

The aminobisphosphonate pamidronate controls influenza pathogenesis by expanding a $\gamma\delta$ T cell population in humanized mice

Wenwei Tu,^{1,3} Jian Zheng,¹ Yinping Liu,¹ Sin Fun Sia,² Ming Liu,⁴ Gang Qin,¹ Iris H.Y. Ng,^{1,2} Zheng Xiang,¹ Kwok-Tai Lam,¹ J.S. Malik Peiris,² and Yu-Lung Lau¹

¹Department of Paediatrics and Adolescent Medicine and ²Department of Microbiology, Li Ka Shing Faculty of Medicine, University of Hong Kong, Hong Kong, China

³Joint Research Center of West China Second University Hospital, Sichuan University, 610041 Chengdu, China

⁴Guangzhou Institute of Respiratory Diseases, Guangzhou Medical College, 510182 Guangzhou, China

There are few antiviral drugs for treating influenza, and the emergence of antiviral resistance has further limited the available therapeutic options. Furthermore, antivirals are not invariably effective in severe influenza, such as that caused by H5N1 viruses. Thus, there is an urgent need to develop alternative therapeutic strategies. Here, we show that human V γ 9V δ 2 T cells expanded by the aminobisphosphonate pamidronate (PAM) kill influenza virus-infected cells and inhibit viral replication *in vitro*. In Rag2^{-/-} γ c^{-/-} immunodeficient mice reconstituted with human peripheral mononuclear cells (huPBMCs), PAM reduces disease severity and mortality caused by human seasonal H1N1 and avian H5N1 influenza virus, and controls the lung inflammation and viral replication. PAM has no such effects in influenza virus-infected Rag2^{-/-} γ c^{-/-} mice reconstituted with V γ 9V δ 2 T cell-depleted huPBMCs. Our study provides proof-of-concept of a novel therapeutic strategy for treating influenza by targeting the host rather than the virus, thereby reducing the opportunity for the emergence of drug-resistant viruses. As PAM has been commonly used to treat osteoporosis and Paget's disease, this new application of an old drug potentially offers a safe and readily available option for treating influenza.

CORRESPONDENCE

Wenwei Tu:

wwtu@hku.hk

OR

J.S. Malik Peiris:

malik@hkucc.hku.hk

OR

Yu-Lung Lau:

lauylung@hkucc.hku.hk

Abbreviations used: CMA, concanamycin A; EthD-2, ethidium homodimer-2; huPBMC, human PBMC; MDM, monocyte-derived macrophage; MOI, multiplicity of infection; PAM, pamidronate.

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

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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 $\gamma\delta$ -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 $\gamma\delta$ -T cells are V γ 9V δ 2 T cells, a major “innate-like” peripheral T cell subset (Carding and Egan, 2002; Beetz et al., 2008).

V γ 9V δ 2 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 V γ 9V δ 2 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 zoledronate, which are commonly used for the treatment of osteoporosis and Paget’s disease, can induce intracellular accumulation of IPP, leading to activation and expansion of human V γ 9V δ 2 T cells (Bonneville and Scotet, 2006; Bonneville et al., 2010; Urban et al., 2010).

Phosphoantigen-expanded V γ 9V δ 2 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 V γ 9V δ 2 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 V γ 9V δ 2 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 V γ 9V δ 2-TCR (Sicard et al., 2005; Bonneville and Scotet, 2006; Born et al., 2006), and whether V γ 9V δ 2 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^{-/-} γ c^{-/-} (Rag2^{-/-} γ c^{-/-}) 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 V γ 9V δ 2 T cell immunity. Our study suggests a novel strategy for treatment of seasonal, zoonotic, and pandemic influenza by using PAM to enhance host V γ 9V δ 2 T cell immunity.

RESULTS

Cytotoxic activity of V γ 9V δ 2 T cells against influenza virus in vitro

We recently demonstrated that IPP-expanded V γ 9V δ 2 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 V γ 9V δ 2 T cells have similar effects on virus-infected cells, we examined their cytolytic activities during the co-culture of PAM-expanded V γ 9V δ 2 T cells with influenza H1N1/A/PR/8/34 (PR/8) virus-infected, autologous MDMs. PAM selectively expanded V γ 9V δ 2 T cells in vitro (Fig. S1). Similar to in our previous study (Qin et al., 2009), V γ 9V δ 2 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). V γ 9V δ 2 T cells significantly inhibited PR/8 H1N1 virus replication in MDMs (Fig. 1 A). In addition, PAM-expanded V γ 9V δ 2 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 V γ 9V δ 2 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

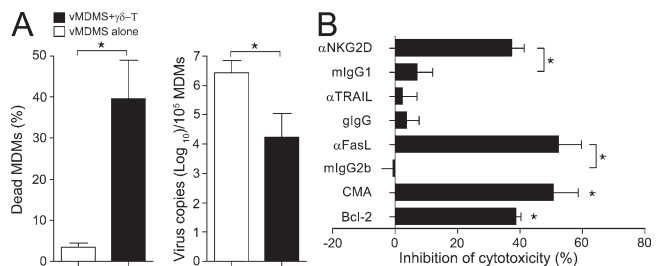


Figure 1. Cytotoxicity of PAM-expanded V γ 9V δ 2 T cells against virus-infected MDMs. (A) MDMs were infected with PR/8 virus at an MOI of 2, and then cultured alone (vMDMs alone) or with purified PAM-expanded V γ 9V δ 2 T cells at a ratio of 1:10 for 6 h. The percentages of dead MDMs among target cells (CD3⁻ population), identified as CD3⁻EthD2⁺ for four different experiments are shown. To determine the viral copy, PR/8 virus-infected MDMs were cultured alone or with purified PAM-expanded V γ 9V δ 2 T cells for 48 h. Total RNA was extracted from both cells and supernatant, and viral matrix gene copies were quantified, followed by reverse transcription. The results were shown as virus gene copies per 10⁵ MDMs for four different experiments. *, P < 0.05. (B) PAM-expanded V γ 9V δ 2 T cells were co-cultured with PR/8 virus-infected MDMs at a ratio of 10:1 for 6 h. The perforin inhibitor CMA, granzyme B inactivator Bcl-2, anti-NKG2D (α NKG2D), anti-TRAIL (α TRAIL), anti-FasL (α FasL) blocking antibodies, or their relevant isotype controls, were used. The cytotoxicity was analyzed by flow cytometry as the percentage of EthD-2⁺ cells in the CD3⁻ population and calculated as the percentage of inhibition relative to those without any treatment. The data shown as mean \pm SEM are representative of four independent experiments. *, P < 0.05 as compared with their relevant isotype control. mlgG1, mouse IgG1; mlgG2b, mouse IgG2b; glgG, goat IgG.

MDMs after TRAIL blocking antibody treatment (Fig. 1 B). To further confirm the involvement of cytolytic granule release in the killing of virus-infected MDMs by V γ 9V δ 2 T cells, the perforin-specific inhibitor concanamycin A (CMA) and the granzyme B inactivator Bcl-2 were used. As shown in Fig. 1 B, the cytolytic activities of V γ 9V δ 2 T cells against PR/8 virus-infected MDMs were significantly abrogated after CMA treatment or Bcl-2 treatment (Fig. 1 B). These results indicate that the cytotoxicity of V γ 9V δ 2 T cells is dependent on NKG2D activation, and mediated by Fas-FasL and perforin-granzyme B pathways.

Generation of humanized mice in Rag2^{-/-} γ c^{-/-} immunodeficient mice

To determine the role of human V γ 9V δ 2 T cells in influenza A infection *in vivo*, we reconstituted Rag2^{-/-} γ c^{-/-} mice with human peripheral mononuclear cells (huPBMCs). Human lymphocytes had stably engrafted in Rag2^{-/-} γ c^{-/-} 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 and 30% 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%), V γ 9⁺V δ 2⁺ T cells (2.9%), CD25^{high}CD4⁺ 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^{-/-} γ c^{-/-} mice with functional human T and B cells, including a similar percentage of V γ 9⁺V δ 2⁺ T cells, after 4 wk of huPBMC transplantation are used for following experiments and referred to as “humanized” mice.

Antiviral activity of human V γ 9V δ 2 T cells against influenza virus infection *in vivo*

Wild-type, Rag2^{-/-} γ c^{-/-}, 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^{-/-} γ c^{-/-} 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 V γ 9V δ 2 T cell-depleted huPBMCs (Fig. S5) showed comparable weight loss (Fig. 3 B).

We used adoptive transfer of human V γ 9V δ 2 T cells to investigate their antiviral activity *in vivo*. Highly purified (>97%) PAM-expanded V γ 9V δ 2 T cells (10⁶ cells/mouse) were adoptively transferred into influenza PR/8 virus-infected humanized mice *i.v.* at day 2, 4, and 6 after infection. Consequently, the frequency of V γ 9V δ 2 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 V γ 9V δ 2 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

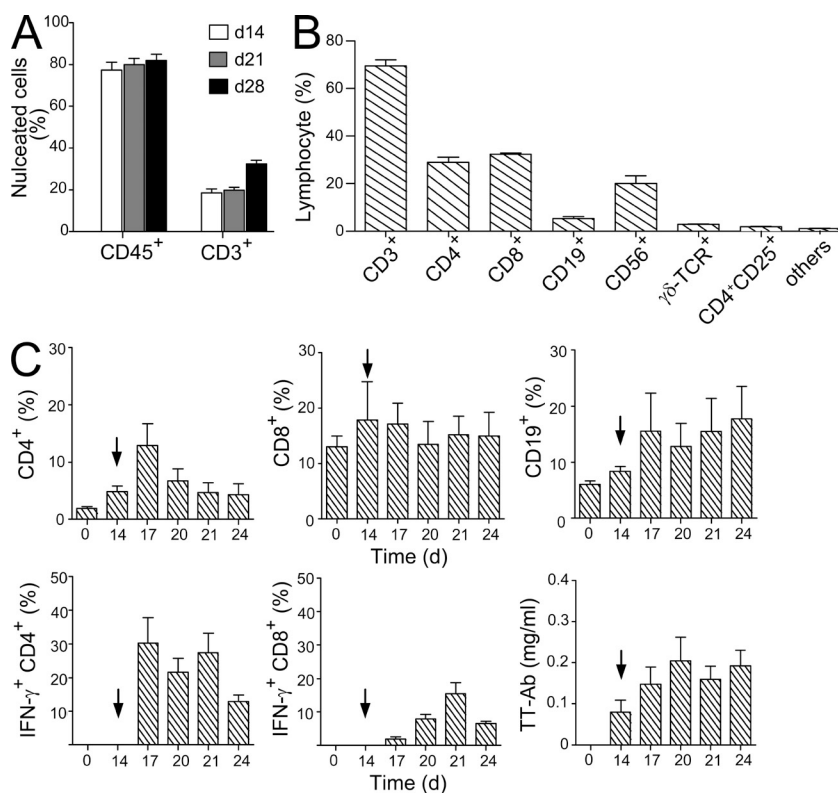


Figure 2. Human lymphocyte engraftment and functional human T and B cell development in huPBMC-transplanted Rag2^{-/-} γ c^{-/-} mice. (A) The percentage of human CD45⁺ cells and CD3⁺ T cells in peripheral blood nucleated cells in mice ($n = 5$) at indicated time after transplantation. (B) The percentage of human lymphocyte subsets in peripheral blood lymphocytes in mice ($n = 5$) at day 28 after transplantation. The data are representative of four independent experiments. (C) A booster of TT vaccine (\downarrow) was given at day 14 after priming in humanized mice 4 wk after huPBMC transplantation. The percentages of CD4⁺ T, CD8⁺ T, CD19⁺ B cells and IFN- γ -producing cells in CD4⁺ and CD8⁺ T cells from peripheral blood nucleated cells, and the levels of serum TT-specific antibodies at indicated times after the first dose of TT vaccination, are shown ($n = 6$). The data shown as mean \pm SEM are representative of two independent experiments.

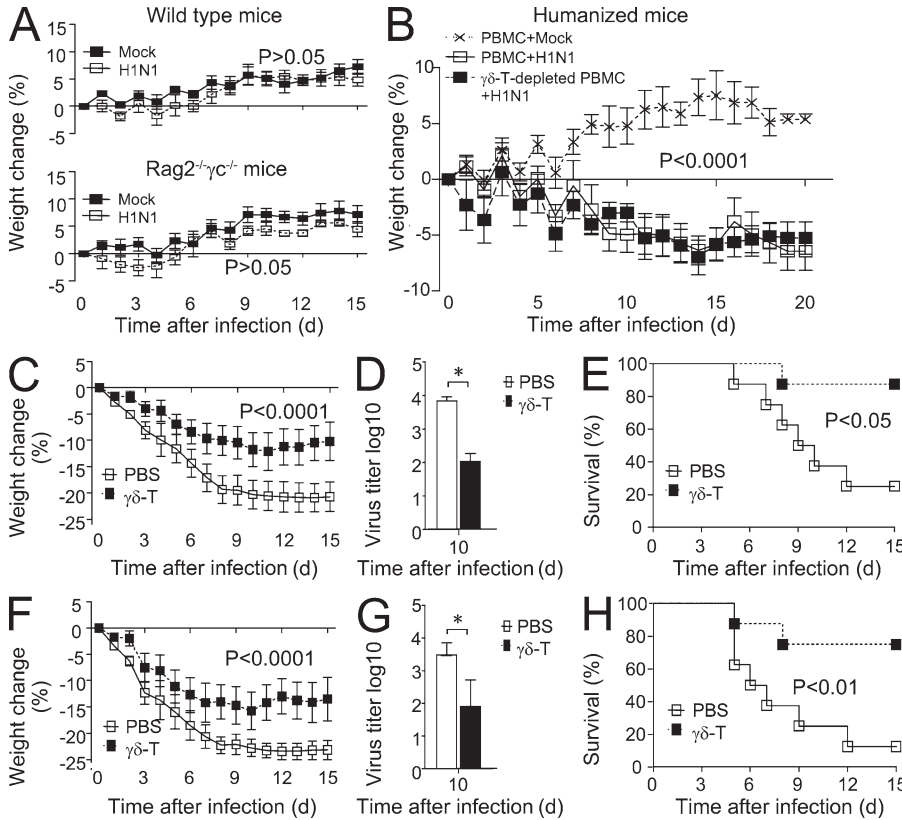


Figure 3. Antiviral activity of human Vγ9Vδ2 T cells against influenza in vivo.

(A) The body weight changes in wild-type and Rag2^{-/-}γc^{-/-} mice after infection with human H1N1 virus or mock (PBS) i.n. (n = 6 per group). (B) The body weight changes in humanized mice and Vγ9Vδ2 T cell-depleted humanized mice (n = 6 per group) after infection with human H1N1 virus i.n. As the control, humanized mice (n = 6) were treated with PBS (Mock). (C–E) After adoptive transfer of in vitro PAM-expanded Vγ9Vδ2 T cells or PBS i.v. at day 2, 4, and 6 after infection, the body weight changes (n = 8 per group; C), lung viral titers (n = 4 per group; D) and survival (n = 8 per group; E) in PR/8 virus-infected humanized mice were measured at indicated time after infection. (F–H) After adoptive transfer of in vitro PAM-expanded Vγ9Vδ2 T cells or PBS i.v. at day 2, 4, and 6 after infection, the body weight changes (n = 8 per group; F), lung viral titers (n = 4 per group; G), and survival (n = 8 per group; H) in PR/8 virus-infected Rag2^{-/-}γc^{-/-} mice were measured at indicated time of after infection. The data are representative of two independent experiments. *, P < 0.05.

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 adoptively 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). In contrast, 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

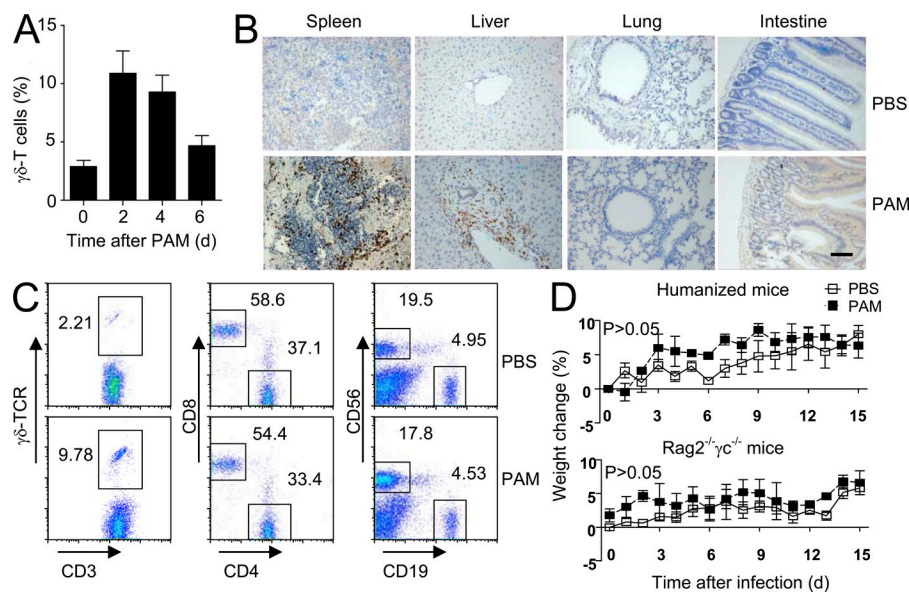


Figure 4. PAM selectively expands human $V\gamma 9V\delta 2$ T cells in humanized mice.

(A) The percentage (mean \pm SEM) of $V\gamma 9V\delta 2$ T cells in peripheral blood lymphocytes in humanized mice ($n = 4$) after 1 dose of PAM (10 mg/kg body weight) via i.p. injection. (B) Accumulation of $V\gamma 9V\delta 2$ T cells (brown) in different tissues at day 2 after 1 dose of PAM or PBS. Representative immunohistochemical sections from spleen, liver, lung, and intestine in uninfected humanized mice stained with an anti-human $V\delta 2$ mAb (7B6). Bar, 100 μ m. (C) Representative data for the percentages of $V\gamma 9V\delta 2$ T cells, CD3⁺ T, CD4⁺ T, CD8⁺ T, CD19⁺ B, and CD56⁺ NK cells in the peripheral blood lymphocytes at day 2 after 1 dose of PAM or PBS in uninfected humanized mice ($n = 4$ per group). (D) Body weight changes of humanized ($n = 5$ per group) or Rag2^{-/-}γc^{-/-} mice ($n = 5$ per group) after PAM or PBS treatment on day 0, 2, 4, and 6. The data are representative of two independent experiments.

on mortality could not be investigated. Whereas no $V\gamma 9V\delta 2$ 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 $V\gamma 9V\delta 2$ 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 $V\gamma 9V\delta 2$ 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 $V\gamma 9V\delta 2$ T cells can also migrate to infected lungs to exert their antiviral activity after avian H5N1 infection. As Rag2^{-/-}γc^{-/-} mice have no $V\gamma 9V\delta 2$ 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 humanized 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 V γ 9V δ 2 T cells

To further determine whether antiviral role of PAM against influenza virus is mediated by V γ 9V δ 2 T cells in humanized mice, humanized mice reconstituted with V γ 9V δ 2 T cell-depleted huPBMCs (Fig. S5) were used. Mice reconstituted with whole huPBMCs or V γ 9V δ 2 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 V γ 9V δ 2 T cell-depleted huPBMCs (Fig. 8). These results demonstrated that the antiviral effect of PAM against influenza virus is mainly mediated by a V γ 9V δ 2 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 V γ 9V δ 2 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 V γ 9V δ 2 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 V γ 9V δ 2 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

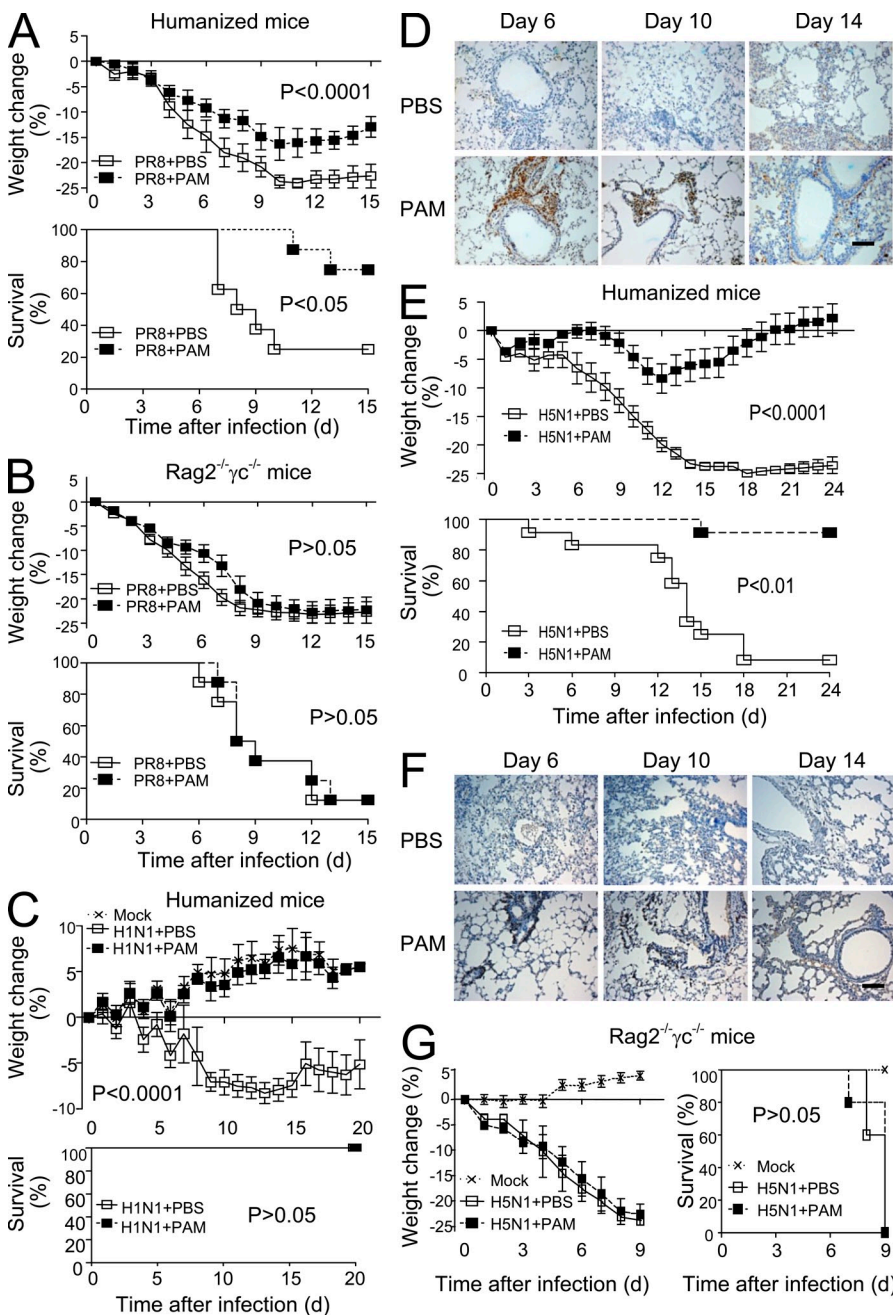


Figure 5. PAM reduces influenza disease severity and mortality. (A and B) Mice were infected with PR/8 virus and treated with PAM or PBS on day 3, 5, 7, and 9 after infection. The body weight change and survival in virus-infected humanized mice ($n = 6$ per group; A), and Rag2^{-/-}γC^{-/-} mice ($n = 6$ per group; B) are shown. (C and D) Humanized mice were infected with human H1N1 virus and treated with PAM or PBS on day 3, 5, 7, and 9 after infection. The body weight changes and survival (C; $n = 6$ per group), and representative immunohistochemical staining for V γ 9V δ 2 T cells (brown) in the lungs (D) at the indicated time after PAM or PBS treatment are shown. (E–G) Mice were infected with avian H5N1 virus and treated with PAM or PBS on day 1, 3, 5, 7, and 9 after infection. The body weight changes and survival (E; $n = 12$ per group) and representative immunohistochemical staining for V γ 9V δ 2 T cells (brown) in the lungs (F) at indicated time after PAM or PBS treatment are shown. The body weight changes and survival in avian H5N1 virus-infected Rag2^{-/-}γC^{-/-} mice ($n = 5$ per group; G) are also shown. The data are representative of two to three independent experiments. Bars, 100 μm.

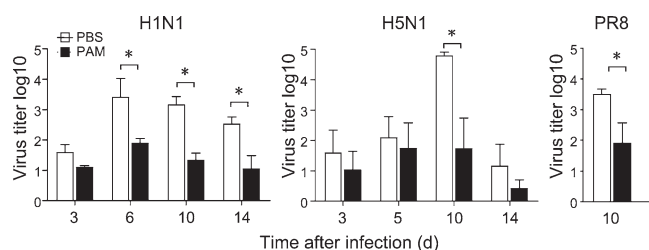


Figure 6. PAM inhibits influenza virus replication in lung. Humanized mice were infected with human H1N1 ($n = 5$ per group), avian H5N1 ($n = 5$ per group), or PR/8 ($n = 4$ per group) viruses, and treated with PAM or PBS as indicated in the legend of Fig. 5. The virus titers in the supernatants of homogenized lung tissue were determined at the indicated times after infection. *, $P < 0.05$. The data shown as mean \pm SEM are representative of two to three independent experiments.

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 V γ 9V δ 2 T cell immune responses to influenza viruses.

$\gamma\delta$ -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 V γ 9V δ 2 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 V γ 9V δ 2 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 V γ 9V δ 2 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 V γ 9V δ 2 T cells into influenza virus-infected immunodeficient Rag2^{-/-} γ c^{-/-} and humanized mice, here we demonstrate for the first time that V γ 9V δ 2 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 V γ 9V δ 2 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 V γ 9V δ 2 T cells in vivo. To the best of our knowledge, this is the first demonstration of the therapeutic effect of PAM on influenza infections.

V γ 9V δ 2 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 V γ 9V δ 2 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 cytotoxicity of V γ 9V δ 2 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 V γ 9V δ 2 T cells also produce large amounts of antiviral cytokines,

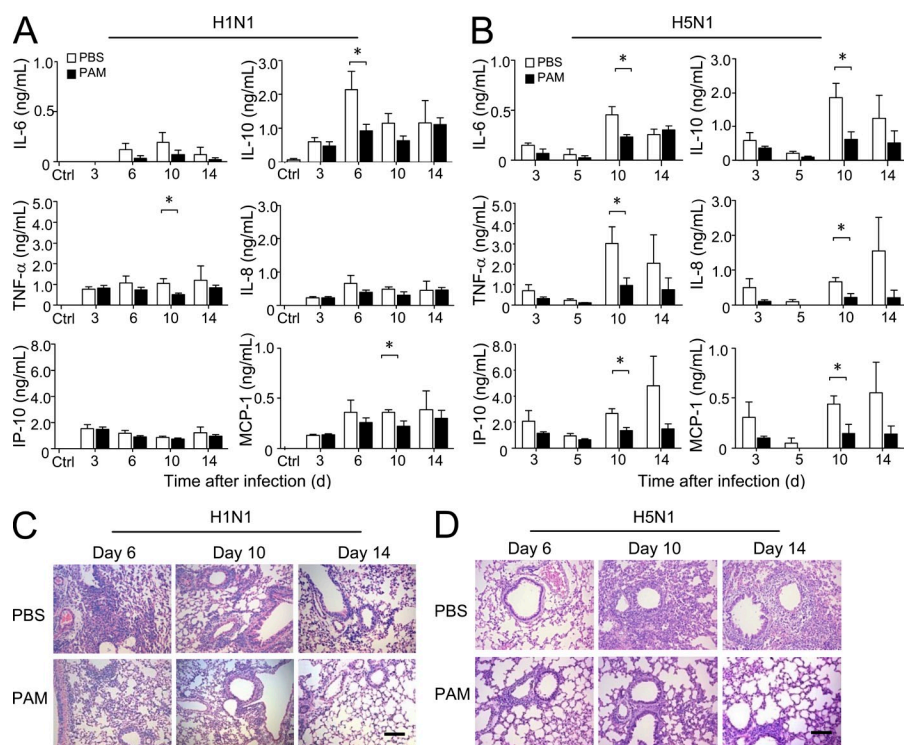


Figure 7. PAM prevents inflammation in infected lung. Humanized mice were infected with human H1N1 ($n = 4$ per group; A), or avian H5N1 ($n = 4$ per group; B) viruses, and treated with PAM or PBS as indicated in the legend of Fig. 5. The levels of human proinflammatory cytokines and chemokines in the supernatants of homogenized lung tissue were determined at the indicated times after infection (A and B). The data are shown as mean \pm SEM. *, $P < 0.05$. (C and D) Histopathologic changes in mouse lung tissues collected at indicated times after infection are shown. Representative histological sections of the lung tissues from human H1N1 (C) and avian H5N1 (D) virus-infected humanized mice after PAM or PBS treatment as indicated in the legend of Fig. 5 were stained with hematoxylin and eosin. Bars, 100 μ m. The data are representative of three independent experiments.

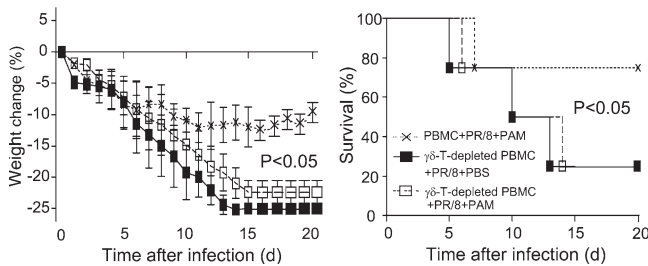


Figure 8. PAM cannot control influenza diseases in humanized mice without V γ 9V δ 2 T cells. V γ 9V δ 2 T cell–depleted huPBMCs were obtained after double depletions of V δ 2 T cells by positive selection with magnetic microbeads. Rag2^{-/-} γ c^{-/-} mice were transplanted with 30×10^6 of huPBMCs or V γ 9V δ 2 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 V γ 9V δ 2 T cells in the mice reconstituted with V γ 9V δ 2 T cell–depleted huPBMCs (Fig. S5). Humanized mice reconstituted with whole huPBMCs (PBMC) or V γ 9V δ 2 T cell–depleted huPBMCs (PBMC- γ δ -T) were infected with PR/8 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 phosphoantigen-expanded V γ 9V δ 2 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 V γ 9V δ 2 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 V γ 9V δ 2 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^{-/-} γ c^{-/-} 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 V γ 9V δ 2 T cells, but did not expand non-V γ 9V δ 2 γ δ T cells. The number of V γ 9V δ 2 T cells was 63.9 times higher than that of non-V γ 9V δ 2 γ δ T cells after 9 d of PAM stimulation (Fig. S1). Although non-V γ 9V δ 2 γ δ T cells could be also activated by PAM, the frequencies of CD69⁺, perforin⁺, and granzyme B⁺ cells in non-V γ 9V δ 2 γ δ T cells were significantly lower than that in V γ 9V δ 2 T cells (Fig. S1). Indeed, our adoptive transfer experiments in Rag2^{-/-} γ c^{-/-} mice (Fig. 3, F–H) have demonstrated that PAM-expanded V γ 9V δ 2 T cells by themselves effectively controlled influenza infection in vivo, as Rag2^{-/-} γ c^{-/-} 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 V γ 9V δ 2 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 V γ 9V δ 2 T cells.

In contrast to our original expectation that the absence of V γ 9V δ 2 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 V γ 9V δ 2 T cell–depleted huPBMCs showed similar disease severity (Fig. 3 B). This is probably because the effector functions of V γ 9V δ 2 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 V γ 9V δ 2 T cells (Fig. 2 and 4B). Our in vitro results showed that influenza virus alone could not expand V γ 9V δ 2 T cells and only partially activated these cells. It was only in the presence of PAM that V γ 9V δ 2 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 V γ 9V δ 2 T cells in influenza infections because the effector functions of V γ 9V δ 2 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 V γ 9V δ 2 T cells by PAM effectively control influenza virus disease.

Notably, human seasonal H1N1 virus could not efficiently infect wild-type or Rag2^{-/-} γ c^{-/-} 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 V γ 9V δ 2 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 V γ 9V δ 2 T cells with phosphoantigens or aminobisphosphonates are in development for the treatment of certain tumors and bacterial infections (Bonneville and Scotet, 2006; Caccamo et al., 2008; Dieli et al., 2008; Huang et al., 2009; Bonneville 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 V γ 9V δ 2 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 V γ 9V δ 2 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 V γ 9V δ 2 T cells and MDMs. PBMCs were isolated from buffy coats of health donors from Hong Kong Red Cross by Ficoll-Hypaque (Pharmacia) gradient centrifugation as previously described (Tu et al., 2006). 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 10% FBS RPMI-1640 medium with 9 μ g/ml of PAM. Recombinant human IL-2 (Invitrogen) was added to a final concentration of 500 IU/ml every 3 d from day 3. After 14-d culture, V γ 9V δ 2 T cells were purified by negative selection with TCR γ/δ^+ T cell isolation kit according to the manufacturer's instruction (Miltenyi Biotec). Human MDMs were generated from mononuclear cells as previously described (Zhou et al., 2006). In brief, adherent monocytes were seeded in 96-well flat-bottomed plates at 10^5 cells/well or in 24-well plates at 5×10^5 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^{-/-} γ c^{-/-} mice were purchased from Taconic and maintained in the Laboratory Animal Unit, the University of Hong Kong. To establish humanized mice models, huPBMCs were isolated from buffy coat preparations of blood from healthy donors from the Hong Kong Red Cross. V γ 9V δ 2 T cell-depleted huPBMCs were obtained

after depletion of V δ 2 T cells by magnetic microbeads (Miltenyi Biotec). 4–5-wk-old male or female Rag2^{-/-} γ c^{-/-} mice were treated with liposomes (VU Medisch Centrum, Amsterdam, the Netherlands) 1 d before transplantation. The sublethally irradiated mice were transplanted *i.p.* with 30×10^6 huPBMCs or V γ 9V δ 2 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 un-adsorbed 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^{-/-} γ c^{-/-} and established humanized mice were infected *i.n.* with human influenza H1N1 virus (25 μ l, $10^{8.4}$ TCID₅₀), PR/8 virus (25 μ l, 10^4 TCID₅₀), or avian influenza H5N1 (25 μ l, $1 \times$ LD₅₀) under anesthesia. For human H1N1 and PR/8 virus infection, a human equivalent dose of PAM (10 mg/kg body weight; Pamisol; 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. V γ 9V δ 2 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. Afterward, nonadherent cells were harvested directly. Adherent cells were detached with 0.25% trypsin-EDTA. All the adherent and nonadherent cells were stained with anti-CD3 to identify V γ 9V δ 2 T cells and ethidium homodimer-2 (EthD-2) to identify dead cells (King, 2000). The cytotoxicity of V γ 9V δ 2 T cells against virus-infected MDMs was assessed by flow cytometry as the percentage of EthD-2⁺ cells in the CD3⁺ population as we did before (Qin et al., 2009).

Quantification of fluA viral copies by RT-PCR. MDMs (10^5) were infected by PR/8 virus at an MOI of 2. 1 h later, un-adsorbed virus was washed away carefully and the MDMs were cultured alone or with 10^6 of V γ 9V δ 2 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'-CTTCTAACCGAGGTCGAAACG-3'; reverse primer, 5'-GGCATTTTGGACAAAGCGTCTA-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 10^5 MDMs.

Blocking assay. V γ 9V δ 2 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 antibodies anti-NKG2D (10 μ g/ml; 1D11; BD), anti-FasL (10 μ g/ml; 100419;

R&D Systems), anti-TRAIL (10 µg/ml; RIK-2; R&D Systems), and their relevant isotype controls were added in the co-culture for blocking NKG2D-, 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-TCRγ9 (B3), anti-TCRδ2 (B6; BD), anti-TCRγδ (5A6.E9), and anti-CD14 (TUK4; Invitrogen). For intracellular staining, cells were fixed, permeabilized, and then stained with anti-IFN-γ, anti-perforin (Pfp, δG9), 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 FACS Aria (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 g 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 MedSystems).

Immunohistochemistry assays of lungs. The lungs, livers, spleens, kidneys, and intestines from Rag2^{-/-}γc^{-/-} 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 (Abcam), or anti-human TCRδ2 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 (Max-Vision; China). Histopathologic score and the number of CD3⁺ and Vδ2⁺ 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 (Dybing 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 Vδ2⁺ 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 Vγ9Vδ2 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 Vγ9Vδ2 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 Vγ9Vδ2 T cells in vitro. Fig. S2 indicates that PAM-expanded Vγ9Vδ2 T cells display cytolytic activities against influenza H3N2, B, and H5N1 viruses. Fig. S3 shows the generation of humanized mice in Rag2^{-/-}γc^{-/-} mice with huPBMCs. Fig. S4 demonstrates human H1N1 virus cannot infect wild-type and Rag2^{-/-}γc^{-/-} mice. Fig. S5 shows the generation of humanized mice with Vγ9Vδ2 T cell-depleted huPBMCs. Fig. S6 shows the numbers of Vγ9Vδ2 T cells in the 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 Vγ9Vδ2 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 Vγ9Vδ2 T cells in peripheral blood lymphocytes in mice after adoptive transfer of Vγ9Vδ2 T cells. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20110226/DC1>.

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