Critical Negative Regulation of Type 1 T Cell Immunity and Immunopathology by Signaling Adaptor DAP12 during Intracellular Infection


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Critical Negative Regulation of Type 1 T Cell Immunity and Immunopathology by Signaling Adaptor DAP12 during Intracellular Infection


Transmembrane signaling adaptor DAP12 has increasingly been recognized for its important role in innate responses. However, its role in the regulation of antimicrobial T cell responses has remained unknown. In our current study, we have examined host defense, T cell responses, and tissue immunopathology in models of intracellular infection established in wild-type and DAP12-deficient mice. During mycobacterial infection, lack of DAP12 leads to pronounced proinflammatory and Th1 cytokine responses, overactivation of Ag-specific CD4 and CD8 T cells of type 1 phenotype, and heightened immunopathology both in the lung and lymphoid organs. DAP12-deficient airway APC display enhanced NF-κB activation and cytokine responses upon TLR stimulation or mycobacterial infection in vitro. Of importance, adoptive transfer of Ag-loaded DAP12-deficient APC alone could lead to overactivation of transferred transgenic or endogenous wild-type T cells in vivo. We have further found that the immune regulatory role by DAP12 is not restricted only to intracellular bacterial infection, since lack of this molecule also leads to uncontrolled type I T cell activation and severe immunopathology and tissue injury during intracellular viral infection. Our study thus identifies DAP12 as an important novel immune regulatory molecule that acts, via APC, to control the level of antimicrobial type 1 T cell activation and immunopathology.


A homodimeric transmembrane signaling adaptor protein, DNA-activating protein of 12 kDa (DAP12),3 that was first identified and cloned by Vivier’s (1) and Lanier’s (2) laboratories in 1997 and 1998, respectively, has a small extracellular domain and an intracytoplasmic ITAM. A number of important immune regulatory receptors such as NKG2D, Ly49, triggering receptor expressed on myeloid cells (TREMds), MDL-1, and CD200R in NK and myeloid cells including dendritic cells and macrophages, were subsequently found to be associated with and signal through DAP12 (3). Originally, DAP12 was identified as a killer-cell activating receptor-associated protein for its important role in NK cell activation (2). Indeed, recently by using DAP12-deficient models, the studies from Vivier and colleagues (4), Raulet and colleagues (5), Colonna and Campbell (6), Cerboni and colleagues (7), Domer et al. (8), and French et al. (9) have provided unequivocal evidence that the DAP12 signaling pathway is critically required for activation of NK cell-killing activities in models of tumor and murine CMV (MCMV) infection to which host defense rests almost solely on innate NK functions (4–9). Since DAP12 is also expressed by innate myeloid dendritic cells, macrophages, and neutrophils, its role in the function of these cell types during innate immune or inflammatory responses has received an increasing attention. A number of earlier studies from us (10) and others (11–13) seem to support an activating or proinflammatory role by DAP12 and as a result, transgenic overexpression of DAP12 leads to enhanced inflammatory responses (14–16), whereas abrogation of the DAP12 pathway dampens such innate inflammatory responses (7, 12, 15, 17). However, more recent experimental studies from Lanier, Colonna and Dalod’s laboratories (18–21) argue against this conviction and have provided the evidence that lack of DAP12 or abrogation of associating TREM-2 molecules may instead lead to more pronounced activation of murine macrophages or dendritic cells in vitro and potentiated innate inflammatory responses during endotoxic or Gram-negative bacterial sepsis in vivo. Such discrepancies may be at least in part accounted for by the fact that the ligands for the majority of DAP12-associating receptors including TREMs, myeloid DAP12-associated protein 1 (MDL-1), and signal regulatory protein β (SIRPβ) still remain unknown (3), and selective engagement of these receptors may lead to differential DAP12-mediated signaling events. And this fact underscores the importance of investigating the role of DAP12 in immune regulation in the context of relevant in vivo models of disease.
Unfortunately, relatively little is known about the role of DAP12 in the regulation of adaptive immunity in general and its role in the regulation of T cell responses to intracellular infection has remained completely unknown. Previous studies from Vivier and Lanier’s laboratories have used two noninfectious models of experimental autoimmune encephalomyelitis (22) and hapten-specific contact dermatitis (23) in DAP12-deficient mice, and demonstrated diminished T cell responses due to insufficient T cell priming by APC. However, these experimental findings may not be reconciliatory with human studies where DAP12-deficient human subjects were found to suffer marked demyelination in certain brain regions accompanied by diffuse brain tissue inflammation (24) and decreased DAP12 expression was also associated with exacerbated systemic lupus erythematosus (25).

To begin the investigation of the role of DAP12 in antimicrobial type 1 immunity, we have recently reported that DAP12 and its associating molecules TREM-1, TREM-2, and MDL-1 were upregulated in murine lung during the course of intracellular mycobacterial infection and their differential regulation of expression in APC upon infection or exposure to type 1 cytokines (26), thus implicating DAP12 in the regulation of antimicrobial type 1 T cell immunity. To investigate the role of this molecule in type 1 T cell activation, we have now examined host defense, T cell responses, and tissue immunopathology in models of intracellular mycobacterial and influenza viral infections in wild-type (WT) and DAP12-deficient mice. By using various approaches, we have unexpectedly found that DAP12 plays a critical negative regulatory role in the development of type 1 T cell responses and tissue immunopathology. Furthermore, we have identified that DAP12-deficient APC display an altered phenotype in response to TLR stimulation and infection in vitro and are responsible for the observed dysregulated T cell activation in vivo.

Materials and Methods

Mice

DAP12-deficient (DAP12KO) mice (27) were fully backcrossed to the C57BL/6 genetic background using the marker-assisted approach in the Speed Congenics Facility of the Rheumatic Diseases Core Center at Washington University. The WT control C57BL/6 mice were purchased from Harlan Laboratories. Both DAP12KO and OT-1 transgenic mice were bred and maintained in the Central Animal Facility at McMaster University (West Hamilton, Canada). All mice were 8- to 12-wk-old and housed in the specific pathogen-free facility. All experiments were conducted in accordance with the guidelines of animal research ethics board of McMaster University.

Pulmonary mycobacterial infection

_Mycobacterium bovis_ bacillus Calmette-Guérin (BCG; Connaught strain) was grown and prepared as previously described (28). Briefly, before each use, the stored mycobacterial bacilli were washed with PBS containing 0.05% Tween 80 twice and passed through a 27-gauge needle 10 times to disperse clumps. Pulmonary mycobacterial infection was elicited by intracheally infecting mice with 0.5 × 10^6 CFU of live bacilli. The level of bacterial burden was determined at the described time points in the lung and spleen by plating serial dilutions of tissue homogenates in triplicate onto Middlebrook 7H10 agar plates containing Middlebrook oleic acid-albumin-dextrose-catalase enrichment (29, 30). Plates were incubated at 37°C for 21 days in semisealed plastic bags. Colonies were then counted, calculated, and presented as log_{10} CFU per organ.

Pulmonary influenza viral infection

Pulmonary influenza infection was elicited by intranasally infecting mice with 1 × 10^6 PFU of a mouse-adapted human influenza A virus (31). At different times, flu Ag-specific CD8 T cell responses were analyzed with the cells obtained from the lung, spleen, and lymph nodes by flu nuclear protein (NP) tetramer immunostaining without culture or by IFN-γ intracellular cytokine staining (ICCS) upon culture with NP peptides. The lung viral infection was quantified by a viral plaque-forming assay. Briefly, Madin-Darby canine kidney cells were grown to 90% confluency and serial diluted homogenized lung supernatants were incubated with confluent Madin-Darby canine kidney cells for 30 min. An enriched agarose medium was added and the cells were incubated at 37°C for 2 days. The viral plaques were fixed with a solution containing 25% acetic acid and 75% methanol before counting.

Peripheral recombinant adenovirus infection

Mice were injected i.m. with 10^5 PFU of a recombinant adenovirus (Ad) expressing the immunodominant MHC class I epitope of chicken egg OVA (SIINFEKL) as previously described (32, 33). Two weeks postimmunization, cells from the lung and lymphoid organs were collected and analyzed by SIINFEKL tetramer immunostaining without culture and by ICCS upon culture with SIINFEKL peptides.

Bronchoalveolar lavage (BAL) and lung histopathology

BAL was conducted as previously described (34–37). BAL fluids were stored at −20°C until cytokine assays. The lung tissue collected at various time postinfection was fixed in 10% formalin. Tissue sections were stained with H&E for conventional histopathologic examination.

Gene expression by RNase protection and real-time RT-PCR assays

For RNase protection assay, total tissue RNA samples were isolated by using TRIzol reagent (Invitrogen Life Technologies). RNase protection
assay was used to assess cytokine mRNA levels and was conducted as previously described (38–40). Briefly, 32P-labeled riboprobes were synthesized using a commercial mouse multiprobe kit (BD Biosciences Pharmingen) containing templates against the following gene transcripts: inducible NO synthase, TNF-α, IL-1β, IL-6, IL-18, MIF, IFN-γ, RANTES, MIP-1α, MIP-2, and a housekeeping gene L32. The riboprobes were hybridized with each RNA sample overnight at 56°C according to the manufacturer’s instructions. The protected RNA fragments were separated using a 5% polyacrylamide gel. Real-time RT-PCR was used to assess T-bet and GATA-3 gene expression as previously described (26). Briefly, total RNA was extracted from T cells using the RNeasy Mini kit (Qiagen). RNA was quantified using the Agilent 2100 Bio-Analyzer machine operated by the 2100 expert software (Agilent). Reverse transcription was performed using Moloney murine leukemia virus reverse transcriptase and random primers (Promega). Mitochondrial ribosomal protein L32, T-bet, and GATA-3 primers and labeled probes were purchased from Applied Biosystems. Transcripts were quantified by real-time PCR on an Applied Biosystems PRISM 7700 Sequence Detector (PerkinElmer Applied Biosystems) using Applied Biosystems predesigned TaqMan Gene Expression assays and reagents. The mRNA abundance was normalized to the amount of housekeeping L32 and is expressed as arbitrary units.

Cytokine protein measurement

The level of cytokines, including IL-12p40, IFN-γ, and TNF-α in culture supernatants or lung lavage fluids, was measured by using an ELISA kit (R&D Systems) or cytometric bead array (BD Pharmingen) according to the manufacturer’s instruction.

NF-κB p65 transcription factor assay

Nuclear extraction was performed with cultured pulmonary APC by using a nuclear extract kit (Trans-AM; Active Motif). By using a quantitative sandwich immunoassay kit (Trans-AM; Active Motif), NF-κB p65 in nuclear extracts was detected according to the manufacturer’s instruction. NF-κB p65 translocation was measured colorimetrically at 450 nm and was normalized to the positive control provided by the kit.

ICC, FACS, tetramer immunostaining, and ELISPOT assay

The frequency of Ag-specific IFN-γ-releasing T cells was determined by ICCS and FACS as previously described (41). Briefly, \( \sim 2 \times 10^6 \) splenocytes or lymph node cells or \( 0.5 \times 10^6 \) BAL cells were cultured
in 96-well U-bottom plates. Cells were either stimulated with Mycobacterium tuberculosis (M.tb) culture filtrate protein (CF) (8 μg/ml) plus M.tb Ag85 complex protein (8 μg/ml) or stimulated with influenza NP ASNENMDTM peptides (1 μg/ml) or OVA SIINFEKL peptides (1 μg/ml) for 18 h and were then treated with GolgiPlug (BD Pharmingen) for an additional 5 h. Cells were washed and blocked with CD16/CD32 in 0.5% BSA/PBS for 15 min on ice, then stained with appropriate Abs against cell surface markers. Cells were then washed, permeabilized, and stained according to the manufacturer’s instructions. For tetramer immunostaining, influenza NP366–374 H-2Db/ASNENMDTM and OVA H-2Kb/SIINFEKL tetramers were used. Stained cells were run on the LSRII or FACSCanto (BD Pharmingen) and analyzed using FlowJo software (Tree Star). The ELISPOT assay was also conducted as previously described (41). Briefly, isolated splenocytes (0.5 × 10^6/well) or lymph node cells (0.25 × 10^6/well) were seeded into a 96-well polyvinylidene difluoride microplate (Millipore) precoated overnight with a mouse IFN-γ capture Ab. Cells were incubated for 24 h with or without antigenic stimulation. The plate was then developed by using standardized streptavidin-conjugated alkaline phosphatase and chromogen method (R&D Systems). The number of IFN-γ-releasing cells was determined by using a dissecting microscope.

**In vitro culture and stimulation of APC**

Pulmonary APC were purified from the airway of naive mice by BAL. Eighty-five to 90% of these airway APC were CD11c+ as determined by FACS. These cells (1 × 10^6/well) were cultured in 96-well plates with or without mycobacterial infection (2 CFU/cell) or stimulation by TLR ligands LPS (1 ng/ml) or CpG, peptidoglycan (PGN), poly(I:C), and Flt C (1 μg/ml). The culture supernatants were collected after 24 h (TLR agonists) or 48 h (mycobacteria) and stored at −20°C until cytokine measurements. In separate experiments, cells were cultured as above and immunostained for FACS analysis of surface markers of APC maturation and activation including CD11c, CD11b, MHC class II, B7.1, and CD40. In other experiments, APC

**FIGURE 3.** Increased activation of mycobacterial Ag-specific type 1 T cells and T-bet expression in the lung and lymphoid organs of DAP12KO mice during pulmonary mycobacterial infection. A, Airway luminal cells were removed at days 14 and 25 postinfection by lung lavage (BAL) and pooled cells from three to four mice per time were cultured in the presence or absence (Unstim) of M.tb CF and M.tb Ag85 complex protein Ags and subjected to IFN-γ ICCS and FACS analysis in conjunction with the use of anti-CD3, CD4, and CD8 Abs. The percentage represents the relative size of T cell populations that are positive for IFN-γ, CD3, and CD4 or CD8. Right panels. The quantification of total numbers of such cells/BAL. The results are representative of three independent experiments. B, Total lymphocytes isolated from mediastinal lymph nodes (LN) and spleens at days 14 and 25 postinfection were cultured in the presence (+Ag) or the absence (−Ag) of mycobacterial Ag M.tb CF or irrelevant OVA Ags (negative control) and subjected to ELISPOT or ICCS to determine the relative frequency of mycobacterial Ag-specific, IFN-γ-releasing T cells. Results are expressed as mean ± SEM of Ag-specific T cells per million cells from triplicate wells per condition for ELISPOT assay or representative data obtained from using pooled lymph nodes for ICCS. C, Total lung tissue RNA isolated at days 14 and 25 postinfection was subjected to a quantitative real-time RT-PCR assay for quantification of T-bet or GATA-3 gene expression, and the data are expressed as arbitrary units after normalization of T-bet or GATA-3 mRNA abundance to the amount of housekeeping L32 mRNA. The results are mean ± SEM of five cDNA samples generated from five mice per group/time.
were cultured in the presence of LPS (1 ng/ml) with or without an IκB kinase β (IKKβ) inhibitor (10 μM) for varying times before being subjected to nuclear extraction and NF-κB p65 detection assay. In vivo assessment of APC functions and T cell activation

CD11c+ APC were purified by a double MACS column purification procedure by positive selection from lung and/or lymphoid tissues of WT and DAP12KO mice primed in vivo with live Mycobacterium bovis BCG bacilli for 14 days. The purity of purified CD11c+ APC was always >90%. The CD11c+ APC were then pulsed in vitro with Mycobacterium CF protein Ags for 24 h. A total of 1.5 × 10^6 Ag-pulsed APC was injected i.m. as a cell vaccine to naive WT and DAP12KO mice. At day 14 postimmunization, splenocytes (0.5 × 10^6/well) were harvested and restimulated with M.tb CF (8 μg/ml) for 18 h and were then treated with GolgiPlug for an additional 5 h, and the level of mycobacterial Ag-specific IFN-γ-producing T cells was assessed by ICCS. In a different set of experiments, 1 × 10^6 CFSE-labeled OT-I-transgenic CD8 T cells were transferred i.v. into naive WT recipient mice. After 2 days, mice were injected intradermal (i.d.) with SIINFEKL peptide-pulsed WT or DAP12KO CD11c+ APC (3.5 × 10^5 per hind footpad). Five days later, local draining popliteal lymph node and spleen cells were harvested, immunostained for Thy1.1/CD8 surface molecules, and analyzed for the extent of proliferation of transferred congenic Thy1.1+ CFSE-labeled OT-I T cells by FACS.

Results

DAP12 deficiency leads to enhanced resistance and granulomatous inflammation in response to pulmonary mycobacterial infection

We have recently shown that expression of DAP12 and its associating molecules are up-regulated both in the lung and APC during pulmonary mycobacterial infection or upon exposure to type 1 cytokines, thus implicating a DAP12 signaling pathway in the regulation of type 1 antimicrobial immunity (26). In the current study, we first investigated the role of DAP12 in host defense against pulmonary mycobacterial infection by using DAP12KO mice. Thus, upon mycobacterial infection, both WT and DAP12KO mice remained healthy without signs of body weight loss or sickness throughout the study. Although the level of mycobacterial infection in the lung and spleen was similar at day 14 both in WT and DAP12KO mice, it was significantly lower in DAP12KO mice than WT mice at day 25 (Fig. 1A). We next examined histopathology in the lung and found that DAP12KO mice were able to mount...
a significant level of granulomatous inflammatory responses as early as day 14 when a minimum of granuloma formation was present in the lung of WT mice (Fig. 1B). A greater level of tissue inflammation still existed in the lung of DAP12KO mice up to day 25 postinfection. Because T cells play a major role in granuloma formation and antmycobacterial host defense, we examined the extent of T cell infiltration in the lung. The total numbers of both CD4+ and CD8+ T cells were markedly higher in the lung of DAP12KO mice at both times compared with WT mice (data not shown). Accompanied with T cell infiltration were markedly increased numbers of MHC class II+CD11b+ and CD11c+ macrophages (data not shown). These data suggest that DAP12 deficiency leads to enhanced antmycobacterial resistance as a result of pronounced granulomatous inflammation.

DAP12 deficiency leads to enhanced proinflammatory and Th1 cytokine responses to mycobacterial infection in the lung

To begin investigating the mechanisms underlying earlier and greater inflammatory responses in the lung of DAP12KO mice, we assessed the level of cytokines and chemokines in vivo first by using a RNase protection assay. At day 14 after infection, there was remarkable up-regulation of gene expression of proinflammatory mediators including inducible NO synthase, TNF-α, IL-1β, IL-6, IL-18, MIF, and IFN-γ and chemokines RANTES, MIP-1α, and MIP-2 in the whole lung tissue of DAP12KO mice compared with WT mice (Fig. 2A). At day 25, although the difference remained, the magnitude of differential expression of these genes was less pronounced between DAP12KO and WT mice (Fig. 2A). To examine whether the level of cytokine protein production increased correspondingly, we elected to examine the protein levels in the BAL fluids of type 1 cytokines TNF-α, IL-12, and IFN-γ given their critical role in antibacterial type 1 T cell immunity (34, 37, 41–45). Indeed, the protein levels of type 1 cytokines, most prominently IL-12 and IFN-γ, were remarkably higher in the lung of DAP12KO mice at day 14 postinfection when they were still barely detectable in the lung of WT control mice (Fig. 2B) while the differences still remained at day 25. Thus, the earlier and more intense lung cellular responses were closely associated with heightened proinflammatory and Th1 cytokine responses.

Heightened activation of Ag-specific type 1 CD4 and CD8 T cells and T-bet expression in DAP12KO mice during mycobacterial infection

Earlier and greater tissue inflammatory cellular and cytokine responses detected in the lung of DAP12KO mice upon mycobacterial infection suggest a role of potential dysregulated activation...
of type 1 T cells characteristic of IFN-γ secretion. To this end, we first examined the level of mycobacterial Ag-specific type 1 CD4 and CD8 T cell responses in the lung. DAP12 deficiency led to an early sharp rise both in the frequency and absolute number of Ag-specific IFN-γ+ CD4 and CD8 T cells in the respiratory tract at day 14 (Fig. 3A) when little responses could be seen in WT mouse lung, consistent with our previous findings (37, 41). At day 25, although cell responses increased in WT mouse lung, they were still markedly greater in the lung of DAP12KO mice (Fig. 3A). The number of mycobacterial Ag-specific CD4 and CD8 T cells was similarly much higher in pulmonary lymph nodes (Fig. 3B, upper and middle panels) and spleen (Fig. 3B, lower panels) of DAP12KO mice than in WT controls at both time points. Given the vital role of transcription factor T-bet in Th1 differentiation and IFN-γ production (46–48), we next examined the expression of T-bet in the whole lung tissue or purified lung-derived Th1 cytokines IFN-γ, TNF-α, and IL-2. The data are expressed as mean ± SEM of cytokine-positive CD8 T cells from three to four mice per group. The numbers in the parentheses represent the fold increase number of tetramer-specific CD8 T cells in DAP12KO mice over WT controls.

We further examined cell activation surface markers of airway APC before and after in vitro stimulation. The majority of both WT and DAP12KO APC were CD11c+CD11b+ in the presence of this inhibitor could completely abolish heightened TNF-α production by LPS-stimulated DAP12KO APC (Fig. 4B). These data suggest that DAP12 deficiency enhances cytokine production in APC via an NFκB-dependent manner.

FIGURE 6. DAP12 deficiency leads to enhanced type 1 T cell activation following peripheral viral infection. A, Mononuclear cells were isolated from the lung, draining lymph nodes (LNs), and spleen of WT and DAP12KO mice 14 days after i.m. infection with a recombinant Ad expressing OVA peptide SIINFEKL and immunostained with SIINFEKL tetramer (Tet+). The data are expressed as mean ± SEM of tetramer-positive CD8 T cells/organ from three to four mice per group. B, The splenocytes isolated as above were stimulated in vitro with SIINFEKL peptide and immunostained for CD8 and intracellular Th1 cytokines IFN-γ, TNF-α, and IL-2. The numbers in the parentheses expressed as mean ± SEM of cytokine-positive CD8 T cells from three to four mice per group.

Enhanced cytokine responses and NF-κB activation of DAP12-deficient APC on TLR stimulation or mycobacterial infection in vitro

It has been demonstrated by us and others that DAP12 is expressed on APC, including both dendritic cells and macrophages, but not in resting or activated T cells (1, 9, 26, 49, 50). Our observation that the level of proinflammatory and Th1 cytokines, including IL-12 and TNF-α, was remarkably enhanced in the lung of DAP12KO mice even at day 14 after mycobacterial infection (Fig. 2) suggests a role of DAP12-deficient APC in type 1 T cell overactivation (Fig. 3). To begin investigating the mechanisms of type 1 T cell overactivation as a result of DAP12 deficiency in our model, we evaluated the phenotypes of lung APC in response to TLR ligand stimulation and mycobacterial infection. As the majority of naive airway macrophages express CD11c and these cells constitute the first line of APC intercepting incoming mycobacteria within the respiratory tract, we examined whether DAP12KO airway APC may differentially respond to stimulation in vitro. We found that DAP12KO APC released more proinflammatory cytokine TNF-α than WT controls upon mycobacterial infection or stimulation with LPS or Fli-c (Fig. 4A). In comparison, neither WT nor DAP12KO APC markedly responded to stimulation by CpG, PGN, and poly(I:C). To investigate the mechanism of heightened cytokine responses by DAP12KO APC, we examined the role of NFκB, a key transcription factor in induction of proinflammatory cytokines in APC, downstream of TLR stimulations (51). To this end, naive airway APC of WT and DAP12KO mice were stimulated with LPS and the level of NFκB (p65) nuclear translocation was measured at various time points. A greater level of p65 activity was detected in DAP12KO APC upon LPS stimulation and it was inhibited in the presence of 10 μM, an optimal concentration, of a specific IKKβ inhibitor (AS602868) which prevents the translocation of NFκB to the nucleus (52, 53) (Fig. 4B). Furthermore, the presence of this inhibitor could completely abolish heightened TNF-α production by LPS-stimulated DAP12KO APC (Fig. 4B). These data suggest that DAP12 deficiency enhances cytokine production in APC via an NFκB-dependent manner.
data suggest that DAP12 deficiency also leads to enhanced surface expression of selected APC activation and maturation molecules, in addition to its effect on cytokine responses.

**DAP12-deficient APC enhances mycobacterial Ag-specific type 1 T cell activation in vivo**

To directly determine the role of DAP12-deficient APC in the overactivation of mycobacterial Ag-specific type 1 T cells in vivo, CD11c+ APC were purified from mycobacterium-infected WT and DAP12KO mice. These in vivo-primed APC were then loaded ex vivo with soluble mycobacterial Ags (M.tb CF). The same number of Ag-loaded WT and DAP12KO APC were then injected i.m. as a cell-based vaccine to immunize naive WT and DAP12KO mice, respectively. At day 14 postimmunization, splenocytes were harvested and the level of in vivo-activated mycobacterial Ag-specific, IFN-γ-releasing CD4+ and CD8+ T
cells were determined by ICCS and FACS analysis. It was found that the number of Ag-specific type 1 CD4 and CD8 T cells was three to four times greater in DAP12KO APC-immunized mice than that in WT APC-immunized controls (Fig. 5A). These data suggest that DAP12-deficient APC could directly augment Ag-specific type 1 T cell activation in vivo.

**DAP12-deficient APC enhances OVA-specific transgenic T cell activation in vivo**

To further dissect the role of DAP12-deficient APC in type 1 T cell overactivation, we used an adoptive OT-I-transgenic T cell transfer model set up in WT hosts that were immunized with either WT or DAP12KO APC. This approach allowed us to address whether DAP12-deficient APC were still able to trigger a differential T cell response in a host where everything else in the immune system was identical to that in a WT host, by starting with the same numbers of APC and Ag-specific T cells. To this end, naïve WT mice were transferred i.v. with CFSE-labeled OT-I CD8 T cells and were subsequently immunized intradermal with SIINFEKL peptide-pulsed WT or DAP12KO CD11c+ APC or a recombinant adenovirus expressing SIINFEKL as a positive control. The extent of transferred transgenic T cell proliferation in the popliteal lymph nodes and spleen was assessed 5 days postimmunization. Immunization with DAP12KO APC led to a remarkably higher percentage of transferred congenic OT-I T cells that had undergone high rounds (≥4) of proliferation than WT APC (90% vs 50%), resulting in three to four times higher numbers of such T cells detected in the lymph nodes (Fig. 5B) and spleen (data not shown). In contrast, OT-I T cells in mice injected with unpulsed APC underwent very limited proliferation (negative control). These data suggest that DAP12-deficient APC alone are sufficient to cause hyperresponses of Ag-specific T cells.

**DAP12 deficiency also leads to enhanced Ag-specific type 1 T cell responses and immunopathology to viral infection**

Having established a regulatory role of DAP12 and APC in type 1 immunity during intracellular bacterial infection, we wondered whether DAP12 would play a similar role in type 1 immune activation during intracellular viral infection. We addressed this question first by using a transgenic viral approach. To this end, WT and DAP12KO were infected i.m. with a recombinant replication-defective adenovirus (AdSIINFEKL) expressing SIINFEKL peptide (a CD8 T cell peptide of chicken OVA). At day 14 after immunization, the level and phenotype of SIINFEKL-specific CD8 T cells were measured by using SIINFEKL tetramer immunostaining and ICCS. We found that the total number of tetramer-specific CD8 cells was significantly higher in the draining lymph node (3.1-fold), spleen (2.1-fold), and the lung (1.8-fold) of immunized DAP12KO mice than in WT mice (Fig. 6A). Upon examination of the cytokine profile using ICCS, we found a much greater number of SIINFEKL-specific CD8 T cells capable of production of type 1 cytokines, IFN-γ, TNF-α, and IL-2, in the spleen of DAP12KO mice (Fig. 6B). The similar pattern of type 1 T cell responses was also observed in the draining lymph nodes and lung tissue (data not shown). Using a slightly different approach by which the same number of purified OT-I-transgenic CD8 T cells were adoptively transferred i.v. to naïve WT and DAP12KO mice and these mice were then injected i.m. with AdSIINFEKL virus, we also found a greater number of congenic OT-I T cells in the spleen at day 7 postimmunization (data not shown). These data together indicate that DAP12 deficiency also leads to heightened type 1 T cell activation following systemic viral infection.

Because the above approach involved only the use of a replication-defective recombinant virus delivered via a systemic route, we set out to address the role of DAP12 in a relevant model of acute respiratory viral infection. To this end, WT and DAP12KO mice were infected via the airway with influenza A virus. We found that while in contrast to chronic mycobacterial infection (Fig. 1A), the levels of acute flu infection were similarly controlled at day 7 and eventually cleared by day 12 in the lung of both WT and DAP12KO mice (Fig. 7A, left panel); DAP12KO, but not WT, mice were severely ill. Thus, although overall WT mice gained their body weight over the course of flu infection (2–8%), on average DAP12KO mice lost 14–17% body weight at days 7 and 12, respectively, despite viral clearance at the latter time (Fig. 7A, right panel). Such sickness in DAP12KO mice was associated with intense tissue inflammation and immunopathology (Fig. 7B). At day 12, lung structural injury indicated by lung remodeling changes including bronchial hyperplasia and tissue fibrinotic responses were evident (Fig. 7B). In comparison, WT mouse lungs had little sign of severe tissue immunopathology. Furthermore, similar to what we have observed with mycobacterial infection, there was a remarkably increased number of flu NP tetramer-specific or NP peptide-specific IFN-γ-producing CD8 T cells both in the lung (Fig. 7C) and lymphoid organs (Table I), as assessed by both flu NP tetramer immunostaining and IFN-γ ICCS, respectively. These findings together suggest that DAP12 is also required for the control of type 1 T cell activation and immunopathology during acute intracellular viral infection.

**Discussion**

DAP12 is a transmembrane signaling adaptor protein primarily expressed in NK and myeloid cells (3, 6, 54). Although the critical role of DAP12 in NK cell activation has been well established, its role in regulating the function of other innate cells such as dendritic cells and macrophages still remains contentious, considered

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**Table I. Increased influenza NP-specific tetramer or IFN-γ CD8+ T cells in the draining lymph nodes and spleens of DAP12KO mice following pulmonary influenza infection**

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<th>Spleen</th>
<th>Lymph Nodes</th>
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<tr>
<td></td>
<td>CD8+ Tetramer</td>
<td>CD8+ IFN-γ</td>
</tr>
<tr>
<td>WT (day 7)</td>
<td>1.68 × 10^4 (0.037%)</td>
<td>1.74 × 10^4 (0.039%)</td>
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<tr>
<td>DAP12KO (day 7)</td>
<td>6.37 × 10^4 (0.082%)</td>
<td>5.14 × 10^4 (0.061%)</td>
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<tr>
<td>WT (day 12)</td>
<td>4.4 × 10^4 (0.51%)</td>
<td>8.5 × 10^4 (0.18%)</td>
</tr>
<tr>
<td>DAP12KO (day 12)</td>
<td>9.1 × 10^4 (0.59%)</td>
<td>1.4 × 10^4 (0.2%)</td>
</tr>
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</table>

*At days 7 and 12 after influenza infection, lymph node cells and splenocytes from WT and DAP12KO mice were isolated and stimulated with influenza NP peptides and immunostained for CD8 and IFN-γ by ICCS. Some cells were also immunostained directly with NP tetramer without Ag stimulation. The data were analyzed by FACS and are expressed as absolute numbers/organ and relative percentages.
to be either proinflammatory (10–17) or anti-inflammatory (18–21) in a number of acute inflammatory or innate immune processes. Because the APC is a critical determinant of the initiation and phenotype of adaptive T cell immunity, conceivably DAP12-mediated signaling in APC may also critically impact on T cell responses. However, little is known about the role of DAP12 in the regulation of T cell activation, particularly during intracellular infection for which T cell-mediated immunity is critically required.

We have recently reported that expression of DAP12 and its associating molecules was increased and was differentially regulated in pulmonary APC by type I cytokines during the course of intracellular mycobacterial infection (26). These findings implicate a role of DAP12 in the regulation of type I antimicrobial immunity. Indeed, we have now for the first time demonstrated that DAP12 is a critical negative regulator of type I T cell activation and immunopathology during intracellular infection elicited by mycobacteria or influenza virus to which immune protection requires type I T cell activation and effector activities (44, 55). We provide further evidence that DAP12 expressed by APC plays a critical role in this process. Of note, our findings contrast the conclusion drawn from the previous two studies using noninfectious models (experimental autoimmune encephalomyelitis and hapten contact hypersensitivity) where DAP12KO mice were found to instead develop a dampened T cell response (22, 23). In these studies, impaired T cell activation in DAP12KO mice is believed to be attributed to insufficient T cell priming, resulting perhaps from reduced APC migration to the lymph nodes (56) or increased tolerogenic APC (57, 58). These findings appear to support the conclusion from some, but certainly not all, in vitro studies that DAP12 plays an activating or proinflammatory role in APC activities (10–12). However, these earlier experimental observations may unlikely explain the findings from human studies where DAP12 deficiency (Nasu-Hakola syndrome) leads to severe demyelination and brain inflammation (24) and that decreased DAP12 expression was correlated with exacerbation of systemic lupus erythematosus (25). Furthermore, more recent experimental studies have suggested an inhibitory role of DAP12 signaling in macrophage or dendritic cell activation (18–22). Our current study conducted by using in vivo models of intracellular bacterial and viral infections demonstrates a much heightened level of type I T cell activation in DAP12-deficient hosts and thus reveals a negative regulatory role by DAP12 in T cell activation and immunopathology. And rather than showing a diminished level of activation, DAP12-deficient APC generate a higher level of activation upon stimulation or infection in vitro and elicit increased T cell activation in vivo. Our findings may be potentially used to explain heightened inflammatory/immune responses observed in the human DAP12 deficiency syndrome and why such human subjects do not suffer increased susceptibility to infections (3, 24). To support our conclusion, DAP12-deficient mice were also recently found to be more resistant to a short-course intracellular listerial bacterial infection, although unfortunately both APC and T cell responses were not investigated in this study (20). Therefore, putting the findings from us and others together, a new notion can be proposed that DAP12 signaling in APC plays diverse roles depending on the nature of model systems. Thus, although it is immune regulatory or suppressive during intracellular infection as we have shown here or anti-inflammatory in such acute inflammatory settings as endotoxiaemia or sepsis caused by extracellular bacterial pathogens, as shown by others (20), it may be proinflammatory or proimmune in autoimmune reaction (22) or proinflammatory in some other in vivo settings of acute inflammation (13).

The fact that a single DAP12 signaling adaptor can lead to either decreased or enhanced inflammatory or immunological outcomes depending on the nature of agents or models may reflect a differential engagement of DAP12 by a great number of DAP12-associating surface molecules including TREMs, MDL-1 and SIRP-β whose cognate ligands have remained unknown (3). In addition, the phenotype of DAP12-bearing APC will further be influenced by differential engagement of TLRs. Such realization suggests the importance of using in vivo models elicited with agents of physiologic relevance to investigate the role of DAP12 and the risk of generalizing the knowledge from one scenario for all. It is tempting to speculate why DAP12 plays an unequivocally activating role in NK activities, whereas it may play a bipolarized role in the activities of APC. It is possible that this adaptor molecule has evolved to ensure robust innate functions to quickly control a relatively transient insult and such functions are often executed by innate cells such as NK cells. This notion is supported by the observations made in models of MCMV infection to which host defense relies largely on NK functions and thus lack of DAP12 leads to impaired NK activities and increased susceptibility (7–9). In this regard, the relationship between recently identified enhanced cytokine responses in DAP12-deficient plasmacytoid dendritic cells and decreased innate host resistance to MCMV infection in DAP12-deficient mice has remained unclear (21). However, in the situation where innate APC are critically required for the initiation and activation of T cell-mediated immunity, the outcome of DAP12 signaling within APC is determined by the nature of Ag or pathogen and its engagement with DAP12-associating molecules that are uniquely expressed on these cells. This may allow a great flexibility in adaptive immunity tailored to Ag or pathogen. Thus, in the event of intracellular infection to which host defense relies largely on T cell activities, APC-associated DAP12 plays a critical regulatory role, working together with other immune regulatory molecules, membrane bound and soluble, to ensure a well-balanced T cell and tissue inflammatory response. As we have demonstrated in the current study, if this important checkpoint is lost, the exaggerated type I T cell activation and tissue immunopathology and injury result. Although such consequence may not necessarily be detrimental to DAP12-deficient hosts in the case of chronic intracellular bacterial infection, it is certainly harmful to the host in the course of acute respiratory viral infection where immunopathology and tissue injury persist beyond viral clearance. Our findings identify DAP12 as an important novel immune regulatory molecule, which via its intracellular signaling properties in APC, regulates the phenotype of APC to control the level of type I T cell activation and immunopathology. We believe that these results enhance our understanding of immune regulatory mechanisms in infectious disease, cancer, and autoimmunity and shall help with the future development of vaccines and immunotherapeutics.

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Disclosures
The authors have no financial conflict of interest.

References


