Vδ2 T cells can directly inhibit viral replication and control influenza disease in vivo (Fig. 3).

Consistent with previous reports about aminobisphosphonates in human and nonhuman primates (Das et al., 2001; Kunzmann et al., 1999; Sicard et al., 2005), we report that PAM can selectively expand Vγ9Vδ2 T cells in humanized mice (Fig. 4). Furthermore, we demonstrate that PAM can inhibit viral replication, attenuate lung inflammation, and control pathology and disease caused by influenza through selective activation and expansion of Vγ9Vδ2 T cells in vivo.

To the best of our knowledge, this is the first demonstration of the therapeutic effect of PAM on influenza infections. Vγ9Vδ2 T cells can mediate elimination of virus-infected cells or tumor cells by cytolytic and noncytolytic antiviral mechanisms (Bukowski et al., 1994; Battistini et al., 2005; Bonneville and Fournié, 2005; Born et al., 2007). Here, we found that PAM-expanded Vγ9Vδ2 T cells can recognize and efficiently kill influenza PR/8 H1N1 virus–infected cells, and thus contribute to virus clearance in vitro (Fig. 1). The cytoxicity of Vγ9Vδ2 T cells against influenza virus–infected cells is dependent on NKG2D activation and is mediated by the Fas–FasL and perforin–granzyme B pathways (Fig. 1). Phosphoantigen-expanded Vγ9Vδ2 T cells also produce large amounts of antiviral cytokines, infiltration of inflammatory lymphocytes, the induction of proinflammatory cytokines and chemokines in the lung, weight loss, and sometimes death (Figs. 5–7). These findings indicate that the humanized mice established here are suitable for the study of human Vγ9Vδ2 T cell immune responses to influenza viruses.

γδ-T cells play critical roles in the defense against infectious pathogens and in tumors (Bukowski et al., 1994; Sciammas and Bluestone, 1999; Battistini et al., 2005; Poccia et al., 2005a; Scotet et al., 2005). In vitro studies have demonstrated broad antiviral activities of human Vγ9Vδ2 T cells against human immunodeficiency virus, hepatitis B virus, herpes simplex virus, vaccinia virus, human cytomegalovirus and SARS coronavirus (Kabelitz et al., 2004; Poccia et al., 2006; Poccia et al., 2005a; Sicard et al., 2005). Previously, we had demonstrated that IPP-expanded Vγ9Vδ2 T cells can efficiently kill human seasonal H1N1- and avian influenza virus H9N2- and H5N1-infected MDMs, and inhibit virus replications in vitro (Qin et al., 2009). In this study, we further showed that PAM-expanded Vγ9Vδ2 T cells have a similar antiviral activity against influenza PR/8 H1N1 virus (Fig. 1) and other strains, such as H3N2, B, and H5N1 viruses (Fig. S2). Importantly, by the use of adoptive transfer of in vitro-expanded Vγ9Vδ2 T cells into influenza virus–infected immunodeficient Rag2−/− γc−/− and humanized mice, here we demonstrate for the first time that Vγ9Vδ2 T cells can directly inhibit viral replication and control influenza disease in vivo (Fig. 3).

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could not prevent weight loss and death caused by influenza infection in vivo, as Rag2<sup>−/−</sup> mice were transplanted with 30 × 10<sup>6</sup> of huPBMCs or Vγ9Vδ2 T cell-depleted huPBMCs i.p. from the same healthy human donor. In contrast with the mice reconstituted with whole huPBMCs, there were no or very few human Vγ9Vδ2 T cells in the mice reconstituted with Vγ9Vδ2 T cell-depleted huPBMCs (Fig. S5). Humanized mice reconstituted with whole huPBMCs (PBMC) or Vγ9Vδ2 T cell-depleted huPBMCs (PBMC-γδ-T) were infected with PR8 virus, and treated with PAM or PBS on day 3, 5, 7 and 9 after infection. The body weight change and survival in virus-infected humanized mice (n = 4 per group) are shown. The data are representative of two independent experiments.

such as IFN-γ (Poccia et al., 2005b). Consistent with a previous report that avian influenza H5N1 is resistant to the antiviral effects of IFN-γ (Seo et al., 2002), we also found that IFN-γ secreted from phohtoantigen-expanded Vγ9Vδ2 T cells only suppressed human H1N1 virus replication, but did not affect avian H5N1 viral replication in vitro (unpublished data). Here, we further found that PAM-expanded Vγ9Vδ2 T cells in vivo migrated to influenza virus–infected lungs of humanized mice and contributed to the control of virus replication and disease (Fig. 5 and 6; and Fig. S6). Therefore, we speculate that during influenza virus infections in these humanized mice, human Vγ9Vδ2 T cells activated and expanded by PAM may clear human H1N1 viruses by directly killing the virus-infected cells and secreting IFN-γ to inhibit virus replication, but for H5N1 infection, the virus clearance may be mainly dependent on their direct killing. The lack of a beneficial effect of PAM in influenza virus–infected Rag2<sup>−/−</sup>γδ<sup>−/−</sup> mice excludes the possibility that this drug has a direct antiviral effect on influenza virus.

Our in vitro study has indicated that PAM largely expanded Vγ9Vδ2 T cells, but did not expand non-Vγ9Vδ2 γδ T cells. The number of Vγ9Vδ2 T cells was 63.9 times higher than that of non-Vγ9Vδ2 γδ T cells after 9 d of PAM stimulation (Fig. S1). Although non-Vγ9Vδ2 γδ T cells could be also activated by PAM, the frequencies of CD69<sup>+</sup>, perforin<sup>+</sup>, and granzyme B<sup>+</sup> cells in non-Vγ9Vδ2 γδ T cells were significantly lower than that in Vγ9Vδ2 T cells (Fig. S1). Indeed, our adoptive transfer experiments in Rag2<sup>−/−</sup>γδ<sup>−/−</sup> mice (Fig. 3, F–H) have demonstrated that PAM-expanded Vγ9Vδ2 T cells by themselves effectively controlled influenza infection in vivo, as Rag2<sup>−/−</sup>γδ<sup>−/−</sup> mice lack both human and murine T, B, and NK cells. Importantly, we also found that PAM could not prevent weight loss and death caused by influenza virus in the mice reconstituted with Vγ9Vδ2 T cell–depleted huPBMCs, whereas PAM could significantly prevent the weight loss and death in the mice reconstituted with whole huPBMCs (Fig. 8). Therefore, our data unequivocally demonstrated that the antiviral role of PAM against influenza virus is mainly mediated by human Vγ9Vδ2 T cells.

In contrast to our original expectation that the absence of Vγ9Vδ2 T cells in humanized mice may allow more severe disease to develop after influenza infection than seen in mice reconstituted with whole huPBMCs, here we found that mice reconstituted with either whole huPBMCs or Vγ9Vδ2 T cell–depleted huPBMCs showed similar disease severity (Fig. 3 B). This is probably because the effector functions of Vγ9Vδ2 T cells are dependent on their numbers and the degree of activation (Bonneville and Fournié, 2005; Bonneville and Scotet, 2006; Bonneville et al., 2010) and the humanized mice contain only a few Vγ9Vδ2 T cells (Fig. 2 and 4B). Our in vitro results showed that influenza virus alone could not expand Vγ9Vδ2 T cells and only partially activated these cells. It was only in the presence of PAM that Vγ9Vδ2 T cells are fully activated and expanded in number and to express sufficient perforin and granzyme B to protect against influenza infection (Fig. S9).

As observed in humans (Beigel et al., 2005; Cheung et al., 2002), different strains of influenza virus cause varying disease severity in humanized mice. Compared with human seasonal H1N1 virus, avian H5N1 virus induced more severe illness, pulmonary pathology, and even death in humanized mice (Fig. 5; Fig. 7, C and D; Fig. S8). Avian H5N1 virus led to higher viral load and induced higher levels of proinflammatory cytokines and chemokines such as IL-6, TNF, and IP-10 in the lungs (Fig. 6 and 7). It is not clear whether the proinflammatory cytokine induction is simply a reflection of the enhanced H5N1 viral replication or whether it contributes to immunopathology itself. In vitro studies in primary human cells have shown that, compared with seasonal influenza viruses, H5N1 virus intrinsically causes enhanced cytokine expression (Cheung et al., 2002; Chan et al., 2005). In the humanized mice, all the T, B, and NK cells derive from human origin, whereas its lung epithelial cells derive exclusively from mouse origin. Thus, influenza infection in these mice is associated with the expression of cytokines/chemokines of human and mouse origin (Fig. 7 and Fig. S7). Because human cytokines may or may not have physiological activity in mouse lung epithelium, this is not a perfect model of influenza pathogenesis. However, this humanized mouse model provides a relevant and valuable model for the study of the role of the innate-like Vγ9Vδ2 T cells in influenza infections because the effector functions of Vγ9Vδ2 T cells do not require conventional antigen presentation in the context of the MHC (Bonneville and Scotet, 2006; Urban et al., 2010). We used this model to demonstrate that expansion and activation of Vγ9Vδ2 T cells by PAM effectively control influenza virus disease.

Notably, human seasonal H1N1 virus could not efficiently infect wild-type or Rag2<sup>−/−</sup>γδ<sup>−/−</sup> mice, but it did successfully infect humanized mice as indicated by the virus replication in the lung and the significant body weight loss (Fig. 3 and 6;
and Fig. S4). Importantly, as for avian H5N1 virus, human seasonal H1N1 virus could infect both human and mouse cells in the lung in humanized mice (Fig. S10), suggesting that the process of humanization may alter the lung tissue tropism for influenza virus in mice. This needs to be investigated by examining the expression of the H1N1 virus receptor in the respiratory tract and lung in humanized mice.

The significance of targeting Vγ9Vδ2 T cells rather than the influenza virus itself lies in their innatelike effector functions that allow them to exhibit their antiviral activities rapidly, regardless of virus subtype and the emergence of drug-resistant strains. The use of a human equivalent dose of PAM can effectively control influenza diseases in humanized mice suggesting it is feasible to translate to human clinical trials. Several protocols based on the in vivo activation of Vγ9Vδ2 T cells with phosphoantigens or aminobisphosphonates are in development for the treatment of certain tumors and bacterial infections (Bon Neville and Sciotet, 2006; Caccamo et al., 2008; Dieli et al., 2008; Huang et al., 2009; Bon Neville et al., 2010).

In summary, our study demonstrates that PAM controls influenza infection in humanized mice by inhibition of virus replication and associated attenuation of inflammation in the lungs through boosting the human Vγ9Vδ2 T cells, and that this beneficial effect is active against viruses of varying subtypes and virulence. Our findings confirm proof of principle for a novel therapeutic approach—using PAM to boost human Vγ9Vδ2 T cell immunity against seasonal, zoonotic, and pandemic influenza viruses. These studies now deserve to be extended to human clinical trials. As PAM is commonly used clinically for the treatment of osteoporosis and Paget’s disease, this new application of an old drug potentially offers a safe and readily available option either alone or in combination with conventional antiviral drugs for the treatment of influenza, especially for combating pandemic or drug-resistant viruses.

MATERIALS AND METHODS

Generation of PAM-expanded Vγ9Vδ2 T cells and MDMs. PBMCs were isolated fromuffy coats of health donors from Hong Kong Red Cross by Ficoll-Hypaque (Pharmacia) gradient centrifugation as previously described (Zhou et al., 2006). In brief, adherent monocytes were seeded in 24-well plates at 5 × 10⁵ cells/well or in 24-well plates at 5 × 10⁶ T cells and MDMs.

Then they were refed by RPMI-1640 supplemented with 5% fetal bovine sera. After 4 days of culture, Vγ9Vδ2 T cells were purified by negative selection with TCR γ/δ T cell isolation kit according to the manufacturer’s instruction (Miltenyi Biotech). Human MDMs were generated from mononuclear cells as previously described (Zhou et al., 2006). In brief, adherent monocytes were seeded in 24-well plates at 5 × 10⁵ cells/well or in 24-well plates at 5 × 10⁶ cells/well. Then they were refed by RPMI-1640 supplemented with 5% autologous serum and allowed to differentiate to macrophages for 14 d. The purity of monocytes, as determined by flow cytometry with anti-CD14 monoclonal antibody, was consistently >90%.

Generation of humanized mice. Rag2−/− γc−/− mice were purchased from Taconic and maintained in the Laboratory Animal Unit, the University of Hong Kong. To establish humanized mouse models, huPBMCs were isolated from buffy coat preparations of blood from healthy donors from the Hong Kong Red Cross. Vγ9Vδ2 T cell–depleted huPBMCs were obtained after depletion of Vδ2 T cells by magnetic microbeads (Miltenyi Biotech). 4–5-wk-old male or female Rag2−/− γc−/− mice were treated with liposomes (VU Medisch Centrum, Amsterdam, the Netherlands) 1 d before transplantation. The sublethally irradiated mice were transplanted i.p. with 30 × 10⁵ huPBMCs or Vγ9Vδ2 T cell–depleted huPBMCs. In general, huPBMCs from 1 buffy coat were used for generation of 8–10 humanized mice. After transplantation, graft-versus-host disease symptoms such as weight loss, temperature changes, and diarrhea were monitored daily. The immune reconstitution of humanized mice was examined at indicated times. All manipulations were performed in compliance with the approval of the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster and the guidelines for the use of experimental animals by the Committee on the Use of Live Animals in Teaching and Research, Hong Kong.

Viruses, infections, and treatment of virus-infected humanized mice. Human influenza virus H1N1 (A/Hong Kong/54/98), mouse-adapted influenza H1N1 (A/PR/8/34), and avian H5N1 (A/Hong Kong/486/97) were used. All the viruses were cultured in Madin-Darby canine kidney cells, as described previously (Qin et al., 2009; Tu et al., 2010). The virus titer was determined by daily observation of cytopathic effect in cells infected with serial dilutions of virus stock; median tissue culture infective dose (TCID₅₀) was calculated according to the Reed-Muench formula. For in vitro experiments, day 14–differentiated MDMs were infected by influenza virus at a multiplicity of infection (MOI) of 2. After 1 h of viral adsorption, the cells were washed by PBS to remove unadsorbed virus.

Humanized mice were separated into mock, PBS-treated, and drug-treated groups and matched according to sex, age, and the source of huPBMCs. 4 wk after transplantation of huPBMCs, RAg2−/− γc−/− and established humanized mice were infected i.n. with human influenza H1N1 virus (25 µl, 10⁴ TCID₅₀), PR/8 virus (25 µl, 10⁴ TCID₅₀), or avian influenza H5N1 virus (25 µl, 1 × 10⁴ ID₅₀) under anesthesia. For human H1N1 and PR/8 virus infection, a human equivalent dose of PAM (10 mg/kg body weight; Pamlol; Hospira Australia Pty Ltd) or an equivalent volume of PBS was injected i.p. on days 3, 5, 7, and 9 after virus infection. For avian H5N1 infection, PAM (10 mg/kg body weight) or an equivalent volume of PBS was injected i.p. on days 1, 3, 5, 7, and 9 after virus infection. Survival was monitored, and the infected mice were weighed daily; mice with >25% weight loss were sacrificed and counted as dying.

Cytotoxic assay. Vγ9Vδ2 T cells (Effector, E) were co-cultured with PR/8 virus–infected MDMs (Target, T) at an E/T ratio of 10:1 for 6 h. Nonadherent cells were harvested and total RNA was isolated using trizol reagent. For virus–infected MDMs (Target, T) at an E/T ratio of 10:1 for 6 h. The neutralization antibody against influenza virus was assessed by flow cytometry as the number of target gene copies per 10⁵ cells in the CD3+ population as we did before (Qin et al., 2009).

Quantification of influenza viral copies by RT-PCR. MDMs (10⁵) were infected by PR/8 virus in an MOI of 2. 1 h later, unadsorbed virus was washed away carefully and the MDMs were cultured alone or with 10⁵ of Vγ9Vδ2 T cells for 48 h. Then the cells and supernatant were harvested and total RNA was extracted by Trizol LS reagent according to the manufacturer’s instructions (Invitrogen). The cDNA was synthesized with oligo(dT)₁₂–₁₈ primer and Superscript II reverse transcription (Invitrogen). Viral matrix gene copies were quantified on the basis of a SYBR green fluorescence signal after PCR (forward primer, 5'-CTTCTACCCGGTGCTGAAGC-3'; reverse primer, 5'-GCCATTTTGGAACAGGCTCTA-3') by ABI PRISM 7700 Sequence Detection System (Applied Biosystems; Qin et al., 2009). Results were expressed as the number of target gene copies per 10⁵ MDMs.

Blocking assay. Vγ9Vδ2 T cells (E) were co-cultured with PR/8 virus–infected MDMs (T) at an E/T ratio of 10:1 for 6 h. The neutralization antibody against influenza virus was assessed by flow cytometry as the number of target gene copies per 10⁵ MDMs.
R&D Systems), anti-TRAIL (10 µg/ml; R&D Systems), and their relevant isotype controls were added in the co-culture for blocking NKGD2-, FasL-, and TRAIL-mediated pathways, respectively (Matarollo et al., 2007). For blocking perforin and granzyme B the perforin inhibitor CMA (1 µg/ml; Sigma-Aldrich) and granzyme B inactivator Bcl-2 (1 µg/ml; R&D Systems) were used as previously described (Zeine et al., 1998). The cytotoxicities were analyzed by flow cytometry as described in cytotoxic assay and calculated as percentage of inhibition relative to those of control.

**Immunization.** 4 wk after transplantation of huPBMCs, established humanized mice were primed with 1.5 limits of flocculation (lf) of TT vaccine (Adventis-Pasteur) subcutaneously in the inguinal pouch region. A booster vaccination of 0.25 lf TT was given in the right hind footpad 2 wk after priming. The numbers of human CD4+ T cells, CD8+ T cells, and CD19 B cells in mouse peripheral blood were counted by FACS analysis, and IFN-γ-producing cells in CD4 and CD8 T cells in peripheral blood were analyzed by intracellular cytokine staining. The serum TT-specific antibody was examined by ELISA.

**Flow cytometric analysis.** Cells were stained for surface markers with the following antibodies: anti-CD3 (HIT3a), anti-TCR9V (B3), anti-TRAIL2 (B6; BD), anti-TCR16 (SA6.E9), and anti-CD14 (TUK4; Invitrogen). For intracellular staining, cells were fixed, permeabilized, and then stained with anti–IFN-γ, anti-perforin (Pfp, BG9), and anti–granzyme B (GrB, GB11) antibodies (BD) or their relevant isotype controls as described previously (Tu et al., 2004; Zheng et al., 2009). All samples were acquired on a FACSArta (BD) and analyzed by FlowJo software (Tree Star).

**ELISA assay.** The concentrations of total human IgG or TT-specific IgG in the serum of humanized mice were determined by quantitative ELISA set (Bethyl Laboratories, Inc.).

**Determination of virus titer and inflammatory cytokines and chemokines.** The lungs from infected humanized mice were harvested at indicated times and homogenized in 2 ml of PBS. After centrifugation at 1,500 rpm for 15 min, the supernatants were collected for determination of virus titer and inflammatory cytokines and chemokines. The concentrations of human and murine cytokines and chemokines were detected and analyzed with human cytokine and chemokine assay kits (Bender MediSystems).

**Immunohistochemistry assays of lungs.** The lungs, livers, spleens, kidneys, and intestines from Rag22/– mice or humanized mice were harvested at indicated times, fixed with 10% formalin, and maintained in 75% ethanol. Sections were prepared according to standard protocols and stained with hematoxylin and eosin, or anti-human CD45, anti–human CD3 (Bcam), or anti–human TCR182 antibody (clone 7B6; gift from M. Bonneville and E. Scotet, Institut National de la Santé et de la Recherche Médicale U892, Paris, France), and visualized by HRP-Polymer anti–mouse/rabbit IHC kit (MaxVision; China). Histopathologic score and the number of CD3+ and V82+ cells in lung tissues was analyzed by two independent observers who were blinded to the treatment. The lung tissue sections were screened, and five fields for each mouse were selected randomly. Histopathologic score was evaluated as previously described (Dybeng et al., 2000). The severity was graded on a scale of 0 to 3: normal, 0; mild, 1; moderate, 2; severe, 3. The nucleated CD3+ and V82+ cells in each area were counted at ×40 magnification and expressed as the number of cells per field (Kuang et al., 2009).

**Statistical analyses.** Data are means ± SEM. Multiple regression analysis was used to test the differences in the body weight changes between PBS and VV9V82 T cell–treated or PAM-treated groups adjusted for time after infection. The differences in cell death and viral copy for in vitro experiments, and viral titers or concentrations of proinflammatory cytokines/chemokines between PBS and VV9V82 T cell–treated or PAM-treated groups were analyzed by unpaired two-tailed Student’s t test. The P value of the difference for survival was determined by Kaplan-Meier log-rank test. P < 0.05 was considered to be significant.

**Online supplemental material.** Fig. S1 demonstrates that PAM selectively expands human V9V82 T cells in vitro. Fig. S2 indicates that PAM-expanded VV9V82 T cells display cytolytic activities against influenza H3N2, B, and H5N1 viruses. Fig. S3 shows the generation of humanized mice in Rag22/– mice with huPBMCs. Fig. S4 demonstrates human H1N1 virus cannot infect wild-type and Rag22/– mice. Fig. S5 shows the generation of humanized mice with V9V82 T cell–depleted huPBMCs. Fig. S6 shows the numbers of V9V82 T cells in lungs of virus–infected humanized mice after treatment with PAM. Fig. S7 shows that effects of PAM on murine proinflammatory cytokines and chemokines in virus–infected lung in humanized mice. Fig. S8 shows the pathology score and infiltrated human CD3 in the lungs of human H1N1 or avian H5N1 virus–infected, PAM-treated humanized mice after infection. Fig. S9 demonstrates that PAM enhances influenza virus–stimulated VV9V82 T cells activation and expression of perforin and granzyme B. Fig. S10 shows that human H1N1 and avian H5N1 viruses infect both human and murine cells in the lung of humanized mice. Table S1 shows the percentages of peripheral blood lymphocyte subsets before and after transplantation in humanized mice. Table S2 shows the frequencies of V9V82 T cells in peripheral blood lymphocytes in mice after adoptive transfer of V9V82 T cells. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20111026/D1.

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Author contributions: W. Tu conceived and designed the study, designed all the experiments, analyzed data, and wrote the paper; J. Zheng designed and performed parts of experiments, and analyzed data; Y. Liu, S.F. Sia, M. Liu, G. Qin, I.H.Y. Ng, Z. Xiang, and K.T. Lam performed parts of experiments; J.S.M. Peiris and Y.-L. Lau conceived and planned the study and co-wrote the paper.

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