Control of Pathogenic CD4 T Cells and Lethal Immunopathology by Signaling Immunoadaptor DAP12 during Influenza Infection

Sarah McCormick, Christopher R. Shaler, Cherrie-Lee Small, Carly Horvath, Daniela Damjanovic, Earl G. Brown, Naoko Aoki, Toshiyuki Takai and Zhou Xing

J Immunol published online 9 September 2011
http://www.jimmunol.org/content/early/2011/09/09/jimmunol.1101050

Supplementary Material
http://www.jimmunol.org/content/suppl/2011/09/09/jimmunol.1101050.DC1

Subscription
Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Control of Pathogenic CD4 T Cells and Lethal Immunopathology by Signaling Immunoadaptor DAP12 during Influenza Infection

Sarah McCormick,*† Christopher R. Shaler,*† Cherrie-Lee Small,*† Carly Horvath,*† Daniela Damjanovic,*† Earl G. Brown,‡ Naoko Aoki,*† Toshiyuki Takai,§ and Zhou Xing*‡

Immunopathology is a major cause of influenza-associated morbidity and mortality worldwide. However, the role and regulatory mechanisms of CD4 T cells in severe lung immunopathology following acute influenza infection are poorly understood. In this paper, we report that the emergence of immunopathogenic CD4 T cells is under the control of a transmembrane immunoadaptor DAP12 pathway during influenza infection. We find that the mice lacking DAP12 have unaltered viral clearance but easily succumb to influenza infection as a result of uncontrolled immunopathology. Such immunopathology is associated with markedly increased CD4 T cells displaying markedly increased cytotoxicity and Fas ligand expression. Furthermore, the immunopathogenic property of these CD4 T cells is transferrable. Thus, depletion of CD4 T cells or abrogation of Fas/Fas ligand signaling pathway improves survival and immunopathology. We further find that DAP12 expressed by dendritic cells plays an important role in controlling the immunopathogenic CD4 T cells during influenza infection. Our findings identify a novel pathway that controls the level of immune-pathogenic CD4 T cells during acute influenza infection.

The Journal of Immunology, 2011, 187: 000–000.

Received for publication April 4, 2011. Accepted for publication August 5, 2011. This work was supported by the Canadian Institutes for Health Research and the Ontario Thoracic Society.

Address correspondence and reprint requests to Dr. Zhou Xing, Department of Pathology and Molecular Medicine, McMaster University, 1280 Main Street West, Room MDCL-4012, Hamilton, Ontario L8S 4K1, Canada. E-mail address: xingz@mcmaster.ca

The online version of this article contains supplemental material.

Abbreviations used in this article: BAL, bronchoalveolar lavage; DAP12, DNAX-activating protein of 12 kDa; DAPKO, DNAX-activating protein of 12 kDa-deficient knockout; DAP12KO, DNAX activating protein of 12 kDa-knockout; FasL, Fas ligand; MOI, multiplicity of infection; wt, wild-type.

Copyright © 2011 by The American Association of Immunologists, Inc. 0022-1767/11S16/00

The Journal of Immunology

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1101050

Copyright © 2011 by The American Association of Immunologists, Inc. 0022-1767/11S16/00

The Journal of Immunology

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1101050
Materials and Methods

Mice

DAP12-knockout (DAP12KO) mice were fully backcrossed to the C57BL/6 genetic background and breeding colony maintained at McMaster University. Eight- to twelve-week-old control wt C57BL/6 mice were purchased from Charles River Laboratories. Rag2−/−/γc−/− (strain 4111) mice were purchased from Taconic Farms (41). All mice were housed in the specific pathogen-free facility at McMaster University, and experiments were conducted in accordance with the guidelines of animal research ethics board of McMaster University.

Primary influenza virus infection

Mice were infected intranasally with 1 × 10^5 or 5 × 10^5 PFU of a mouse-adapted A/FM1/47 (H1N1) influenza A virus (42) in 25 μl as described previously (43–45). The mice were monitored daily for the signs of illness, body weight changes, and mortality.

Detection of DAP12 protein expression in the lung

wt mice were infected with influenza, and lungs were collected in lysis buffer (0.5% Triton-X100, 50 mMTris [pH 8.0], 140 mM NaCl, and 10 mM EDTA) containing protease inhibitors (Sigma-Aldrich, Oakville, ON, Canada). Equal volumes of lung homogenates were immunoprecipitated with rabbit anti-DAP12 Abs bound to protein A-Sepharose beads (GE Healthcare, Piscataway, NJ) (28). Lysates were run on SDS-15% polyacrylamide gels under reducing conditions, blotted onto Immobilon-P (Millipore, Bedford, MA), and probed with rabbit anti-DAP12 Ab, followed with a goat, and rabbit Ab conjugated to Alexa 680 (Molecular Probes, Burlington, ON, Canada). Blots were scanned and quantitatively analyzed using the Odyssey imaging system (Licor, Lincoln, NE).

Lung histopathology, immunohistochemistry, and bronchoalveolar lavage

At various time points following infection, mice were sacrificed, and lungs were perfused with 10% formalin, sectioned, and H&E stained. The extent of immunopathological changes in the lung was semi-quantified by blinded scoring of H&E sections from multiple samples per time point. Three fields per section were analyzed and scored on a scale of 0–5 for the relative severity of epithelial damage and airway plugs, epithelial cell metaplasia/hyperplasia, inflammation around the conducting airways, and inflammation in the lung parenchyma. Immunohistochemical staining for CD3+ T cells was carried out on formalin-fixed sections. To study airway luminal cells, bronchoalveolar lavage (BAL) was carried out as described previously (43, 45, 46). Supernatants from BAL samples were collected and stored at −20°C. Total cell numbers in BAL were enumerated, and differential cell counts were determined using Wright–Giesma stained cytocentrifuged BAL specimens.

Tissue mononuclear cell isolation and culture

Spleens and lungs were removed aseptically, and the intra-airway luminal cells were removed from the lung by exhaustive lavage. Lungs were collected and processed to single-cell suspension by collagenase digestion as described previously (43, 45, 46). Spleens were processed to single-cell suspensions by mechanical disruption as described previously (43, 45, 46). All cells were cultured in RPMI 1640 supplemented with 10% FBS, 1% l-glutamine, 1% penicillin, and streptomycin.

Influenza tetramer and intracellular cytokine immunostaining and flow cytometric analysis

Freshly isolated cells were cultured in vitro in a U-bottom 96-well plate and stimulated with either influenza CD8 peptide NP (ASNNEDTM) for 6 h or whole UV-inactivated flu virus for 18 h at 37°C 5% CO2. GolgiPlug (BD Biosciences) was added for the final 6 h. Cells were immunostained with CD3-PerCPCy5.5, CD4-PECy7, CD8a-allophycocyanin Cy7, IFN-γ-allophycocyanin, and Fasl-biotin (all Abs purchased from BD Pharmingen, Mississauga, ON, Canada). Tetramer flow cytometric analysis was carried out using the immunodominant CD8 T cell peptide (ASNNEDTM) of influenza NP bound to the C57BL/6 MHC class I allele H-2D^d (Texas A&M University, College Station, TX). Stained cells were then run on a FACS Canto and analyzed on FlowJo software (version 6.3.4; Tree Star).

Analysis of cytokines and chemokines in BAL

Cytokine or chemokine contents in BAL were measured by Quantikine ELISA (R&D Systems, Minneapolis, MN) or by Luminex (Medicorp, Montreal, QC, Canada) according to the manufacturer’s protocol.

In vivo intratracheal CTL assay

A modified in vivo CTL assay was developed from our previously established protocol (43, 47). Briefly, the CTL target cells splenocytes from naive C57BL/6 were cultured in media alone or pulsed with UV-inactivated flu viruses (multiplicity of infection [MOI] = 10 prior to infection) overnight at 4°C. Unpulsed splenocytes were labeled with 0.5 μM CFSE (CFSEhigh), and UV-flu pulsed splenocytes were labeled with 5 μM CFSE (CFSElow). One million CFSEhigh and CFSElow cells were injected intratracheally in 40 μl via the surgically exposed trachea into the airway of flu-infected and naïve mice. Twenty-four hours following infection, CTL target cells were retrieved by exhaustive BAL. The extent of in vivo lysis of the transferred CFSE-labeled target cells was determined according to the loss of CFSE dye by flow cytometry as described previously (43).

In vitro CTL assay

The Promega CytoTox nonradioactive cytotoxicity assay (Promega, Madison, WI) was used to measure CD4 T cell CTL in vitro. Briefly, the CTL target splenocytes from naïve C57BL/6 mice were cultured in media alone or pulsed with UV-inactivated flu viruses (MOI = 10) overnight at 4°C, and 40,000 cells were plated in a U-bottom 96-well plate 2 h prior to coculture. CD4 T cells purified from flu-infected lungs (9 d) were incubated for 24 h with target cells in various T cell/target ratios. Supernatants were harvested, and the amount of LDH released into the culture media was measured according to the manufacturers’ protocol. The following formula was used to calculate the percent target lysis: experimental LDH — effector spontaneous LDH — target spontaneous LDH maximum LDH — target spontaneous LDH.

Anti-flu Ab titration by ELISA

Ninety-six-well Nunc Immuno plates were coated with 2.5 μg/ml flu-infected MDCK cell lysate and incubated overnight at 4°C. Wells were washed 4 times with 0.002% Tween 20 in PBS and blocked with 1% BSA for 1 h at 37°C. The plate was washed and serially diluted BAL, serum, or lung homogenates were added and incubated for 1 h at 37°C. The plate was washed four times, and biotinylated goat anti-mouse IgA, IgG1, or IgG2A (BD Biosciences) was added and incubated for 1 h at 37°C. The plate was washed four times, and strepavidin-alkaline phosphatase (Sigma-Aldrich) was added and incubated for 30 min. The plate was washed, and phosphatase activity was measured by adding p-nitrophenyl phosphate to 10% diethanolamine (Sigma-Aldrich), 0.02% NaN3, and 0.01% MgCl2·6 H2O (pH 9.8) for 30 min. The reaction was stopped by adding 2 N NaOH, the OD was measured at 405 nm, and the Ig titer was determined, based on the following formula: Ab titer = (ODsample reading × dilution)/0.05.

In vivo T cell depletion and Fasl. blocking

T cell depletion was carried out by administering 200 μg of a monoclonal anti-CD4 (OK1.5) and/or anti-CD8 (2.43) i.p. in 250 μl PBS at 4 and 9 d postinfection (45). Fasl. neutralization was carried out by delivering 50 μg anti-Fasl mAb (BD Pharmingen) intranasally every other day, starting at 4 d postinfection. Isotype control Ab or PBS was administered to the control mice in each experiment.

Adaptive CD4 T cell transfer

CD4 T cells from influenza-infected wt or DAP12KO lungs were isolated by positive selection using a double-column MACS purification protocol as specified by the manufacturer (Miltenyi Biotec, Auburn, CA). Cell purity was determined to be >95% by flow cytometry. Two million CD4 T cells from either wt or DAP12KO mice were adaptively transferred to Rag2−−/−γc−/− mice via the tail vein. Six hours following T cell transfer, mice were challenged intranasally with 2 × 10^7 PFU influenza virus. Mice were monitored daily for signs of illness and body weight changes.

Immune analysis and adoptive transfer of lung APC

Naïve lung APC from wt and DAP12 mice were isolated by positive selection of CD11c+ cells using the EasySep separation kit, according to the manufacturer’s protocol (StemCell Technologies, Vancouver, BC, Canada). Cell purity was determined to be >90% by flow cytometry. Two million CD4 T cells from either wt or DAP12KO mice were adaptively transferred to Rag2−−/−γc−/− mice via the tail vein. Six hours following T cell transfer, mice were challenged intranasally with 2 × 10^5 PFU influenza virus. Mice were monitored daily for signs of illness and body weight changes.
described above. Some cells were resuspended in serum-free media and transfectected with a replication-deficient adenovirus gene transfer vector expressing DAP12 (48) or the control vector. Cells were incubated at 37˚C for 3 h and then washed three times before being resuspended in complete media. Cell were pulsed overnight with UV inactivated flu (MOI = 10 prior to UV inactivation). One million CD11c+ cells were injected into the hind quadriceps muscles of naive wt and DAP12KO mice. Immunized mice were sacrificed 14 d following APC transfer, and the popliteal lymph nodes and spleen were isolated and Ag-specific CD4 T cell responses evaluated as described above.

**Statistical analysis**

Survival data were compared with a log-rank test using GraphPad (Prism, La Jolla, CA). All other analysis to compare groups was carried out using an unpaired, two-tailed Student \( t \) test.

**Results**

**DAP12 protein expression is upregulated in the lung of influenza-infected wt hosts**

DAP12 expression was previously shown to increase in response to chronic intracellular bacterial infection in the lung and is involved in modulating macrophage responsiveness to proinflammatory cytokines (28). To determine whether DAP12 may play a role in host defense against influenza viral infection, wt C57BL/6 mice were infected with influenza A virus, and 3, 5, and 9 d postinfection lungs were analyzed for the presence of DAP12 protein in lung tissue by immunoprecipitation and Western blotting. In agreement with the previous findings (28), DAP12 protein was found constitutively expressed in the lung at the predicted size of 12 kDa (Supplemental Fig. 1). However, influenza infection enhanced its expression by 3 d postinfection (126% increase over day 0) and elevated DAP12 protein expression sustained between days 5 (120% increase over day 0) and 9 postinfection (126% increase over day 0) (Supplemental Fig. 1). As expected, no DAP12 protein expression could be detected in the lungs of DAP12-deficient (KO) mice. These results suggest that DAP12 is involved in anti-influenza immunity.

**Hosts lacking DAP12 are susceptible to infection by a nonlethal dose of influenza virus**

To investigate the role of DAP12 pathway in host defense against primary respiratory influenza infection, DAP12KO as well as control wt mice were infected with influenza A virus. Mice were monitored daily for changes in body weight as well as symptoms of illness. In contrast to their wt counterparts, as early as 2 d postinfection, DAP12KO mice began showing greater signs of illness over wt controls, as indicated by significant body weight losses and deteriorating overall health status, and they lost body weight progressively out to 9 d postinfection (Fig. 1A). As a result, some of DAP12KO mice started to die (reaching the end point) from 2 d onward and by 8 d, ∼60% of them succumbed, in contrast to only ∼2% overall mortality rate in wt controls (Fig. 1B). DAP12KO mice that survived beyond 9 d postinfection were able to recover. These data indicate that the hosts lacking DAP12 are remarkably susceptible to an otherwise nonlethal dose of respiratory influenza infection.

**Hosts lacking DAP12 display exaggerated lung immunopathology following influenza infection**

To examine the cause of increased morbidity and mortality in infected DAP12KO mice, we examined histopathological changes in the lung over the course of influenza infection. In the lung of wt
mice, only mild inflammation was present at 3 d postinfection, and it increased marginally by 5 d with no sign of severe immunopathology seen up to 9 d (Fig. 1C). Lung inflammation in these mice was predominantly mononuclear. In sharp contrast, by 3 and 5 d postinfection, the lungs of DAP12KO mice had intense inflammation, which was characterized by neutrophilic and mononuclear infiltration around the major airways and blood vessels, extensive airway epithelial injury (sloughing), and inflammatory plug formation within the medium- and small-size airways (Fig. 1C, Table I). By 9 d, the lungs of the surviving DAP12KO animals still demonstrate necrotic foci and signs of tissue remodeling including bronchial epithelial hyperplasia and goblet cell metaplasia (Fig. 1C). Small patchy areas of intense inflammation and lymphocytic aggregates were still seen in the lungs of some DAP12KO animals by 18 d postinfection (data not shown). These observations suggest severe immunopathology and lung injury to be the basis of increased morbidity and mortality in infected DAP12KO animals.

**Hosts lacking DAP12 have an uncompromised capability to clear influenza virus from the lung**

We next examined whether pronounced lung immunopathology and mortality in influenza-infected DAP12KO mice was due to impaired viral clearance. Thus, we quantified and compared the level of viral burden in the lungs of wt and DAP12KO mice at days 3, 5, and 9 following infection. Of interest, we found no differences in the level of viral infection between wt and DAP12KO animals at various time points, and of importance, DAP12KO animals cleared the virus from the lung as efficiently as the wt controls (Fig. 1D). To examine whether the markedly increased morbidity and mortality of infected DAP12KO animals could be due to uncontrolled influenza virus dissemination from the lung to other tissue sites, we examined the viral titer in the spleen, kidney, liver, or brains of both wt and DAP12KO mice and detected no viruses in any of these tissue sites (data not shown). These results together suggest that, first, DAP12 is not required for host resistance to influenza viral infection, and second, the severe immunopathology and tissue injury seen in infected DAP12KO animals is not due to impaired viral clearance.

**Increased inflammatory responses in the lung of influenza-infected DAP12-deficient hosts**

To begin investigating the potential mechanisms underlying uncontrolled immunopathology seen in influenza-infected DAP12KO animals, we first examined the composition of inflammatory cell types, particularly neutrophils and total lymphocytes in the lung of DAP12KO animals (Fig. 2), in keeping with their severe tissue inflammation and immunopathology (Fig. 1C). The major difference in neutrophils was seen at early time points (days 3 and 5), whereas the difference in lymphocytes was seen at later time points (days 5 and 9) (Fig. 2B, 2C).

As influenza infection is able to cause exaggerated cytokine responses, which may directly cause exaggerated tissue inflammation and immunopathology in the lung (49, 50), we examined whether heightened inflammatory cellular responses seen in the lung of infected DAP12KO animals might be associated with dysregulated proinflammatory cytokine responses. Although we found the levels of proinflammatory cytokines or chemokines IL-1β, TNF-α, MIP-1α, MCP-1, keratinocyte chemoattractant, and IFN-γ-inducible protein-10 in the lung to be largely comparable at various time points between infected wt and DAP12KO mice (Supplemental Fig. 2), TNF-α and IFN-γ-inducible protein-10 levels were moderately higher around 5 d postinfection in the lung of DAP12KO animals than in the lung of wt controls (Supplemental Fig. 2B, 2F). We also compared the levels of type 1 IFN-β levels in the lung but did not find the difference between wt and DAP12KO mice (data not shown). Because NO has been implicated in influenza infection (51), we measured NO in the lung and found the levels of induced NO production to be comparable between wt and DAP12KO mice (data not shown). Taken together, these findings suggest that although there are significantly higher levels of inflammatory cellular responses in the lung of infected DAP12KO animals, the severe illness and immunopathology in these hosts are unlikely accounted for by differentially regulated proinflammatory cytokine responses.

**Increased IFN-γ-producing, cytotoxic CD4 T cells in the lung of influenza-infected DAP12-deficient hosts**

To further investigate the mechanisms underlying uncontrolled immunopathology seen in influenza-infected DAP12KO animals, we examined T cell responses in the airway lumen (BAL) as well as in the lung interstitium in greater detail at 3, 5, and 9 d following influenza infection. The total number of CD4 T cells in the airway lumen increased sharply in DAP12KO mice over wt controls shortly postinfection (3 d) and remained significantly elevated at later time points, particularly at 5 d postinfection (Fig. 3A). A similar trend was observed in the lung interstitium of DAP12KO mice (Fig. 3C). To assess the effector activities of these CD4 T cells, we examined CD4 T cell IFN-γ production by intracellular cytokine staining. Compared with wt controls, there were markedly greater numbers of flu Ag-stimulated IFN-γ+ effector CD4 T cells in the airway lumen (BAL) and lung interstitium of DAP12KO mice at days 3, 5, and 9 postinfluenza infection (Fig. 3B, 3D).

To further evaluate the effector function of dysregulated CD4 T cell responses in the lung of infected DAP12KO animals, we developed an in vivo intratracheal CTL assay to assess the ability of

---

**Table I. Lung immunopathology by influenza infection in wt and DAP12KO hosts**

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Days</th>
<th>Epithelial Sshelling and Airway Plugs</th>
<th>Epithelial Metaplasia/ Hyperplasia</th>
<th>Large Airway Inflammation</th>
<th>Parenchymal Inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>3</td>
<td>+++</td>
<td>—</td>
<td>+/—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>—</td>
<td>—</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>DAP12KO</td>
<td>3</td>
<td>+</td>
<td>—</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>+++</td>
<td>—</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>+++</td>
<td>—</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

At various time points following influenza infection, mice were sacrificed, and their lungs fixed in 10% formalin before being sectioned and H&E stained. Blinded semiquantification of the extent of lung pathology was carried out by examining lung sections from three to nine animals per time point. Severity of pathological change is expressed as significant (+++), moderate (++), mild (+), and absent (—).
experiments. Macrophages (DAP12KO mice following pulmonary influenza infection. Influenza-specific target lysis observed in the lung of previously described methodology (47). There was a very low level of influenza-infected animals, based on our delivered to the airway of influenza-infected animals, (splenocytes) were CFSE labeled and subsequently intratracheally these CD4 T cells to recognize and lyse influenza-infected target (Fig. 2).

FIGURE 2. Increased inflammatory cellular responses in the lung of DAP12KO mice following pulmonary influenza infection. Influenza-infected wt and DAP12KO mice were subject to BAL, and the number of macrophages (A), neutrophils (B), and lymphocytes (C) in the airway was enumerated on cytospins by differential cell counting. Data are expressed as the mean ± SEM of 4–10 mice/group/time from three independent experiments. *p < 0.05, **p < 0.01, ††p < 0.001.

these CD4 T cells to recognize and lyse influenza-infected target cells. To this end, in vitro flu Ag-pulsed CTL target cells (splenocytes) were CFSE labeled and subsequently intratracheally delivered to the airway of influenza-infected animals, based on our previously described methodology (47). There was a very low level of influenza-specific target lysis observed in the lung of infected wt animals at both days 5 and 9 (Fig. 3E). In contrast, there was a much greater level of influenza-specific target lysis observed in the lung of DAP12KO mice at these time points postinfection (Fig. 3E). No CTL could be detected at 3 d postinfection in either strain of mice (data not shown). To verify that the observed cytotoxicity was mediated specifically by CD4 T cells, we depleted CD8 T cells in vivo by using an anti-CD8 mAb prior to CTL target delivery. We found that the removal of CD8 T cells from the lung did not attenuate the level of influenza-specific target lysis (Fig. 3F), indicating that the dysregulated CD4 T cells in the lung of DAP12KO animals acquired potent cytotoxic capabilities during influenza infection. Furthermore, with an in vitro CTL assay and the same number of effector cells, the CD4 T cells purified from the whole lungs of flu-infected DAP12KO mice (9 d) resulted in a significantly greater rate of target lysis at 10:1 and 2:1 T cell/target cell ratios than the wt counterparts (Fig. 3G).

Different from increased CD4 T cells in the lung of DAP12KO animals, we found the total CD8 T cell numbers in the lung to be comparable between infected wt and DAP12KO animals (Supplemental Fig. 3A). However, there were greater numbers of NP tetramer+ (Supplemental Fig. 3B) or IFN-γ+ CD8 T cells (Supplemental Fig. 3C) in the lung of DAP12KO animals. Because DAP12 deficiency has been found to cause decreased regulatory T cell functions (cytokine release and cytotoxicity), which persisted well beyond the time of viral clearance in the lung of influenza-infected animals lacking DAP12.

CD4 T cells, but not CD8 T cells, are responsible for increased susceptibility and lung immunopathology of influenza-infected DAP12-deficient hosts

Our data thus far suggest a role of exaggerated T cell responses in increased mortality and severe immunopathology of influenza-infected DAP12KO animals. To investigate whether T cells and if so, which T cell subset, were causally linked to increased mortality and severe immunopathology, we infected DAP12KO mice with a lower dose of influenza virus to retard the morbidity of DAP12KO hosts. At 4 d postinfection, we depleted either CD8 T cells or CD4 T cells by i.p injection of mAb (Fig. 4A). As expected, infected DAP12KO mice receiving control treatment were severely ill and by days 8, only ~30% of them survived (Fig. 4A). In comparison, the DAP12KO mice depleted of CD8 T cells did not have an improved survival rate over the control group. In contrast, unexpectedly the depletion of CD4 T cells remarkably improved the health status and uplifted the survival rate of infected DAP12KO mice up to ~90% (Fig. 4A), which was similar to the survival rate in infected wt hosts (Fig. 1B). On the basis of this, we further examined the relationship of the survival rate with the extent of tissue immunopathology. As expected, similar to what we observed in the previous experiments (Fig. 1C), the control DAP12KO lungs had severe tissue inflammation, bronchial epithelial injury/sloughing, and hyperplasia (Fig. 4B) in keeping with their poor survival (Fig. 4A). In comparison, the DAP12KO animals depleted of CD8 T cells had even worse lung immunopathology and injury (Supplemental Fig. 4A). In stark contrast, in accordance with their markedly enhanced survival rates, the DAP12KO animals depleted of CD4 T cells had markedly reduced tissue inflammation and bronchial epithelial injury/ sloughing in the lung (Fig. 4B, Table II).

As CD4 T cells are known to contribute to anti-influenza virus host defense (1, 2, 22), we next examined the relationship between CD4 T cell depletion-improved survival and influenza viral clearance in the lung. We found no significant difference in the level of viral burden in the lungs of control and CD4 T cell-depleted DAP12KO animals up to 7 d postinfection (Fig. 4C). However, although the control DAP12KO mice completely cleared the virus from the lung by 9 d postinfection in consistence with the data in Fig. 1D, the CD4 T cell-depleted DAP12KO counterparts had delayed viral clearance, and a complete viral clearance was not seen until 14 d postinfection (Fig. 4C). We also observed a similarly delayed viral clearance in the lung of CD8 T cell-depleted DAP12KO animals (Supplemental Fig. 4B). As
CD4 T cell-mediated helper function is considered important to Ab responses, we addressed whether the delayed viral clearance seen in CD4 T cell-depleted DAP12KO mice (Fig. 4C) was related to potentially diminished Ab titers. We found that the levels of flu-specific IgA, IgG1, and IgG2a were comparable between the control DAP12KO and CD4 T cell-depleted DAP12KO mice at various time points postinfection (Fig. 4D–F). This may be because T cells were not depleted until 4 d postinfection (Fig. 4A) and because DAP12KO hosts had an accelerated CD4 Th function (being markedly elevated as early as day 3 as shown in Fig. 3B). These data suggest that the delayed viral clearance in CD4-depleted DAP12KO mice was unlikely due to altered Ab titers.

The above findings indicate that the dysregulated CD4 T cells, but not CD8 T cells, are the culprit for increased mortality and severe lung immunopathology of influenza-infected DAP12KO animals. Furthermore, the data suggest that although both CD4 and CD8 T cell subsets are involved in anti-influenza host defense (viral clearance), they possess the distinct immunopathogenic potential. Thus, dysregulated CD4 T cells may be more immune-destructive than CD8 counterparts.

The immunopathogenic potential of dysregulated CD4 T cells from influenza-infected DAP12-deficient hosts is transferrable to DAP12-competent hosts

To further examine the effect of dysregulated CD4 T cells on immunopathology in influenza-infected DAP12KO animals, we questioned whether the immunopathogenic potential of CD4 T cells acquired in a DAP12-deficient tissue environment would still be maintained in a DAP12-competent wt tissue environment. To this end, total CD4 T cells were purified using a MACS column protocol from wt and DAP12KO mice 7 d postinfluenza infection. Purified wt CD4 T cells or those from DAP12KO animals were then adoptively transferred i.v to naive Rag2−/− mice that were subsequently infected with influenza virus (Fig. 5 experimental schema). In contrast to the mice receiving the CD4 T cells from wt hosts, the mice receiving the CD4 T cells from DAP12KO hosts were ill, suffering a progressively worsening body weight loss (Fig. 5A).

Upon examination of H&E-stained lung tissue sections, we found that although there was intense inflammatory infiltration in the peribronchial and perivascular areas in the lungs of Rag2−/−
mice receiving wt CD4 T cells, a much greater extent of such inflammation was seen in the lung of the mice receiving DAP12KO host-derived CD4 T cells, and such inflammation was accompanied by severe bronchial epithelial injury and the formation of intrabronchial inflammatory plugs (Fig. 5B), reminiscent of the immunopathology and tissue injury seen in the lung of infected DAP12KO animals (Fig. 1C). The severe immunopathology and injury caused by transferred DAP12KO host-derived CD4 T cells was in accord with severe illness of these mice (Fig. 5A). To understand the topographical relationship of pronounced lung tissue inflammation and injury in these animals with the adoptively transferred CD4 T cells, we examined the localization of CD4 T cells in lung tissue by immunohistochemistry. Indeed, immunohistochemical staining for CD3 T cell marker revealed the greater accumulation of T cells in the peribronchial and perivascular areas of the lungs of Rag2<sup>−/−</sup> mice receiving wt CD4 T cells, a much greater extent of such inflammation was seen in the lung of the mice receiving DAP12KO host-derived CD4 T cells, and such inflammation was accompanied by severe bronchial epithelial injury and the formation of intrabronchial inflammatory plugs (Fig. 5B), reminiscent of the immunopathology and tissue injury seen in the lung of infected DAP12KO animals (Fig. 1C). The severe immunopathology and injury caused by transferred DAP12KO host-derived CD4 T cells was in accord with severe illness of these mice (Fig. 5A). To understand the topographical relationship of pronounced lung tissue inflammation and injury in these animals with the adoptively transferred CD4 T cells, we examined the localization of CD4 T cells in lung tissue by immunohistochemistry. Indeed, immunohistochemical staining for CD3 T cell marker revealed the greater accumulation of T cells in the peribronchial and perivascular areas of the lungs of Rag2<sup>−/−</sup> mice receiving CD4 T cells was in accord with severe illness of these mice (Fig. 5A). To understand the topographical relationship of pronounced lung tissue inflammation and injury in these animals with the adoptively transferred CD4 T cells, we examined the localization of CD4 T cells in lung tissue by immunohistochemistry. Indeed, immunohistochemical staining for CD3 T cell marker revealed the greater accumulation of T cells in the peribronchial and perivascular areas of the lungs of Rag2<sup>−/−</sup> mice receiving CD4 T cells was in accord with severe illness of these mice (Fig. 5A). To understand the topographical relationship of pronounced lung tissue inflammation and injury in these animals with the adoptively transferred CD4 T cells, we examined the localization of CD4 T cells in lung tissue by immunohistochemistry. Indeed, immunohistochemical staining for CD3 T cell marker revealed the greater accumulation of T cells in the peribronchial and perivascular areas of the lungs of Rag2<sup>−/−</sup> mice receiving CD4 T cells was in accord with severe illness of these mice (Fig. 5A). To understand the topographical relationship of pronounced lung tissue inflammation and injury in these animals with the adoptively transferred CD4 T cells, we examined the localization of CD4 T cells in lung tissue by immunohistochemistry. Indeed, immunohistochemical staining for CD3 T cell marker revealed the greater accumulation of T cells in the peribronchial and perivascular areas of the lungs of Rag2<sup>−/−</sup> mice receiving

### Table II. Lung immunopathology by influenza infection in DAP12KO and CD4-depleted DAP12KO hosts

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Days</th>
<th>Epithelial Soughing and Airway Plugs</th>
<th>Epithelial Metaplasia/ Hyperplasia</th>
<th>Large Airway Inflammation</th>
<th>Parenchymal Inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>+++</td>
<td>–</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>++</td>
<td>–</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>CD4 depleted</td>
<td>9</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>++</td>
<td>–</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

At various time points following influenza infection, mice were sacrificed, and their lungs fixed in 10% formalin before being sectioned and H&E stained. Blinded semiquantification of the extent of lung pathology was carried out by examining lung sections from three to nine animals per time point. Severity of pathological change is expressed as significant (++++)+, moderate (+++), mild (+), and absent (–).
DAP12KO-derived CD4 T cells compared with the lung of mice receiving wt CD4 T cells (Fig. 5B). Of importance, many T cells in the former were seen permeating not only the damaged bronchial epithelium but also the intrabronchial inflammatory plugs (Fig. 5B). Morphometric quantification of lung tissue infiltrated by T cells revealed markedly much more T cells in the lungs of influenza virus-challenged Rag2<sup>−/−</sup>/γc<sup>−/−</sup> mice that received DAP12KO-derived CD4 T cells than in the lungs of influenza-challenged mice receiving wt CD4 T cells or in the lungs of unchallenged mice or of virus-challenged mice receiving no CD4 T cells (Fig. 5C). This suggests that adoptively transferred DAP12KO-derived CD4 T cells underwent greater expansion and activation in the lung of severe immune-deficient hosts in response to influenza viral Ag stimulation. These findings together indicate that the immunopathogenetic potential of CD4 T cells in influenza-infected DAP12KO animals is transferrable and that these CD4 T cells can retain their robust immunopathogenicity in a lung environment that is otherwise identical to that of wt DAP12-competent hosts.

Hyperactivated CD4 T cells of influenza-infected DAP12-deficient hosts mediate lethal lung immunopathology via Fas/FasL pathway

Markedly increased numbers of IFN-γ-producing, cytotoxic CD4 T cells (Fig. 3A–D) accounted for the morbidity and severe immunopathology (Fig. 4A, 4B) in the lung of influenza-infected DAP12KO animals. To further dissect the mechanisms by which these CD4 T cells cause severe lung immunopathology, we examined their surface expression of T cell activation markers CD44, CD25, and CD69 at various time points. We found much greater numbers of CD4 T cells in the lung of infected DAP12KO animals that expressed high levels of CD44 (Fig. 6A), CD25, and CD69 (data not shown) than in the lung of infected wt animals. Because CD4 T cells are known to express FasL and have been implicated in CD4 T cytotoxicity and tissue injury (53), we examined FasL expression on CD4 T cells. The level of FasL expression was found markedly increased on aerial luminal (Fig. 6B) and lung interstitial (Fig. 6C) CD4 T cells in infected DAP12KO animals at various time points over that in infected wt animals.

Given markedly increased CD4 T cell FasL expression (Fig. 6B, 6C) and CD4 T cell-mediated cytotoxicity (Fig. 3E, 3F) and their connection to increased morbidity/mortality (Figs. 4, 5) in infected DAP12KO animals, we next investigated the role of Fas/FasL pathway in CD4 T cell-mediated morbidity and lung immunopathology in DAP12KO animals. Blockade of Fas/FasL interaction by mAb-mediated FasL neutralization was found to markedly increase survival of influenza-infected DAP12KO animals (Fig. 6D), which was associated with improved lung immunopathology (Fig. 6F, Table III). In comparison, IFN-γ blockade failed to improve the survival of influenza-infected DAP12KO animals (data not shown). The above findings together indicate that dysregulated cytotoxic CD4 T cells in influenza-infected DAP12KO animals lead to severe lung immunopathology and morbidity via Fas/FasL signaling pathway.

DAP12 expressed in Ag presenting cells is critical to controlling influenza Ag-specific CD4 T cell responses

Myeloid APC, NK cells, and neutrophils are the major cell types that express DAP12 (24, 25). We next examined whether DAP12 expressed by myeloid APC played a major role in regulating CD4 T cell activation following influenza infection. We first evaluated the immune phenotype of lung APCs from wt and DAP12KO animals. We found that CD11c<sup>+</sup>CD11b<sup>+</sup> APCs isolated from naive DAP12KO mice produced more TNF-α in response to influenza virus infection, but not to stimulation by UV-inactivated influenza viruses, than wt controls (Fig. 7A). These APCs also produced higher levels of TNF-α upon stimulation with TLR7 ligand imiquimod. Exposure to viable or UV-inactivated influenza viruses upregulated
APC surface expression of T cell costimulatory CD80 (Fig. 7B) and MHC class II molecules (Fig. 7C) to a greater extent than wt controls. The extent of upregulation of these molecules by imiquimod, however, did not differ between DAP12KO and wt APCs.

To evaluate the in vivo role of DAP12 expressed in lung APCs in regulating influenza Ag-specific CD4 T cell activation, APCs isolated from the lung of naive wt or DAP12KO animals were loaded overnight with influenza viral Ags. These APCs were then injected i.m. to immunize either naive wt or DAP12KO mice that were sacrificed for examination of Ag-specific CD4 T cell responses 14 d postimmunization (Fig. 7 experimental schema). wt APCs when used to immunize wt mice (wt→wt) were found to prime a level of CD4 T cell responses (Fig. 7D). In comparison, DAP12KO APCs when used to immunize either wt (DAP12-KO→wt) or DAP12KO (DAP12KO→DAP12KO) mice triggered significantly higher levels of CD4 T cell activation (Fig. 7D).

To examine whether reconstitution of DAP12 expression in DAP12KO APCs could revert heightened CD4 T cell activation, we isolated DAP12KO APCs and transduced them with a recombinant viral-based DAP12 gene transfer vector or a control vector before in vivo injection. We found that only immunization with the DAP12 gene-reconstituted DAP12KO APCs (DAP12-KO→DAP12KO + DAP12), but not with the nonreconstituted DAP12KO APC control (DAP12KO→DAP12KO), brought down the level of Ag-specific CD4 T cell activation (Fig. 7E). These data together suggest that DAP12 expressed by lung APCs plays a critical role in regulating the level of influenza Ag-specific CD4 T cell activation.

Table III. Lung immunopathology by influenza infection in DAP12KO and FasL-neutralized DAP12KO hosts

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Days</th>
<th>Epithelial Metaplasia/ Hyperplasia</th>
<th>Large Airway Inflammation</th>
<th>Parenchymal Inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9</td>
<td>+++++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>++++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>FasL neutralized</td>
<td>9</td>
<td>+/-</td>
<td>++</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
</tr>
</tbody>
</table>

At various time points following influenza infection, mice were sacrificed, and their lungs fixed in 10% formalin before being sectioned and H&E stained. Blinded semiquantification of the extent of lung pathology was carried out by examining lung sections from three to four animals per time point. Severity of pathological change is expressed as significant (+++++), moderate (+++), mild (+), and absent (-).
FIGURE 7. DAP12 expressed by lung APCs is critically required for regulating CD4 T cell activation during influenza infection. Purified lung CD11c+ APCs were stimulated with influenza virus, UV-inactivated virus, or imiquimod for 48 h. The level of TNF-α was measured in culture supernatants by ELISA (A). Cell surface expression of T cell costimulatory molecule CD80 (B) and MHC class II (C) was measured by flow cytometry. To investigate the differential ability of lung DAP12-expressing and DAP12-deficient APCs to engage and activate CD4 T cells in vivo, UV-inactivated influenza virus-loaded CD11c+ APCs isolated from wt or DAP12KO mouse lungs were injected i.m. to naive wt or DAP12KO mice (wt→wt, DAP12KO→wt, and DAP12→DAP12). The extent of flu Ag-specific CD4 T cell activation was assessed 14 d after APC immunization (experimental schema) (D). To examine whether reconstitution of DAP12 expression in DAP12-deficient APCs could restore the control of flu Ag-specific CD4 T cell responses, the CD11c+ APCs isolated from the lung of DAP12KO mice were transduced with a recombinant adenovirus-based DAP12 gene transfer vector (DAP12KO→DAP12KO + DAP12) or a control viral vector (DAP12KO→DAP12KO − DAP12). All APCs were then pulsed with flu Ags and injected i.m. to naive DAP12KO mice and flu Ag-specific CD4 T responses analyzed as described above (E). Data are expressed as mean ± SEM of 10 mice/group from two independent experiments. *p < 0.05.

Discussion

In this paper, we report a previously unrecognized immune mechanism of severe influenza immunopathology and morbidity that is mediated solely by dysregulated immunopathogenic CD4 T cells. We find that although these immunopathogenic CD4 T cells are capable of heightened IFN-γ production, they lead to severe lung immunopathology and tissue injury via their robust FasL expression and CTL. We reveal that the level of such influenza-specific immunopathogenic CD4 T cells is under the control of transmembrane signaling immunoadaptor DAP12 expressed by APC. Of importance, the immune regulatory role of DAP12 pathway during influenza infection is unique because it is only required for controlling the level of immunopathogenic CD4 T cells but not for influenza viral clearance.

Influenza A viruses are the most common cause of flu epidemics and pandemics. Influenza represents a typical scenario in which the balance between viral pathogen clearance (host defense) and tissue immunopathology gets readily tipped. Thus, the collateral lung immunopathology and tissue injury often accounts for the morbidity and fatality of influenza (2, 54). Unfortunately, the immune regulatory mechanisms of influenza immunopathology have just begun to be understood, and enhanced knowledge in this regard is disparately needed for developing effective preventative and therapeutic strategies coping with frequent global flu epidemics/pandemics. It is known that influenza infection activates both CD8 and CD4 T cells (1, 2, 4). However, compared with CD4 T cells, the potential role of CD8 T cells in lung immunopathology has received much greater attention as CD8 T cells are considered the most critical effectors for influenza viral clearance (1, 2). For instance, the immune arsenals including IFN-γ, TNF-α, and cytotoxic molecules used by CD8 T cells for influenza viral clearance were also found involved in the development of lung immunopathology (2). Compared with CD8 T cells, both the role and the regulatory mechanisms of CD4 T cells in influenza-associated immunopathology has remained largely elusive for many years due in part to the lack of immunoreagents and their smaller number and quicker decline post-infection (1, 2). And as a result, the relative contribution of CD8 and CD4 T cells to influenza-associated lung immunopathology cannot be adequately assessed. The progress in this regard is being hampered further by many of the immune mechanisms shared for both influenza viral clearance and influenza-associated immunopathogenesis.

In the current study, we have observed that the development of immunopathogenic CD4 T cells during influenza infection is tightly controlled by the DAP12 signaling pathway. Thus, the removal of DAP12 results in a remarkable emergence of dysregulated CD4 T cells with increased expression of FasL and cytotoxicity. The immunopathogenic property of such CD4 T cells is transferrable to a DAP12-competent lung environment, causing marked immunopathological changes that are not seen with the wt animal-derived CD4 T cells. Furthermore, the immunopathogenic CD4 T cells were found topographically associated with the areas of airway epithelial damage and bronchial inflammatory plugs. These findings together suggest that the exertion of CD4 T cell-mediated immunopathogenicity does not depend on other inflammatory or immunologic signals that may have potentially altered as a result of DAP12 deficiency. A previous study found the...
NK cell function in murine cytomegalovirus-infected DAP12KO mice to be impaired (38) and such altered antiviral innate function because of DAP12 deficiency may be the reason as to why the much increased cytotoxic CD4 T cell responses in flu-infected DAP12KO hosts observed in our current study did not result in increased viral clearance. We believe that the severe airway injury observed in the lung of DAPKO most likely resulted from both markedly increased numbers and activation status (e.g., enhanced FasL expression) of CD4 T cells. Among several approaches of CD4 T cell functional analyses we undertook are the adoptive CD4 T cell transfer and in vitro CTL assay. Although the both approaches compared the function of the same number of purified total CD4 T cells isolated from infected wt and DAP12KO lungs, the presence of greater frequencies of flu-specific CD4 T cells in DAP12KO preparations still disallows us to assign the observed functional difference only to increased activation status. The definitive way of dissecting the relative contribution of increased quantity and quality of CD4 T cells in DAP12KO hosts entails the future use of the reagents that allow the purification of flu Ag-specific CD4 T cells. The relatively low overall increased in vitro CTL activities by the CD4 T cells of DAP12KO hosts are in line with the small but significantly increased in vivo CTL by these cells and are likely due to the functional difference between cytotoxic CD4 and CD8 T cells. It is also noteworthy that lack of DAP12 leads to heightened activation of both CD4 and CD8 T cells. However, we find that only the depletion of CD4 T cells, but not CD8 T cells, improves lung immunopathology and survival. This indicates that dysregulated CD4 T cells can be even much more deleterious or immunopathogenic than CD8 counterparts. In our view, this could be the reason as to why in the normal circumstances the CD8 T cell is the most critical effector in influenza viral clearance, and the CD4 T cell activation is under tight control of the DAP12 pathway, a mechanism that is not required for influenza viral clearance.

Although the precise molecular mechanisms by which DAP12 signaling pathway regulates CD4 T cell activation in influenza remain to be fully elucidated, our current data support a mechanism mediated by DAP12-expressing APC. Indeed, we found flu-infected DAP12KO APC to produce heightened type 1 cytokines and express increased MHC class II and B7.1. Furthermore, adoptively transferred flu Ag-loaded DAP12KO APC, but not DAP12-competent APC, markedly increased Ag-specific CD4 T cell activation in vivo. These data suggest that DAP12 pathway controls CD4 T cell responses via regulating the production of T cell-activating cytokines and Ag presentation by APC. Previous studies have shown that lack of DAP12 in APC led to the increased TLR signaling via enhanced Syk-ERK and PI3K activation (32–35).

We find the dysregulated CD4 T cells to cause severe lung immunopathology and illness via increased Fas/FasL interaction and CTL. FasL expression was also previously shown to be a major mechanism by which CD4 T cells directly lyse tissue cells, causing significant tissue injury and remodeling in other models of inflammation (41, 55). We find that markedly increased FasL expression is not only limited to the CD4 T cells in the lung, because we also find high FasL-expressing CD4 T cells in the local draining lymph nodes of infected DAP12KO hosts (data not shown), suggesting a profoundly altered CD4 T cell programming. This conviction is supported further by our observation that the immunopathogenic property including elevated FasL expression of CD4 T cells is maintained after they are transferred into naive DAP12-competent animals. The markedly improved survival and immunopathology of infected DAP12KO animals following the blockade of Fas/FasL interaction comes with a price of delayed influenza viral clearance, similar to the outcome of CD4 T cell depletion. Thus, different from the current belief that CD4 T cells contribute indirectly to influenza viral clearance, our findings together suggest a direct role of CD4 T cells in viral clearance. Admittedly, although we found the depletion of CD4 T cells (from day 4 postinfection) in DAP12KO mice to be inconsequential to anti-flu Ab responses to a low dose of flu viral infection, the requirement of CD4 Th function for Ab responses could be different when larger doses of flu virus are used. However, our findings do suggest that the mechanisms by which CD4 T cells cause influenza immunopathology differ in several aspects from those by CD8 counterparts. Indeed, CD8 T cell-mediated immunopathology is often associated with delayed viral clearance (2), whereas we have shown in this paper that CD4 T cell-mediated severe immunopathology is independent of viral clearance in the lung. Furthermore, CD8 T cell-mediated influenza immunopathology is primarily caused by CD8 T cell-derived IFN-γ, TNF-α, or perforin/granzyme-mediated CTL (1, 2, 5, 19, 20), whereas immunopathogenic CD4 T cells do so primarily via FasL-mediated CTL. Our current study has provided further evidence that DAP12 expressed by APCs plays a critical role in regulating the level of immunopathogenic CD4 T cells during influenza infection. This DAP12 pathway of immune regulation is not required for influenza viral clearance because DAP12 deficiency has no effect in this process. The revelation of this novel immune regulatory pathway in influenza immunopathology adds to the much needed knowledge required for developing the potential intervention strategies that will more specifically dampen influenza immunopathology with relatively little effect on viral clearance. In this respect, disruption of T cell OX40 interaction with OX40L on APCs was recently found to specifically ameliorate lung immunopathology following primary influenza infection (18). In contrast, costimulation modulation via blocking CD28 signaling specifically dampened immunopathology in prior flu-infected lungs mediated by a secondary memory CD4 T cell response (56). Furthermore, anti-influenza CD8 T cells were recently found to release anti-inflammatory cytokine IL-10, which helped control the extent of immunopathology (12).

Our current observation that lack of DAP12 leads to the emergence of highly immunopathogenic CD4 T cells during influenza infection also suggests that the functionally redundant DAP10 pathway (25) cannot compensate for the loss of DAP12 function. The important immune regulatory role of DAP12 signaling pathway has been increasingly recognized (24–27). Although earlier studies suggest a proinflammatory nature of DAP12 signaling specifically dampened immunopathology in prior flu-infected lungs mediated by a secondary memory CD4 T cell response (56). Furthermore, anti-influenza CD8 T cells were recently found to release anti-inflammatory cytokine IL-10, which helped control the extent of immunopathology (12).

The novel findings from our current study together with the previous reports suggest an emerging paradigm that the functional outcome of DAP12 signaling pathway is largely defined by the nature of Ags or infectious agents and that the conclusion derived from mere in vitro studies can be misleading. Thus, the role of DAP12 pathway may be proimmune when the host is exposed in vivo to inert Ags (39) or extracellular infectious agents or isolated pattern recognition receptor ligands (31, 37, 58). However, when the host is exposed to the intact intracellular infectious agents such as murine cytomegalovirus (35), Listeria monocytogenes (34), Mycobacterium bovis (36), or influenza A virus (current study), DAP12 plays a critical immune suppressive role. As many innate immune receptors associate with and signal through DAP12 and the ligands for many of these receptors still remain unknown, identifying the potential ligands will help develop the effective immunotherapeutic strategies for fine-tuning host antimicrobial responses.
Acknowledgments
We thank Jeanette Boudreau for technical assistance in i.v. injection and Dr. Wayne Yokoyama at Washington University School of Medicine for the provision of DAP12−/− breeder mice on C57BL/6 background.

Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1.

Constitutive and influenza-inducible DAP12 protein expression in wt BL/6 mouse lung. The level of DAP12 protein was examined in the lungs of wt mice in response to influenza infection by immunoprecipitation and western blotting. Equal amounts of lung tissues were subjected to immunoprecipitation and run on an SAS-gel in denaturing conditions. The lung tissue from DAP12KO mice was used as a negative control. Two wt BL/6 animals were set up for each time point post-influenza infection. Recombinant DAP12 protein was used a positive control.
Cytokine and chemokine levels in the bronchoalveolar lavage fluids of wt and DAP12KO mice following influenza infection. At various time points following infection, lungs were isolated and subject to bronchoalveolar lavage (BAL). BAL fluids were measured by ELISA or multiplex cytokine assay for IL-1 (a), TNF-α (b), MIP-1α (c), MCP-1(d), KC (e) and IP-10 (f). Data are expressed as mean±SEM of 3-5 mice/group/time point from 2 independent experiments. *p<0.05
Supplemental Figure 3.

Increased CD8 T cell responses in the lungs of influenza-infected DAP12KO mice. Lungs of wt and DAP12KO mice harvested at various time points following infection were subject to bronchoalveolar lavage. The total number of CD8 T cells in the BAL was determined by flow cytometry (a). Influenza tetramer-specific CD8 T cells were determined by tetramer immunostaining (b). The number of IFN-γ-secreting CD8 T cells was determined by intracellular cytokine staining (c). Data are expressed as mean ± SEM of 5-18 mice/group/time point from 1-3 independent experiments. * p<0.05, ** p<0.01
Supplemental Figure 4.

Depletion of CD8 T cells fails to improve lung immunopathology in influenza-infected DAP12KO mice. At various time points post-infection, lungs from control-treated and CD8 T cell-depleted DAP12KO mice were processed, H&E stained and histopathologically examined (a). Furthermore, lungs harvested from these mice were also subject to influenza viral plaque forming (pfu) assay. Data are expressed as mean±SEM of 5-8 mice/group/time point from 2-3 independent experiments. ‡‡ p<0.005