REGULATION OF PRIMARY T CELL RESPONSES TO INTRACELLULAR INFECTION IN THE LUNG

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Abstract

Pulmonary infections caused by viruses, bacteria, mycobacteria and fungi, are a leading cause of death world-wide. Intracellular pathogens such as influenza virus and *Mycobacterium tuberculosis* live inside host cells, making it difficult for the host to eliminate the pathogen. Adaptive T cell immune responses are required to clear intracellular pathogens. Rapid T cell priming and recruitment of effector T cells to the lung is critical to eliminating the pathogen and ultimately host survival, however, unchecked T cell responses can be detrimental. The research in this thesis examines T cell priming during pulmonary intracellular infections and examines the consequences of impaired/enhanced T cell responses at the lung mucosa.

Mycobacterium tuberculosis (*M.tb*), the bacterium that causes tuberculosis evades detection by the host and delays T cell priming. This delay is believed to allow *M.tb* to establish chronic infection. We show that DAP12 deficient mice prime T cell responses days sooner than wt controls, resulting in enhanced control of virulent *M.tb*. Enhanced T cell priming in DAP12 deficient mice is due to increased antigen presentation by professional antigen presenting cells in the local draining lymph nodes. These findings indicate that accelerating antigen presentation in the lymph node and T cell priming can dramatically improve host resistance to tuberculosis infection.

Influenza infection in DAP12 deficient hosts also results in enhanced T cell activation however caused lethal lung pathology. We identified a previously unidentified role for CD4 T cells expressing FasL causing immunopathology. Furthermore, we show that

DAP12 deficient antigen presenting cells are responsible for priming hyperactivated CD4 T cells and contribute to influenza mortality.

Because T cell responses are so important in host defence against intracellular infection we examined the feasibility of using ex-vivo manipulated antigen presenting cells as a vaccine to generate T cell responses in the lung. Collectively these findings shed light on the factors which regulate host immune responses in the lung following intracellular infection and provide evidence that antigen presenting cells can be manipulated ex-vivo to elicit protective immune responses at the lung mucosa.

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List of Abbreviations

Ab; antibody

ADC; alveolar dendritic cell

Ag; antigen

AICD; activation induced cell death

AM; alveolar macrophage

APC; allophycocyanin

APCs; antigen presenting cells

BAL; bronchoalveolar lavage

BALT; bronchus-associated lymphoid tissue

BCG; *Mycobacterium bovis* Bacille Calmette Guérin

Bcl; B-cell lymphoma

CCL; CC chemokine ligand

CD; cluster of differentiation

CDC; Centers for Disease Control

CFU; colony forming unit

CR; complement receptor

CTL; cytotoxic T lymphocytes

DAP12; DNAX activating protein of 12 kDa

DC; dendritic cell

EAE; experimental autoimmune encephomyelitis

ELISA; enzyme linked immunosorbant assay

FACS; fluorescent activated cell sorting

FITC; fluorescin isothiocyanate

HA; influenza hemagglutinin

HIV; Human Immunodeficiency Virus

ICCS; intracellular cytokine staining

iDC; intra-epithelial dendritic cell

IFN; interferon

Ig; immunoglobulin

IL; interleukin

i.n.; intranasal

IP-10; interferon gamma-induced protein 10 kDa

i.t.; intratracheal

KC; keratinocyte-derived chemokine

KO; knockout

LAM; lipoarabinomannan

LCMV; lymphocytic coriomeningitis virus

LN; lymph node

MBL; mannose binding lectin

MCP; monocyte chemotactic protein

MDR; multidrug resistant

MHC; major histocompatibility complex

MIP; macrophage inflammatory protein

mLN; mediastinal lymph node

MPO; myeloperoxidase

MR; mannose receptor

MOG; myelin oligodendrocyte glycoprotein

M.tb ; Mycobacterium tuberculosis

NA; influenza neuraminidase

NF-κB; nuclear factor-κB

NK; natural killer

NO; nitric oxide

NP; influenza nucleoprotein

NS1; influenza non-structural protein 1

NS2; influenza non-structural protein 2

PAMP; pathogen associated molecular pattern

pDC; plasmacytoid dendritic cell

PE; phycoerthyrin

PFU; plaque forming units

PGN; peptidoglycan

PMN; polymorphonuclear cells

PRR; pattern recognition receptor

RANTES; regulated upon activation, normal T-cell expressed, and secreted cytokine

RNP; nibonucleoprotein

ROS; reactive oxygen species

STAT; signal transducer and activator of transcription

TB; tuberculosis

T-bet; T box expressed in T cells

TCR; T cell receptor

TGF; transforming growth factor

Th; T helper

TLR; toll like receptor

TNF; tumour necrosis factor

TRAIL; TNF-α related apoptosis inducing ligand

WHO; world health organization

Chapter1: Introduction

1.1 Intracellular pulmonary infections

The mammalian lung is a highly evolved structure that facilitates oxygenation of the blood and removal of carbon dioxide waste. The lung can be divided into 2 anatomical sections, the upper respiratory tract and the lower respiratory tract. The upper respiratory tract is composed of the nasal cavity, pharynx, larynx and the trachea and these structures primarily serve to filter and conduct air to the lower respiratory tract. The lower respiratory tract includes the bronchus, bronchioles, alveolar ducts and alveoli where gas exchange occurs. Because of the architecture, the upper respiratory tract is chronically exposed to potentially pathogenic agents including bacteria, viruses, fungi, allergens and pollutants and is colonized by a variety of commensal organisms including bacteria, fungi and viruses. In contrast, the lower respiratory tract is relatively sterile. A number of intrinsic host defence mechanisms such as sneezing, coughing, mucous production and mucociliary flushing are highly effective at preventing most organisms from reaching the lower respiratory tract. Penetration of these barriers by viruses and bacteria such as influenza and *Mycobacterium tuberculosis* cause significant disease worldwide. Infections of the lower respiratory tract are the 4th leading cause of death in women globally. Dealing with intracellular infections is unique because in contrast to other extracellular pathogens which can be cleared by neutralizing antibodies and innate immune cells, clearance of intracellular pathogens requires destruction of infected host cells. This has serious implications for lung function; too much destruction will damage the alveoli and impair gas exchange leading to illness/death, whereas failure to kill infected cells will allow the pathogen to take over and destroy the lung. Therefore the

host must maintain a balance between pathogen elimination and preventing excess damage that could lead to impaired lung function.

1.1.1 Influenza A virus infection

Influenza virus infection is a pressing global concern. Influenza infects 5-15% of the world's population annually, leading to 3-5 million cases requiring hospitalization and 250 000 to 500 000 deaths per year (1). Disease caused by influenza infection results in a spectrum of symptoms ranging from asymptomatic infection to febrile illness causing sudden onset of high fever, malaise, headaches, cough and extensive inflammation of the airways and pneumonitis. Symptoms appear 3-5 days after exposure and last for 7-10 days. Seasonal influenza is generally mild, primarily infecting the aged and the young, resulting in more severe illness in those least able to defend themselves. In contrast, pandemic influenza typically causes severe illness in healthy adult populations.

Influenza A virus, the most common pathogen to cause overt disease in humans, is a member of *Orthomyxoviridae* family. Influenza is spread through the inhalation of infectious aerosols or mucosal contact with contaminated surfaces. Influenza replicates in the superficial mucosal tissues, primarily infecting the epithelial cells of the trachea and bronchioles (2). The virus particle is roughly round, enveloped with glycoproteins hemagglutinin (HA) and neuraminidase (NA) embedded in the envelope and anchored to the viral particle by the M2 protein (Figure 1). Influenza enters the host cell by HA binding sialic acid on host target cells triggering endocytosis of the viral particle. The endosome acidifies by fusing with the lysosome, triggering a conformational change of the M2 protein which forms a pore in the endocytic membrane, allowing the viral

ribonucleo complex (vRNP) to be released into the cytoplasm. The vRNP is comprised of the influenza genome (8 segments of single stranded (ss) RNA encoding 10-11 genes), the nucleoprotein (NP), small amounts of the non-structural protein 2 (NS2) (AKA nuclear export protein) and the polymerase complex (PA, PB1, PB2) and the M2 protein. Once released into the cell cytoplasm, the vRNP travel to the nucleus of the host cell where viral gene replication will begin within 1 hour of infection (3,4). NP is highly conserved in influenza virus as it is critical to stabilize viral RNA and divert host transcriptional machinery to the vRNA. Because of its essential function in viral replication and it is so highly conserved amongst viral strains, NP is often a target for host adaptive immune responses and is a promising target for novel anti-viral therapies (5). In the nucleus, the polymerase complex synthesizes a positive (+ve) sense RNA strand from the negative (-ve) sense viral RNA (vRNA) which is transported to the cytoplasm for gene translation by host ribosomes (3,4). Newly synthesized proteins are either secreted through the Golgi apparatus to the cell surface or are transported back to the nucleus to be packaged with vRNA in progeny virus (4). Meanwhile, the non-structural protein 1 (NS1) participates in stabilizing vRNA so that multiple copies of the whole genome can be synthesized for viral progeny. vRNA associates with NP, NS2, M1 and the polymerase complex to form progeny vRNPs and migrate to the cell membrane where HA and NA cluster and allow progeny virus to bud from the cell (3,4). The newly formed virus buds from the cell, taking some of the host membrane as its envelop and is released from the cell through the cleavage of sialic acid from the cellular membrane by NA (3,4).

Influenza viruses infect all species on the planet. Human tropic influenza viruses also infect a wide range of other hosts including birds, cats and pigs. Pandemic influenza strains are a result of genetic recombination of HA and NA gene segments between influenza strains from different species. There is an estimated 16 HA and 9 NA subtypes that have the potential to recombine to form novel, possibly pandemic influenza strains. Pandemic influenza differs from the seasonal influenza strains because they either spread very rapidly through the population or cause robust immune activation and worse lung pathology. Throughout history, the human population has been periodically attacked by influenza pandemics. The 1918 Spanish influenza pandemic that killed an estimated 20-100 million people, is reported to be a result of recombination of human and swine influenza strains resulting in an H1N1 virus that was not only highly virulent, causing fatal lung pathology, but was capable of rapid person to person transmission (6-8).

Vaccination efforts have significantly lowered the annual disease burden caused by influenza infection. Vaccines typically elicit neutralizing antibody responses against HA and NA although adaptive cellular immune responses may also contribute to the effectiveness of the vaccine. Designing a vaccine against influenza poses unique challenges for two reasons; first, since influenza viruses so readily undergo genetic rearrangement, yearly vaccination is required to optimally protect the population, although antibody responses elicited by previous vaccination or influenza exposure may provide limited protection. Second, the yearly formulation of the vaccine must be decided months prior to flu season, forcing vaccine companies to make an educated guess as to

which strains of influenza will predominate, sometimes resulting in an ineffective vaccine. While vaccination provides good protection against yearly strains, the emergence of pandemic influenza strains is highly unpredictable. For many years there was serious concern that an avian H5N1 influenza strain which devastated the migratory bird populations in Europe and Asia would make the jump to the human population causing a severe pandemic. H5N1 is a threat to public health because it is highly virulent in both birds and humans and infection leads to high viral load. H5N1 viruses readily infects both structural as well as immune cells in the lung, and is believed to cause "cytokine storm", an overproduction of pro-inflammatory cytokines, leading to tissue destruction and severe illness (9-11). In spite of all the attention surrounding avian influenza, the World Health Organization (WHO) reports that avian influenza has only been confirmed in 562 patients since 2003 and only in people that are in close contact with birds. Although H5N1 can cause severe disease in humans, in light of its inability to spread from human to human in the last 8 years of close monitoring suggests it is does not pose a great threat to the general population at present. In contrast, in 2009 a porcine H1N1 influenza virus emerged without warning and rapidly spread through the human population causing wide-spread illness. The 2009 influenza attracted a lot of attention and generated legitimate concern over the preparedness of health care systems to support a major infectious disease outbreak, for example and H5N1 influenza strain, and the availability of anti-viral drugs. Retrospective analysis of the pandemic 2009 H1N1 infection (H1N1/09) revealed an estimated 45% of the American population had been infected with the virus, markedly more people than seasonal influenza (12). H1N1/09 appears to have caused severe lung

pathology only in the immune-compromised, however, it predisposed an estimated 40% of infected individuals to secondary bacterial infection (12, 18). According to the Centers for Disease Control (CDC), the mortality rate in the 2009-2010 influenza season was twice as high as recent years. The general perception is that the 2009-2010 influenza season was worse than in past years with more patients suffering from persistent pneumonia than usual. Contradictory findings exist with regards to the virulence of the virus. Some studies suggest that there was only weak induction of cytokine responses to H1N1/09, while others suggest that there were appreciably increased pro-inflammatory responses compared to other yearly influenza strains (13-17). In the coming years, characterization of the virus and the host immune response to this virus will be characterized and provide us with insight into the mechanisms that allowed the virus to be transmitted so efficiently from human to human. Whether it was because of excessive media coverage or the wide-spread illness caused by H1N1/09, our most recent encounter with a pandemic influenza and the ongoing concerns about highly virulent avian influenza highlights the importance of continuing to study the underlying cause of severe illness during influenza and mechanisms that lead to lung pathology.

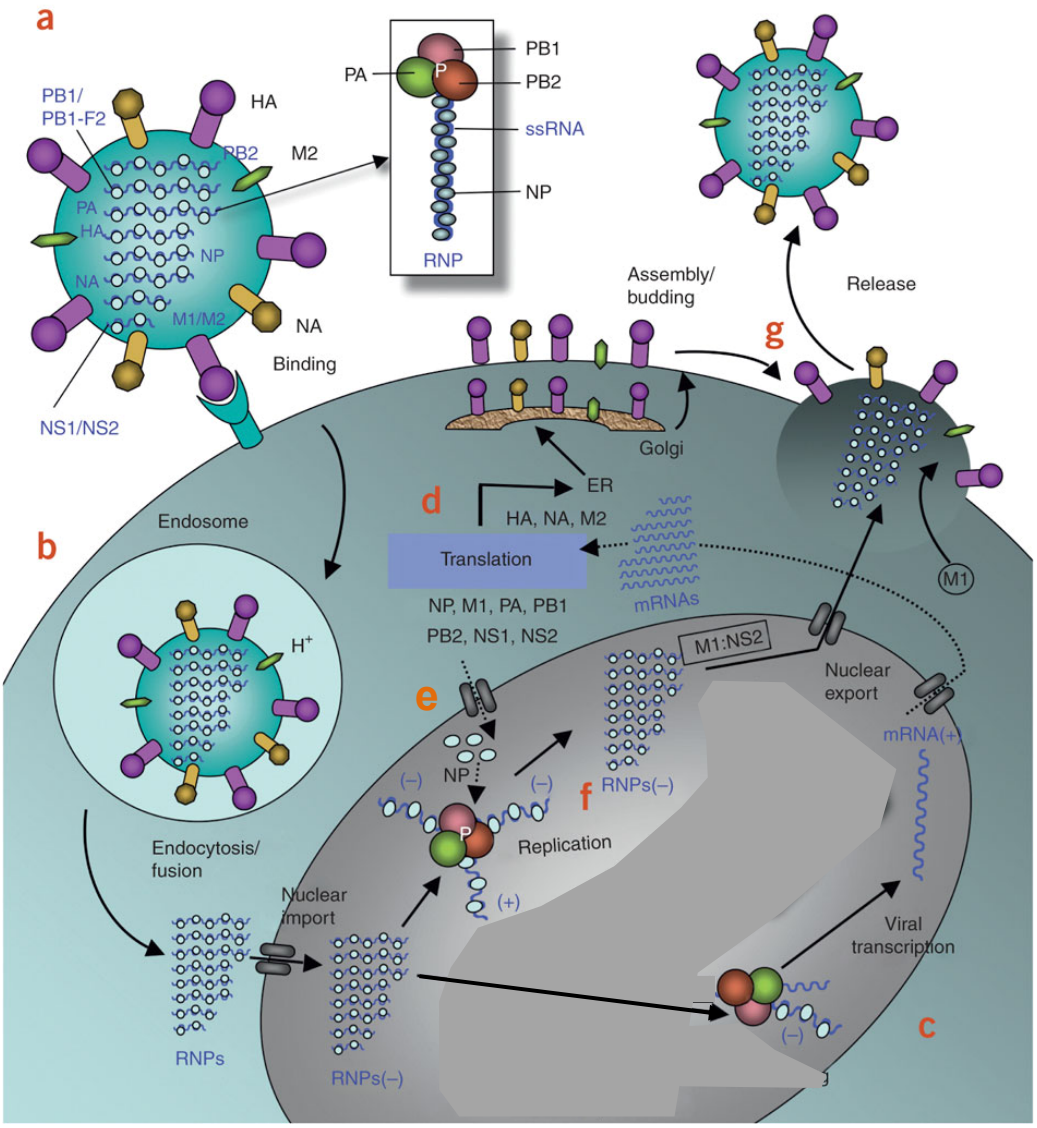


Figure 1. Influenza A virus life cycle. The influenza viral particle is covered by lipid bilayer envelope, contained within the particle are 8 ssRNA genomic segments, each of which is associated with the viral RNA polymerase (PB1, PB2, PA) and nucleoproteins (NPs) to form the vRNPs (a). The vRNP is anchored by the M1 protein to the viral particle scaffolding. Embedded in the envelope are HA and NA spikes which are anchored to the viral particle by the M2 protein (a). HA binds sialic acid on the surface of the host cells, triggering endocytosis of the virus into the endocytic vesicle. Acidification of the endosome triggers the fusion of the viral and endosomal membranes and allows the M2 protein to form a pore in the endosomal membrane releasing the vRNP into the cytoplasm (b). NP targets the vRNP to the cell nucleus where the viral polymerase replicates –ve sense vRNA into +ve sense mRNA for gene translation (c). Viral mRNAs are transported to the cytoplasm for translation into viral proteins (d). Viral surface proteins HA, M2 and NA are processed in the endoplasmic reticulum (ER), processed in

the Golgi and transported to the cell membrane, whereas vRNP proteins NP, M1 and NS2 are transported back to the nucleus (e). The viral polymerase then begins the process of synthesizing multiple copies of –ve sense vRNA to be packaged in progeny virus. NP is required for the two-step process of replicating vRNA (-ve sense vRNA → +ve sense RNA → -ve sense vRNA) and is associated with vRNA throughout synthesis (f). The resulting vRNPs associate with M1–NS2 complex to form the vRNPs and is exported to the cell surface where the progeny viral particle is assembled and bud out of the host cell (g). NA of progeny virus cleaves host cell sialic acid releasing the virus from the host cell. Adapted from Das et al. 2010. Nature Structural & Molecular Biology 17:530-538.

1.1.2. Tuberculosis

Mycobacterium tuberculosis (*M.tb*), the causative agent of tuberculosis (TB) has infected one-third of the world's population. It is estimated that there are 9.2 million new cases of TB every year and 1.7 million deaths due to TB (19,20). Most people contract TB early in life, prior to teenage years. Infection with *M.tb* does not always result in active disease; 90% of people living with TB will not develop clinical disease. The incidence of TB varies throughout the world with the greatest disease burden in sub-Saharan Africa, India, China, and the islands of Southeast Asia and Micronesia where an estimated 1 in 100 persons are infected (19, 21-22). A single infected person with active TB has the potential to spread the infection to 10-15 people every year (23). Many factors contribute to the high prevalence of TB in some areas of the world including poverty, human immunodeficiency virus (HIV) co-infection and the emergence of multidrug resistant strains (MDR) and extensive drug resistant (XDR) strains of TB (21, 24-26). In Canada, the incidence of TB had been decreasing steadily over the last 50 years; however, the rate of decline has slowed. This is due to a disproportionate increase in the number of cases of TB in foreign-born Canadians and "at risk" Canadian-born populations, particularly the native-Indian population and Canadian health care workers who are never vaccinated. The currently available vaccine *Mycobacterium bovis* Bacille Calmette Guérin (BCG) provides protection against childhood forms and disseminated TB but fails to provide protection against adult pulmonary TB. While 80% of the world's population is vaccinated during infancy, vaccination against TB is no longer common practice in North America, leaving much of the population at risk to community acquired TB infection.

Antibiotic therapy is the only treatment for TB, however the protocol is lengthy and has serious toxic side-effects. In recent years, drug resistant strains of *M.tb* have emerged making treatment increasingly difficult and in some cases impossible. The chronic nature of *M.tb* infection, inefficacy of the current vaccine in combination with the cumbersome, drawn out antibiotic therapy makes *M.tb* a highly successful human pathogen and serious health threat.

Mycobacteria are aerobic, facultative intracellular bacteria. *M.tb* is a highly successful pathogen because of its resistance to environmental degradation, chemical disinfectants as well as mammalian host immunity. The cell wall of *M.tb* contributes greatly to its virulence. The cell wall is complex of tightly packed layers of mycolic acid, free lipids, peptidoglycan (PGN) and lipoarabinomannan (LAM) along with several enzymes which form a hydrophobic barrier responsible for the superior resistance against penetrating antibiotics as well as other chemicals. One of the dominant virulence proteins secreted by *M.tb* is the antigen 85 (Ag85) complex. Ag85 is essential for the maintenance and assembly of the cell wall making it an ideal target for new therapies as well as vaccination strategies (27-29). *M.tb* infection is acquired through inhalation of infectious droplets leading to infection of alveolar macrophages (AM) in the alveolar space and to a lesser extent lung resident DCs. *M.tb* uses numerous receptors to initiate phagocytosis into the host cell, namely complement receptors (CR) 1 and CR3, mannose receptor (MR) as well as CD14 (30,31). *M.tb* is taken up in the phagosome where the host cell will attempt to degrade the bacteria by fusing the phagosome with the acidified lysosome. Mycobacteria

have the unique ability to evade degradation by host cells by either blocking phago-lysosome fusion or by escaping the endosome to live in the host cell cytoplasm. LAM has potent immuno-modulatory properties inhibiting phagosome maturation and fusion with the lysosome as well as blocking antigen presentation by major histocompatibility complexes (MHC) (32-34). All of these immune evasion strategies allow *M.tb* to evade host innate anti-microbial defences and establish a chronic infection. In contrast to some other pathogens which infect, replicate rapidly and spread to new hosts, *M.tb* takes a slower approach. *M.tb* is a very slow growing bacterium doubling every 21-41 hours assisting the bacteria to evade host detection (35,36). Mycobacteria also have the ability to migrate away from the superficial mucosal surface into the deep tissues and enter a latent phase of infection. In this phase, bacteria do not actively replicate and maintain only a basal level of metabolic activity conducive to survival.

Treatment of TB is a complicated drawn out process that involves the use of 3 antibiotics for 6-12 months. Lack of patient compliance with the drug regime makes it difficult to treat TB under in the best circumstances and almost impossible in developing countries. Failure to complete the full course of antibiotics is directly contributing to the emergence of drug resistant strains of TB. In 2008, there were 440 000 new MDR-TB cases and 150 000 deaths due to MDR-TB and XDR-TB has been reported in every country around the world (37). The emergence of drug resistant strains of TB has complicated the global effort to control TB. It is becoming increasingly clear that vaccine strategies and therapies to boost host defence against TB infection are going to become important in coming

years. In spite of over 5000 years of co-evolution, we do not have a clear understanding of the factors that determine susceptibility to TB infection and how the host-pathogen interaction can be manipulated to tip the scales in the favour of host survival.

1.2 Host defense against intracellular infection

1.2.1 Primary immune response against influenza A virus infection

Innate immune responses against Influenza A virus

Influenza virus infection is detected by innate pattern recognition receptors (PRRs), specifically toll like receptors (TLRs) and retinoic acid inducible gene-I (RIG-I) (38). Human TLR8 and murine TLR7 localize to the endosomal membrane where they can interact with ssRNA from influenza virus (39). RIG-I is found in the cell cytoplasm and also senses single stranded RNA (40-42). Type-1 interferons (IFN) α/β are released by infected epithelial cells as well as macrophages and dendritic cells (DCs) that reside within the airway. The innate IFN response is important in host defence against pulmonary viral infection because it promotes an "anti-viral state" in adjacent cells, inhibiting transcription and translation of host genes making cells non-permissive to viral infection and limiting viral replication. The innate immune response to influenza leads to the production of pro-inflammatory cytokines and chemokines in 2 distinct waves (Figure 2). The first wave is initiated by the infected epithelial cells resulting in low levels of TNF (tumour necrosis factor)-α, interleukin (IL)-1, IL-6, IL-8, MCP-1(monocyte chemotactic protein-1), IP-10 (interferon gamma-induced protein 10 kDa) and RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted) (Figure 2) (43,44). These inflammatory mediators recruit leukocytes (monocytes/macrophages, DCs, neutrophils) and innate like lymphocytes (natural killer (NK) cells and gamma delta T cells) to the site of infection. In spite of the type 1 IFN response, influenza continues to

replicate in epithelial cells resulting in the release of viral progeny. Immune cells recruited to the site of infection can become infected with influenza virus, and amplify inflammation by secreting a second wave of pro-inflammatory cytokines TNF-α, IL-1β, IL-6, IL-18 and IFN-α/β and chemokines RANTES, MIP (macrophage inflammatory protein)-1α, MCP-1, MCP (monocyte chemotactic protein)-3, IP-10 resulting in the influx of additional innate immune cells and adaptive lymphocytes (Figure 2) (43,44).

One of the first immune cells recruited during any infection is the neutrophil. Not traditionally thought of as important during viral infection, the function of neutrophils in viral infection is controversial. Some reports suggest that neutrophils play a regulatory role in influenza infection by aiding in viral clearance and helping re-establish homeostasis by scavenging cellular debris and apoptotic bodies (45,46). On the other hand, neutrophils are potent sources of myeloperoxidase (MPO) which is associated with lung tissue damage, suggesting excessive neutrophil responses may actually perpetuate virus induced lung damage (47-50). Macrophages recruited to the lung following influenza infection are susceptible to infection but do not support viral replication. Instead they play a role in amplifying inflammation by secreting pro-inflammatory cytokines/chemokines (43,44). Thus macrophages play a two-fold role in influenza infection by limiting viral replication and amplifying inflammation (51,52). DCs are recruited into the infected lung within 24-48 hours of infection where they may become infected with influenza or acquire viral antigens from dead cells (53,54). DCs play a critical role in T cell priming which is discussed in detail in section 1.3. Plasmacytoid

DC (pDC) is a unique subset of cells that are functionally distinct from conventional DCs. pDCs are predominantly found in the lung and play a crucial role in response to viral infection by secreting type 1 IFNs, assisting in early control of viral replication (55). In addition, pDCs help co-ordinate the anti-viral immune response in the lung by activating NK cells and modulating T cell priming (55,56).

NK cells are rapidly recruited to the lung following influenza infection to participate in control of controlling influenza virus. NK cells are early sources of IFN-γ (24-48 hours post infection) which stimulates the production of nitric oxide (NO) by macrophages, limiting viral replication and contributes to antigen presentation and chemokine production (57-60). In addition, NK cells can directly kill virally infected cells through a perforin-granzyme dependent mechanism and the secretion of IFN-γ and TNF-α (61-64).

Innate anti-viral responses are highly effective in limiting viral replication and prevent viremia. Inflammatory responses in the lung result in recruitment of innate effector cells to control viral replication and begin the process of eliminating virally infected cells. Although innate immune responses are important for early viral control, adaptive immune responses are essential for elimination of influenza virus

Adaptive immune responses against influenza A virus infection

Influenza specific CD4 and CD8 T cell responses are primed in the draining lymph node (LN) 3-4 days after infection. Effector T cells are recruited back to the lung 5-7 days post infection by chemokines (IP-10, MIG and RANTES). CD8 T cells are the most abundant lymphocyte to be recruited to the lung following influenza infection and are essential for

viral clearance, as shown by the observation that CD8 deficient mice have impaired viral clearance (65-67). CD8 cytotoxic T lymphocytes (CTL) have several tools available to combat viral infection including the ability to trigger apoptosis of infected cells through perforin-granzyme, Fas-L and TRAIL (tumour-necrosis factor (TNF)-related apoptosis-inducing ligand) (68-70). In addition, IFN-γ and TNF-α secreted by CD8 T cells help to eliminate virally infected cells. Compared to CD8 T cell responses, CD4 T cell responses are both slower to prime and lower in magnitude making it difficult to determine their precise role in anti-influenza immunity. CD4 T cells are thought to play a supportive role in anti-influenza responses by augmenting CD8 T cell responses and assisting in B cell priming/isotype switch. Indeed, CD4 deficient mice have impaired viral clearance attributed to delayed CD8 T cell priming and antibody responses (71-73). In addition to their supportive role in anti-influenza immune responses, polyfunctional CD4 T cells secreting numerous cytokines including IFN-γ, TNF-α, IL-2 and IL-17 (74-77). These effector functions confer the ability of CD4 T cells to clear virus in an antibody-independent manner, suggesting CD4 T cells may play a direct role in eliminating virally infected cells and provide protection against lethal secondary influenza infection (76, 78).

Primary influenza virus infection results in robust activation of highly specific B cell and Ab responses. B cells acquire viral antigen through their B cell receptor (surface bound immunoglobulin (Ig) M), antigen is internalized and presented to activated CD4 T cells by MHC-II in a process called linked recognition. CD40L on CD4 T cells binds CD40 on B cells and in conjunction with type 1 cytokine IFN-γ, drives the production of IgG isotype Abs. Abs serve a number of functions in influenza virus infection; first Ab

responses can readily neutralize influenza virus by blocking both HA and NA epitopes. Second, Abs can opsonise viral particles targeting them for phagocytosis by macrophages and/or neutrophils. Third, Ab responses can actually inhibit viral budding from host cells, blocking viral spread. Ab responses are critical for protection against secondary viral challenge and are the basis of anti-influenza vaccine strategies.

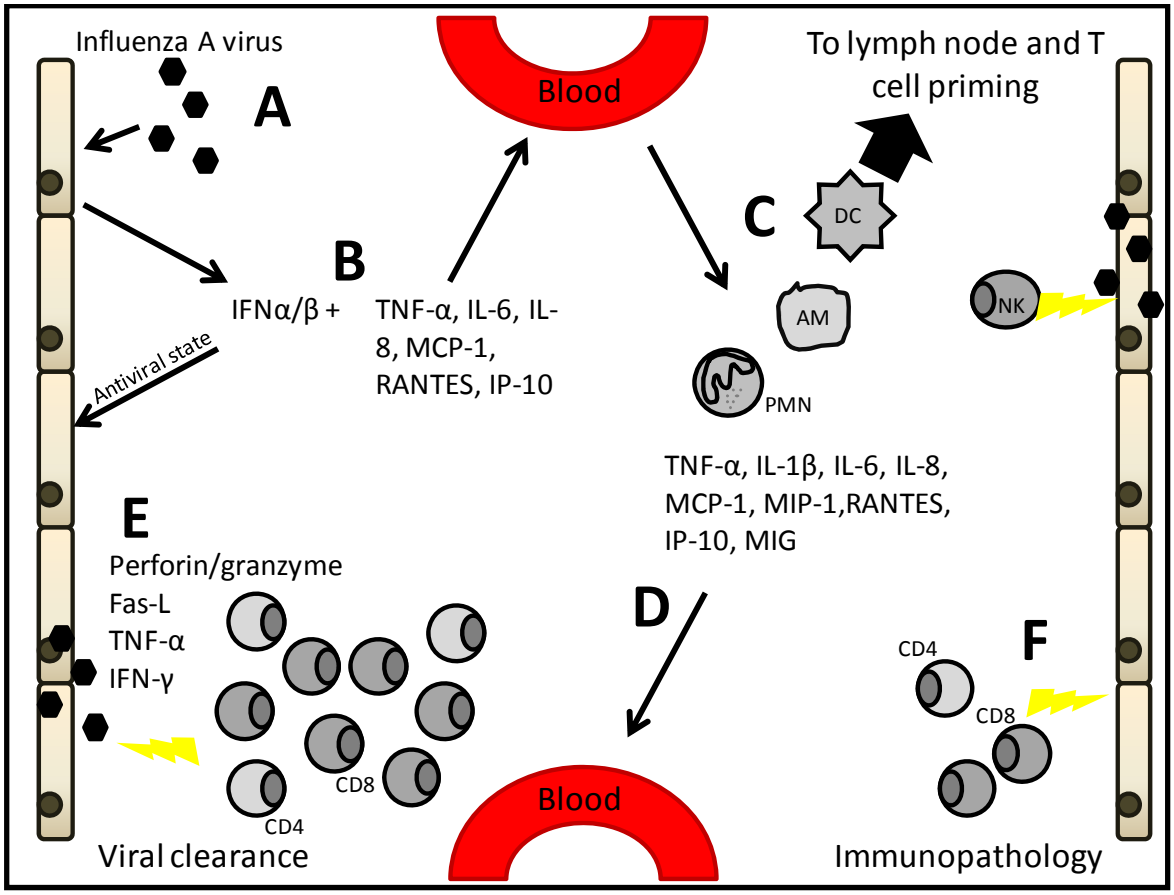


Figure 2. Innate and adaptive immune responses during influenza A virus infection. Influenza virus infects epithelial cells of the lung (A) and rapidly trigger a type 1 IFN response resulting in the production of IFNα/β and pro-inflammatory cytokines and chemokines (B). Pro-inflammatory cytokines/chemokines recruit neutrophils (PMN) followed by macrophages and DCs from the blood (C). Newly recruited innate immune cells are activated directly by influenza virus or become activated by necrotic/apoptotic cells leading to a second wave of pro-inflammatory cytokines (D). Adaptive immune responses are initiated when DCs acquire antigen in the lung and migrate to the local draining LN to prime T cells. Lung resident NK cell and NK cells recruited from the blood can directly lyse influenza infected cells and play an important role in limiting viral burden early during infection until adaptive immune responses can be recruited. Effector CD8 and CD4 T cells are recruited to the lung from the blood to eliminate influenza virus infection through the secretion of cytotoxic cytokines TNF-α and IFN-γ. CTLs directly lyse virally infected cells through perforin/granzyme, FasL and TRAIL (E). Both CD8 and CD4 T cells contribute to lung tissue immunopathology through the same effector mechanisms that clear viral infection (F). Created by S. McCormick, 2011.

Lung immunopathology in influenza virus infection

Fatal cases of human influenza infection such as those during the 1918 H1N1 pandemic and more recently in the 2009 H1N1 epidemic are often due to destruction of lung tissues by host immune responses. The same anti-influenza immune responses necessary to clear viral infection may also contribute to lung immunopathology characterized by cytopathic damage of bronchial and alveolar epithelial cells, necrotizing bronchiolitis and metaplasia of the larger airways (79). Both innate and adaptive immune components can contribute to lung pathology. Virulent strains of influenza may trigger hypercytokinemia (cytokine storm) leading to excessive infiltration of immune cells, pneumonia, respiratory failure and death. Overproduction of NO by macrophages and neutrophils may contribute to non-discriminate tissue destruction and pneumonia (80,81). Neutrophils also have the ability to produce MPOs which degrade the extracellular matrix leading to holes in the lung tissues and severe lung dysfunction. While innate immune responses certainly may initiate lung damage, it is the adaptive immune response that is responsible for much of the tissue destruction. To date, the majority of lung immunopathology has been blamed on CD8 T cells. CD8 T cells have the potential to contribute to tissue damage through a variety of effector T cell functions. Perforin/granzyme and FasL are not generally thought to contribute to immunopathology, but rather effector cytokines TNF-α and IFN-γ are believed to have the greatest immunopathogenic potential (82). This is likely because these cytotoxic effector functions require effector T cells to directly recognize viral peptide presented by MHC class I molecule. The contribution of TNF-α and IFN-γ to influenza induced lung immunopathology is difficult to ascertain because of the

pleotropic nature of these cytokines. Indeed, in a murine model of respiratory syncytial virus infection, depletion of TNF-α ameliorated lung pathology and improved survival (83-84). In contrast, TNF-α deficient mice developed uncontrolled immunopathology and fibrotic remodelling following influenza infection due to unchecked T cell activation and IFN-γ production by activated CD4 and CD8 T cells (unpublished data). These findings together with similar observations in a variety of models suggest TNF-α has the potential to cause or regulate immunopathology although the mechanisms remain unclear (85-89). Similarly, neutralization of IFN-γ results in decreased cellular responses, impaired viral clearance and dysregulated CTL numbers which are hypothesized to contribute to lung pathology (66, 90-91). Determining the contribution of cytokines such as TNF-α and IFN-γ to immunopathology is difficult because the same mechanisms that lead to viral clearance are the cause of pathology. Furthermore, these seemingly pro-inflammatory cytokines have potent immunoregulatory effects by limiting the magnitude of the T cell response. CD4 T cells also have the potential to cause tissue injury. Although there are fewer CD4 than CD8 T cells recruited to the lung during primary influenza infection, memory CD4 T cells persist in the lung, local draining LN causing exaggerated inflammatory responses upon secondary influenza exposure (92). The contribution of CD4 T cells to lung immunopathology during primary influenza infection has been largely overlooked. In other pulmonary diseases, CD4 T cells contribute directly to lung destruction and remodelling leading to impaired lung function (93,94). IL-17 producing CD4 T cells (Th17) have been implicated in influenza immunopathology although they do not appear to contribute directly to viral clearance (48, 95). IL-17 is a potent

neutrophil chemotactic cytokine. Recruitment of excessive neutrophils to the influenza infected lung leads to the generation of oxidized phospholipids which have previously been shown to play a critical role in acute lung injury in viral infections (75). Because of their effector functions during viral infection T cells have the potential to cause severe lung damage if left unchecked. Several regulatory mechanisms exist to prevent aberrant T cell responses and are discussed below in Section 1.2.4.

1.2.2 Primary immune response against mycobacterial infection

Innate immune responses against mycobacterial infection

M.tb infection is acquired by inhalation of infectious droplets leading to deposition of bacilli in the terminal airspaces of the lower respiratory tract. AMs are the first to come into contact with the bacterium and are instrumental in initiating an immune response. *M.tb* will spend the majority of its life hiding within the macrophage. Indeed, *M.tb* infection can only be eliminated when macrophages are activated to degrade intracellular bacteria. Often times the macrophage cannot accomplish this alone and requires additional innate and adaptive immune cells to provide stimulatory cytokines in order to eliminate infection. *M.tb* is a highly complex pathogen with many components such as mycolic acid of the cell wall, PGN, LAM, 19kDa protein and cord factor that can be sensed by extracellular as well as intracellular PRRs on AM (96-98). TLR2 expressed on the surface of AM recognizes extracellular bacteria through LAM and the secreted 19 kDa protein resulting in the production of TNF-α (99,100). In vivo studies reveal TLR2KO mice are more susceptible to *M.tb* infection and develop worse lung pathology in the first weeks following infection indicating TLR2 is essential for host defence against *M.tb* infection (101-103). TLR4 and TLR6 form heterodimers with TLR2 suggesting that they may play a role in sensing *M.tb* infection and host resistance. Indeed TLR4 and TLR 6 deficient mice show some increased susceptibility to *M.tb* infection. However, they are not as susceptible to *M.tb* infection as TLR2 deficient mice pointing to TLR2 as the dominant innate immune sensing molecule of extracellular *M.tb* bacilli (102-104). TLR9 is also important for an effective anti-mycobacterial immune response. TLR9 is

embedded in the membrane of the endosome and allows the host cell to sense bacterial DNA from degraded bacteria. TLR9 deficient mice have impaired ability to produce IL-12 and IFN-γ and have impaired ability to mount sustained adaptive immune responses leading to impaired bacterial control (105-107). In spite of the clear ability of host cells to sense *M.tb* in vitro, in vivo pro-inflammatory responses following primary mycobacterial infection are not detected until 7 days post infection (unpublished data).

TNF-α serves a dual function in *M.tb* infection, first it initiates the inflammation cascade, loosening tight junctions of endothelial cells and promoting cellular infiltration and second, TNF-α promotes the production of antimicrobial mediators such as reactive oxygen species (O^-, NO, H_2O_2) to eliminate intracellular bacteria and promote apoptosis. AMs secret numerous chemokines including MIP-1α/β, MCP-1, IP-10 and RANTES which recruit additional monocytes/macrophages, DCs and neutrophils to the site of infection. In cases where infected macrophages are unable to eliminate intracellular bacteria, innate immune cells cluster around the infected macrophage to form an innate granuloma. The innate granuloma serves to wall off the infection, forming a physical barrier against bacterial dissemination. With time this granuloma will mature and form the basis for the immune granuloma that will contain bacteria for the lifetime of the host.

The role of neutrophils in host defence against *M.tb* infection is contentious. Some studies provide evidence that neutrophils help control early bacterial replication (108-111) while others suggest there is little/no protective role for neutrophils in *M.tb* infection (112-114).

We and others have shown that neutrophils predominate the lung inflammation 5-7 days post infection and preceded influx of a large number of macrophages and DCs (unpublished data) (115,116). Neutrophils certainly participate in the formation of the innate granuloma through the secretion of chemokines (IP-10, MCP-1, MIP-1α/β) (117), activate macrophages through the secretion of cytokines (TNF-α, IL-12, IFN-γ) (117-119), modulate antigen presentation by DCs (109) and even cause lung pathology (109). Most recently, it was shown that phagocytosis of *M.tb*-infected apoptotic neutrophils by DCs amplified naive T cell activation by accelerating DC migration to the LN (115). Taken together these findings indicate that neutrophils likely play a very important role in host defence against *M.tb* infection by supporting the inflammatory process, capturing bacteria and bacterial components and may influence the outcome of the adaptive immune response by activating DCs as well as providing potent Th1 stimulating cytokines.

Innate lymphocytes such as NK, NKT and γδ T cells are also implicated in early host defence against *M.tb* infection. In vitro analysis of NK cells indicates that NK cells can directly lyse *M.tb* infected cells and direct CTL to lyse infected macrophages (120,121). Furthermore NK cells secrete IFN-γ to activate macrophages to overcome pathogen driven immune evasion leading to increase antigen presentation on MHC (122,123). In vivo studies indicate that NK cells are the primary source of IFN-γ during the first 2 weeks following infection and may play a minimal role in bacterial control (124). Similarly, NKT cells are reportedly involved in early IFN-γ cytokine secretion during

intracellular infections (125). Innate γδ T cells were recently identified to secrete IL-17 in response to *M.tb* infection (126-128). The contribution of these cells in resistance to *M.tb* is not yet known although elimination of IL-17 results in impaired granuloma formation and enhanced lung pathology (129). The precise contribution of innate lymphocytes in anti-*M.tb* immunity has been largely overlooked for many years. Investigation into their role in early and sustained anti-*M.tb* responses will be important in developing novel strategies to improve vaccination and therapeutic strategies.

In most pulmonary infections, the innate immune response is without a doubt critical in host defence until adaptive immune responses can be recruited. This is particularly true in *M.tb* infection where there is such a dramatic lag time in initiating an adaptive immune response. It is widely accepted that although long term control of *M.tb* requires robust activation of cellular adaptive immune responses, these responses are never able to completely clear the bacteria. An estimated 10% of persons exposed to TB do not appear to contract the disease and are apparently resistant to infection. In this case, the innate response is thought to sufficient to eliminate the infection before the bacteria can subvert the immune responses and establish a chronic infection. This notion has shifted research efforts to understanding the innate host factors that limit bacteria replication immediately upon infection and investigate factors that regulate adaptive T cell responses in order to better design therapeutic interventions.

Adaptive immune responses against *M.tb* infection

Cellular adaptive immune responses are critical for protection against *M.tb* infection. In contrast to viral and other intracellular bacterial infections, the protective adaptive immune response is dominated by CD4 T cells as opposed to CD8 T cells. Absent or impaired CD4 T cell responses, for example during end stage HIV infection, are associated with increased susceptibility to *M.tb* infection and loss of bacterial control (130-134). *M.tb* has evolved mechanisms to evade degradation by macrophages in order to prolong survival by inhibiting phagolysosome formation or escaping into the cytoplasm (135). This leaves the host with 2 potions to eliminate the bacteria; overcome *M.tb* induced modulation or undergo apoptosis, eliminating the bacteria in the process.

M.tb primed effector CD4 T cells secrete potent immuno-stimulatory cytokines IFN-γ and TNF-α in order to stimulate infected macrophages to form the phagolysosome thereby exposing intracellular bacteria to low pH and anti-microbial peptides NO and ROS (130, 136). CD4 T cells are also able cause apoptosis of infected macrophages by secreting high concentrations of TNF-α, IFN-γ and the Fas-FasL pathway (137,138). CD8 T cells also contribute to bacterial control through the secretion of IFN-γ and TNF-α and also have the potential to stimulate macrophage apoptosis through perforin/granzyme and Fas-FasL (138,139). Effector T cell responses arrive in the lung 14-21 days after initial infection (Figure 3) (140,141). Once in the lung, T cell localize to the innate granuloma where they secrete IFN-γ and TNF-α. In humans, T cells wall off infection from the rest of the lung by forming a lymphocytic cuff surrounding infected macrophages. In mice, T lymphocytes intersperse among infected macrophages to maintain bacterial control.

Cytokine secretion is absolutely critical in host defence against *M.tb*. Optimal NO production and an anti-microbial function of macrophages requires both cytokine signals from IFN-γ and TNF-α (142). Suboptimal production of either one of these cytokines results in impaired NO production and bacterial control (89, 142-144).

A new subset of CD4 T cells secreting IL-17 (Th17) was recently identified in several intracellular infections. Because γδ T cells also produce IL-17, teasing apart the contribution of Th17 cells vs. γδ T cell has been difficult. In models of adoptive transfer aimed at minimizing the contribution of γδ T cells, Th17 cells were capable of partially compensating for IFN-γ deficiency, suggesting that they may play a direct role in bacterial control (145).

In addition to delayed innate immune responses, T cell priming and effector T cell recruitment is delayed following pulmonary mycobacterial infection compared to viral or other intracellular bacterial infection. In humans with known TB exposure, systemic T cell responses begin to be detected at 42 days post infection. In mice, T cell priming does not occur in the LN unit 8-9 days post infection (Figure 3) (140,141). In addition to delayed T cell priming, recruitment of effector T cells into the lung is also delayed to 14 to 21 days post infection. In contrast, during viral infection such as influenza infection, T cell priming can be detected in the LN 3-5 days post infection followed by effector T cell recruitment between 5 and 7 days post infection (Chapter 3). Delayed T cell responses will be discussed in depth below. The mechanisms contributing to delay T cell priming

are an area of intense interest in the hopes of identifying strategies to improve T cell priming and ultimately protective immune responses.

B cell responses are primed as a result of *M.tb* infection and Ab responses are detectable in humans as well as animal models. Neutralizing and opsonising Abs have the potential to bind *M.tb*, however, because of the thick bacterial wall and waxy layer, Ab responses are not very effective in controlling bacterial spread.

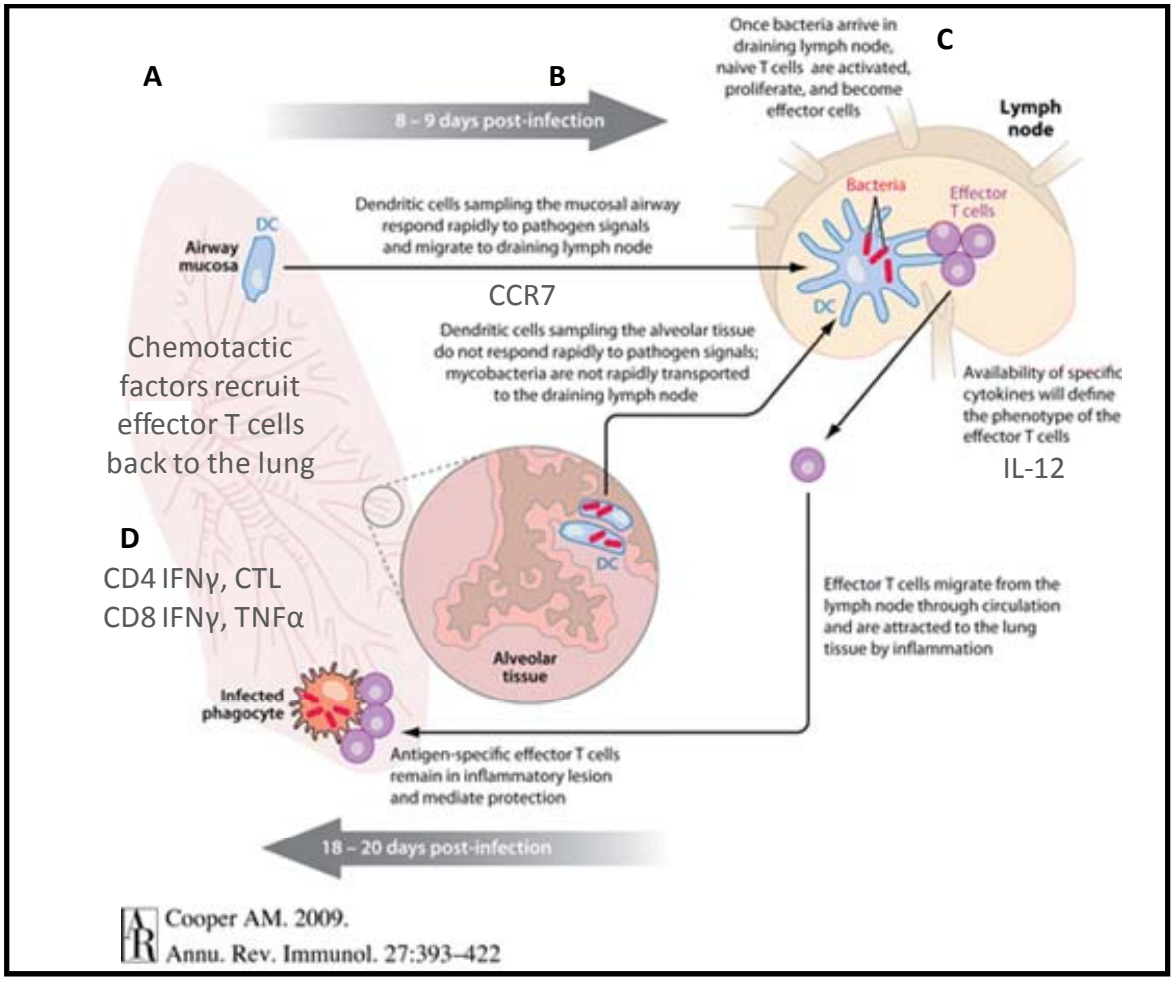


Figure 3. Current understanding of the kinetics of T cell priming during pulmonary *M.tb* infection. *M.tb* is deposited in the lower airways where it infects AM and DCs (A). Several studies report that bacteria do not disseminate to the local draining LN until 9 days post infection, *M.tb* infected DCs undergo a process of maturation whereby they migrate to the LN and shuttle live bacteria in the process (B). The arrival of DCs harbouring *M.tb* immediately precedes naive T cell activation (C). Effector T cells are recruited back to the lung and the site of infection where they activate the infected macrophage to produce anti-microbial compounds nitric oxide (D). Adapted from Cooper, AM. 2009. Cell-Mediated Immune Responses in Tuberculosis. Adapted from Cooper. 2009. *Annu Rev Immunol*. 27:393-422.

1.2.3 Initiation of adaptive immune responses

T cell priming is carried out by DCs, a professional APC specialized to be more efficient at naïve T cell priming than either macrophages or B cells (146). DCs are myeloid derived cells, with a unique morphology of branched projections that allow them to interact at multiple points with other immune cells i.e. T cells and B cells. DCs enter the tissues from the blood as immature DCs where they populate every tissue in the body forming a network designed to sense and respond to attack from the outside world.

There are several types of DCs found in the alveolar space and lung. Alveolar DCs (ADCs) reside in the airway and are the first to encounter antigen (116, 147-148). Like AM, ADCs use TLRs to sample inhaled antigen and respond to pathogens/dangerous matter in the air. ADCs are specialized to priming naïve T cells whereas AMs are better equipped to activate secondary T cell responses (149). Lung tissue is populated by a network of DCs that reside within tissues as well as associated with the conducting airways. A unique population of DCs was recently found interdigitated with epithelial cells termed intra-epithelial DC (iDC) that have the unique ability to project their dendrites into the airway lumen to sample antigen (150-152). Several other APC populations have been identified in the lung and are reviewed in Appendix I. Pulmonary DCs play a very important role in immune surveillance because they are the front line defense against pathogens. DCs sample inhaled antigen though pinocytosis of small molecules, this is contrast to macrophages which can phagocytose whole organisms and larger molecules. DCs sense pathogens through TLRs and secrete pro-inflammatory

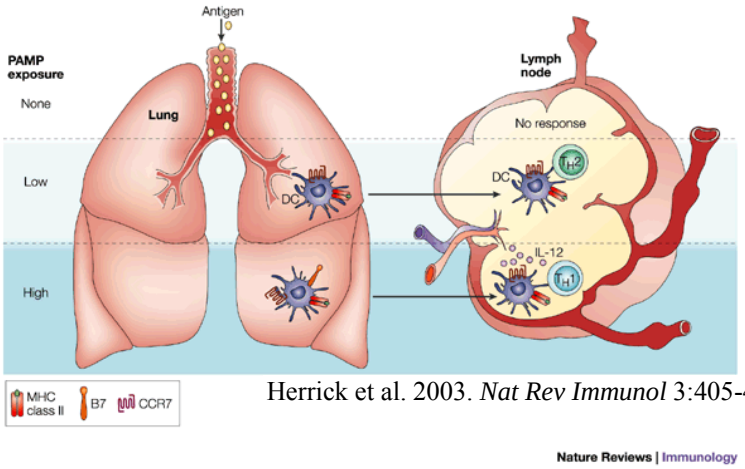
cytokines in response to PAMPS. Upon detection of bacteria, viruses and/or fungi, DCs undergo a process of maturation and leave the lung for the LN within 12 hours in order to rapidly prime T cell responses (153).

DC maturation begins immediately following pathogen recognition. TLR signaling stimulates transcription factors to enter the nucleus leading to expression of genes necessary for T cell priming including chemokine receptors, immuno-stimulatory cytokines, MHC molecules and co-stimulatory molecules (CD80/B7-1 and CD86/B7-2). T cell priming occurs in the local draining LN, for the lung this is the mediastinal LN (mLN). One of the first molecules to be upregulated by maturing DCs is cc-chemokine receptor (CCR) 7, allowing DCs to exit the airway/lung through the lymphatics and home to the T cell rich zones of the LN in response to chemokine (c-c motif) ligand 19 (CCL19) and CCL21 (Figure 4A) (154-157). Cytokines secreted by the mature DC are important for optimal T cell priming as well as T cell polarization. IL-12, a type 1 immune polarizing cytokine, is secreted by DCs in response to intracellular pathogens as well as strong TLR signaling and is indispensible for Th1 and CD8 T cell priming (Figure 4A). Recently IL-12 was shown to optimize CCR7 dependent migration to the LN (158,159). Migration of DCs to the LN is a rapid process, with mature DCs appearing in the LN as little as 24 hours following infection (158). The efficiency of this migration process has a profound impact on the subsequent immune response. Failure of DCs to migrate to the LN severely impairs T cell priming and more often than not results in

decreased lung function and pathology due to formation of inducible bronchus associated lymphoid tissue (iBALT) and T/B cell priming in the tissues (160-162).

Once in the LN, DCs migrate to the T cell zone where peptide fragments of ingested antigen is presented to CD4 and CD8 T cells by MHC class II and MHC class I respectively. The DC and T cell come into close contact allowing the T cell receptor (TCR) to bind MHC presenting foreign peptide. The DC-T cell interaction is stabilized by the CD4/CD8 molecule providing T cells with a stimulatory signal (Figure 4B). Co-stimulatory molecules CD80 and CD86 provide a second stimulatory signal resulting in phosphorylation of the immuno-tyrosine activating motif (ITAM) on the cytoplasmic tails of the T cell signaling complex. In the presence of IL-12, TCR signaling preferentially activates transcription factor T-bet and transcription of effector genes such as IFN-γ, TNF-α, IL-2, perforin, granzyme and Fas-L amongst others (163-166). Newly activated T cells will undergo several rounds of expansion before exiting the LN and migrating via the blood to the infected tissue.

A



Herrick et al. 2003. *Nat Rev Immunol* 3:405-412.

B

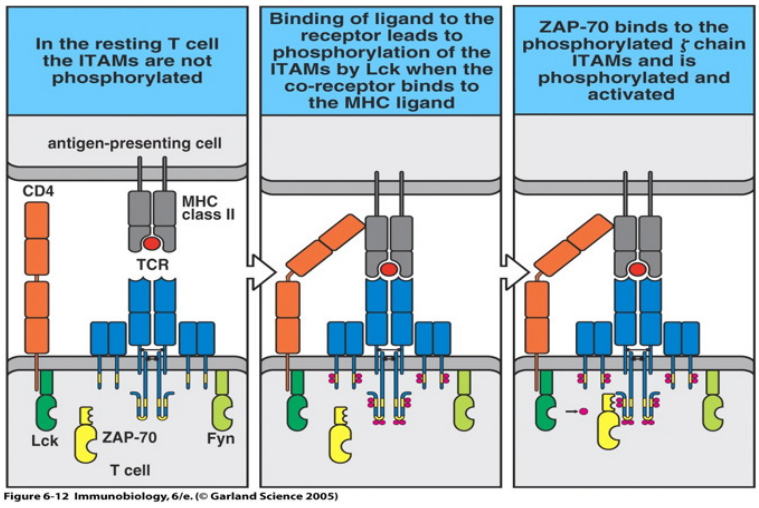


Figure 4. T cell priming by pulmonary dendritic cells. Pulmonary DCs sense infection through TLRs and initiate an adaptive immune responses. High concentrations of PAMPS results in strong TLR signaling and the production of IL-12 resulting in Th1 T cell priming whereas low concentration of PAMPS do not stimulate IL-12 production by DCs leading to Th2 polarized T cell responses. Upon maturation, DCs upregulate CCR7 expression and migrate from the lung to the draining LN (A). MHC class II bound to pathogen derived peptide on DCs interacts with the naïve TCR in the LN. Engagement of the TCR along with co stimulatory molecules leads to phosphorylation of ITAM on the TCR signaling complex and activation of the naïve T cell (B). Adapted from Herrick et al. 2003. *Nat Rev Immunol* 3:405-412 (A) and Janeway et al. 2005. Immunobiology 6th edition: The Immune System in Health and Disease, Garland Science (B).

1.2.4 Regulation of adaptive cellular immune responses

Unchecked T cell responses inevitably lead to tissue destruction and respiratory failure. T cell responses are regulated by a number of mechanisms. Although expansion of antigen specific T cells is required to generate sufficient T cells for pathogen clearance, overexpansion can be detrimental. TNF-α serves a dual purpose in intracellular infection, first by activating macrophages but also by limiting the extent of T cell proliferation (89). Another mechanism limiting effector T cell responses and preventing inappropriate tissues damage is to eliminate effector T cells after pathogen clearance. The lifespan of an effector T cell is therefore limited by intrinsic pro-apoptotic signals. Effector T cells are eliminated by activation induced cell death (AICD) and ultimately apoptosis (167). In activated T cells, expression of Bcl-2 interacting mediator of death (Bim) promotes activation of caspases and cell death, limiting the lifespan of effector T cells (168). In contrast, expression of Bcl-2 can help resist cell death. Some T cells can become the pool of memory T cells which may persist indefinitely. Effector T cells also express Fas which makes them susceptible to FasL mediated killing but other effector T cells (169,170). These mechanisms ensure that the effector T cell do not persist after the infection is cleared and helps return the host to homeostasis.

Regulatory T cells (Tregs) modulate the immune response by inhibiting effector T cell functions. Tregs secrete immuno-suppressive cytokines such as IL-10 and transforming growth factor (TGF)-β which inhibit IFN-γ secretion and prevent the cytolytic functions of CD4 and CD8 T cells (171,172). In addition, IL-10 and TGF-β have inhibitory effects

on APCs including DCs, providing a 2-tiered approach to minimizing T cell activation (172,173). Tregs can directly inhibit T cell responses in a contact dependent manner (172,173) although these mechanisms require further investigation. Tregs control the extent of T cell priming during pulmonary viral infection, such as influenza, and limit CTL functions in vivo (173,174). These functions are especially important during chronic viral infection, such as herpes simplex virus or hepatitis infection where small amounts of viral antigen persist for years and can cause slow destruction of essential tissues. The same actions of Tregs that preserve host tissues may also promote viral persistence leading to debate whether the production of IL-10 by T cells is advantageous for the host or the virus. Clinical findings indicate that regulatory T cells are over expanded in the elderly, providing one possible explanation for the decreased/ineffective immune responses and increased mortality associated with influenza infection (175). To date, these observations have not been definitively correlated with disease outcome and the precise role of Tregs during influenza infection in healthy adults is not yet defined. Although influenza virus infection does not lead to chronic infection, Tregs may limit acute lung pathology during highly virulent influenza infection and thus may be key to improving survival.

Tregs have been documented in human TB, where increased frequency of circulating IL-10 producing T cells is linked with increased risk of reactivation of latent TB (176-178). It is reasonable to speculate that Tregs are present in the granuloma and function to limit T cell activation leading to long term maintenance of an immunosupressed environment.

This immune dampening is likely essential to preserve host tissues and prolong survival. Indeed experimental depletion of regulatory T cell populations leads to mostly enhanced bacterial control and at the expense of increased lung pathology. Tregs have also been implicated in decreased effectiveness of the BCG vaccine by interfering with protective immune responses (179,180).

Regulating immune responses is an important feature of host defence, especially during intracellular infection of the lung. Not to be overlooked is the contribution of tissue macrophages in regulating host T cell responses. Both macrophages and DCs have the potential to secrete immunomodulatory cytokine IL-10 in response to TLR stimulation (181). Indeed macrophages play a critical role in regulating the level of immune activation in the granuloma, preventing destruction of host lung tissues (182). Similarly, DCs secrete IL-10 in response to many TLR ligands and live pathogens in order to limit the extent of T cell activation and prevent destruction of host tissues (183). While direct modulation of adaptive cellular immune responses usually prevent immunopathology, in some situations overactivation T cells at the time of priming causes irrevocable tissue damage and intrinsic immunoregulatory mechanisms cannot prevent immunopathology.

1.3 Role of dendritic cells in adaptive immune responses

Maturation of pulmonary DCs by influenza virus is the first step in priming adaptive T cell responses. DC maturation will ultimately dictate the outcome of an immune response. Weak activation of DCs and subsequent incomplete maturation of DCs may lead to impaired/delayed T cell priming or inappropriate Th2 skewing whereas heightened DC activation may contribute to accelerated or increased T cell priming (184).

Pathogenic T cell responses leading to lethal tissue destruction and respiratory failure have been documented in influenza infection, however, it is not known what factors may precipitate these responses. One of the possibilities is dysregulated T cell priming as a result of alterations in APC populations. There is evidence that highly pathogenic pandemic strains of influenza greatly enhance the level of cytokines and APCs recruited to the airway (185,186). These findings suggest that the virus has the potential to directly influence a number of factors including the extent of antigen presentation, the level of co-stimulation and the amount of cytokine produced by DCs at the time of priming. The observation that influenza can directly influence DC activation and maturation, and by extension T cell responses, has important implications when trying to identify the therapies to improve survival in cases of sever influenza infection and merits further investigation.

During pulmonary *M.tb* infection, delayed T cell priming has serious consequences for bacterial control. Because of the immune-evasive strategies of the bacteria and the low level of immune activation in vivo, it was suggested that the delay in T cell priming was

due to delayed antigen availability in the LN in a timely manner. Indeed the consensus in the field is that bacteria do not appear in the LN until 8-9 days post infection (Figure 3). Careful inspection of the current literature and our own observations clearly indicate that viable mycobacterial bacilli are present in the local draining LN prior to 5 days post infection, albeit in low numbers, refuting the hypothesis that lack of early T cell priming is due to lack of antigen availability in the LN (unpublished data) (140). It was further suggested that delayed T cell priming could be accelerated by increasing the antigenic dose. Increased bacterial inoculation does not dramatically accelerate T cell priming (unpublished data). These observations taken together with the findings that pro-inflammatory responses in the lung are similarly delayed following *M.tb* infection lead us to the hypothesis that delayed T cell priming could be attributed to impaired activation of DCs and/or impaired migration of DCs to the LN. If indeed these hypotheses hold true, enhanced activation of DCs and accelerated migration of DCs to LN should accelerate T cell priming.

Modulating the host DC response will have a profound impact on resistance to intracellular infections. Vaccinologists have long appreciated the importance of enhancing DC activation in vaccine efficacy. Numerous approaches have attempted to optimize vaccine delivery in the hopes of priming a more robust protective immune response and subsequent immune protection. Cytokine adjuvants that target DCs like GM-CSF, IL-12 and IL-15 all enhance vaccine induced immunity although this approach has serious safety limitations when vaccinating the human populations (187-190). Recombinant viral vaccines like adenoviruses and vesicular stomatitis virus show promise

because they preferentially infect APCs, simultaneously delivering antigens for presentation to T cells while providing the necessary TLR signals to upregulate pro-inflammatory cytokine. A more direct approach that gained favour in recent years is direct manipulation of DCs ex vivo for subsequent antologous immunization. Most often employed in cancer vaccinology, ex vivo manipulated DCs show promise as vaccines and therapeutic treatment strategies in situations where mainstream treatment fails (Reviewed in Appendix I and Appendix II).

1.4 DNAX activating protein of 12kDa

Signaling adaptor molecule DNAX activating protein of 12kDa (DAP12) is a signal adaptor molecule found in the plasma membrane of myeloid cells (macrophages, DCs, neutrophils) and NK cells (191-195). The functional DAP12 signaling molecule is made up of 2 homodimers each containing an ITAM and docking sites for signal transduction molecules (Figure 4). DAP12 does not have an extracellular binding domain or a ligand binding domain but non-covalently associates with numerous ligand receptors that are not capable of signaling alone. DAP12 was first described in natural killer (NK) cells for its ability to bind to and signal for killer activating receptors NKp44 and NKG2D (191-192, 196). DAP12 signaling is critical for NK cell cytotoxicity and cytokine production during viral infection (197,198). In myeloid cells, DAP12 non-covalently associates with numerous receptors including signal regulatory protein (SIRP)-1β, triggering receptor on myeloid cells type-1 (TREM-1), TREM-2, TREM-3 as well as myeloid DAP12-associating lectin (MDL-1) (199-204). Interestingly enough, the function of these receptors are still unclear and the ligands for these receptors have not yet been identified.

DAP12 was initially described as a pro-inflammatory molecule (199-201, 205). However, substantial evidence indicates that DAP12 negatively regulates TLR induced pro-inflammatory responses in macrophages and dendritic cells (202-204, 206). We have further demonstrated that DAP12 negatively regulates cytokine and chemokine responses in the lung during mycobacterial infection (207). TLR signaling in the absence of DAP12 results in increased Syk signaling, ERK1/2 phosphorylation and NF-κB translocation to

the nucleus suggesting the increased pro-inflammatory response in the absence of DAP12 is a failure to turn off pro-inflammatory signals (203). In line with in vitro data, in vivo studies indicate that TREM-1 is involved in host defence against infection by amplifying the inflammatory cytokine response (208,209). To complicate matters, a limited number of studies suggest that TREM-2 plays a more anti-inflammatory role by limiting inflammation and promoting DC survival by blocking pro-apoptotic signaling (210). How the same signaling cascade leads to such opposing biological effects remains to be elucidated.

The contribution of DAP12 to T cell priming is equally conflicting. Early reports in a murine model of EAE showed impaired MOG T cell priming and subsequently enhanced resistance of DAP12 deficient mice to EAE (211). More recently, it was shown that the observed decrease in T cell priming was due to inefficient MHC class II recycling on the surface of APCs and enhanced ubiquitin-targeted MHC degradation (212). We were the first to report the contradictory evidence that DAP12 deficiency enhanced T cell responses (207). We report not only enhanced T cell priming by DAP12 deficient APCs but also enhanced bacterial control. In line with our findings, Sumpter et al. show that DAP12 deficient liver DCs stimulate enhanced T cell proliferation indicating DAP12 helps maintain homeostasis by inhibiting inappropriate activation of T cells against commensal bacteria (213). DAP12 signaling is complex and the precise role DAP12 plays in controlling host immune responses against intracellular infection remains unclear.

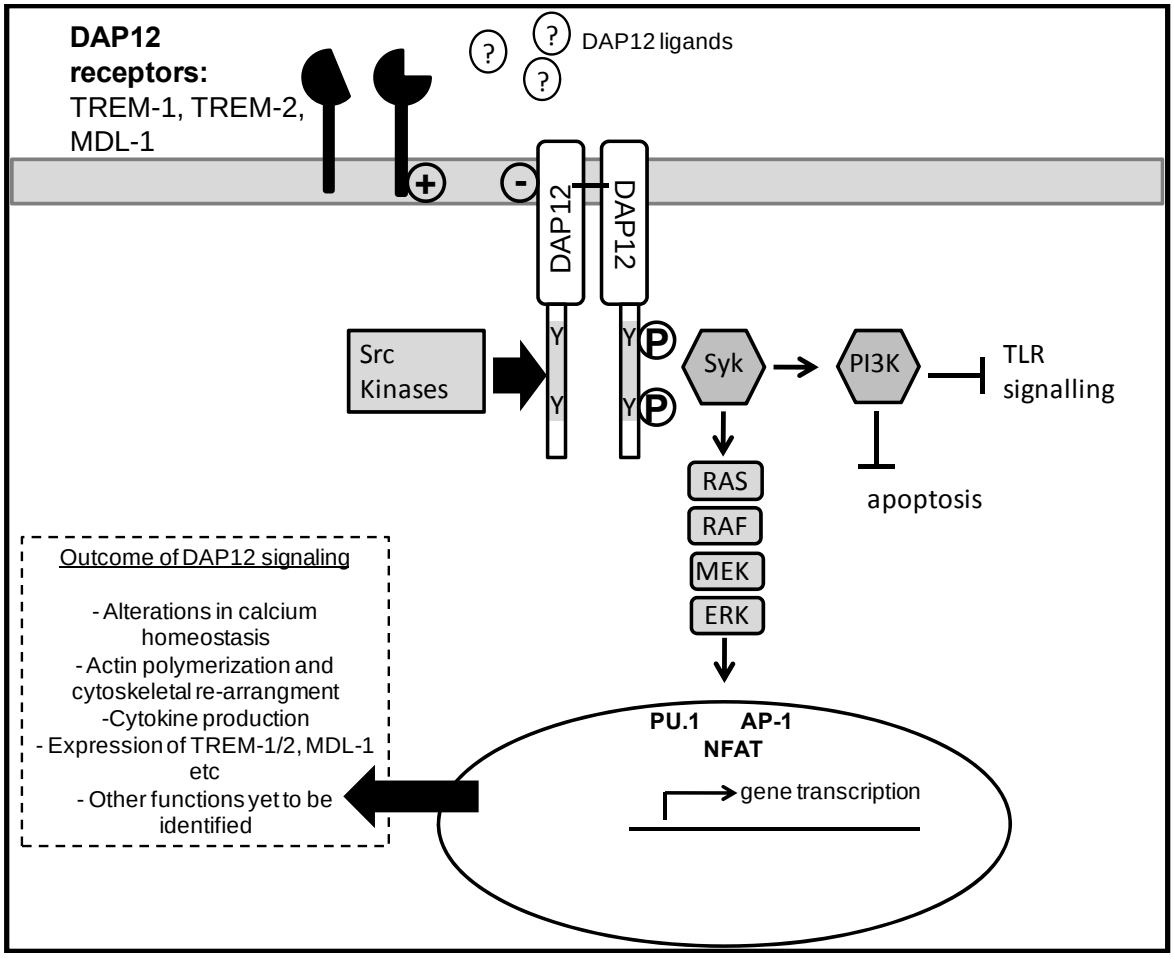


Figure 5. Simplified DAP12 signaling pathway. DAP12 forms a homodimers in the plasma membrane and non-covalently associated with cell surface receptors. The ITAM motifs of DAP12 become phosphorylated by Src kinase leading to recruitment of Syk family of kinases. DAP12 signaling results in nuclear translocation of a number of transcription factors. DAP12 signaling inhibits TLR singling and apoptosis through PI3 kinase. Created by S. McCormick, 2011.

1.5 Thesis Objectives

T cells are primed in the LN by professional APCs, most often DCs, leading to expansion of antigen specific effector T cells which disseminate throughout the body and into tissues in order to control and eliminate pathogens. The APC - T cell interaction is complex and has a profound impact on the kinetics, amplitude and effector function of the T cell response. A great deal of research has gone into understanding the basic mechanics of the APC – T cell interaction, however, host factors that may enhance T cell priming are less well defined. The aim of this thesis was to examine the biological consequences of enhanced T cell responses during primary *M.tb* and influenza infection.

Rapid T cell priming following pulmonary infection is essential for pathogen control and ultimately survival. In TB, T cell priming is delayed leading to impaired bacterial control. In Chapter 2, the outstanding issues surrounding the biological consequences of delayed T cell priming were addressed. We hypothesized that earlier antigen presentation and accelerated T cell priming would improve host defense against primary *M.tb* infection. Using a model of primary *M.tb* infection in DAP deficient and wt mice we found that enhanced T cell responses lead to enhanced bacterial control. Furthermore, we found that accelerated T cell priming was due to enhanced APC maturation and antigen presentation in the local draining LN.

It is well known that adaptive CD8 T cell responses are important for host defense against influenza virus infection. In Chapter 3, the biological consequence of enhanced anti-influenza CD4 T cell activation was examined. Using a model of pulmonary influenza infection in DAP12 deficient and wt mice, we found enhanced CD4 T cell responses in DAP12 deficient hosts were associated with lethal lung immunopathology. Pathogenic CD4 T cell responses were a result of altered T cell effector functions in T cells primed by DAP12 deficient APCs.

APCs are critical for priming T cell responses and improving the efficacy of vaccines against *M.tb* and influenza infection has largely focused on enhancing APCs maturation and antigen presentation. Recently, ex vivo manipulation of APCs for the purpose of generating a vaccine has garnered a great deal of interest. Indeed, DC based vaccines have been used to prime T cell responses in cancer immunotherapy, although the applicability of this vaccine formulation had not been examined in eliciting protective immune responses at the lung mucosa. In Chapter 4, the ability of ex vivo manipulated DCs to prime protective T cell responses against TB was evaluated. This study shows that enhancing the level of APC maturation leads to enhanced T cell priming and protective immune responses in the lung. These findings provide evidence that DCs can be manipulated to safely prime T cell responses priming at the lung mucosa.

In Chapter 5, the implications of these findings are summarized and discussed along with future directions.

Chapter 2: Accelerated T cell priming leads to enhanced protection against pulmonary *Mycobacterium tuberculosis* infection in DAP12 deficient hosts.

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Accelerated T cell priming leads to enhanced protection against pulmonary *Mycobacterium tuberculosis* infection in DAP12 deficient hosts.

The timing of T cell priming has profound consequences on host resistance to infection Delayed T cell priming in humans and mice following pulmonary mycobacterial infection is hypothesized to lead to impaired bacterial control and chronic tuberculosis. Here, using a model of pulmonary *M.tb* infection in DAP12 deficient and wt mice, we found that accelerated T cell priming leads to enhanced bacterial control in DAP12 deficient hosts. The accelerated T cell priming can be attributed to earlier and enhanced antigen presentation by DAP12 deficient APCs in the draining LN. Furthermore, APCs from DAP12 deficient mice are more activated, secrete higher levels of immune activating cytokines, express higher levels of T cell co-stimulatory molecules leading to enhanced T cell priming. These findings indicate enhanced T cell priming improved bacterial control and DAP12 has a profound regulatory effect on the kinetics of T cell priming by controlling the level of APC maturation during *M.tb* infection.

I designed, executed all the experiments, analyzed and interpreted the data. I generated all figured and wrote the manuscript. Dr. Zhou Xing established the foundation for the project and provided general guidance and supervision. Christopher (Ryan) Shaler provided invaluable technical assistance and scientific discussion. Carly Horvath and Mangalakumari (Mathy) Jeyanathan executed the virulent *M.tb* challenge in the Level III biocontainment facility. Daniela Damjanovic provided technical support by running the Luminex assay. A special thanks to Anna Zganiacz for all her support and work in Level III.

Accelerated T cell priming leads to enhanced protection against pulmonary *Mycobacterium tuberculosis* infection in DAP12 deficient hosts.

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Running title: Accelerated T cell priming in DAP12 KO mice following tuberculosis infection

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Abstract

Delayed T cell priming and recruitment to the lung during pulmonary *M.tb* infection is hypothesized to lead to impaired bacterial control and chronic infection. Delayed T cell priming is hypothesized to be due to impaired antigen presentation. We observed that DAP12 deficient mice are better able to control virulent *M.tb* challenge than wt mice. Enhanced bacterial control in DAP12 deficient mice was associated with accelerated T cell priming in the LNs of DAP12 deficient mice and subsequently enhanced recruitment of T cells to the infected lung days sooner than in wt control mice. Such accelerated T cell priming in DAP12 deficient mice was due to enhanced antigen presentation by CD11c+ antigen presenting cells in the draining LN 5 days post infection. Furthermore we show that DAP12 deficient lung derived antigen presenting cells are highly activated in response to *M.tb* infection, leading to enhanced antigen presentation. Here we provide evidence that earlier T cell responses improve bacterial control and enhanced activation of pulmonary antigen presenting cells contribute to improved host resistance to TB infection.

Introduction

Mycobacterium tuberculosis (*M.tb*), the causative agent of tuberculosis (TB), infects one third of the world population causing 2 million deaths annually. *M.tb* primarily infects antigen presenting cells (APCs) in the alveolar space, most often the alveolar macrophage (AM), where it evades innate host defence mechanisms in order to establish chronic intracellular infection. Mycobacteria modulate the host immune response by stimulating the production of immunoregulatory cytokine IL-10 which inhibits effector immune responses and promotes a most hospitable growing environment for the bacteria (1). Priming adaptive immune responses and robust recruitment of effector CD4 T cells to help activate infected macrophages is crucial for controlling bacterial dissemination and replication. Recruitment of activated T cells to the lungs of mycobacterially infected animals is delayed until 15-20 days post infection (2-4), compared with T cell recruitment during viral infection or other intracellular bacterial infection such as *Listeria monocytogenes* where T cells appear 4-9 days post infection (5-8). Delayed T cell responses are believed to allow unchecked bacterial replication and dissemination of bacteria throughout the body. Indeed there is an inverse relationship between susceptibility to *M.tb* infection and the Th1 immune response (9,10). We and others have reported that Balb/c and C3H/HeJ mice are more susceptible to mycobacterial infection than C57Bl/6 mice as a result of lower Th1 responses (11-13). These findings lead to the hypothesis that accelerating T cell priming will result in earlier recruitment of T cell to the lung and enhanced bacterial control, however, this hypothesis has yet to be adequately investigated.

Delayed effector T cell response following *M.tb* infection is a result of delayed T cell priming in local draining lymph node (LN) (2, 4, 14). It is widely believed that live bacteria are necessary to prime T cell responses in the LN but priming does not occur until 11 days post infection (3-4, 15). Wolf et al. went on to show that DCs were highly susceptible to *M.tb* infection and upon infection had an impaired ability to migrate to the local draining LN in order to present antigen to T cells (16). Taken together these findings lead to current belief that pulmonary DCs acquire *M.tb* bacilli in the lung and upon infection, migrate to the draining LN where they prime T cell responses, however, this process is delayed due to the immunomodulatory properties of *M.tb*. It is hypothesized that this delayed antigen presentation by professional APCs is the cause of the delayed effector T cell responses.

DNAX activating protein of 12kDa (DAP12) is a signaling adaptor molecule containing 2 cytoplasmic ITAM signaling motifs expressed by innate immune cells including macrophages, DCs, neutrophils and NK cells (17-20). A number of receptors associate with DAP12 including triggering receptor expressed on myeloid cells (TREM)-1, 2, myeloid DAP12 associating lectin (MDL)-1 on myeloid cells and activating receptors (NKp44, NKp46 and NKG2D) on NK cells (17-22). In APCs, DAP12 signaling limits the amplitude pro-inflammatory cytokine released in response to pathogen associated molecular patterns (PAMPs) by regulating the level Erk phosphorylation (23,24). We and others have shown DAP12 has profound regulatory effects on adaptive immune responses by negatively regulating DC activation and subsequent T cell activation (25,26). We have previously shown that DAP12 deficient mice mount a more robust T cell response to

pulmonary *Mycobacterium bovis* infection due to enhanced T cell priming by DAP12 deficient DCs (25). In this study, we aimed to determine if the increased T cell responses observed in DAP12 deficient mice were due to accelerated T cell priming by APCs in the local draining LN and elucidate the role of accelerated T cell priming host defense against *M.tb* infection. We provide evidence that T cell priming is accelerated in *M.tb* infected DAP12 deficient mice and accelerated recruitment of effector CD4 T cell responses into the lung leads to enhanced bacterial control. Furthermore we provide evidence that heightened activation of DAP12 deficient APCs leads to enhanced antigen presentation in the draining LNs days sooner than in wt controls.

Materials and Methods

Mice

DAP12-gene knockout (DAP12KO) mice were fully backcrossed to the C57BL/6 genetic background and the breeding colony is maintained at McMaster University. Age and sex matched wild type (wt) C57BL/6 control mice were purchased from Harlan. Transgenic OT-I and OT-II mice were purchased from Taconic (27-29). Age and sex matched Balb/c mice were purchased from Harlan. 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.

Pulmonary mycobacterial infection

Mycobacterium tuberculosis H37Ra (ATCC 25177; *M.tb*) was provided by Dr. Marcel Behr and grown as previously described (30). Immediately prior to infection, frozen mycobacteria were washed with PBS containing 0.05% Tween 80 once followed by 2 PBS washes and passed through a 27 gauge needle 10 times to disperse clumps. Infection was carried out by modified intratracheal infection with $0.5x10^6$ CFU. Briefly, mice were anaesthetized and suspended by the front teeth from a suspension wire; the tongue was pulled out and to the side of the mouth using sterile forceps in order to open up the trachea and 40µl of PBS containing bacilli instilled at the back of the throat. Mice were allowed to inhale 5 times before being removed from the apparatus and allowed to recover. 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 OADC enrichment. Plates were incubated at 37°C for 17 days in semisealed plastic bags. Colonies were counted, calculated, and presented as $\log_{10}$ CFU per organ.

Challenge with virulent *M.tb* H37Rv (ATCC 27294) was carried out in the level III bio-containment facility at McMaster University. Bacteria were prepared as described above and 10 000 CFU delivered intranasally to naive mice. The level of bacterial burden in the lungs and spleens of mice at 2, 4 and 8 weeks post challenge was determined by plating serial dilutions of tissue homogenates as described above. Plates were incubated at 37°C for 17 days in semisealed plastic bags. Colonies were counted, calculated, and presented as $\log_{10}$ CFU per organ.

Lung pathology and mononuclear cell isolation for evaluation of immune activation

At 2 and 4 weeks following virulent *M.tb* challenge, mice were sacrificed and fixed in 10% formalin, sectioned and H&E stained. Lungs and mediastinal lymph nodes (LN) were removed aseptically and the intra-airway luminal cells were removed from the lung by exhaustive bronchoalveolar lavage (BAL) as previously described (31). Supernatants from BAL samples were collected and stored at –20°C. Lungs were processed to single cell suspension by collagenase digestion as previously described (31). All cells were cultured in RPMI 1640 supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin and streptomycin.

Analysis of cytokines and chemokines in BAL fluid and cell culture supernatants

Cytokine or chemokine contents in BALF or cell culture supernatants were measured by Quantikine ELISA (R&D Systems, Minneapolis, Minnesota) or by Luminex (Medicorp, Montreal, Quebec) according to the manufacturer's protocol.

Intracellular cytokine staining and flow cytometric analysis

Freshly isolated cells were cultured in vitro in a U-bottom 96 well plate and stimulated with *M.tb* culture filtrate (10ug/ml) and UV inactivated *Mycobacterium bovis* Bacille Calemette-Guerin (BCG) for 18 hours at 37°C 5% CO_2. Golgi–plug (BD Biosciences) was added for the final 6 hours. Cells were immunostained with CD3-PerCpCy5.5, CD4-PECy7, CD8α-APCCy7 and IFNγ-APC (all antibodies purchased from BD Pharmingen, Mississauga, Ontario). Stained cells were then run on a FACS Canto and analyzed on FlowJo software (version 6.3.4; Tree Star Inc).

In vitro analysis of antigen presenting cells

Naive lung antigen presenting cells (APCs) from wt and DAP12 mice were isolated by positive selection of CD11c+ cells using either MACS column purification kit (Miltenyi Biotec Inc, Auburn, California) or the EasySep separation kit (STEMCELL Technologies, Vancouver British Columbia) according to the manufacturers protocol. Cell purity was determined to be greater than 90% by flow cytometry. For APC characterization, cells were infected or stimulated in vitro with *M.tb* H37Ra (MOI = 5 CFU) or LPS (1ug/ml) for 48 hours. Cell culture supernatants were harvested for cytokine assay or the cells were immunostained for cell surface markers including CD11c-APC, CD11b-PECy7, IA^b-Biotin and CD86-Alexa 700 (all antibodies purchased from BD Pharmingen). Stained cells were then run on a LSRII and analyzed on FlowJo software (version 6.3.4; Tree Star Inc).

Ex vivo quantification of antigen presentation by lung and LN derived APCs

In order to evaluate the extent of antigen presentation by APCs during pulmonary mycobacterial infection, chicken ovalubumin (OVA; 2.5ug) was administered alone or co-administered with *M.tb* intratracheally to wt and DAP12KO mice. At various time points following infection, mice were sacrificed and lung or LN CD11c+ cells were isolated as described above and co-cultured in a ratio of 1:5 with CFSE labelled OT-II or OT-I cells purified by CD4 positive or CD8 positive MACS selection or (Miltenyi Biotec Inc, Auburn, California) according to the manufacturer's protocol. Cells were cultured at 37°C 5% CO_2 for 72 hours or 48 hours and then immunostained for CD3-PerCpCy5.5, CD4-PECy7, CD8α-APC and CFSE dilution in T cells analyzed by flow cytometry. A mixed lymphocyte reaction was set up at the same time as transgenic OT proliferation

experiments. In this case, CD11c+ cells were co-cultured in a ratio of 1:5 with CFSE labelled Balb/c T cells purified by negative Pan T cell isolation (Miltenyi Biotec Inc, Auburn, California). Purified lung and LN derived CD11c+ cells were phenotyped at the time of antigen presentation assays by immunostaining for cell surface markers including CD11c-APC, CD11b-PECy7, IA^b-Biotin and CD86-Alexa 700. Stained cells were run on the LSRII and their phenotype analyzed using FlowJo software. In separate experiments, the level of *M.tb* antigen present in lung and LN CD11c+ cells was measured by ex vivo co-culture with BB7 hybridoma cells. The CD4 BB7 T cell hybridoma was kindly provided by Drs. Henry Boom and Clifford Harding (Case Western Reserve University, Cleveland, Ohio). BB7 cells recognize *M.tb* Ag85B (241-256) when bound to IA^b and produce IL-2 in response to antigen stimulation (32-33). LN and lung derived CD11c+ cells were isolated as described above and co-cultured with BB7 cells grown to log-phase at a ratio of 1 APC: 2 T cells for 23 hours and sups harvested and stored at -20°C until cytokine measurement could be carried out. IL-2 levels were measured by ELISA (R&D Systems).

Confocal microscopy

Naive lung derived CD11c+ cells were purified as described above and cultured at 37°C for 1 hour. Cells were pulsed with *M.tb* H37Ra and at various time points following infection, cells were harvested, washed with PBS and a cytospin centrifuge was used to mount cells onto a slide for staining. Cells were fixed in 2% paraformaldehyde for 1 hour and blocked in (5% normal goat serum, 5% BSA, 0.1% Triton-X) for 2 hours to prevent non-specific staining. Cells were washed with 5% Tween 20 PBS and probed with an

NFκB primary antibody (p65 rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, California) at room temperature for 2 hours. Cells were washed and stained with a secondary antibody (AlexaFluor 488 conjugated goat anti-rabbit, Molecular Probes, Mississauga, Ontario) at room temperature for 1 hour. Cells were washed and counterstained with propidium iodide (Molecular Probes, Mississauga, Ontario) for 5 minutes before being coverslipped with Vectashield (Vector Laboratories, Burlingame, California) hard mounting media. The extent of NFκB nuclear translocation was visualized using the Carl Ziess LSM 510 confocal microscope and images were analyzed using LSM510 image software.

Results

Enhanced control of virulent M.tb in DAP12 deficient lungs

We previously showed that DAP12 deficient animals were better able to control pulmonary infection with attenuated *Mycobacterium bovis* Bacille Calmette Guerin infection (25). To determine if KO mice were similarly better able to control virulent *M.tb* infection, DAP12 deficient and wt animals were challenged with virulent *M.tb* H37Rv. Both strains of mice remained healthy and steadily gained weight over the course of infection although DAP12 deficient mice gained significantly more weight than wt controls (data not shown). Bacterial burden in the lungs and spleens of DAP12 deficient and wt mice was similar 2 weeks post infection (Figure 1A), and significantly lower in DAP12 deficient lungs 4 weeks post infection (Figure 1B). It is important to note that the level of bacterial burden in the lungs of DAP12 deficient mice declined between 2 and 4

weeks (1 log reduction) whereas the bacterial burden in the wt mice did not change. Bacterial burden in the spleens of DAP12 deficient animals was consistently lower compared to wt animals, although the level of bacterial burden did not change over the 4 week infection. Histopathological examination of lung sections 2 weeks post infection revealed wt mouse lungs had only mild inflammation, with small patchy areas of monocytic infiltration (Figure 1C). DAP12 deficient lungs had more diffuse inflammation and clusters of lymphocytic aggregates associated with the large conducting airways and adjacent blood vessels (Figure 1C). The inflammation in the parenchyma in wt lungs intensified between 2 and 4 weeks and numerous granulomas were found throughout the lungs (Figure 1D). In sharp contrast, no granuloma could be found in the DAP12 deficient mouse lung at 4 weeks post infection. Instead, there was diffuse inflammation throughout lung parenchyma which was predominantly lymphocytes and neutrophils (Figure 1D).

Kinetics of bacterial control in DAP12 deficient and wt mice

Having observed enhanced bacterial control in the DAP12 deficient lungs 4 weeks post infection but not earlier, we sought to better understand how DAP12 deficient mice managed to better control bacterial replication by mapping bacterial burden during the first month following infection. Furthermore, we needed to establish an infection protocol that would allow us to carry out immune analysis outside of the Level 3 biocontainment facility. To this end, DAP12 deficient and wt control mice were challenged with *M.tb* H37Ra, a strain of *M.tb* that has been attenuated through the silencing of the RD1 region of the genome which contains numerous virulence factors (34). At 3, 7, 10, 14, 21 and 28

days post infection the bacterial burden in the lung and spleen was enumerated by colony assay. In line with our observations with virulent *M.tb*, bacterial titres were similar in DAP12 deficient and wt mouse lungs between 1 day (data not shown) and 21 days post infection (Figure 2A). In both strains of mice, bacterial titres rose steadily for the first 21 days suggesting bacterial replication was unchecked by the host immune response. At 28 days post infection bacterial burden in the lungs of DAP12 deficient mice was significantly lower than in wt mice. This difference was a result of DAP12 deficient mice halting bacterial replication in contrast to wt mice where bacteria continued to grow between 21 and 28 days post infection. Bacterial dissemination to the spleen occurred at the same time in both mouse strains, with bacteria first appearing in the spleen at 7 days post infection (Figure 2B). Systemic bacterial burden in the spleen mirrored that in the lung, with similar bacterial titres between DAP12 deficient and wt mice up to 21 days post infection and enhanced bacterial control in DAP12 deficient spleens 28 days post infection (Figure 2B). Taken together these data indicate DAP12 deficient mice are able to arrest bacterial replication between 21 and 28 days post infection whereas wt mice cannot effectively control bacterial replication. In addition, we found that infection with attenuated *M.tb* indeed recapitulates the observations made using the virulent *M.tb* strains. The similar kinetics of infection between the virulent and attenuated *M.tb* strains provides us with a suitable model to examine the immune response in DAP12 deficit hosts.

Enhanced inflammation in the airways of DAP12 deficient hosts

To begin investigating the mechanisms leading to enhanced bacterial control in DAP12 deficient mice, we examined the inflammation in the airways of DAP12 deficient and wt controls. To this end, we examined the composition of the inflammatory infiltrates in the airway at 3, 7, 10, 14 and 21 days post infection. Three days following infection, DAP12 deficient mice had markedly more total cells in the airway lumen than wt controls (Figure 3A). The total number of cell in the airway lumen contracted slightly between 3 and 7 days post infection and then steadily increased over the course of infection. Morphological analysis of the cells recovered from the airway revealed significantly increased number of monocytes in the airway of DAP12 deficient mice 3 days post infection (Figure 3B). By 14 days post infection the inflammation in the airways of DAP12 deficient mice became predominantly neutrophilic, accounting for 40-60% of cellular infiltrates between 7 and 21 days post infection (Figure 3C). In contrast, the inflammation in wt mouse airway was predominantly monocytic, accounting for 60-90% of infiltrating cells between 7 and 21 days post infection. Only very low numbers of lymphocytes were observed in the airway lumens of DAP12 deficient and wt mice prior to 10 days post infection and increased dramatically between 10 and 21 days post infection (Figure 3D). We found that the overall level of inflammation in the airway of DAP12 deficient and wt mice was similar following *M.tb* infection, although neutrophils predominated the inflammation in DAP12 deficient mice.

Accelerated T cell priming in the LNs of DAP12KO mice

It has been reported that T cell priming is delayed during *M.tb* infection leading to delayed recruitment of effector T cells and unchecked bacterial replication for the first 20 days (2). CD4 Th1 cells are especially important in host resistance to infection due to their ability to activate macrophages to kill intracellular bacteria through the secretion of potent activating cytokines IFN-γ and TNF-α. Having previously examined only T cell responses at 14 days post infection and beyond (25) we sought to determine if the increased T cell numbers in DAP12 deficient mice was due to accelerated T cell priming and effector T cell recruitment. To assess the kinetics of the T cell priming, LNs from DAP12 deficient and wt mice were isolated 3, 7, 10, 14 and 21 days post infection and the total number of T cells as well as the number of antigen specific T cells secreting IFN-γ was quantified by ICCS. The total number of CD4 T cells (Figure 4A) and antigen specific IFN-γ secreting T cells (Figure 4B) in the LN of DAP12 deficient mice expanded markedly between 3 and 7 days post infection. In contrast, CD4 T cell responses remained very low in the LNs of wt mice prior to 14 days post infection. These findings indicate CD4 T cell priming occurs approximately 7 days sooner in DAP12 deficient hosts than in wt controls. Of note, we observed markedly more antigen specific CD4 T cells in DAP12 deficient mice compared to wt controls which are in line with our previous observations. Having demonstrated that T cell priming does indeed occur earlier in DAP12 deficient mice, we then sought to determine if primed T cells are also recruited earlier to the infected lung. To this end, we quantified the CD4 T cell response in the lung and airway lumen. CD4 T cells began accumulating in the DAP12 deficient lung tissue 7 days post infection (Figure 4C and D) and in the airway between 14 and 21 days post

infection (Figure 4E and F). Here we show that CD4 T cell priming occurs 7 days sooner in DAP12 deficient LNs and reaches a higher magnitude than wt controls. *M.tb* specific CD4 T cells appear in the lungs of DAP12 deficient mice 7 days post infection, 7 days earlier than in wt lungs. In addition to the early arrival of CD4 T cells, both the frequency and magnitude of the IFN-γ CD4 T cell responses in DAP12 deficient mice was strikingly elevated over wt controls.

Selectively elevated chemokines in the DAP12 deficient airway

In light of the increased protection against *M.tb* infection observed in DAP12 deficient mice, we aimed to determine if increased chemokine production in the airway could contribute to increased recruitment of immune cells to the lung. To this end, we measured the level of chemokines present in the BAL fluid 3, 7, 10, 14 and 21 days post infection. We found the levels of macrophage/DC recruiting chemokines MIP-1α (Figure 5A), MIP-1β (data not shown) and MCP-1 (Figure 5B) remained only slightly above naive controls, in both strains of mice until 14 days post infection at which point we found markedly elevated chemokine levels in DAP12 deficient hosts. The level of neutrophil chemotactic factor KC (Figure 4C) was strikingly elevated in DAP12 deficient hosts throughout infection. In contrast, neutrophil chemokine MIP-2 levels remained low and were similar between mouse strains throughout infection (Figure 5D), suggesting the increased neutrophil responses observed in DAP12 deficient host can be attributed to enhanced KC chemokine response in DAP12 deficient hosts. Having observed heightened T cell responses in DAP12 deficient mice we examined the levels of several T cell recruiting

chemokines. T cell recruiting chemokines remained low prior to 10 days post infection. IP-10 (Figure 5E), MIG (Figure 5F) and RANTES (Figure 5G) were elevated in DAP12 deficient hosts became highly elevated over wt controls at 14 and 21 days post infection. Together these findings indicate heightened chemokine responses in DAP12 deficient host contribute to enhanced inflammation and host resistance to *M.tb* infection.

Enhanced T cell effector cytokine responses in DAP12 deficient hosts

Cytokine production in response to *M.tb* antigen in the tissues is required for macrophage activation and elimination of intracellular bacteria. To determine if the increased CD4 T cell recruitment in the DAP12 deficient lungs and airways translated into increased cytokine production at the site of infection, we quantified the amount of cytokine in the supernatants of LN cell cultures and the BAL fluid. In line with our ICCS data, no IFN-γ could be detected in the LN supernatants prior to 7 days post infection (Figure 6A). DAP12 deficient LN cells released staggering amounts of IFN-γ beginning at 7 days post infection. Wt cells in contrast, produced only very low levels of IFN-γ at all time points examined. Cytokine analysis from BAL fluids revealed similar amounts of IFN-γ produced by lung derived cells from DAP12 deficient and wt mice 3 through 10 days post infection (Figure 6B). Higher levels of IFN-γ were present in the DAP12 deficient lung cultures 14 and 21 days post infection, indicating the enhanced level of CD4 T cells recruited to the lung correlates with enhanced cytokine production. Since Th1 cells were so strikingly elevated in DAP12 deficient lungs we sought to determine if the increased CD4 T cells might also translate into heightened levels of other T cell cytokines. To this

end we measured the level pro-inflammatory cytokine TNF-α (Figure 7A), Th1 polarizing cytokine IL-12 (Figure 7B) and Th17 cytokine IL-17 (Figure 7C). In line with the IFN-γ data, there was enhanced TNF-α and IL-12 in the lungs of DAP12 deficient lungs 14 and 21 days post infection. IL-17 levels were significantly elevated in DAP12 deficient lungs 10 through 21 days post infection. In light of the universally enhanced level of Th1 cytokines, we asked if the enhanced CD4 T cell activation could be attributed to dysregulated CD4 T cell activation leading to both Th1 and Th2 polarized cells. To address this, we measured Th2 cytokines IL-4 (Figure 7D), IL-5 and IL-13 (data not shown). Similar levels of Th2 cytokines were observed in both DAP12 deficient and wt mice. Immunoregulatory cytokine IL-10 has important roles in not only controlling T cell effector functions but also the magnitude of the immune response. Increased IL-10 producing regulatory T cells leads to impaired bacterial control and unchecked bacterial replication (35). In this situation the enhanced Th1 activation could be attributed to decreased immunoregulatory cytokines in DAP12 deficient hosts. To rule out this possibility, we measured IL-10 in the BAL fluid and found no difference in the amount of IL-10 in DAP12 deficient and wt mice (Figure 7E). Together these findings suggest the CD4 T cell activation DAP12 deficient mice is a result of expansion of specific T helper subsets, specifically Th1 and Th17 subsets and is not a result of decreased immunoregulatory cytokines.

Increased antigen presentation in the draining LNs of DAP12 deficient hosts

Having observed earlier T cell priming in the draining LNs of DAP12 deficient mice, we hypothesized that accelerated T cell responses could be due to accelerated migration of APCs to the draining LNs. To begin to test our hypothesis, we carried out experiments using ovalbumin (OVA) specific transgenic T cells (OT) to assess the level of antigen in the LNs of DAP12KO and wt mice during *M.tb* infection. To this end, we treated mice with either soluble OVA alone or co-delivered with *M.tb* and 3, 5, 7 and 14 days later purified CD11c cells from the mediastinal LNs and co-cultured them with either purified transgenic OT cells or MHC mis-matched T cells (see schema Figure 8A). Phenotypic analysis of LN derived CD11c cells from mice treated with OVA alone revealed heightened MHC class II expression on DAP12 deficient cells compared to wt controls (Figure 8B), *M.tb* infection further increased MHC expression on both DAP12 deficient and wt APCs. Co-stimulatory molecule CD86 expression was only marginally increased on DAP12KO cells compared to wt at 7 days post infection and highly increased in *M.tb* infected DAP12 deficient APCs 14 days post infection (Figure 8C). In order to better determine if the increased MHC expression observed by flow cytometry would translate into greater T cell activation, we measured the ability of these cells to drive allogenic T cell proliferation. At 3 days post infection, only baseline levels of T cell proliferation was observed in both strains of mice (data not shown). At 5 and 7 days post infection, DAP12 deficient CD11c cells elicited greater CD4 T cell proliferation (Figure 8D) and CD8 T cell proliferation (Figure 8F) than wt controls. This level of proliferation was further enhanced by *M.tb* infection indicating DAP12 deficient APCs express higher levels of MHC expression by 5 days post infection and are superior T cell activators early during

infection. By 14 days post infection, the level of T cell proliferation had normalized between the mouse strains suggesting APCs in the LNs of wt mice do indeed upregulate MHC expression later than DAP12 deficient APCs. The level of OVA transported to the LNs was measured by assessing the level of OT-II and OT-I proliferation in response to purified CD11c cells from the LN. No antigen specific T cell proliferation could be detected in the LNs at 3 days in either strain of mice (data not shown). LN derived DAP12 deficient CD11c cells drove slightly greater OT-II proliferation at 5 and 7 days than wt CD11c cells and markedly enhanced OT-II proliferation 7 days post infection (Figure 8E). No CD8 OT-I proliferation could be detected 5 days post infection (Figure 8G). CD11c cells from DAP12 deficient mice drove greater OT-I proliferation at 7 and 14 days compared to wt controls. Taken together these data provide evidence that in DAP12 deficient hosts, activated APCs expressing higher levels of MHC class I and II arrive in the LN between 3 and 5 days post infection while the arrival of activated wt APCs arrive between 7 and 14 days post infection. Furthermore, DAP12 deficient APCs in the LN present antigen better than their wt counterparts.

We went on to confirm these findings using a transgenic CD4 T cell hybridoma that produces IL-2 in response to *M.tb* antigen 85B. DAP12 deficient LN derived CD11c APCs stimulated greater IL-2 production than wt CD11c APCs indicating they present more *M.tb* antigen 7 days post infection (Figure 9A). Similarly, DAP12 deficient lung derived CD11c APCs stimulated greater IL-2 production than wt counterparts indicating DAP12 deficient APCs are superior antigen presenters compared to wt (Figure 9B).

Taken together these data suggest that DAP12 deficient APCs arrive in the draining LN earlier than wt APCs.

Prolonged NF-κB nuclear translocation in DAP12 deficient APCs

We have previously reported that DAP12 deficient CD11c cells mount a heightened inflammatory response to TLR ligands as well as attenuated *M. bovis infection* (25) however, we have never examined the response to *M.tb* infection. First we sought to determine if DAP12 deficient APCs were similarly hyperactivated in response to *M.tb* infection in a dose dependent manner. CD11c cells from naive DAP12 deficient and wt mouse lungs were purified and treated with varying concentrations of *M.tb* for 48 hours. As expected, DAP12 deficient APCs produced greater amounts of TNF-α in response to low concentrations of *M.tb* than wt controls, with the greatest cytokine production in cells treated with *M.tb* at an MOI=5 (Figure 10A). MHC class II expression was only marginally increased in DAP12 deficient CD11c cells treated with *M.tb* at an MOI of 5 and 10 but not with an MOI of 1. These data indicated that CD11c cells treated with an MOI=5 were optimal for studying the effects of *M.tb* infection in vitro. In order to understand why DAP12 deficient APCs produce such an exaggerated inflammatory response to *M.tb* we examined the speed and duration of NF-κB translocation in purified lung APCs. CD11c cells were purified from the lungs of naive DAP12 deficient and wt mice and immunostained for NF-κB before being examined by confocal microscopy. NF-κB translocated to the nucleus within 30 min of *M.tb* exposure in both DAP12 deficient and wt APCs. Nuclear NF-κB could reliably be found in the nucleus of DAP12 deficient

APCs 6 hours following *M.tb* treatment, hours longer than in wt controls. These data provide some evidence that the enhanced activation observed in DAP12 deficient APCs is due to prolonged activity of NF-κB in the nucleus and suggests it may have a profound impact on gene transcription although this remains to be examined.

Discussion

Delayed T cell priming has been observed in both animal models as well as human infection and has a major impact on bacterial control and dissemination. In mouse models, adaptive immune responses are not observed in the lungs until 18-20 days following infection, allowing for bacterial expansion. It is believed that this delay puts the host at a disadvantage, rendering it incapable of clearing the infection. Here we provide for the first time direct evidence that DAP12 deficient mice are better able to control virulent *M.tb* infection. Based on our prior observations that DAP12 deficient hosts mount a heightened T cell response to the vaccine strain *M. bovis* 14 days post infection and APCs were highly activated in response to *M. bovis*, we wondered whether the enhanced T cell responses were actually due to accelerated T cell priming. We found that antigen specific CD4 T cells in the LNs of DAP12 deficient hosts expanded 7 days sooner than in wt controls, indicating T cell priming indeed occurred earlier in the absence of DAP12. Furthermore, recruitment of effector T cells to the lung and airway was also accelerated in DAP12 deficient hosts, provided evidence that earlier T cell priming leads to enhanced protection against *M.tb* challenge. In both strains of mice, the arrival of effector T cell responses precedes obvious decline in bacterial burden in the lungs by 7

days. This is in contrast to other intracellular infections such as influenza virus or *Listeria monocytogenes* where the appearance of effector adaptive immune responses was closely followed by pathogen control (Chapter 3) (5, 7-8). We do not yet understand why there is such a delay between the appearance of adaptive immune responses but may speculate that *M.tb* infected macrophages require a great deal of cytokine activation in order to overcome immune suppression imposed by the pathogen. While these data provide compelling evidence that accelerated T cell priming is the direct cause of enhanced bacterial control in DAP12 deficient mice, further experiments are necessary to definitely rule out the contribution of other immune components. To address these issues we plan to inhibit T cell priming in DAP12 deficient mice prior to 10 days post infection using a CTLA-4 fusion antibody, thus normalizing the timing of T cell recruitment to the lung, and examining the ability of DAP12 deficient and wt mice to control bacterial burden. A complementary approach will be to block the recruitment of effector T cells to the lung in DAP12 deficient and wt mice prior to 14 days using lymphocyte inhibitor FTY720 and assess the ability of these mice to control bacterial burden. Because DAP12 deficient mice mount heightened CD4 T cell responses which may enhance bacterial control, we plan to adoptively transfer a fixed number of effector T cells from DAP12 deficient or wt mice to the lungs of immune-deficient (Rag2KO) mice prior to challenge, 7 days post challenge and 14 days post challenge and then compare the bacterial load in the lungs and spleens of these mice. These experiments will allow us to definitively prove that earlier recruitment of activated CD4 T cells is directly responsible for enhanced bacterial control.

In addition to the enhanced IFN-γ production in DAP12 deficient mice, we observed enhanced IL-17 cytokine levels in the BAL fluid. The contribution of IL-17 in host defence against *M.tb* is not well defined although IL-17 has been implicated in granuloma formation and in some cases controlling bacterial replication (36). Still others report IL-17 plays a pathogenic role in *M.tb* infection leading to lung pathology (37). In this study, it is not clear if the increased IL-17 is a result of increased γδ T cells or CD4 Th17 responses although based on the markedly increased CD4 T cell numbers in the lungs and airways of DAP12 deficient mice we may speculate that Th17 responses are elevated. It is not yet known if the IFN-γ positive cells are also positive for IL-17. It has been reported that DAP12 deficient mice have decreased regulatory T cell numbers (38), in light of the enhanced level of activated CD4 T cells, it is possible that there are decreased regulatory T cells in DAP12 deficient mice leading to unchecked T cell activation. Based on our observations in a similar model of influenza infection we find no major differences in the number of regulatory T cells in DAP12 deficient mice. Additionally, we have found similar levels of IL-10 in the lungs of *M.tb* infected DAP12 deficient mice, suggesting regulatory T cells are indeed functional, although this remains to be investigated.

DCs are professional antigen presenting cells specialized in T cell priming. During mycobacterial infection, there is a correlation between the amount of mycobacteria in the LN and the extent of T cell activation (2). Since mycobacteria are believed to be brought to the LN by APCs, as a result the more bacteria in the LN is thought to correlate with

greater APC migration. We aimed to determine if the accelerated T cell priming in the LN was due to earlier accumulation of antigen in the LNs of DAP12 deficient mice. Indeed APCs from DAP12 deficient LN triggered greater T cell proliferation at 5 and 7 days post infection than wt APCs. This observation, along with the findings that there were more CD11c APCs in the LNs of DAP12 deficient mice suggest that APCs in DAP12 deficient hosts migrate to the local draining LN sooner than their wt counterparts. These findings are being confirmed using other experimental approaches including using *M.tb* Ag85B transgenic T cell to measure Ag85B levels in LN derived CD11c cells and tracking the arrival of mycobacteria in the LN using green fluorescent protein tagged *M.tb*. In addition to earlier APC arrival, DAP12 deficient APCs are more activated in response to *M.tb* infection, producing enhanced levels of pro-inflammatory cytokines compared to wt APCs. We provide evidence that DAP12 deficient APCs also remain activated for longer than wt controls providing early evidence that DAP12 deficient DCs prime greater T cell responses by becoming overactivated in response to mycobacteria. Indeed over production of some pro-inflammatory cytokine such as IL-12p40 may even enhance the ability of DAP12 deficient DC to migrate to the draining LNs (31). Our data appear to confirm the hypothesis that accelerated T cell responses following *M.tb* infection enhance resistance to virulent *M.tb* infection and are a direct result of enhanced antigen presentation by APCs in the local draining LN.

We found that compared to wt mice, DAP12 deficient mice generate earlier and more pronounced innate immune responses in response to *M.tb* infection. Inflammation in the

DAP12 deficient lung was highly neutrophilic and did not result in granuloma formation. The increased neutrophil recruitment is likely caused by the enhanced production of the chemokine KC throughout infection and further amplified by IL-17 after 10 days post infection and may play a role in the production of other inflammatory chemokines responsible for the recruitment of conscripted immune cells. Recruitment of neutrophils is a normal consequence of any infection. The role of neutrophils in resistance to *M.tb* infection has been largely overlooked until a recent study suggested neutrophils may in fact play a significant role in innate defence susceptibility to mycobacterial infection (39). Indeed neutrophils have been reported to shuttle live mycobacteria to the draining LN faster than macrophages or DCs (40). This may have a significant impact in our model, where neutrophils predominate the lung inflammation and T cell responses are primed days sooner than expected. It is possible the increased neutrophil numbers accelerate the accumulation of mycobacteria in the draining LN leading to enhanced T cell priming. These findings highlight a previously unidentified role for neutrophils in host resistance against TB. DAP12 is expressed in neutrophils and reportedly amplifies inflammatory responses through TREM-1 signaling (41-44). The role of DAP12 in controlling neutrophil responses during *M.tb* infection merits further investigation.

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Figures Legends

Figure 1. Control of virulent *M.tb* in DAP12 deficient hosts. DAP12KO and wt mice were challenged i.n. with 10 000 CFU of virulent *M.tb* H37Rv. At 2 weeks (A) and 4weeks (B) post infection, bacterial burden in the lung and spleen was quantified by colony-forming assay. At 2 weeks (C) and 4 weeks (D) lungs of *M.tb* infected mice were formalin-fixed and underwent H&E staining for histological examination. Data are the mean ± SEM of 7-8 mice/group/time point. * $p<0.05$

Figure 2. Early bacterial control in DAP12 deficient mice. The kinetics of infection in DAP12KO and wt mice was assed following *M.tb* H37Ra infection. At various time points following infection the bacterial burden in the lungs (A) and spleens (B) was assessed by colony-forming assay. Data are mean ± SEM of 5-10 mice/group/time point from 1-2 independent experiments. * $p<0.05$

Figure 3. Increased neutrophilic inflammation in the lung of DAP12KO mice following pulmonary *M.tb* infection. At various time points following *M.tb* infection, DAP12KO and wt mice were subject to bronchoalveolar lavage and the total cell numbers (A), number of macrophages (B), neutrophils (C) and lymphocytes (D) in the airway were enumerated on cytospins by differential cell counting. Data are expressed as the mean ± SEM of 5–10 mice/group/time from 2 independent experiments. *$p<0.05$, **$p<0.01$, # $p<0.001$

Figure 4. Accelerated CD4 T cell priming and heightened T cell responses in DAP12KO mice. The kinetics of CD4 T cell priming was assessed following *M.tb* infection of DAP12KO and wt mice. At 3, 7, 10, 14 and 21 days post infection, airway lumenal cells were collected by BAL and LN as well as lung cells were processed to a single cell suspension. The expansion of CD4 T cells in the LN (A), lung (C) and airways (E) was measured by immunostaining and FACS analysis. At the same time points, the number of

CD4 IFN-γ secreting T cells was quantified by ICCS. LN cells (B), whole lung cells (D) and BAL cells (F) were stimulated ex vivo with *M.tb* culture filtrate and crude BCG for 18 hours followed by 6 hours in the presence of Golgi-plug. Cells were immunostained and analysed by flow cytometry. Data are mean ± SEM of 5-10 mice/group/time point from 2 independent experiments. * $p<0.5$, ** $p<0.001$, # $p<0.005$

Figure 5. Chemokine and cytokine responses in the BAL fluid of *M.tb* infected mice. At various time points following infection, lungs from *M.tb* infected mice underwent conventional lavage and BAL fluid stored at -20°C until analysis could be carried out. Cytokine responses were measured by multiplex cytokine assay for MIP-1α (A), MCP-1 (B), KC (C), MIP-2 (D), IP-10 (E), MIG (F) and RANTES (G) . Data are expressed as mean ± SEM of 3-5 mice per group per time point. * $p<0.5$, ** $p<0.001$

Figure 6. Enhanced IFN-γ responses in DAP12KO mice. At various time points following infection, airway lumenal cells were collected by BAL fluid from conventional lavage was collected and stored at -20°C until analysis could be carried out. LN cells were processed to a single cell suspension and cells stimulated with *M.tb* culture filtrate and crude BCG for 48 and then supernatants were harvested and stored at -20°C until analysis could be carried out. The amount of IFN-γ in the cell culture supernatants of LN derived cells (A) and in the BAL fluid (B) was measured my ELISA. Data are mean ± SEM of 4-5 mice/group/time point from1-2 independent experiments. * $p<0.05$

Figure 7. Heightened type 1 and Th17 cytokine responses in DAP12KO mice. At various time points following infection, lungs from *M.tb* infected mice underwent conventional lavage and BAL fluid stored at -20°C until analysis could be carried out. Cytokine responses were measured by multiplex cytokine assay for TNF-α (A), IL-12p40 (B), IL-17 (C), IL-4 (D) and IL-10 (E). Data are expressed as mean ± SEM of 4-5 mice per group per time point. * $p<0.5$, ** $p<0.001$

Figure 8. Heightened T cell proliferation driven by LN derived DAP12 deficient antigen presenting cells. The level T cell proliferation elicited by DAP12KO and wt APCs in the LN was assessed at various time points following delivery of OVA alone or with *M.tb* i.t (A). The activation status of freshly isolated CD11c+CD11b+ APCs in the LN was examined by processing LNs to a single cell suspension and immunostaining for MHC class II (B) and co-stimulatory molecule CD86 (C) for flow cytometric analysis. CD11c+ cells were isolated from LNs by MACS purification and co-cultured with CFSE labelled purified T cells from either MHC-mismatched (D and F) mice or OT-II (E) or OT-I (G) transgenic mice. The level of CFSE dilution was assessed by flow cytometry 96 hours (CD4 T cells) and 48 hours (CD8 T cells) later. Data are from 1 experiment.

Figure 9. Antigen presentation by LN and lung derived CD11c+ APCs**.** DAP12KO and wt mice were infected with *M.tb* i.t and 7 days later, LN (A) and lung (B) CD11c cells were purified by MACS. Purified CD11c cells were cultured alone or with BB7 cells for 24 hours. The level of IL-2 in the supernatant was measured by ELISA. Data are representative of 2 independent experiments.

Figure 10. Enhanced cytokine responses and prolonged NF-κB translocation in DAP12 deficient lung antigen presenting cells. CD11c cells were purified naive DAP12KO or wt lungs by MACS and cultured alone or in the presence of varying concentrations of live *M.tb* for 48 hours. The level of TNF-α in the cell supernatant was measured by ELISA (A). Data are mean ± SEM of cells plated in triplicate. The effect of *M.tb* on lung derived CD11c cells was assessed by measuring the surface expression of MHC class II (B) by flow cytometry following culture alone or with varying concentrations of live *M.tb* after 48 hours. NF-κB translocation was examined by confocal microscopy following in vitro culture of DAP12KO and wt lung derived CD11c cells with *M.tb* (H37Ra). Purified cells were stimulated for 0 minutes to 6 hours with *M.tb* (MOI=5) before being harvested and

cyto-centrifuged onto glass slides. Cells were fixed and immunostained before being scanned (C). Data are representative of 2 independent experiments.

Figures

Figure 1.

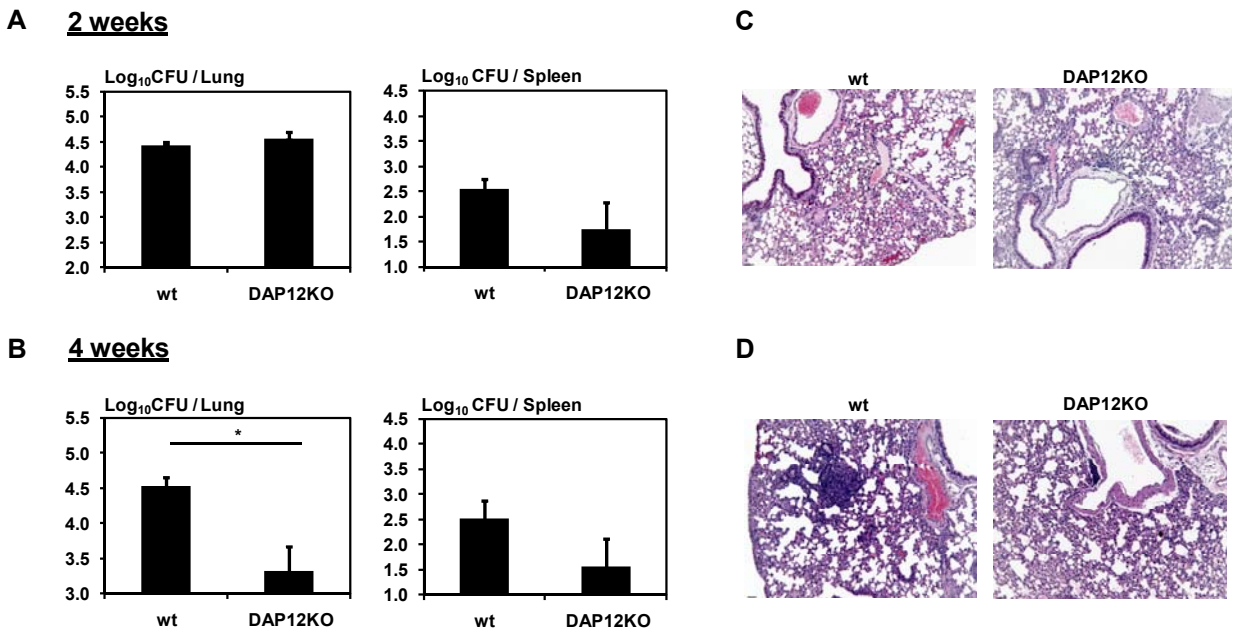


Figure 2.

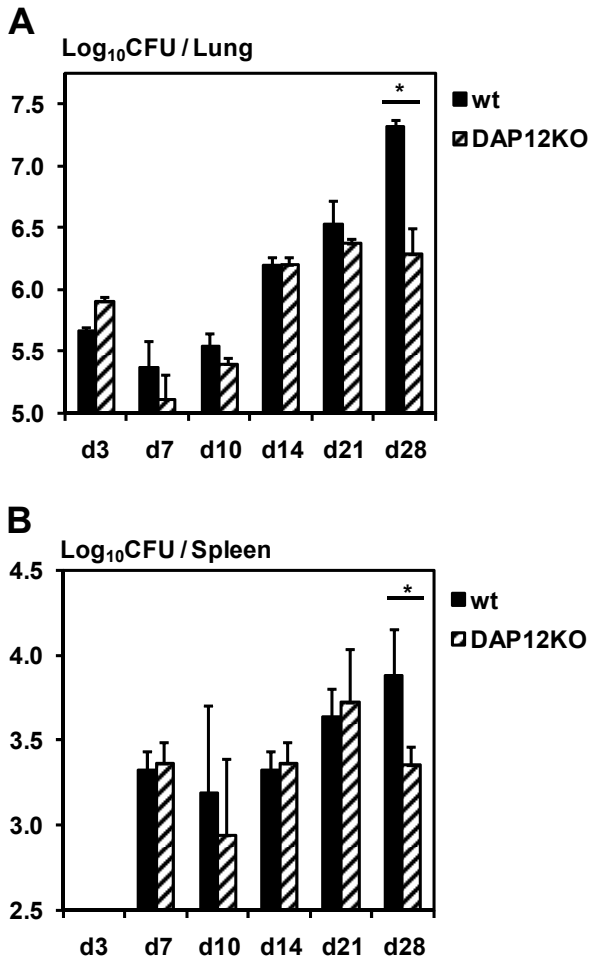

A
Log10CFU / Lung
7.5
7.0
6.5
6.0
5.5
5.0
*
wt
DAP12KO
d3
d7
d10
d14
d21
d28
B
Log10CFU / Spleen
4.5
4.0
3.5
3.0
2.5
*
wt
DAP12KO
d3
d7
d10
d14
d21
d28

Figure 3.

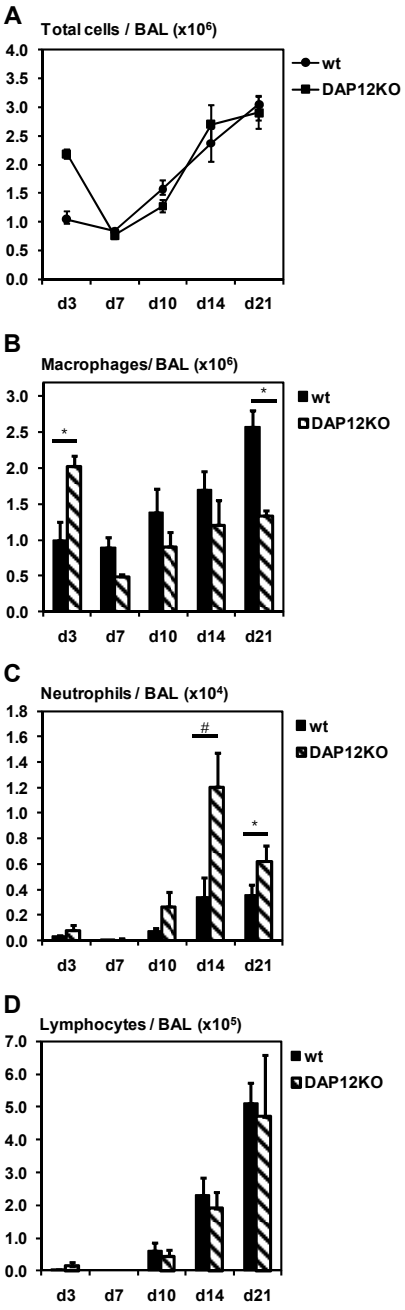

A
Total cells / BAL (x10⁶)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
d3 d7 d10 d14 d21
wt
DAP12KO
B
Macrophages/ BAL (x10⁶)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
d3 d7 d10 d14 d21
wt
DAP12KO
C
Neutrophils / BAL (x10⁴)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
#
*
d3 d7 d10 d14 d21
wt
DAP12KO
D
Lymphocytes / BAL (x10⁵)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
d3 d7 d10 d14 d21
wt
DAP12KO

Figure 4.

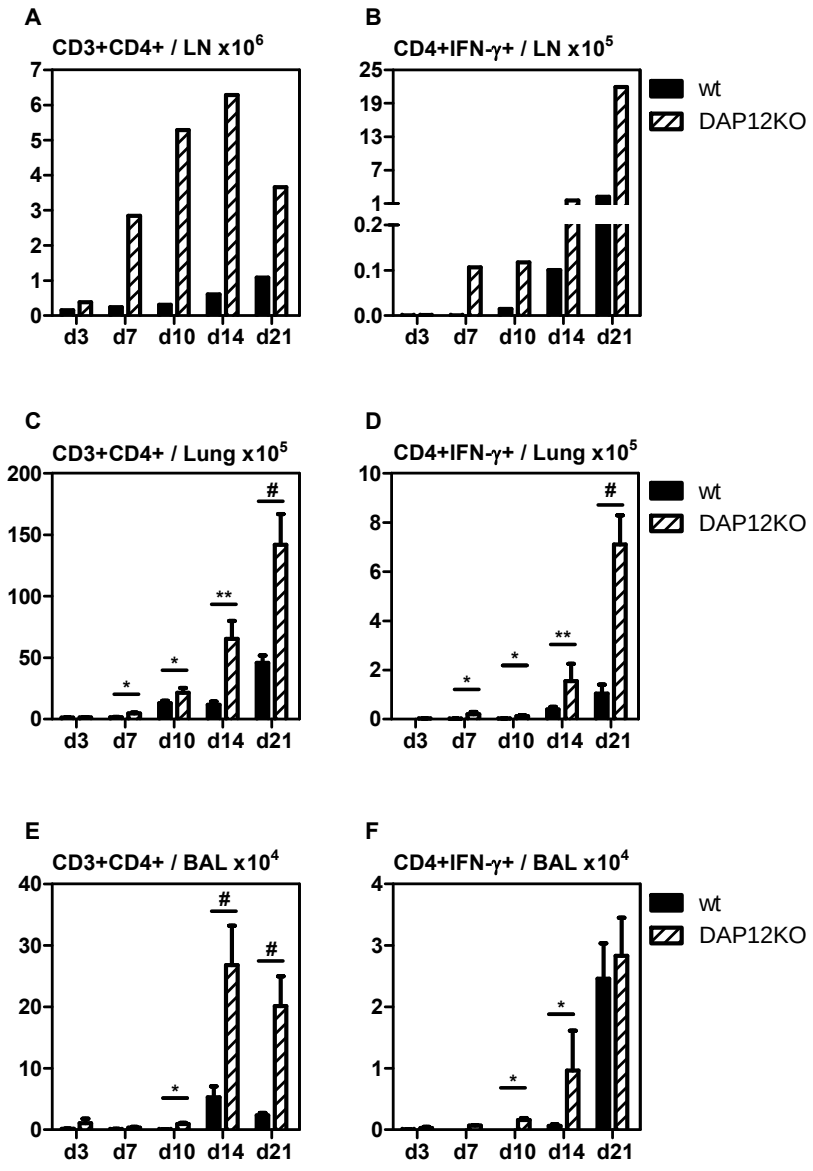

A
CD3+CD4+ / LN x10^6
B
CD4+IFN-γ+ / LN x10^5
wt
DAP12KO
C
CD3+CD4+ / Lung x10^5
D
CD4+IFN-γ+ / Lung x10^5
E
CD3+CD4+ / BAL x10^4
F
CD4+IFN-γ+ / BAL x10^4
d3 d7 d10 d14 d21

Figure 5.

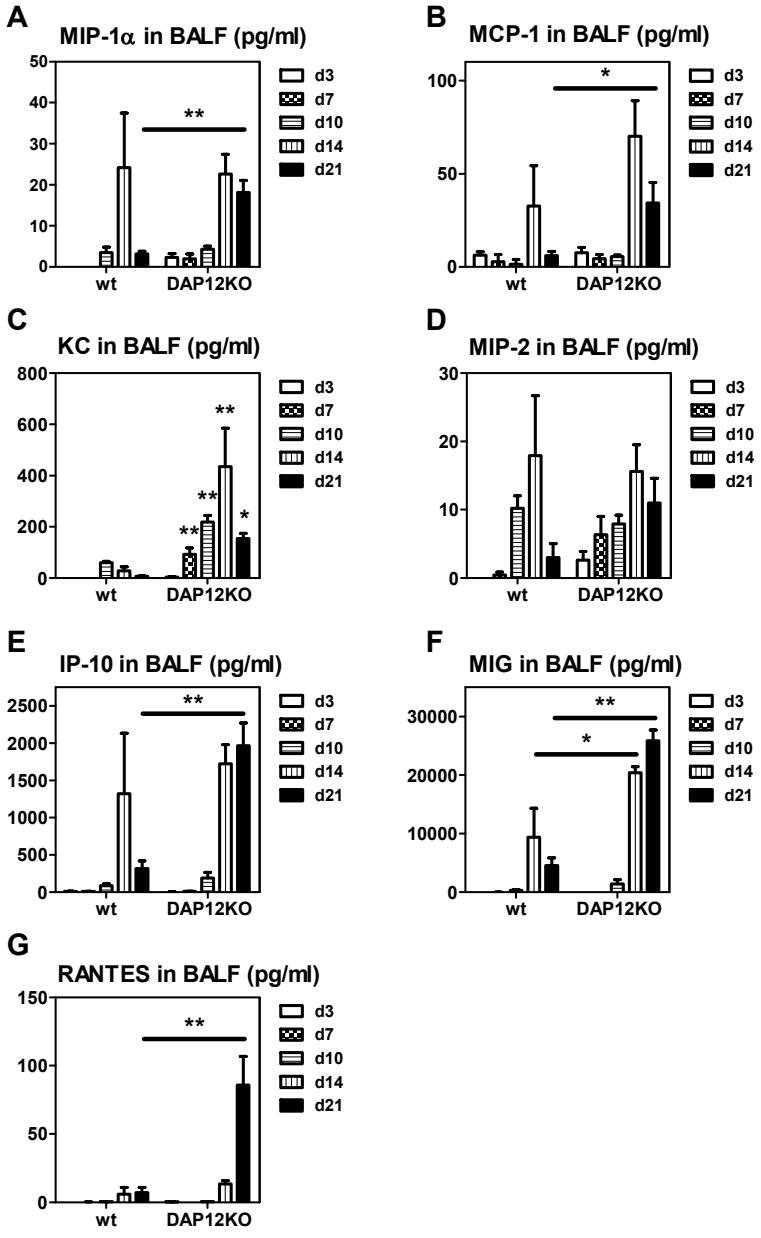

A
MIP-1α in BALF (pg/ml)
B
MCP-1 in BALF (pg/ml)
C
KC in BALF (pg/ml)
D
MIP-2 in BALF (pg/ml)
E
IP-10 in BALF (pg/ml)
F
MIG in BALF (pg/ml)
G
RANTES in BALF (pg/ml)
d3
d7
d10
d14
d21
wt
DAP12KO

Figure 6.

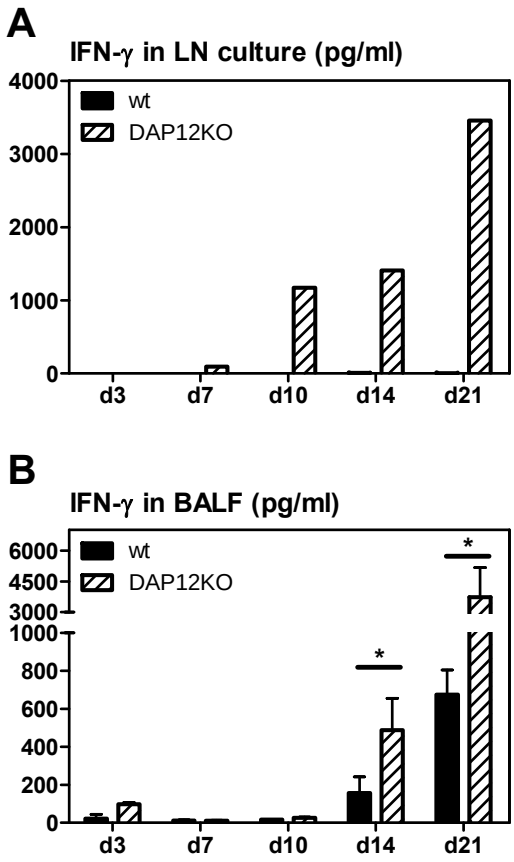

A
IFN-γ in LN culture (pg/ml)
4000
3000
2000
1000
0
wt
DAP12KO
d3
d7
d10
d14
d21
B
IFN-γ in BALF (pg/ml)
6000
4500
3000
1000
800
600
400
200
0
wt
DAP12KO
*
*
d3
d7
d10
d14
d21

Figure 7.

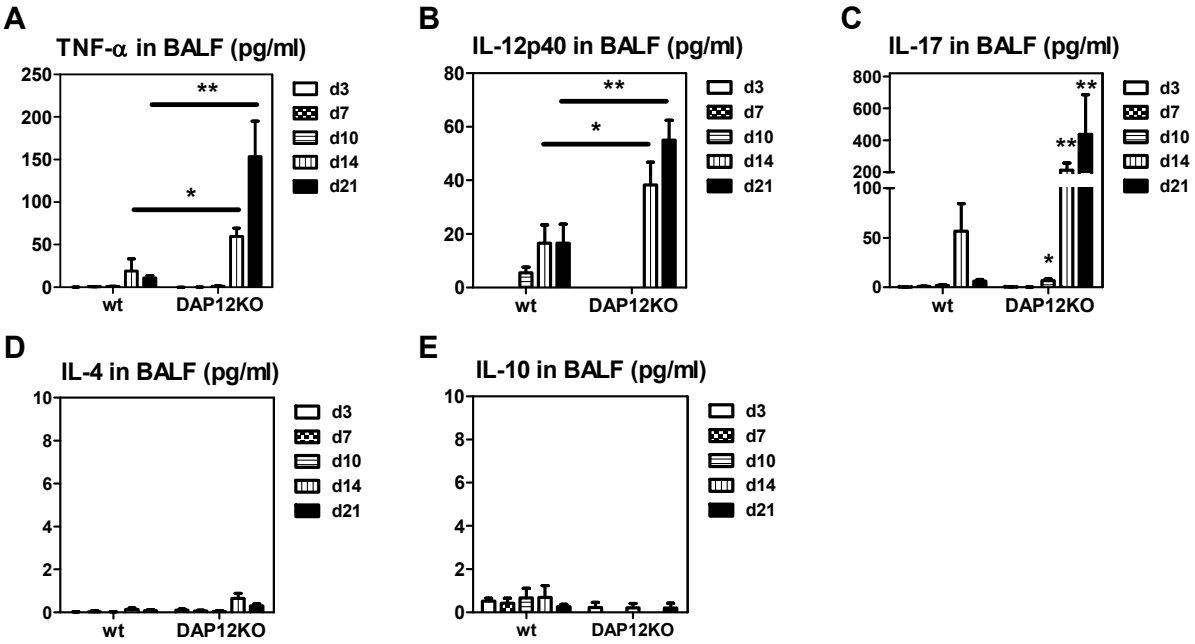

A
TNF-α in BALF (pg/ml)
250
200
150
100
50
0
**
*
wt
DAP12KO
d3
d7
d10
d14
d21
B
IL-12p40 in BALF (pg/ml)
80
60
40
20
0
**
*
wt
DAP12KO
d3
d7
d10
d14
d21
C
IL-17 in BALF (pg/ml)
800
600
400
200
100
50
0
**
**
*
wt
DAP12KO
d3
d7
d10
d14
d21
D
IL-4 in BALF (pg/ml)
10
8
6
4
2
0
wt
DAP12KO
d3
d7
d10
d14
d21
E
IL-10 in BALF (pg/ml)
10
8
6
4
2
0
wt
DAP12KO
d3
d7
d10
d14
d21

Figure 8.

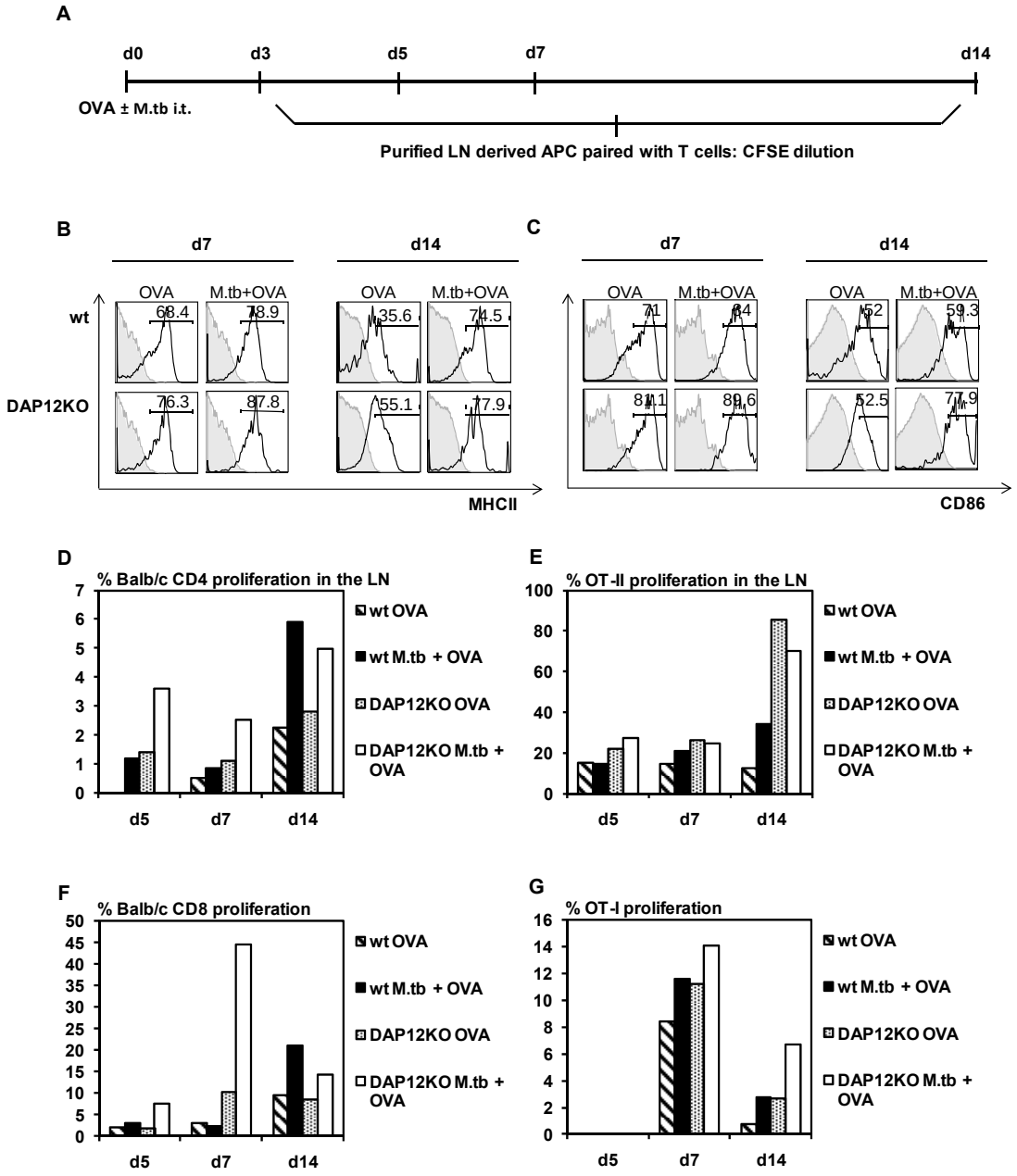

A
d0
d3
d5
d7
d14
OVA ± M.tb i.t.
Purified LN derived APC paired with T cells: CFSE dilution
B
d7
d14
OVA
M.tb+OVA
wt
DAP12KO
68.4
78.9
35.6
74.5
76.3
87.8
55.1
77.9
MHCII
C
d7
d14
OVA
M.tb+OVA
71
84
52
59.3
81.1
89.6
52.5
77.9
CD86
D
% Balb/c CD4 proliferation in the LN
wt OVA
wt M.tb + OVA
DAP12KO OVA
DAP12KO M.tb + OVA
d5
d7
d14
E
% OT-II proliferation in the LN
F
% Balb/c CD8 proliferation
G
% OT-I proliferation

Figure 9.

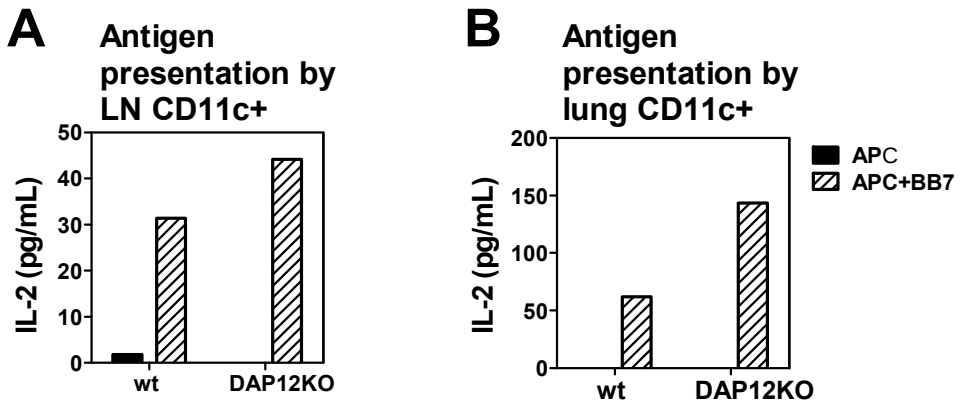
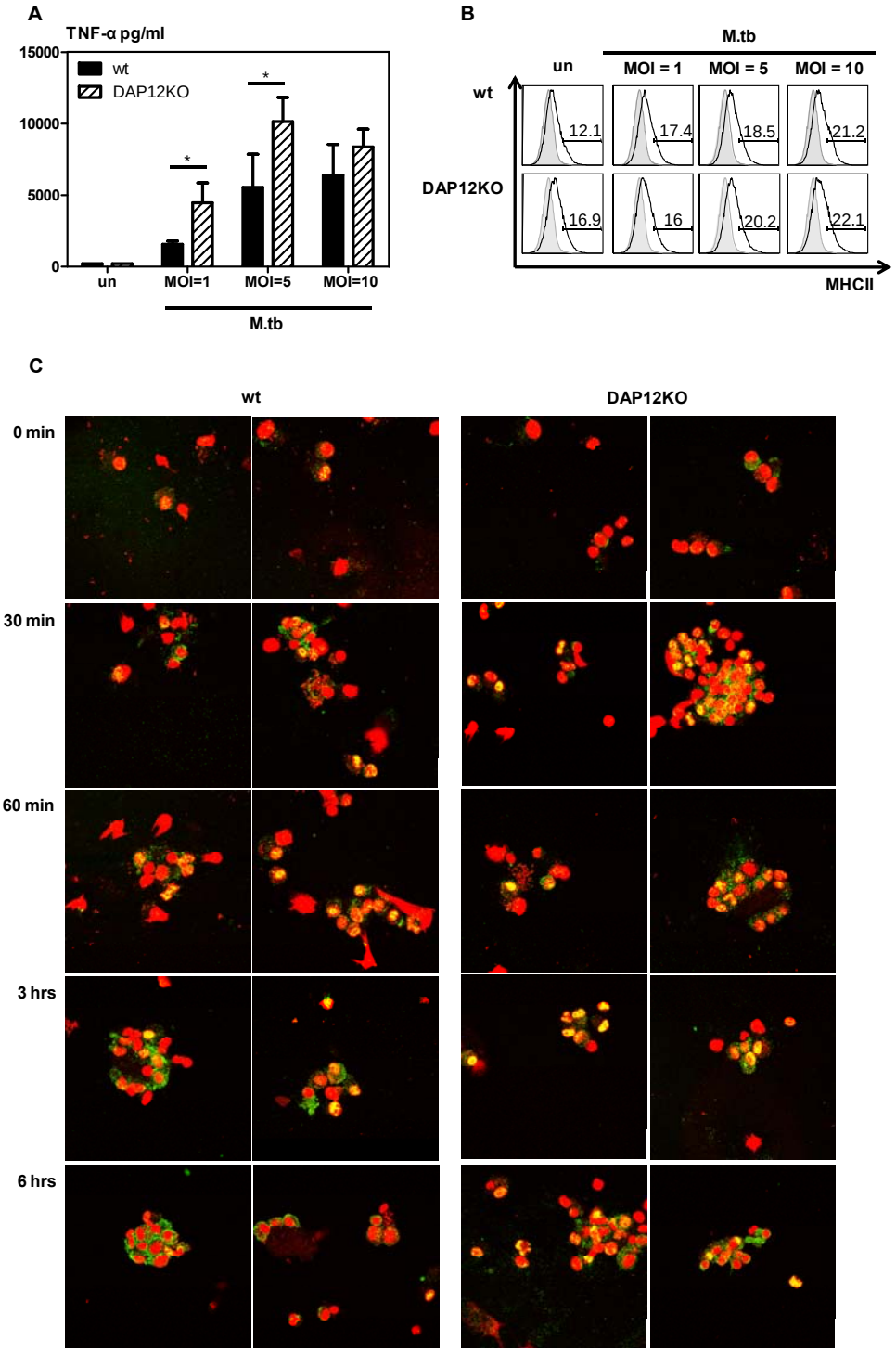

A
Antigen presentation by LN CD11c+
IL-2 (pg/mL)
50
40
30
20
10
0
wt
DAP12KO
B
Antigen presentation by lung CD11c+
IL-2 (pg/mL)
200
150
100
50
0
wt
DAP12KO
APC
APC+BB7

Figure 10.


A
TNF-α pg/ml
15000
10000
5000
0
wt
DAP12KO
*
*
un
MOI=1
MOI=5
MOI=10
M.tb
B
M.tb
un
MOI = 1
MOI = 5
MOI = 10
wt
DAP12KO
12.1
17.4
18.5
21.2
16.9
16
20.2
22.1
MHCII
C
wt
DAP12KO
0 min
30 min
60 min
3 hrs
6 hrs

Chapter 3: Control of pathogenic CD4 T cells and lethal immunopathology by signaling immuno-adaptor 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

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Control of pathogenic CD4 T cells and lethal immunopathology by signaling immuno-adaptor DAP12 during influenza infection

T cell responses against influenza virus are necessary to eliminate infection however excessive T cell responses have been linked to lung pathology. Here using a model of influenza A H1N1 infection in DAP12 deficient and wt mice we found that DAP12 deficient mice succumb to lethal lung immunopathology due to heightened CD4 T cell responses. We found that CD4 T cells primed by DAP12 deficient APCs expressed higher FasL levels and were capable of greater cytotoxicity than wt controls. These findings suggest that DAP12 plays a regulatory role in controlling the amplitude of the T cell response and protects the host from the development of pathogenic T cell responses.

I designed, executed the experiments, analyzed and interpreted the data. I generated all figures and wrote the manuscript. Dr. Zhou Xing established the foundation for the project and provided general guidance and supervision. Christopher (Ryan) Shaler provided invaluable technical assistance and scientific discussion. Carly Horvath provided technical assistance with experiment execution and Daniela Damjanovic provided technical support by running the influenza antibody ELISA. Early Brown provided the influenza virus. Naoki Aoki provided support with the Western Blot. Toshiyuki Takai provided the DAP12KO mice. A special thanks to Jeanette Boudreau for her excellent hands and to Anna Zganiacz for all her support.

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The Journal of Immunology

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.**

The world is constantly confronted by influenza epidemics or even pandemics. Lung immunopathology is the major cause of influenza-associated morbidity and mortality (1, 2). Upon entering the lung, the influenza virus infects both bronchial epithelial and APC such as dendritic cells (3). Flu Ag-laden APC activate Ag-specific CD8 and CD4 T cells in the bronchial-associated lymphoid tissue and draining lymph nodes (4). It is well-known that CD8 T cells play a central and direct role in flu viral clearance by their cytokine release and cytolytic activities (5–9). The role of CD4 T cells in flu viral clearance is believed to be mostly indirect via their augmenting effects on CD8 T and B lymphocytes (1, 7, 10, 11).

Anti-flu host defense is always accompanied by varying, sometimes fatal, lung immunopathology but much still remains to be understood about the immune regulatory mechanisms of flu-associated immunopathology (2, 12). It has been widely believed that the same mechanisms are shared by both anti-flu host defense and immunopathogenesis (2, 13–16). Indeed, the cytotoxic molecules and cytokines produced by anti-flu CD8 T cells also contribute to lung immunopathology and tissue injury (5, 6, 8, 17–20). However, little is understood about the role of CD4 T cells in lung immunopathology and, in particular, the mechanisms that govern the generation of immunopathogenic CD4 T cells during influenza infection (1, 21–23).

DNAX-activating protein of 12 kDa (DAP12) is a newly identified, evolutionarily highly conserved two ITAM-containing signaling adaptor molecule expressed only in innate immune cells such as dendritic cells, macrophages, as well as NK cells (24–27). A number of innate immune receptors including the triggering receptor expressed on myeloid cells, MDL-1 and NKG2D, are associated with and signal though DAP12 (24–29). Although initially identified to be a proinflammatory or immune-activating molecule, recent emerging evidence suggests a dual role of DAP12 signaling pathway, depending on the target immune cell type, the nature of Ags or infectious agents or the variation in different animal models (28–40). However, the precise role of DAP12 pathway in the regulation of immunopathogenic T cell subsets and immunopathology in acute influenza infection remains unclear.

In this study, by using an experimental model of influenza infection in wild-type (wt) and DAP12-deficient knockout (DAPKO) mice, we have investigated the regulatory immune mechanisms of anti-flu T cells, particularly CD4 T cells and their relationship with lung immunopathology. We find that although DAP12 pathway is not required for flu viral clearance, it is critically required for negatively regulating the level of immunopathogenic CD4 T cells. Thus, dysregulated CD4 T cells express high levels of Fas ligand (FasL), become cytotoxic, and cause severe lung immunopathology via FasL signaling pathway. Thus, our findings identify a novel DAP12-mediated immune regulatory pathway that is required to control CD4 T cell-induced immunopathology independent of influenza viral clearance.

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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.

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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. $Rag2^{-/-}\gamma c^{-/-}$ (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 5×10^4 PFU of a mouse-adapted A/FM/1/47 (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 X-100, 50 mM Tris [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 anti-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 semiquantified 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 (ASNENMDTM) for 6 h or whole UV-inactivated flu virus for 18 h at 37°C 5% CO_2. GolgiPlug (BD Biosciences) was added for the final 6 h. Cells were immunostained with CD3-PerCpCy5.5, CD4-PECy7, CD8α-allophycocyanin Cy7, IFNγ-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 (ASNENMDTM) of influenza NP bound to the C57BL/6 MHC class I allele $H\text{-}2D^b$ (Texas A&M University, College Station, TX). Stained cells were then run on a FACSCanto 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 inactivation) overnight at 4°C. Unpulsed splenocytes were labeled with 0.5 μM CFSE ($CFSE^{low}$), and UV flu-pulsed splenocytes were labeled with 5 μM CFSE ($CFSE^{high}$). One million $CFSE^{high}$ and $CFSE^{low}$ cells were injected intratracheally in 40 μl via the surgically exposed trachea into the airway of flu-infected and naive mice. Twenty-four hours following injection, 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 naive 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 LHD − target spontaneous LDH/target maximum LDH − target spontaneous LDH.

Anti-flu Ab titer quantification 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% NaN_3, and 0.01% $MgCl_2 \cdot 6\ H_2O$ (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 = (OD_{405} 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 (GK1.5) 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.

Adoptive 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 adoptively transferred to $Rag2^{-/-}\gamma\text{-}c^{-/-}$ mice via the tail vein. Six hours following T cell transfer, mice were challenged intranasally with 2×10^4 PFU influenza virus. Mice were monitored daily for signs of illness and body weight changes.

Immune analysis and adoptive transfer of lung APC

Naive 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. For APC characterization, cells were infected or stimulated in vitro with either flu virus (MOI = 0.5), UV-inactivated flu virus (MOI = 10 prior to UV inactivation), or imiquimod (5 μg/ml) (Imgenex, San Diego, CA) for 48 h. Cell culture supernatants were harvested for cytokine assay, or the cells were immunostained for cell surface markers including CD11c-PECy7, CD11b-allophycocyanin, IA^b-biotin, and CD80-PE (all Abs purchased from BD Pharmingen). For adoptive APC transfer, cells were isolated as

described above. Some cells were resuspended in serum-free media and transfected 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. 1*A*). 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. 1*B*). 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

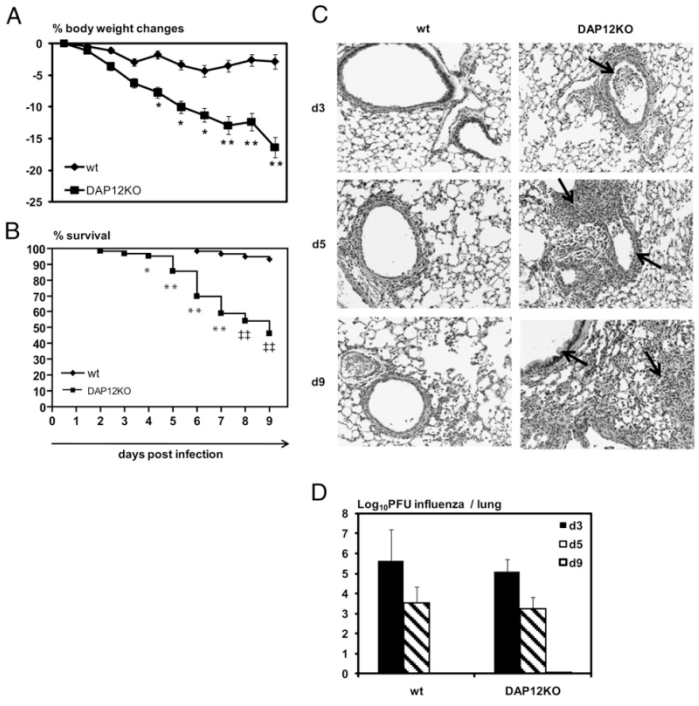

FIGURE 1. DAP12KO mice are more susceptible to pulmonary influenza infection than wt control mice. DAP12KO and wt mice were infected with influenza A virus and monitored daily for changes in body weight (*A*) and survival rate (*B*). Body weight data are mean ± SEM change and survival data are collected from 10 independent experiments, n = 18–55 mice/group. Lung pathology was examined by H&E staining of lung sections from wt and DAP12KO mice at various time points following infection, and the representative histopathologic images are shown (*C*). Arrows indicate inflammatory plug in airway (*top right*), epithelial damage including sloughing and neutrophilic inflammation (*middle right*), goblet cell hyperplasia and lymphocytic clustering (*bottom right*). Original magnification ×10. Viral burden in the lungs of influenza-infected mice was assessed by plaque forming assay (*D*), and the data are expressed as mean ± SEM of five independent experiments, n = 8–12 mice/group/time point. $^*p < 0.05$, $^{**}p < 0.01$, $^{\ddagger\ddagger}p < 0.001$.

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. 1*C*). 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. 1*C*, 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. 1*C*). 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. 1*D*). To examine whether the markedly increased morbidity and mortality of infected DAP12KO animals could be due to uncontrolled influenza viral 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 cellular infiltrates in the airway at days 3, 5, and 9 following infection. Compared with wt animals, there were significantly greater numbers of major 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. 1*C*). 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. 2*B*, 2*C*).

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. 2*B*, 2*F*). 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. 3*A*). A similar trend was observed in the lung interstitium of DAP12KO mice (Fig. 3*C*). 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. 3*B*, 3*D*).

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

Mouse Strain	Days	Epithelial Sloughing and Airway Plugs	Epithelial Metaplasia/ Hyperplasia	Large Airway Inflammation	Parenchymal Inflammation
wt	3	+	—	+/—	—
	5	—	—	++	++
	9	—	+	++	+
DAP12KO	3	++++	—	+++	++
	5	+++++	—	++++	+++
	9	—	++++	+++++	+++++

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 (—).

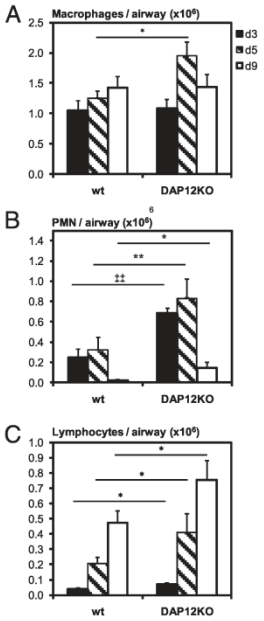

FIGURE 2. Increased inflammatory cellular responses in the lung of DAP12KO mice following pulmonary influenza infection. Influenza-infected wt and DAP12KO mice were subject to BAL, and the number of macrophages (*A*), neutrophils (*B*), and lymphocytes (*C*) in the airway was enumerated on cytospins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

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. 3*E*). 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. 3*E*). 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. 3*F*), 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. 3*G*).

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. 3*A*). However, there were greater numbers of NP tetramer^{+} (Supplemental Fig. 3*B*) or IFN-γ^{+} CD8 T cells (Supplemental Fig. 3*C*) in the lung of DAP12KO animals. Because DAP12 deficiency has been found to cause decreased regulatory T cells in an autoimmune model (52), we examined Foxp3^{+} regulatory T cells in the lung and found no difference between the wt and DAP12KO mice. Also, we did not identify any difference in the level of immune-regulatory cytokines IL-10 and TGF-β (data not shown).

Although Ab responses may play a less important role than cytotoxic T cell responses in flu viral clearance during primary infection, we compared flu-specific Ab titers in wt and DAP12KO mice. We found that different from dysregulated T cell responses in DAP12KO mice, the titers of flu-specific IgA in BAL and IgG1 and IgG2A in serum were raised to very similar levels at all time points examined in both wt and DAP12KO mice (Fig. 3*H–J*).

The above data together reveal the emergence of an unusual dysregulated CD4 T cell population capable of robust effector 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. 4*A*). As expected, infected DAP12KO mice receiving control treatment were severely ill and by days 8, only ~30% of them survived (Fig. 4*A*). 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. 4*A*), which was similar to the survival rate in infected wt hosts (Fig. 1*B*). 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. 1*C*), the control DAP12KO lungs had severe tissue inflammation, bronchial epithelial injury/sloughing, and hyperplasia (Fig. 4*B*) in keeping with their poor survival (Fig. 4*A*). In comparison, the DAP12KO animals depleted of CD8 T cells had even worse lung immunopathology and injury (Supplemental Fig. 4*A*). 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. 4*B*, 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. 4*C*). However, although the control DAP12KO mice completely cleared the virus from the lung by 9 d postinfection in consistence with the data in Fig. 1*D*, the CD4 T cell-depleted DAP12KO counterparts had delayed viral clearance, and a complete viral clearance was not seen until 14 d postinfection (Fig. 4*C*). We also observed a similarly delayed viral clearance in the lung of CD8 T cell-depleted DAP12KO animals (Supplemental Fig. 4*B*). As

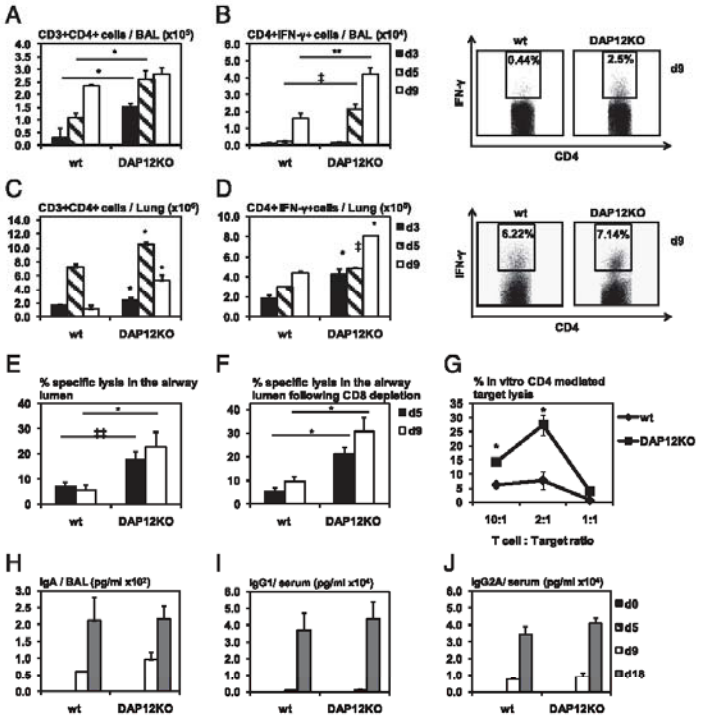

FIGURE 3. CD4 T cell effector activities are increased in the airway (BAL) and lung tissue of influenza-infected DAP12KO mice. The lungs of infected mice harvested at various times postinfection were subject to BAL and lung tissue mononuclear cell isolation. The total number of CD4 T cells in the BAL (*A*) and lung (*C*) was determined by flow cytometric analysis. The number of IFN-γ–secreting CD4 T cells with or without flu Ag stimulation was determined by intracellular cytokine staining and flow cytometric analysis of pooled BAL (*B*) or lung cells (*D*). Data are expressed as the mean ± SEM of 5–18 mice/group/time from three independent experiments. The extent of CTL activity in the airway lumen was measured by in vivo CTL assay at 5 and 9 d postinfection (*E*). The level of CD4 T cell-mediated CTL was assessed by in vivo CTL in mice depleted of CD8 T cells 2 d before the target cell delivery (*F*). Data are expressed as the mean percentage of Ag-specific target cell lysis ± SEM of 8–12 mice/group/time. The cytolytic capability of lung CD4 T cells was also assessed by in vitro CTL assay (*G*), and the results are expressed as the mean percentage of Ag-specific target cell lysis ± SEM of triplicate determinations. Flu-specific IgA titers in the BAL (*H*) and flu-specific IgG1 (*I*) and IgG2A (*J*) titers in the serum were determined by ELISA. Data are expressed as mean Ab titer ± SEM of four to five mice per group per time point. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{\ddagger}p < 0.005$, $^{\ddagger\ddagger}p < 0.001$.

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. 4*C*) 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. 4*D–F*). This may be because T cells were not depleted until 4 d postinfection (Fig. 4*A*) and because DAP12KO hosts had an accelerated CD4 Th function (being markedly elevated as early as day 3 as shown in Fig. 3*B*). 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 $Rag2^{-/-}\gamma c^{-/-}$ 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. 5*A*).

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 $Rag2^{-/-}$

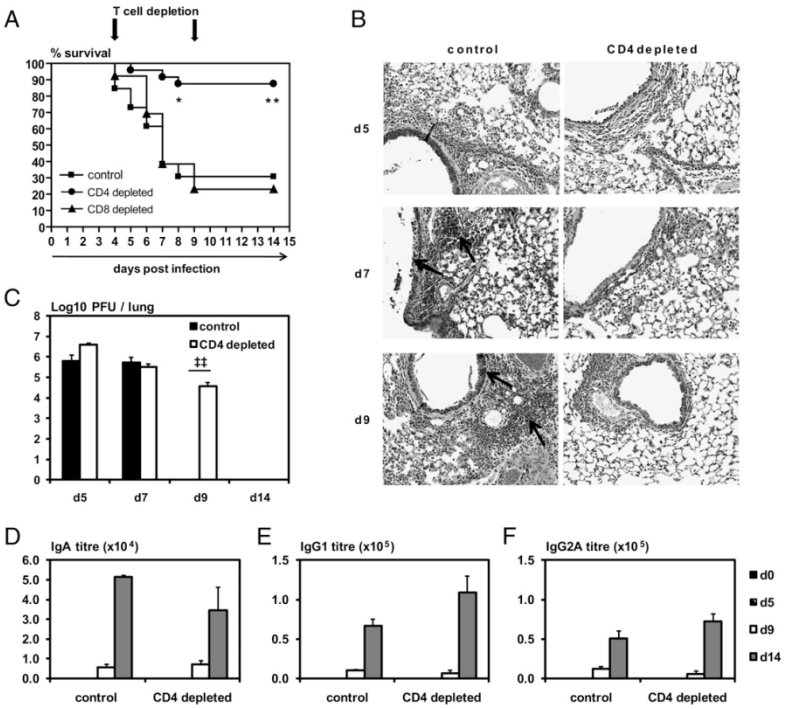

FIGURE 4. Depletion of CD4 T cells, but not CD8 T cells, improves the survival and lung immunopathology of influenza-infected DAP12KO mice. DAP12KO mice were infected with influenza virus, and at 4 and 8 d postinfection, they were depleted of CD8 or CD4 T cells i.p injection of anti-CD8 or CD4 mAb or control treatment. Mice were monitored daily for changes in body weight and survival (*A*). In separate experiments, infected mice were treated as in *A* and sacrificed at various time points postinfection for histopathologic assessment (*B*) (original magnification ×10) and flu viral titration (*C*). Arrows indicate epithelial damage leading to sloughing and airway inflammatory plugs or lymphocytic aggregates. The titers of flu-specific IgA (*D*), IgG1 (*E*), and IgG2A (*F*) in the lung were measured by ELISA. Data are expressed as the mean ± SEM of five to eight mice per group per time from two to three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{\ddagger\ddagger}p < 0.005$.

$\gamma c^{-/-}$ 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. 5*B*), reminiscent of the immunopathology and tissue injury seen in the lung of infected DAP12KO animals (Fig. 1*C*). The severe immunopathology and injury caused by transferred DAP12KO host-derived CD4 T cells was in accord with severe illness of these mice (Fig. 5*A*). 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 $Rag2^{-/-}\gamma c^{-/-}$ mice receiving

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

Mouse Strain	Days	Epithelial Sloughing and Airway Plugs	Epithelial Metaplasia/ Hyperplasia	Large Airway Inflammation	Parenchymal Inflammation
Control	5	++++	–	+++	+++
	7	+++++	++	++++	++++
	9	++	++++	+++++	+++++
CD4 depleted	5	++	–	++	++
	7	–	–	++	++
	9	–	+/–	+	+

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 (–).

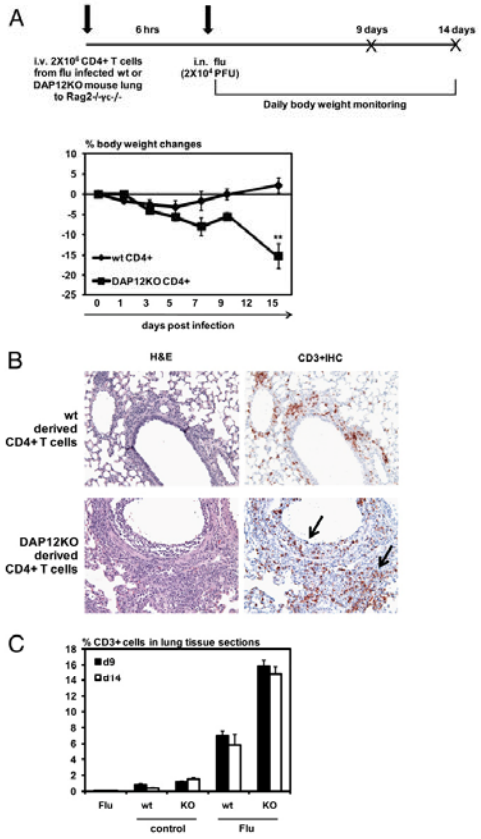

FIGURE 5. Adoptive transfer of CD4 T cells purified from the lung of influenza-infected DAP12KO mice but not those from infected wt mice causes the morbidity and severe lung immunopathology in the recipient DAP12-competent mice. CD4 T cells from influenza-infected DAP12KO and wt mice were purified, and equal numbers were transferred i.v. to naive immunodeficient $Rag2^{-/-}\gamma c^{-/-}$ mice, which were subsequently challenged with influenza virus. The body weight changes were monitored daily (*A*), and the data are expressed as mean body weight change ± SEM of 5–10 mice/group, representative of two independent experiments. At 9 and 14 d postinfection, mice were sacrificed. The extent of lung immunopathology was examined by H&E staining and the localization of transferred T cells was assessed by immunohistochemistry (CD3 + immunohistochemistry [IHC]) (*B*). Original magnification ×10. Arrows indicate the accumulation of T cells in the inflammatory plugs within the airway and within intense lung inflammatory foci. The negative control of IHC was included as IHC^{-}. Serial IHC sections were analyzed and the extent of T cell infiltration was quantified by morphometric analysis (*C*), and the data are expressed as mean area staining positive for $CD3^{+}$ T cells per lung section ± SEM of 8–10 mice/group/time point from two independent experiments.

DAP12KO-derived CD4 T cells compared with the lung of mice receiving wt CD4 T cells (Fig. 5*B*). Of importance, many T cells in the former were seen permeating not only the damaged bronchial epithelium but also the intrabronchial inflammatory plugs (Fig. 5*B*). Morphometric quantification of lung tissue infiltrated by T cells revealed markedly much more T cells in the lungs of influenza virus-challenged $Rag2^{-/-}\gamma c^{-/-}$ 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. 5*C*). 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 immunopathogenic 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. 3*A–D*) accounted for the morbidity and severe immunopathology (Fig. 4*A*, 4*B*) 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. 6*A*), 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 airway luminal (Fig. 6*B*) and lung interstitial (Fig. 6*C*) 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. 6*B*, 6*C*) and CD4 T cell-mediated cytotoxicity (Fig. 3*E*, 3*F*) 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 the survival of influenza-infected DAP12KO animals (Fig. 6*D*), which was associated with improved lung immunopathology (Fig. 6*E*, 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 $CD11c^{+}CD11b^{+}$ 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. 7*A*). These APCs also produced higher levels of TNF-α upon stimulation with TLR7 ligand imiquimod. Exposure to viable or UV-inactivated influenza viruses upregulated

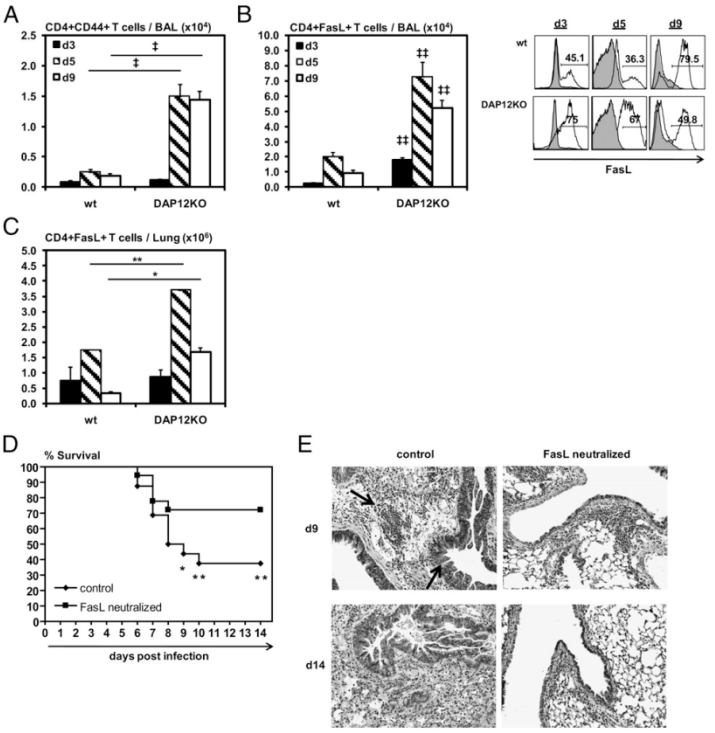

FIGURE 6. Blockade of FasL signaling pathway in overactivated CD4 T cells improves the survival and lung immunopathology of influenza-infected DAP12KO mice. Influenza-infected wt and DAPKO mice were sacrificed at various time points, and the level of overactivated CD4 T cells expressing CD44 in the airway (BAL) (*A*) or FasL in BAL (*B*) and lung tissue (*C*) was evaluated by flow cytometry. Data are expressed as mean PFU per organ ± SEM of five to eight mice per group per time point from two independent experiments. The role of FasL signaling pathway in CD4 T cell-mediated severe morbidity and immunopathology was examined by using a mAb to block FasL (FasL depleted). The control mice received only the treatment with isotype control Ab (*D*). Mice were monitored daily for body weight changes and survival rate (n = 7–10 mice/group). Lung immunopathology was assessed at designated time points (*E*). Original magnification ×10. Arrows indicate severe bronchial epithelium injury and hyperplasia or interstitial lymphocytic aggregates. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{\ddagger}p < 0.005$; $^{\ddagger\ddagger}p < 0.001$.

APC surface expression of T cell costimulatory CD80 (Fig. 7*B*) and MHC class II molecules (Fig. 7*C*) 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. 7*D*). 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. 7*D*).

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 − DAP12), brought down the level of Ag-specific CD4 T cell activation (Fig. 7*E*). 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

Mouse Strain	Days	Epithelial Metaplasia/ Hyperplasia	Large Airway Inflammation	Parenchymal Inflammation
Control	9	+++++	+++	++++
	14	+++++	++++	++++
FasL neutralized	9	+/–	++	++
	14	–	+	+/–

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 (–).

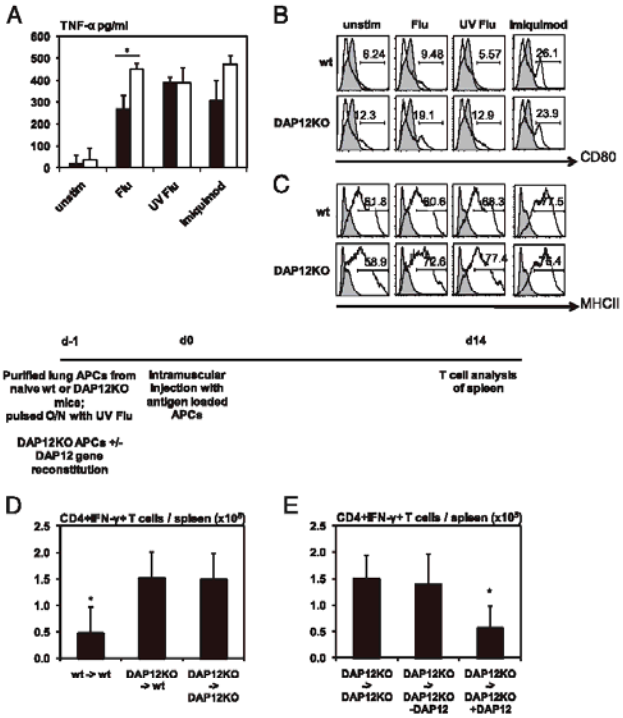

FIGURE 7. DAP12 expressed by lung APCs is critically required for regulating CD4 T cell activation during influenza infection. Purified lung CD11c$^+$ APCs were stimulated with influenza virus, UV-inactivated virus, or imiquimod for 48 h. The level of TNF-α was measured in culture supernatants by ELISA (*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, DAP12-KO→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 pathway (29, 31, 37–39, 57, 58), mounting evidence indicates its anti-inflammatory or immune regulatory role (32, 34–36, 40, 59). 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 monocytogene* (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.

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Disclosures

The authors have no financial conflicts of interest.

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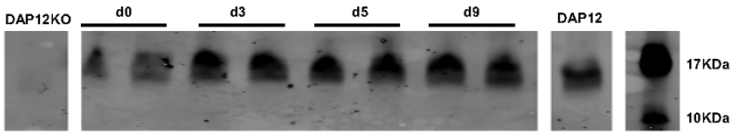
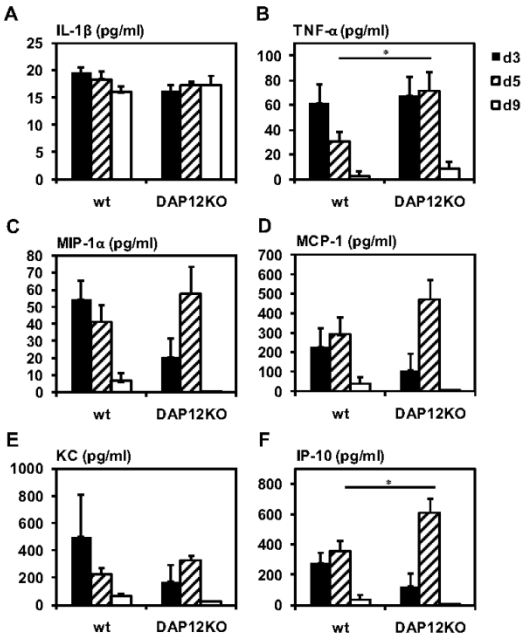
Supplemental Figure 1.



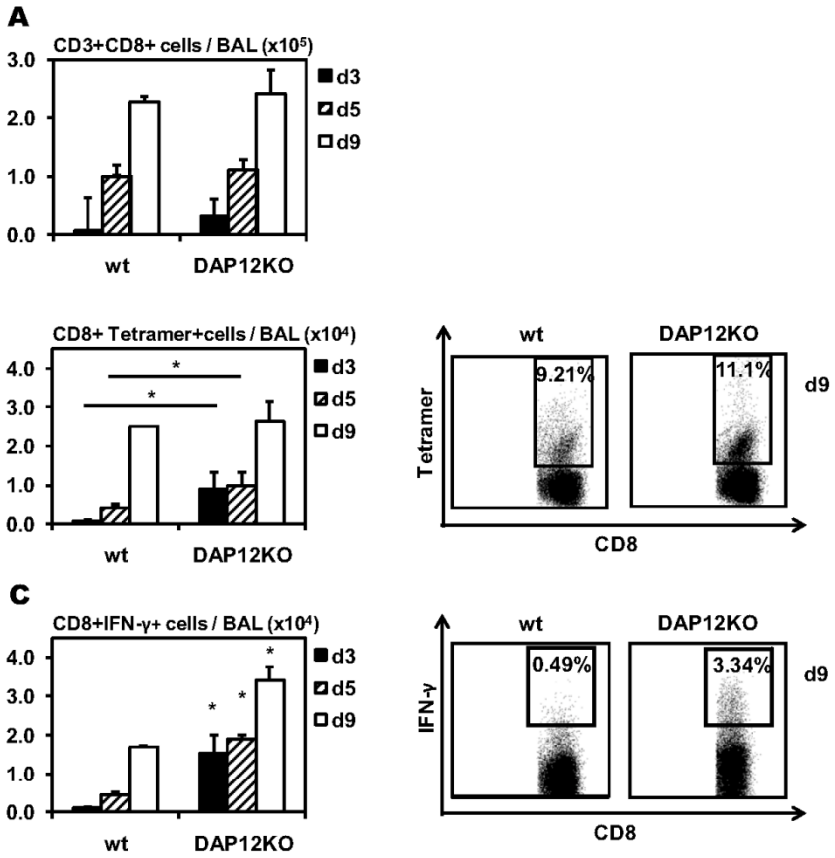
Constitutive and influenza-inducible DAP12 protein expression in wt BL/6 mouse lung. The level of DAP12 protein was examined in the lungs of wt mice in response to influenza infection by immunoprecipitation and western blotting. Equal amounts of lung tissues were subjected to immunoprecipitation and 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.

Supplemental Figure 2.



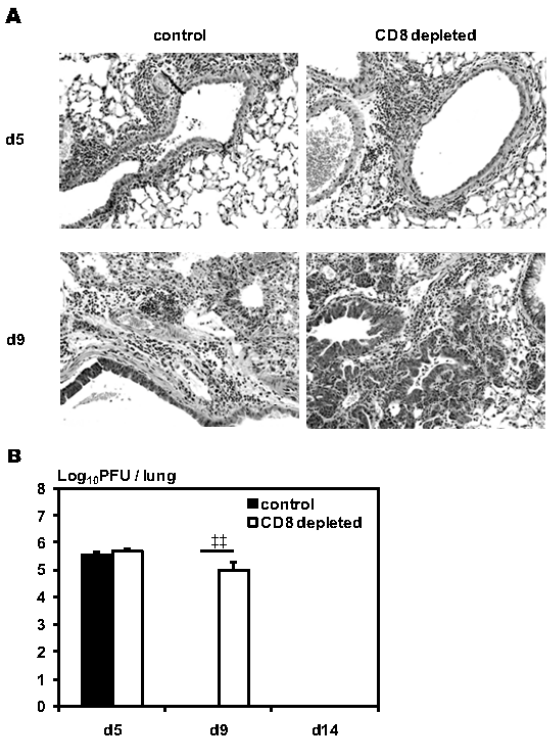
Cytokine and chemokine levels in the bronchoalveolar lavage fluids of wt and DAP12KO mice following influenza infection. At various time points following infection, lungs were isolated and subject to bronchoalveolar lavage (BAL). BAL fluids were measured by ELISA or multiplex cytokine assay for IL-1 (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. ‡‡ $p<0.005$

Chapter 4: Mucosally delivered dendritic cells activate T cells independently of IL-12 and endogenous APCs

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Mucosally delivered dendritic cells activate T cells independently of IL-12 and endogenous APCs

DCs are sentinel cells that specialize in T cell priming. DCs can be manipulated ex vivo to present antigen, and secrete immunostimulatory factors providing a safe way to generate vaccines against infectious disease and cancer. The applicability of a mucosally delivered DC based vaccine had not been addressed. By virally transducing DCs ex vivo we were able to show that vaccine DCs rapidly migrated to the local draining LN in an IL-12 dependent manner within 24 hours of delivery and primed protective CD4 and CD8 T cell responses in the airway and lung parenchyma. The observed T cell activation was a direct result of antigen presentation and activation by vaccine DCs rather than relying on endogenous host DCs. Our observations indicate that mucosal delivery of vaccine DCs represents an effective approach to enhance mucosal T cell immunity.

I designed, executed the experiments, analyzed and interpreted the data. I generated figures and wrote the manuscript. Dr. Zhou Xing established the foundation for the project and provided general guidance and supervision. Dr. Michael Santosuosso provided invaluable technical support and aided in experimental design. Dr. Cherrie-Lee Small provided technical assistance and scientific discussion. Christopher (Ryan) Shaler provided technical assistance. Xizhong Zhang, Mangalakumari (Mathy) Jeyanathan and Jingyu Mu carried out the virulent *M.tb* challenge in the Level III biocontainment facility. Shunsuke Takenaka provided technical support and scientific discussion. Patricia Ngai provided technical assistance. Jack Gauldie and Yonghong Wan provided scientific support and people that helped establish the techniques.

The Journal of Immunology

Mucosally Delivered Dendritic Cells Activate T Cells Independently of IL-12 and Endogenous APCs[1]

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In vitro manipulated dendritic cells (DC) have increasingly been used as a promising vaccine formulation against cancer and infectious disease. However, improved understanding of the immune mechanisms is needed for the development of safe and efficacious mucosal DC immunization. We have developed a murine model of respiratory mucosal immunization by using a genetically manipulated DC vaccine. Within 24 h of intranasal delivery, the majority of vaccine DCs migrated to the lung mucosa and draining lymph nodes and elicited a significant level of T cells capable of IFN-γ secretion and CTL in the airway lumen as well as substantial T cell responses in the spleen. And such T cell responses were associated with enhanced protection against respiratory mucosal intracellular bacterial challenge. In comparison, parenteral i.m. DC immunization did not elicit marked airway luminal T cell responses and immune protection regardless of strong systemic T cell activation. Although repeated mucosal DC delivery boosted Ag-specific T cells in the airway lumen, added benefits to CD8 T cell activation and immune protection were not observed. By using MHC-deficient vaccine DCs, we further demonstrated that mucosal DC immunization-mediated CD8 and CD4 T cell activation does not require endogenous DCs. By using IL-12-deficient vaccine DCs, we also observed that IL-12$^{-/-}$ DCs failed to migrate to the lymph nodes but remained capable of T cell activation. Our observations indicate that mucosal delivery of vaccine DCs represents an effective approach to enhance mucosal T cell immunity, which may operate independent of vaccine IL-12 and endogenous DCs. ***The Journal of Immunology,*** **2008, 181: 2356–2367.**

Dendritic cells (DC)[3] are highly specialized APCs that form a network over both the interior and exterior surfaces of the body, constituting an important part of host defense against invading pathogens (1). DCs are the most potent APC critically involved in the initiation of innate and adaptive immune responses to pathogens, particularly at mucosal surfaces (2). Thus, in vitro manipulation of DCs and subsequent in vivo administration have been exploited as an important strategy for immunization against cancer or infectious disease.

In vitro/ex vivo-manipulated DCs have been used to activate T cells in vivo in the field of immunotherapy. For instance, autologous patient-derived DCs loaded ex vivo with tumor Ags were demonstrated to be able to break tolerance and activate T cells in tumor-bearing patients (3–6). Furthermore, DC-based immunotherapy is also being explored to enhance pathogen-specific immune responses in immunocompromised individuals, such as those infected with HIV (7). In this regard, there are two advantages of using in vitro-manipulated DC vaccine; 1) to restore anti-HIV immunity, the in vivo otherwise deactivated DCs can be reactivated and loaded with HIV epitopes ex vivo and thus enabled to activate T cells upon being readministered back to the same host; and 2) the same DCs can be potentially manipulated to carry or express Ags of multiple opportunistic pathogens such as mycobacteria and *Aspergillus* that often cause lethal secondary infection in HIV hosts (8, 9). Although both experimental and clinical studies suggest that such DC-based vaccines are safe and promising, their immune-activating and protective efficacy has remained questionable.

Like any other immunization strategies, both the mode of Ag expression and the route of delivery play an important role in determining the efficacy of DC-based immunization strategy. We and others have shown that virally transduced or gene-modified DCs are superior at in vivo T cell activation over peptide- or whole-protein-loaded DCs (10–13). Because many of the infectious diseases are acquired via the mucosal route, it is believed that compared with the systemic or parenteral route, mucosal immunization at the site of pathogen entry may elicit the most protective immunity. Indeed, mounting experimental evidence supports this notion (13–16). Although live organism-based vaccines including genetically modified viruses or attenuated pathogens may be directly used for the purpose of mucosal immunization, they could be unsafe and ineffective, particularly in immunocompromised hosts. In this regard, DC immunization represents an attractive approach which is not only safe and repeatable for mucosal application but is also able to effectively deliver Ags to activate adaptive immune cells, thus overcoming the limitations of impaired mucosal immunity in immunocompromised hosts.

To date, a small number of in vitro-manipulated DC formulations have been explored for the purpose of respiratory mucosal

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[3] Abbreviations used in this paper: DC, dendritic cell; BCG, Bacillus Calmette-Guérin; i.n., intranasal; MHCII, MHC class II; MHCI, MHC class I; BAL, bronchoalveolar lavage; LN, lymph node; cLN, cervical LN; mLN, mediastinal LN; pLN, popliteal LN; i.t., intratracheal; ICCS, intracellular cytokine staining; wt, wild type.

immunization only with limited success. For instance, only a short term of protection against *Mycobacterium tuberculosis* was accomplished following intranasal (i.n.) mucosal immunization with the DCs infected with live *Mycobacterium bovis* bacillus Calmette-Guérin (BCG; Ref. 17). Likewise, DCs loaded in vitro with heat-killed *Bordetella pertussis* were only modestly effective in priming lung mucosal immune responses (18). Furthermore, i.n. delivery of the DCs genetically modified with plasmid DNA encoding a microbial Ag, only minimally enhanced T cell responses and protection from *Coccidioides* challenge (8, 19). Such limited success in mucosal DC immunization is believed to be attributable at least in part to our lack of knowledge in DC trafficking, immunogenic differences by mucosal and parenteral routes of delivery, and the mechanisms involved in DC mucosal immunization.

In this study, we have used the in vitro genetically manipulated DCs as a tool and murine i.n. mucosal immunization as a model system to specifically address: 1) where vaccine DCs travel post-mucosal delivery; 2) how well such delivered DCs may activate Ag-specific T cells both locally and systemically and how it compares to the parenteral route of delivery; 3) whether DC-activated T cell responses translate to immune protection; and 4) the role of DC-associated MHC class I (MHCI) and II (MHCII) molecules and IL-12 in T cell activation.

Materials and Methods

Mice and reagents

Female BALB/c and C57BL/6 mice (6–10 wk old) were purchased from Harlan Laboratories. Congenic CD45.1$^+$ mice were purchased from The Jackson Laboratory. C2DBN12 (MHCII$^{-/-}$) mice were purchased from Taconic. β_2-Microglobulin-CD8 double-knockout (MHCI$^{-/-}$) and IL-12 p40 knockout mice (IL-12$^{-/-}$) were bred in house at McMaster University (Hamilton, Ontario, Canada). All animals were housed in a specific-pathogen-free facility in the Central Animal Facility at McMaster University in accordance with the McMaster Animal Research Ethics Board. The construction and amplification of a replication deficient (E1/E3 deleted) recombinant adenovirus encoding the gene for Ag 85A has been previously described (11) and was used to transduce bone marrow derived dendritic cells. All viruses were purified and stored at −70°C until needed. Purified *M. tuberculosis* Ag 85 complex and *M. tuberculosis* culture filtrate proteins were provided by Colorado State University (Fort Collins, CO) through funds from the National Institute of Allergy and Infectious Disease (Contract 1-A1-75320). Two synthetic Ag85A peptides on a BALB/c background (H-2^d) were used. The MHCI-specific peptide (MPVGCQSSF) and MHCII-specific peptide (LTSFLPGWLQANRHVKPTGS) were synthesized by Dalton Chemical Laboratories (Toronto, Ontario, Canada). All proteins were dissolved in DMSO and stored at −20°C until needed.

Generation of bone marrow-derived DCs and preparation of DC-based vaccine

Bone marrow cells were harvested from the femurs and tibiae of naive BALB/c mice as previously described (11) and cultured in RPMI 1640 containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 40 pg/ml recombinant murine GM-CSF. Three and six days after initial culture, cells were replenished with fresh medium supplemented with GM-CSF. On day 7, DCs were infected with AdAg85A (DCAdAg85A) at a multiplicity of infection of 100. After 4 h of incubation at 37°C, all DCs were harvested by scraping the bottom of culture plates with a spatula, washed thee times with PBS, and used to immunize mice.

Immunization, preparation of DC vaccines, and in vivo trafficking

Vaccines were prepared immediately before immunization and kept on ice until use. All DCs were delivered either i.n. or i.m. Immunizations were conducted by using 1.0×10^6 DCs/mouse in 30 μl of PBS for i.n. injections and 0.5×10^6 DCs/mouse in 100 μl of PBS for i.m. injections. A higher dose was used for i.n. delivery considering the loss of some of i.n. delivered materials to the gastrointestinal tract. Anesthetized mice were allowed to breath in the 30 μl containing vaccine DCs, or DC vaccine suspension was injected into the hind legs (50 μl each leg). For in vivo trafficking studies, either autologous bone marrow-derived DCs or congenic CD45.1$^+$ DCs were prepared as described and labeled with 5 μM CFSE. Cells were counted by trypan exclusion, and 1×10^6 live cells were delivered i.n. in 30 μl of PBS. At 24-h intervals, mice were sacrificed and bronchoalveolar lavage (BAL), lung interstitium, cervical lymph nodes (cLN), mediastinal lymph nodes (mLN), popliteal LN (pLN), and spleen were isolated. Lung tissue, cLN, mLN, and pLN were digested with collagenase for 1 h to release APC populations from the connective tissue and processed to single-cell suspensions. Cells were stained for CD45.1 and/or CD11c and analyzed for the presence of CD45.1$^+$ or CFSE-labeled CD11c$^+$ populations by flow cytometry. Live *M. bovis* BCG (0.5×10^6 CFU/mouse; Connaught Laboratories) was injected s.c. around both hind legs, as a positive control of vaccination in *M. tuberculosis* challenge experiments.

In vivo i.v. and intratracheal (i.t.) CTL assays

The in vivo CTL assay was conducted as previously described (11, 14). Briefly, splenocytes from naive female BALB/c mice were isolated the night before each in vivo cytotoxicity assay. Spleens were removed into complete medium (RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 50 μM 2-ME). Splenocyte suspension was filtered through a 55-μm pore size nylon membrane before being centrifuged at 1500 rpm for 5 min. Pellets were resuspended in 2 ml/spleen of RBC lysis buffer (R&D Systems) and incubated at room temperature for 12 min. Approximately 30 ml of PBS were added after the 12-min incubation to stop the RBC lysis. The whole splenocytes were filtered through a 55-μm pore size nylon membrane, centrifuged at 1500 rpm for 5 min, and resuspended at 20×10^6 cells/ml in complete medium. For the in vivo cytotoxicity assay, the splenocytes were pulsed with either the CD8 or CD4 T cell Ag85A peptide (10 μg/ml) and incubated overnight at 4°C. Such splenocytes used as CTL target cells were then resuspended at 20×10^6 cells/ml in PBS containing 5% FBS. The Ag85A peptide-pulsed splenocytes were labeled with 5 μM CFSE and denoted CFSE^{high}, and the unpulsed splenocytes were labeled with 0.5 μM CFSE (CFSE^{low}) for 5 min at room temperature in unlit conditions. The cells were washed twice to remove any free CFSE with 5% FBS-PBS and then once with PBS. Both CFSE^{high} and CFSE^{low} cell populations (5×10^6 of each per mouse for i.v. CTL, 1×10^6 of each per mouse for i.t. CTL) and were mixed in a 1:1 ratio (in a total of 200 μl volume for i.v CTL and 40 μl for i.t. CTL) and injected into DC-vaccinated mice. A naive mouse was also injected with peptide-pulsed, CFSE-labeled cells and used as unprimed control for calculation (see equation at end of paragraph). After 6 h (for the CD8 CTL assay) or 24 h (for the CD4 CTL assay) following target cell injection, either splenocytes (i.v CTL) or BAL cells (i.t. CTL) were isolated. The in vivo lysis of the target cells was determined according to the extent of loss of CFSE dye by flow cytometry. Up to 1×10^6 events were collected for analysis. To calculate Ag-specific, CD8 or CD4 T cell-mediated lysis, the formula used was: percentage of specific lysis = [1 − (ratio unprimed/ratio primed) × 100], where ratio is (percentage CFSE^{low}/percentage CFSE^{high}).

Cell isolation for characterization of immune activation following vaccination

Immunized mice were sacrificed 2, 6, or 12 wk postimmunization to examine immunogenicity. Spleens and lungs were removed aseptically and the intra-airway luminal cells were removed from the lung by exhaustive lavage as previously described (14, 15). Briefly, the mouse lung was lavaged five times with 1.8 ml of PBS through a polyethylene tube cannulated into the trachea to ensure maximal recovery. After lavage, the lungs were perfused through the left ventricle with Hanks' buffer to remove RBC from the vasculature. The lungs were then cut into small piece (>1 mm × 1 mm) and incubated with collagenase type 1 (Sigma-Aldrich) for 1 h at 37°C. Lung fragments were then crushed through a 100-μm pore size filter (18). Cells were collected and enumerated on a hemocytometer after dilution in Turks white blood cell counting buffer. Spleen cells were isolated as previously described (12). All isolated cells (spleen, lung, and airway-luminal) were then resuspended in RPMI 1640 supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin and streptomycin.

FACS, intracellular cytokine staining, and tetramer staining

These procedures were conducted as previously described (11, 14, 15, 20). Briefly, single-cell suspensions from immunized mice of spleen, lung, and airway-luminal cells were obtained as described above. Cells were cultured in a U-bottom 96-well plate at a concentration of 20×10^6 cell/ml, and airway-lumen-derived cells were cultured at a concentration of 5×10^6

cells/ml. Cells were cultured in the presence of Golgi plug (10 μg/ml brefeldin A; BD Pharmingen) and either no stimulation, Ag85A CD4 or CD8 T cell peptides at a concentration of 1 μg/well for 6 h. Cells cultured with Ag85 complex and *M. tuberculosis* culture filtrate at a concentration of 10 μg/ml in the absence of Golgi plug for the first 18 h followed by 6 h in the presence of Golgi plug. Cells were then washed and blocked with CD16-CD32 in 0.5% BSA-PBS for 15 min on ice and then stained with the appropriate surface Abs. Cells were then washed, permeabilized, and stained according to the manufacturer's instructions included in the ICCS kit (BD Pharmingen). The following Abs were used: CD3-CyChrome (BD Pharmingen); CD4-PE-Cy7 (BD Pharmingen); CD8a-allophycocyanin-Cy7 (BD Pharmingen); and IFN-γ-allophycocyanin (BD Pharmingen). Stained cells were then run on a FACS Canto, and 250,000 events were collected per sample (BD Pharmingen) and analyzed on FlowJo software (version 6.3.4; Tree Star). Tetramer flow cytometric analysis was conducted using the immunodominant CD8 T cell peptide (MPVGGQSST) of Ag85A bound to the BALB/c MHCI allele H-2L^d which was ordered from Texas A&M University (College Station, TX). Cells were washed and blocked with CD16-CD32 in 0.5% BSA-PBS for 15 min on ice, then stained with tetramer for 1 h in the dark at room temperature, and then washed and stained with surface Abs. Stained cells were then run on a FACS Canto, and 250,000 events are collected per sample (BD Pharmingen) and analyzed on FlowJo software.

Immune protection against pulmonary M. tuberculosis *challenge*

M. tuberculosis (H37Rv strain) (ATCC 27294) was grown in Middlebrook 7H9 broth supplemented with Middlebrook OADC enrichment (Invitrogen), 0.002% glycerol, and 0.05% Tween 80 for ~10–15 days and then aliquoted and stored in −70°C until needed as previously described (14, 15, 20). Before each use, *M. tuberculosis* bacilli were washed with PBS containing 0.05% Tween 80 twice and passed through a 27-gauge needle 10 times to disperse clumps. Immunized and non-immunized mice were infected i.n. with 10,000 CFU of *M. tuberculosis* at either 4 or 6 wk postimmunization in the Level III Containment Facility of McMaster University. The level of bacterial burden was determined 4 wk postchallenge in the lung and spleen by plating serial dilutions of tissue homogenates in triplicates onto Middlebrook 7H10 agar plates containing Middlebrook OADC enrichment. 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.

Detection of M. tuberculosis *Ag85A by Western blotting*

Western blot was used to detect both intracellular and secreted forms of Ag85A from AdAg85A-infected cells as previously described (21). Briefly, bone marrow-derived DCs were infected with a multiplicity of infection of 100 AdAg85A for 6 h and were washed with 5% FBS-PBS to remove free virus. Cells were cultured in 12-well plates for up to 7 days. Medium was replenished every other day as needed to ensure that cells remained viable. On the day of harvest, cell supernatants were collected and spun to remove any cellular contamination. Cells were lysed with 0.05% Triton in water and immediately frozen. A 10% SDS gel was cast, and samples were loaded immediately. Membranes were probed with a mouse anti-Ag85A mAb and HRP-conjugated anti-mouse IgG.

Statistical analysis

All statistics were performed with Microsoft Excel using a two-tailed *t* test assuming equal variances. Individual *p* values are included in the text.

Results

In vivo distribution of vaccine DCs following i.n. administration

Although cell-based mucosal immunization strategies are an increasingly popular approach to generating protective immunity against pathogens, there is a lack of information on the distribution of vaccine cells in the mucosal and nonmucosal compartments following i.n. mucosal delivery. To this end, we prepared CD45.1$^+$ congenic vaccine DCs transduced to express an immunogenic *M. tuberculosis* Ag Ag85A by using a recombinant adenoviral vector (DCAdAg85A) for i.n. delivery. At various times post-DC delivery, cells isolated from various tissue compartments were analyzed for the frequency of *CD45.1*+ DCs. Maximal numbers of vaccine DCs were detected in the airway lumen (Fig. 1*A*) and the lung interstitium (Fig. 1*B*) at 24 h. These airway luminal and lung mucosa-associated populations underwent contraction

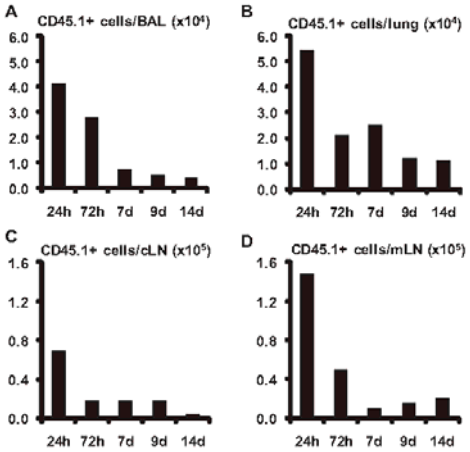

FIGURE 1. Localization of virally transduced vaccine DCs following i.n. administration. Congenic CD45.1-expressing DCs were transfected with AdAg85A (multiplicity of infection, 100), and 1×10^6 cells were delivered i.n. to BALB/c mice. At 24-h intervals, mice were sacrificed, and the cells isolated from BAL (*A*), lung interstitium (*B*), cLN (*C*), and mLN (*D*) were examined by flow cytometry for quantification of CD45.1$^+$CD11c$^+$ DCs. Data are expressed as the mean value of two mice per time, representative of two independent experiments. d, Day.

over the 14-day period examined (Fig. 1, *A* and *B*), and a small population of residual vaccine DCs could be detected 14 days after i.n. delivery. Vaccine DCs could be detected as early as 24 h following i.n. delivery in the cLNs (7.5% of total recovered DCs), which drain the nasal mucosa and in the mLNs (16% of total recovered DCs) which drain the lung (Fig. 1, *C* and *D*). Vaccine DCs migrated to these lymph nodes (LN) contracted rapidly, and the total number of vaccine DCs declined sharply by 72 h. A small but stable population of vaccine DCs could be detected up to 14 days, particularly in mLNs. In sharp contrast, no appreciable CD45.1$^+$ congenic vaccine DCs could be detected in the distal lymphoid organs including the spleen and pLNs at any time point (data not shown). Similar kinetics of vaccine DC trafficking were also observed by using CFSE-labeled autologous DCs (data not shown). These data suggest that some of the i.n. delivered vaccine DCs have the ability to migrate rapidly from the airway lumen to the local draining LNs and lung interstitium while a portion of these cells remain within the airway lumen.

Ag-specific T cell responses within the airway lumen and lung interstitium following i.n. administration of vaccine DCs

Having examined the distribution of vaccine DCs, we set out to determine the kinetics of activation, phenotype, and pulmonary distribution of Ag-specific T cells in response to i.n. DC immunization. We also compared i.n. with parenteral i.m. route of immunization. We paid special attention to the number of airway luminal T cells that have been shown to be essential in providing protection against pathogenic mucosal challenge (14, 22). Mice were immunized i.n. or i.m. with DCAdAg85A and sacrificed at 2, 6, and 12 wk postimmunization. We first analyzed the number of total CD4$^+$ and CD8$^+$ T cells in the airway lumen by FACS. The levels of CD4$^+$ and CD8$^+$ T cells in the airway lumen of i.m. DCAdAg85A-immunized mice were very small and insignificant at various time points (Table I). In contrast, i.n. DCAgAg85A

Table I. *Total CD4$^+$ and CD8$^+$ T cells recruited into the airway lumen following i.m. or i.n. delivery of vaccine DCAdAg85A*[a]

	CD4$^+$			CD8$^+$		
	2 wk	6 wk	12 wk	2 wk	6 wk	12 wk
DCAdAg85A i.m.	0.34 ± 0.03[b]	0.21 ± 0.01	0.12 ± 0.001	2.73 ± 0.19	0.37 ± 0.03	0.42 ± 0.29
DCAdAg85A i.n.	2.89 ± 0.45	0.61 ± 0.24	0.70 ± 0.20	8.34 ± 0.13	2.00 ± 0.8	1.42 ± 0.42

[a] The total numbers of CD4$^+$ and CD8$^+$ T cells, gated on CD3$^+$ T cells in the BAL fluids, were quantified by FACS. Data are expressed as the average number ± SEM of six to nine mice per time per group.
[b] All numbers are $\times 10^4$.

induced much more total CD4$^+$ and CD8$^+$ T cells into the airway lumen than i.m. immunization (8.5- and 3-fold more CD4$^+$ and CD8$^+$ T cells at 2 wk; 3- and 5.4-fold more at 6 wk; and 5.8- and 3.3-fold more at 12 wk, respectively; Table I). Next, the number of Ag-specific T cells in the airway lumen was quantified by tetramer staining and ICCS for IFN-γ. DCAdAg85A immunization i.n. elicited a significant population of CD8$^+$tetramer$^+$ T cells in the airway lumen at 2 wk postimmunization (Fig. 2*A*). This population contracted between 2 and 6 wk postimmunization (from 2.18 × 10^4 cells to 0.48 × 10^4 cells/BAL; >5.5-fold reduction). However, it remained stable up to 12 wk (0.33 × 10^4 cells/BAL; Fig. 2*A*). To understand the functional capacity of such Ag-specific airway luminal T cells elicited by i.n. DC immunization, the number of IFN-γ-producing CD8$^+$ T cells was enumerated by ICCS. The kinetic pattern of IFN-γ-secreting cells mirrored the tetramer$^+$ cells, although the absolute number of IFN-γ-secreting cells was lower than tetramer$^+$ cells. CD8$^+$IFN-γ$^+$ T cell responses peaked at 2 wk (1.62 × 10^4 cells/BAL) declined markedly by 6 wk (0.25 × 10^4 cells/BAL) and further contracted by 12 wk (0.02 × 10^4 cells/BAL) after immunization (Fig. 2*B*). An i.t. CTL was conducted as previously described to further characterize the function of airway luminal CD8$^+$ T cells (14, 22). CD8 T cell-mediated CTL activity was greatest 2 wk (17.1%) after i.n. DC immunization, and low levels of CTL activity could still be detected up to 12 wk (2.5%; Fig. 2*C*). We also determined the level of CD4 T cell activation in the airway lumen following i.n. DC immunization. A significant population of CD4$^+$IFN-γ$^+$ T cells could be detected in the airway at all time points examined (Fig. 2*D*). Similar to CD8$^+$ T cells, the number of CD4$^+$IFN-γ$^+$ T cells peaked at 2 wk postimmunization and declined by 6 wk; only a small population could be detected at 12 wk post-i.n. DCAdAg85A delivery. Compared with T cell responses in the airway lumen, different kinetics of T cell responses were observed in the lung interstitium post-i.n. DC vaccine delivery. Similar numbers of total CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cells were found in the lung interstitium between the two immunization regimens at all time points examined (data not shown). The levels of CD8$^+$tetramer$^+$ cells, IFN-γ-secreting CD8$^+$ T cells and IFN-γ-secreting CD4$^+$ T cells in the lung interstitium peaked at 12, 6, and 6 wk, respectively (Fig. 3). Also, similar to CD4 T cell responses in the airway lumen (Fig. 2*D*),

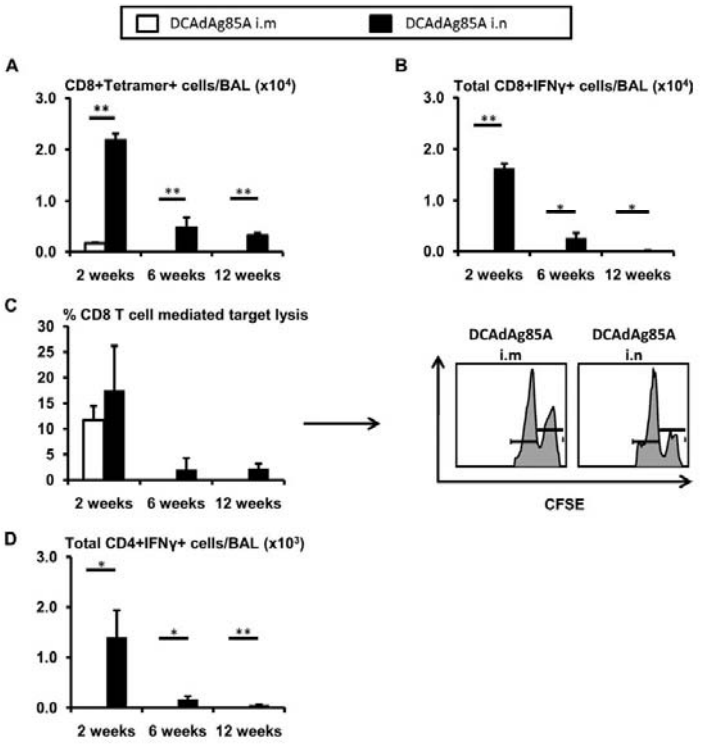

FIGURE 2. T cell responses in the airway lumen following i.m. or i.n. DCAdAg85A immunization. BALB/c mice were immunized and scarified 2, 6, or 12 wk later. Total airway luminal cells were obtained by exhaustive BAL. Cells were stained directly with tetramer (*A*) or cultured in the presence of Ag85A CD8 T cell peptide (*B*) or CD4 T cell peptide (*D*) for 6 h and in the presence of Golgi plug. Cells were then stained and analyzed by flow cytometry. CD8 T cell-mediated i.t. CTL activity (*C*) was measured by transferring CFSE-labeled Ag85A CD8 T cell peptide-loaded target splenocytes to the lungs of vaccinated mice. Lungs were lavaged 6 h later, and the relative loss of CFSE-labeled cells was examined by flow cytometry. The representative histograms of week 2 CTL analysis are presented. Data represent the means ± SEM of six to nine mice per time per group from two to three independent experiments. *, $p < 0.05$; **, $p < 0.01$.

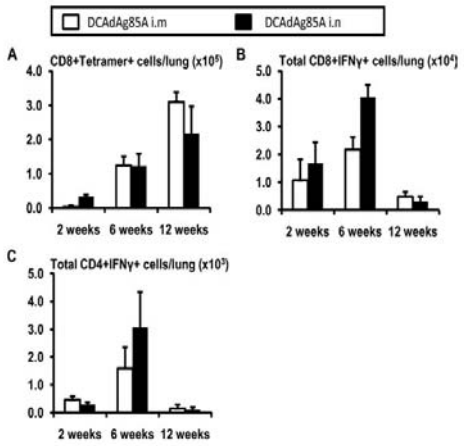

FIGURE 3. T cell responses in the lung following i.m. or i.n. DCAdAg85A immunization. BALB/c mice were immunized and scarified 2, 6, or 12 wk later. Total lung cells were obtained by collagenase digestion and mechanical disruption of lung tissue. Cells were stained directly with tetramer (*A*) or cultured in the presence of Ag85A CD8 T cell peptide (*B*) or CD4 T cell peptide (*C*) for 6 h and in the presence of Golgi plug. Cells were then stained and analyzed by ICCS and flow cytometry. Data represent the means ± SEM of six to nine mice per time per group from two to three independent experiments.

CD4 T cell responses in the lung interstitium almost vanished at 12 wk (Fig. 3*C*).

In contrast to the i.n. route of immunization, i.m. DC delivery resulted in a much smaller population of $CD8^+$tetramer$^+$ T cells in the airway lumen detectable only at 2 wk postimmunization (Fig. 2*A*). Furthermore, there were no detectable $CD8^+$IFN-γ^+ (Fig. 2*B*) or $CD4^+$IFN-γ^+ (Fig. 2*D*) T cells in the airway lumen following i.m. DC immunization at any time point examined. Consistent with the small $CD8^+$tetramer$^+$ T cell population in the airway lumen of i.m. DC-immunized mice at 2 wk (0.16×10^4 cells/BAL), there was a modest level of CD8 T cell-mediated CTL detectable only at 2 wk (11.7%) and no further responses could be measured at 6 and 12 wk (Fig. 2*C*). However, in contrast to the absence or minimal numbers of T cells in the airway lumen triggered by i.m. DC delivery, there was a significant presence of Ag-specific T cells in the lung interstitium (Fig. 3). Both the levels and kinetics of T cell responses in lung interstitium by i.m. DC delivery were comparable with those by i.n. DC delivery (Fig. 3). Taken together, these data demonstrate that mucosally delivered vaccine DCs result in much more potent Ag-specific, IFN-γ-producing T cell responses within the airway lumen than parenterally delivered vaccine DCs, but both have a similar capability to generate reservoirs of Ag-specific T cells in the mucosal-associated lung interstitial compartment.

Ag-specific T cell responses in the distant lymphoid organs following i.n. administration of vaccine DCs

It is believed that besides the elicitation of T cell responses at the mucosa, an effective vaccine is also expected to generate Ag-specific T cells in the systemic lymphoid organs to effectively control the systemic dissemination of pathogens from the mucosa as well as to provide a rich peripheral reservoir of Ag-specific T cells that may be mobilized to the mucosal site upon mucosal pathogen exposure. For these reasons, we also examined the level of systemic T cell responses following i.n. DC immunization. We first quantified the total $CD4^+$ and $CD8^+$ T cells in the spleen following i.n. and i.m. DCAdAg85A vaccination (Table II). Compared with i.n. DC delivery, i.m. DC immunization resulted in more total $CD4^+$ and $CD8^+$ T cells at both 2 and 6 wk but similar T cell numbers in the spleen at 12 wk (Table II). We then quantified the number of Ag-specific T cells in the spleen following i.n. DC immunization. A population of $CD8^+$tetramer$^+$ T cells were detected in the spleen at 2 wk and steadily increased in numbers from 2 to 6 wk and maintained between 6 and 12 wk (Fig. 4*A*). Ag-specific $CD8^+$IFN-γ^+ T cells were also readily detectable in the spleen of i.n. DC-immunized mice at various time points following immunization (Fig. 4*B*). We further assessed CD8 T cell-mediated CTL activity in the spleens of i.n. DC immunized mice by using a systemic in vivo CTL assay (14, 22). Low levels of CD8 CTL were measurable in the spleens by 2 wk which steadily increased between 2 and 12 wk post-i.n. immunization (Fig. 4*C*). We also observed a relatively small yet stable population of Ag-specific $CD4^+$IFN-γ^+ T cells in the spleen at all time points examined (Fig. 4*D*) and similar low levels of CD4 T cell-mediated CTL activity in the spleen (data not shown).

Compared with i.n. mucosal immunization, parenteral i.m. DC immunization was expected to result in a greater level of T cell activation in the spleen. Indeed, the number of $CD8^+$tetramer$^+$ and $CD8^+$IFN-γ^+ T cells in the spleen of i.m. DC-immunized mice was in general higher than in i.n. DC-immunized mice, although the number of tetramer$^+$ T cells was similar at 12 wk (Fig. 4, *A* and *B*). This was associated with significantly higher levels of CD8 T cell-mediated CTL in the spleen, particularly at 2 and 6 wk (Fig. 4*C*). In contrast, the magnitude of CD4 T cell responses in the spleens of i.m. and i.n. DC-immunized mice was similar (Fig. 4*D*). The level of CD4 T cell-mediated CTL activity was also similar in the spleen of both i.m. and i.n. DC-immunized mice (data not shown). Together, these data suggest that although not as potent as parenteral delivery, respiratory mucosal DC immunization could lead to substantial CD4 and CD8 T cell responses in the systemic lymphoid organs in addition to its unique strength in eliciting intra-airway luminal T cell responses.

Enhanced local and systemic immune protection from pulmonary bacterial challenge by i.n. administration of vaccine DCs

To establish the relevance of improved T cell responses by i.n. mucosal DC immunization, we assessed the immune protective

Table II. *Total $CD4^+$ and $CD8^+$ T cells in the spleen following i.m. or i.n. delivery of vaccine DCAdAg85A*[a]

	$CD4^+$			$CD8^+$		
	2 wk	6 wk	12 wk	2 wk	6 wk	12 wk
DCAdAg85A i.m.	1335.67 ± 0.83[b]	1390.07 ± 34.05	1680.05 ± 13.38	1669.79 ± 185.97	1699.04 ± 11.53	1063.54 ± 11.53
DCAdAg85A i.n.	1144.54 ± 6.62	1124.31 ± 10.02	1731.49 ± 13.08	1279.48 ± 10.04	1540.53 ± 3.93	1234.56 ± 14.37

[a] The total numbers of $CD4^+$ and $CD8^+$ T cells, gated on $CD3^+$ T cells in the spleen, were quantified by FACS. Data are expressed as the average number ± SEM of six to nine mice per time per group.
[b] All numbers are $\times 10^4$.

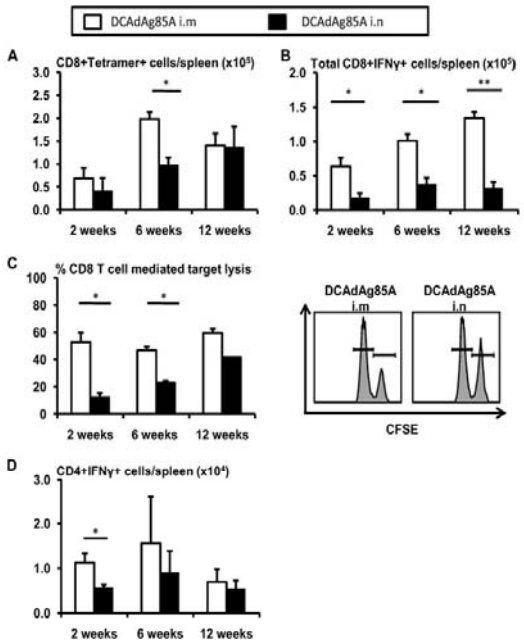

FIGURE 4. T cell responses in the spleen following i.m. or i.n. DCAdAg85A immunization. BALB/c mice were immunized and sacrificed 2, 6, or 12 wk later. Whole splenocytes were processed to single-cell suspensions. Cells were stained directly with tetramer (*A*) or cultured in the presence of Ag85A CD8 T cell peptide (*B*) or CD4 T cell peptide (*D*) and Golgi plug for 6 h. Cells were then stained and analyzed by ICCS and flow cytometry. Systemic CTL responses were measured by i.v. delivery of CFSE-labeled, Ag85A CD8 T cell peptide-pulsed splenocytes (*C*). The representative histograms of week 2 CD8 CTL analysis are presented. Mice were sacrificed 6 h later, and the splenocytes were analyzed for the relative loss of CFSE-labeled cells. Data represent the means ± SEM of six to nine mice per group from two to three independent experiments. *, $p < 0.05$; **, $p < 0.01$.

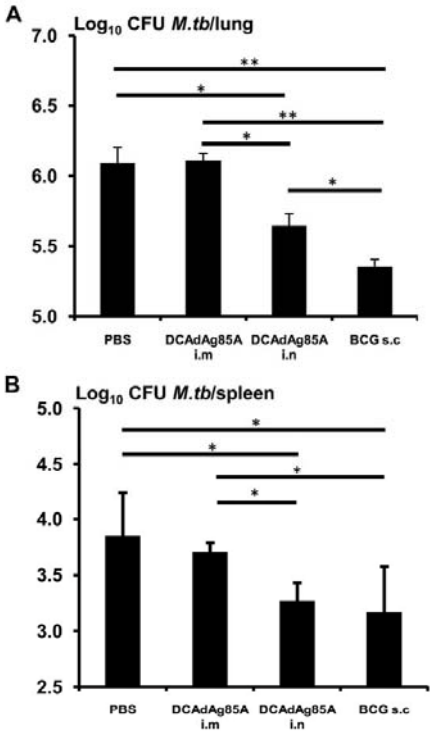

FIGURE 5. Immune protection against pulmonary *M. tuberculosis* (*M.tb*) challenge by i.n. delivery of vaccine DCs. Mice were immunized with DCAdAg85A i.n. or i.m. or with BCG s.c. (positive control) or PBS (naive control). Mice were challenged i.n. with virulent *M. tuberculosis* 6 wk postimmunization. Four weeks after challenge, mice were sacrificed, and the level of *M. tuberculosis* infection in the lung (*A*) and spleen (*B*) was enumerated by colony-forming assay. Data represent the means ± SEM of three to five mice per group. *, $p < 0.05$; **, $p < 0.001$.

capacity by i.n. DC immunization and compared this with i.m. parenteral DC immunization. Because the model microbial Ag expressed by adenoviral vector-transduced vaccine DCs in this study is an immunogenic *M. tuberculosis* Ag, the i.n. or i.m. DC-immunized mice were challenged with virulent *M. tuberculosis*. Compared with naive controls, i.m. DC immunization provided little protection against pulmonary pathogenic challenge as measured by bacterial colony assay in the lung and spleen (Fig. 5*A*). In contrast, i.n. DC immunization provided an enhanced level (half-log) of protection against challenge, although it does not appear to be as potent as BCG immunization, a standard positive control set up in parallel in this study (Fig. 5*A*). Furthermore, i.n. DC immunization also led to an enhanced level of systemic immune protection in the spleen, and this level of protection was comparable with that by BCG control (Fig. 5*B*). These data demonstrate that the Ag-specific T cell responses elicited by mucosally administered, gene-modified vaccine DCs can lead to enhanced host defense both at local and systemic tissue compartments.

Repeatability of respiratory mucosal delivery of vaccine DCs

Repeated systemic delivery of vaccine DCs has been attempted for cancer immunotherapy with some success in activating tumor-associated Ag-specific T cells (7, 23). Here, after having investigated the immunogenicity and protective efficacy of a single mucosal DC delivery, we examined the possibility of repeated administrations of vaccine DCs by using a homologous prime-boost immunization regimen. To this end, mice were immunized either i.n. or i.m. with vaccine DCs as described above. After 2 wk, mice were boosted i.n. with the same vaccine DCs. Four weeks after i.n. boosting, mice were sacrificed and the number of tetramer$^+$ CD8$^+$ T cells in the airway lumen and spleen was quantified by flow cytometry. DC boosting i.n. of i.m. or i.n. primed mice resulted in significant expansion of the number of tetramer$^+$ CD8 T cells in the airway lumen, and such enhanced levels of tetramer$^+$ CD8 T cell responses were comparable in i.m. and i.n. primed mice. However, i.n. DC boosting did not significantly increase the number of CD8$^+$IFN-γ^+ T cells in the airway lumen of i.m. primed mice, and it even decreased such cells in i.n. primed mice (Fig. 6*B*). In comparison, i.n. DC boosting increased CD4$^+$IFN-γ^+ cells in the airway lumen of either i.m.- or i.n.-primed mice (Fig. 6*C*). In the lung interstitium, i.n. DC boosting did not increase tetramer$^+$ CD8 T cells (Fig. 6*D*) and as in the airway lumen, it led to significantly lower numbers of CD8$^+$IFN-γ^+ cells (Fig. 6*E*), whereas it had little enhancing effect on CD4$^+$IFN-γ^+ cells (Fig. 6*F*). In the spleen, whereas i.n. DC boosting substantially increased tetramer$^+$ CD8$^+$ T cells above the levels generated by either i.m. or i.n.

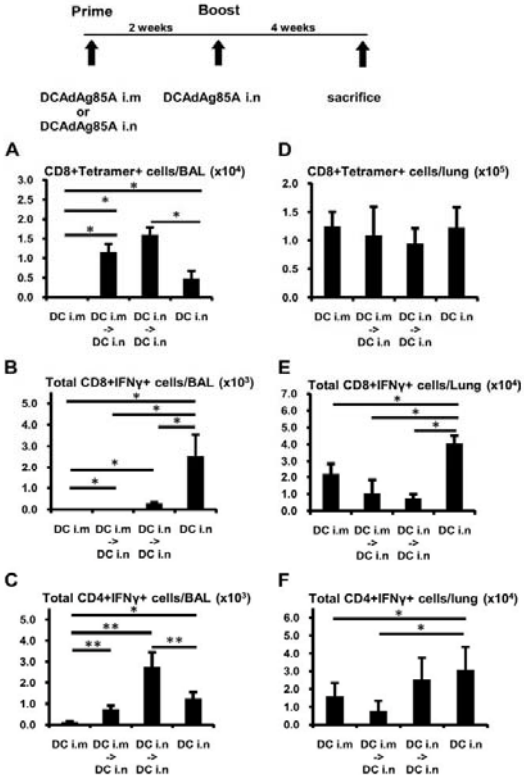

FIGURE 6. Expansion of Ag-specific T cells by repeated homologous DC immunization. Mice were immunized either i.n. or i.m. with DCAdAg85A; 2 wk later, some of the mice were boosted i.n. with DCAdAg85A. Total airway luminal cells were obtained by exhaustive BAL, and lung interstitial cells were digested with collagenase before mechanical disruption to single-cell suspensions. BAL (*A*) and lung interstitial cells (*D*) were stained directly with tetramer or cultured in the presence of Ag85A CD8 T cell peptide (*B* and *E*, respectively) or CD4 T cell peptide (*C* and *F*, respectively) and Golgi plug for 6 h. Cells were then stained and analyzed by ICCS and flow cytometry. Mice boosted with DCAdAg85A were compared with single immunization controls. Data represent the mean value ± SEM of three to five mice per group from two independent experiments. *, $p < 0.05$; **, $p < 0.01$.

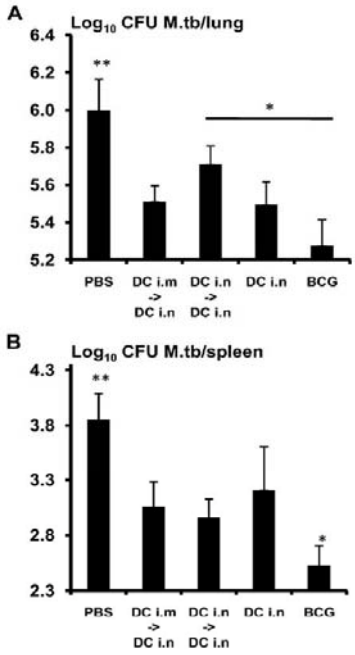

FIGURE 7. Immune protection against pulmonary *M. tuberculosis* (*M.tb*) challenge by single i.n. DCAdAg85A immunization or by DCAdAg85A prime-boost immunization. Mice were immunized either i.m. or i.n. with DCAdAg85A; 2 wk later, some of these mice were boosted i.n. with DCAdAg85A. Additional mice were set up as controls with BCG s.c. or PBS at the time of priming. Mice were challenged i.n. with virulent *M. tuberculosis* 4 wk postimmunization. Four weeks after challenge, mice were sacrificed, and the level of *M. tuberculosis* infection in the lung (*A*) and spleen (*B*) was determined by colony-forming assay. Data represent the means ± SEM of five to six mice per group. *, $p < 0.05$; **, $p < 0.001$ compared with all other groups.

prime immunization, it led to decreased $CD8^+IFN\text{-}\gamma^+$ and $CD4^+IFN\text{-}\gamma^+$ T cells (data not shown).

To determine whether repeated respiratory mucosal DC immunizations may further enhance the level of protection generated by a single mucosal immunization, mice were prime-boosted as described above and challenged with *M. tuberculosis* at 4 wk post-DC boosting (Fig. 6). In consistency with the findings described (Fig. 5), single i.n. DCAdAg85A immunization provided significantly enhanced protection against *M. tuberculosis* challenge (Fig. 7; DC i.n.) compared with naive controls. Furthermore, i.n. DC boosting significantly improved the protection offered by parenteral i.m. DC priming (Fig. 7; DC i.m>DC i.n.). However, i.n. DC boosting did not further markedly enhance the level of protection over that by single i.n. DC immunization (DC i.n. > DC i.n.). These data together suggest that although repeated respiratory mucosal delivery of vaccine DCs may enhance microbial Ag-specific $tetramer^+$ CD8 T cells in the airway lumen, they cannot further enhance the activation and protective potential of these CD8 T cells.

Role of endogenous DCs in T cell activation by mucosally delivered vaccine DCs

Experimental evidence has suggested that endogenous DCs may be required for optimal T cell, particularly CD4 T cell, activation following the systemic delivery of vaccine DCs (24). Whether this may be the case for mucosally delivered vaccine DC-mediated $CD4^+$ T cell activation still remains unclear. To address this question, we first examined whether AdAg85A-infected vaccine DCs could release the soluble form of Ag85A protein, considering that soluble microbial Ags could easily be picked up by endogenous DCs which may then activate T cells. We found that both cell-associated and soluble Ag85A protein could be detected by Western blotting as early as 24 h after AdAg85A infection of DCs (Fig. 8*A*) and as late as 7 days (data not shown). Because both cell-associated and -secreted forms of Ag85A could be detected from the cultures of vaccine DCs, we sought to examine whether endogenous APCs could be involved in vaccine DC-mediated $CD8^+$ and $CD4^+$ T cell activation. To address this, C57BL/6 background MHCI-deficient or MHCII-deficient DCs were prepared, infected with AdAg85A, and used to immunize naive wild-type (wt) C57BL/6 mice i.n.. In this case, if the endogenous APCs were significantly involved in T cell activation, we would still expect to

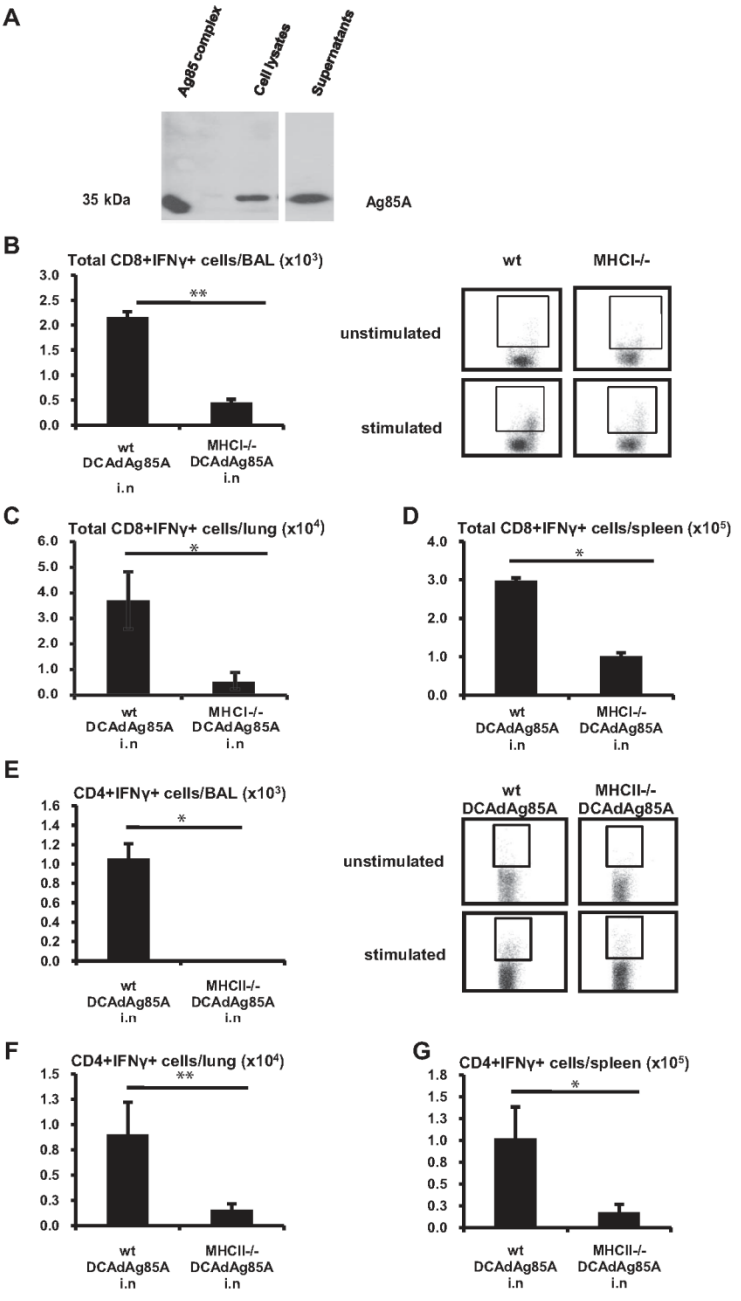

FIGURE 8. Impaired $CD8^+$ and $CD4^+$ T cell activation by i.n. immunization with MHC-deficient vaccine DCs. DCs were infected with AdAg85A for 24 h, and both cell-associated and soluble Ag85A proteins were detected by Western blotting (*A*). Purified Ag85 complex protein was run as a positive control in Western blot. In separate experiments, AdAg85A-infected wt and MHCI or $MHCII^{-/-}$ vaccine DCs were delivered i.n. to wt C57BL/6 mice, and mice were sacrificed 2 wk later. Total airway luminal cells (*B* and *E*) were obtained by exhaustive BAL, whole-lung interstitial cells were obtained by collagenase digestion and mechanical disruption to single-cell suspensions (*C* and *F*), and whole spleen cells were processed to single-cell suspensions (*D* and *G*). Cells were cultured with *M. tuberculosis* Ags for 12 h followed by an additional 6 h in the presence of Golgi plug and then immunostained for ICCS. Ag-specific IFN-γ-producing CD4 and CD8 T cells were analyzed by flow cytometry. The representative histograms of $CD8^+IFN\text{-}\gamma^+$ (*B*) and $CD4^+IFN\text{-}\gamma^+$ (*E*) T cell responses in BAL are also displayed. Data represent the means ± SEM of seven to nine mice per group from two independent experiments. *, $p < 0.01$; **, $p < 0.001$.

see unchanged CD8 and CD4 T cell activation, respectively. To first examine the role of endogenous DCs in CD8 T cell activation, MHCI-deficient DC vaccine was delivered i.n. to naive wt mice. Compared with wt DC vaccination, CD8 T cell activation by MHCI-deficient DC vaccination was severely impaired as we observed sharply reduced numbers of $CD8^+IFN\text{-}\gamma^+$ T cells in both the airway lumen (Fig. 8*B*) and lung interstitium (Fig. 8*C*) of these mice. The number of such Ag-specific activated CD8 T cells was also significantly reduced in the spleen (Fig. 8*D*). In contrast, the numbers of $CD4^+IFN\text{-}\gamma^+$ T cells in all tissue compartments of $MHCI^{-/-}$ DC-immunized mice was comparable with those in wt DC-immunized mice (Table III). We next examined the role of endogenous DCs in CD4 T cell activation following i.n. delivery of MHCII-deficient DC vaccine. Similar to impaired immunogenicity by MHCI-deficient DC vaccination, we observed no detectable $CD4^+IFN\text{-}\gamma$-producing T cells in the airway lumen of wt

Table III. *Comparison of $CD4^+$ IFN-γ- and $CD8^+$ IFN-γ-producing T cell responses following i.n. immunization with wt, $MHCI^{-/-}$, or $MHCII^{-/-}$ DCAdAg85A*[a]

Type of Immunization	$CD4^+$ BAL	$CD4^+$ Lung	$CD4^+$ Spleen	Type of Immunization	$CD8^+$ BAL	$CD8^+$ Lung	$CD8^+$ Spleen
wt DCAdAg85A	0.149 ± 0.018[b]	2.055 ± 0.429	23.576 ± 11.631	wt DCAdAg85A	0.0926 ± 0.047	1.451 ± 0.784	23.576 ± 11.631
$MHCI^{-/-}$ DCAdAg85A	0.2185 ± 0.031	3.309 ± 0.992	24.610 ± 8.775	$MHCII^{-/-}$ DCAdAg85A	0.0477 ± 0.002	1.161 ± 0.481	14.554 ± 7.082

[a] At 2 wk after DCAdAg85A immunization, BAL, lung, and spleen cells were isolated, stimulated, and analyzed by ICCS and FACS. Data are expressed as the average number ± SEM of four to five mice per group.
[b] All numbers are $\times 10^6$.

mice immunized i.n. with MHCII-deficient vaccine DCs, in sharp contrast to enhanced CD4 T cell responses by wt DC vaccination (Fig. 8*E*). Similarly, few $CD4^+$IFN-γ^+ T cells could be detected in the lung interstitium (Fig. 8*F*) and in the spleen (Fig. 8*G*). However, the numbers of $CD8^+$IFN-γ-producing T cells in all examined tissue sites of these mice remained comparable with those by wt DC vaccination (Table III). These data indicate that mucosally delivered wt vaccine DCs can directly activate both Ag-specific CD8 and CD4 T cells by and large independently of endogenous DCs.

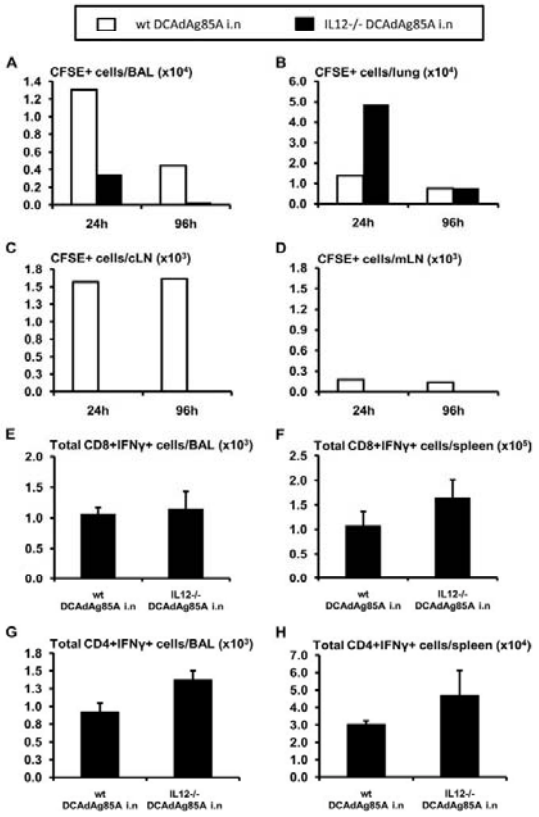

FIGURE 9. Immune activation by $IL\text{-}12^{-/-}$ vaccine DCs. wt C57BL/6 and $IL12^{-/-}$ DCs were transfected with AdAg85A and CFSE labeled. Vaccine DCs were delivered i.n. to wt C57BL/6 mice. At 24 and 96 h, mice were sacrificed and the BAL (*A*), lung interstitium (*B*), cLN (*C*), and mLN (*D*) were examined by flow cytometry for $CFSE^+CD11c^+$ DCs. Data represent the means ± SEM of two mice per group. In separate experiments, mice immunized with wt or $IL\text{-}12^{-/-}$ DCAdAg85A were sacrificed 2 wk after i.n. immunization. Total airway luminal cells were obtained by exhaustive BAL (*E* and *G*), and whole spleens were processed to single-cell suspensions (*F* and *H*). Cells were cultured in the presence of *M. tuberculosis* Ags for 12 h, followed by an additional 6 h with Golgi Plug, and immunostained for ICCS and $CD8^+$IFN-γ^+ and $CD4^+$IFN-γ^+ T cells were analyzed by flow cytometry. Data represent the means ± SEM of three to six mice per group.

Role of type 1 cytokine IL-12 in vaccine DC migration and T cell activation

Little is known about the role of vaccine DC-derived cytokines in T cell activation. However, endogenous APC-derived IL-12 has recently been shown to be critically involved in APC migration and type 1 T cell activation (25, 26); alternatively, vaccine DCs engineered to overexpress IL-12 was found to be more immunogenic to $CD4^+$ T cell activation (27). We have previously shown that virally transduced vaccine DCs displayed increased amounts of IL-12 (11). To investigate whether vaccine DC-derived IL-12 was required for in vivo T cell activation, DCs deficient in IL-12 ($IL\text{-}12^{-/-}$ DCs) isolated from C57BL/6 $IL\text{-}12^{-/-}$ mice were transduced with AdAg85A and compared with wt C57BL/6 DCs for their transmigration to the local LNs and their ability to subsequently activate CD4 and CD8 T cells following i.n. administration to naive C57BL/6 mice. $IL\text{-}12^{-/-}$ DCs showed no impairment in the ability to up-regulate MHCII and costimulatory B7 molecules as well as to produce proinflammatory cytokine TNF-α (data not shown). In agreement with the data by Khader et al. (25), we found that whereas similar to wt controls, $IL\text{-}12^{-/-}$ vaccine DCs could readily be detected in the airway and lung interstitium (Fig. 9, *A* and *B*), these cells in stark contrast to their wt counterparts, failed to migrate to the local LNs draining the nasal mucosa (cLN) and the lung (mLN; Fig. 9, *C* and *D*). Moreover, no $CFSE^+$ vaccine DCs could be detected in the distal lymphoid organs including the spleen and pLNs at any time (data not shown). In light of these findings, we further examined the ability of $IL\text{-}12^{-/-}$ DCAdAg85A to prime naive T cells in vivo. Thus, 2 wk after i.n. delivery, mice were sacrificed, and the number of Ag-specific IFN-γ-secreting $CD8^+$ and $CD4^+$ T cells in the BAL and spleen was quantified by ICCS. To our surprise, similar to wt controls, $IL12^{-/-}$ vaccine DCs activated Ag-specific $CD8^+$IFN-γ^+ and $CD4^+$IFN-γ^+ T cells in both the airway lumen (Fig. 9, *E–G*) and the spleen (Fig. 9, *F–H*). Similar numbers of Ag-specific $CD8^+$ and $CD4^+$ T cells were also detected in the lung and draining LNs (data not shown). These data suggest that although mucosally delivered IL-12-deficient vaccine DCs demonstrate an impaired ability to migrate to the draining LNs, the local microenvironment is still permissible for them to prime Ag-specific $CD8^+$ and $CD4^+$ T cell responses.

Discussion

A plethora of studies have investigated the efficacy of in vitro/ex vivo manipulated DCs as a platform to induce antimicrobial disease immunity. Many of these studies used parenteral immunization strategies to generate high levels of immune activation in the systemic compartment. However, these strategies cannot elicit effective protection against mucosal pathogen challenge. This has led to the investigation of mucosal DC vaccination as a strategy to prime local responses to confer protection against pathogenic challenge at the mucosal sites such as the lung. Only a very limited number of studies have examined the profile of immune activation following i.n. DC immunization and reported only modest protective responses. In this regard, DCs infected in vitro with BCG activated T cell responses in the lung, but this level of immune activation was protective only within the short term (17). Although plasmid DNA-transduced DCs activated IFN-γ responses following mucosal DC immunization, neither the cellular source of the IFN-γ nor the kinetics of immune activation was examined (8, 19). To date, there has been a lack of comprehensive understanding of the immune activation and mechanisms at mucosal surfaces following DC immunization. The general lack of understanding of the mechanisms involved in DC immunization hampers further vaccine development and translation of anti-microbial DC immunization strategies from bench to bedside.

In this study, we addressed the outstanding issues in the field of mucosal DC vaccination including vaccine formulation, localization, kinetic T cell activation, protection, and mechanisms of immune activation. In addition, for the first time we conducted a direct comparison of parenteral and mucosal DC immunization strategies. The DCs virally transduced with a recombinant adenovirus expressing an *M. tuberculosis* Ag, which has previously been demonstrated to elicit superior immune responses over the peptide- or protein-pulsed counterparts (11, 28), is used as a model vaccine for immunization against an invasive intracellular pathogen. It has been known that resistance to many respiratory pathogens requires strong type 1 polarized immune responses characterized by IFN-γ and CTL responses in the airway lumen. Therefore, to profile the immunity and protective efficacy of i.n. and i.m. DC immunization, the immune responses elicited in the airway lumen, lung, and spleen by both parenteral and mucosal vaccination strategies were directly compared. DC immunization i.n. effectively elicited the sustained $CD4^+$ and $CD8^+$ T cell populations capable of IFN-γ and CTL responses in the airway lumen. Parenteral immunization primed a very small and short-lived CD8 T cell population in the airway lumen with only low CD8 CTL activities. Both i.n. and i.m. DC immunization activated $CD4^+$ and $CD8^+$ T cell responses in the spleen although i.m. immunization did tend to prime higher levels of T cell activation at this site than i.n. immunization. Associated with the higher magnitude of T cell responses within the respiratory mucosa by i.n. immunization was enhanced host defense against intracellular bacterial challenge, in contrast to the lack of local protection in the lung by parenteral immunization despite slightly improved protection in the spleen. Our study thus provides the first evidence that respiratory mucosal DC immunization results in effective mucosal T cell responses and improved host resistance against an intracellular pathogen. We admit that compared with 0.7- to 0.8-log reduction of bacterial burden in the lung by a live organism-based BCG vaccine, a standard control for TB vaccine evaluation used in this study, respiratory mucosal DC immunization led to a smaller reduction in bacterial burden (0.5 log). Although further studies are warranted to improve its efficacy, DC-based vaccine is safer and may be potentially used for immunizing HIV-infected hosts to which BCG vaccine is unsafe.

Considering repeated immunization is a common strategy to boost pre-existing T cell responses, we further evaluated the expansion of Ag-specific T cells in the airway lumen and spleen following either parenteral or mucosal prime-mucosal boost immunization. Mucosal boosting efficiently boosted Ag-specific $tetramer^+$ CD8 T cells as well as IFN-γ-secreting CD4 T cells in the airway lumen after either parenteral or mucosal DC prime immunization. However, we found that mucosal DC boosting either did not further expand the number of IFN-γ-secreting CD8 T cells by i.m. DC priming or even reduce such activated CD8 T cells following i.n. DC priming. A similar phenomenon was also observed in the lung. Thus, the lack of further enhanced CD8 T cell activation by mucosal DC boosting represents a mechanism underlying its inability to further enhance immune protection offered by single mucosal DC immunization. We have recently documented a critical role by airway luminal CD8 T cells in immune protection against respiratory intracellular bacterial pathogens (14, 22). Although at this point, it still remains to be fully understood as to why i.n. DC boosting fails to enhance CD8 T cell activation as judged by Ag-stimulated CD8 T cell IFN-γ responses, it is possible that repeated DC immunization causes a shift of airway luminal CD8 T cells from a predominantly effector phenotype to a memory pool. This speculation is supported by our finding that although DC boosting increases $tetramer^+$ CD8 T cells, it does not enhance or even reduce CD8 IFN-γ producers in the airway lumen. We further observed that repeated DC immunization does not increase the number of regulatory $CD4^+FoxP3^+$ or IL-10-secreting T cells (data not shown). These findings together suggest that many factors regulate secondary T cell responses and that preexisting T cell immunity can modulate the effectiveness of boosting vaccine likely by altering APC Ag expression and T cell-activating capacities (29, 30). In recent years repeated DC immunization strategies have been attempted for cancer immunotherapy with only limited success (31–34). Our current study not only provides mechanistic insights into such DC immunization modality but also highlights the need to further fine tune DC-based vaccination strategies.

Because the immunocompromised conditions, particularly HIV infection, are often associated with seriously diminished DC number and function (35–39), immunization with viral or bacterial vectored vaccines will not be an effective strategy. However, DC-based vaccines may be catered in vitro, before in vivo immunization, to potentially overcome the impaired function of the mucosal immune system in such hosts by delivering target Ags and costimulation signals for T cell activation. Nonetheless, such a strategy may still offer only a limited benefit if the endogenous DCs are heavily involved in vaccine DC-mediated immune activation. On the basis of this consideration, our current study investigated whether virally transduced vaccine DCs were still able to activate $CD8^+$ and $CD4^+$ T cells independently of endogenous APC populations in the lung and draining LNs. We observed that mucosal immunization with MHC-deficient vaccine DCs failed to lead to detectable $CD8^+$ and $CD4^+$ T cell activation. This observation suggests that mucosally delivered vaccine DCs are the primary driver of T cell priming and that endogenous APC populations, even if they do acquire soluble Ags released from wt vaccine DCs or the fragments of apoptotic/necrotic vaccine DCs, play a minor role in T cell priming. That mucosally delivered vaccine DCs activate T cells independently of endogenous APCs contrasts the previously observed significant contribution by endogenous APCs following parenteral DC immunization (23, 24, 40–42).

Following a similar line of clinical consideration, we investigated whether the vaccine DCs with some level of immunodeficiency, i.e., cytokine deficiency, could still go on to activate T cells

after mucosal administration. We found that although the vaccine DCs deficient in type 1 cytokine IL-12 failed to migrate to the local draining LNs, consistent with a previous study (25), surprisingly both $CD8^+$ and $CD4^+$ T cell responses remained unimpaired. As the failure of IL-$12^{-/-}$ DCs to migrate to LNs resulted in impaired T cell activation as previously shown (25), our current study draws a different conclusion that migration of DCs to LNs is not required for T cell activation. Such a discrepancy might be due to the fact that we analyzed directly the activation of endogenous naturally generated T cells whereas adoptively transferred OVA-specific transgenic T cells were analyzed in the previous report (25). The fact that we demonstrated the presence of only a small portion (~1%) of transferred vaccine DCs in LNs, in keeping with our previous study (43) and the presence of a significant portion of these APCs in the lung mucosal tissue suggests that vaccine DC-mediated T cell activation might have occurred in lung mucosal lymphoid tissues outside of the LNs, thus lending further support to the conclusion from some previous studies by others. However, our study for the first time derives such conclusion from a wt host with intact draining LNs, different from the previous studies conducted in the mice lacking draining LNs (44, 45). Furthermore, considering the importance of IL-12 in the development of Th1 development (26, 46), our current finding with IL-$12^{-/-}$ vaccine DCs also suggests that this cytokine may be provided by bystander cells in the respiratory mucosa for T cell activation.

In summary, our study has established that respiratory mucosal delivery of genetically modified vaccine DCs is an effective immunization modality for the generation of antimicrobial T cell immunity. We report critical findings that mucosally delivered vaccine DCs can activate both CD8 and CD4 T cells independent of endogenous DCs and vaccine DC-derived IL-12. These features, together with their efficiency in transmigrating to the draining LNs as well as mucosal tissue, support its great promise for immunization in immunocompromised hosts.

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Disclosures

The authors have no financial conflict of interest.

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Chapter 5: Summary of Major Findings and Discussion

Primary T cell responses must strike a fine balance between eliminating the pathogen and preserving host tissues. The aim of this thesis was to examine the biological consequences of enhanced T cell priming during primary *M.tb* and influenza virus infection. Here, both the positive and negative consequences of enhanced T cell priming were examined.

Enhanced T cell priming enhances host resistance to pulmonary TB

Delayed T cell responses in TB have a negative impact on overall bacterial control. It is widely believed that because the host adaptive response is so slow to prime, bacteria have the opportunity to grow and reach a point where even robust T cell responses cannot efficiently eliminate the bacteria. In Chapter 2, we showed that effector T cell responses were primed by 7 days post infection in DAP12 deficient hosts, days sooner than in wt hosts. The earlier T cell priming in DAP12 deficient mice is due to enhanced antigen presentation in the local draining LNs by APC. Furthermore, APCs from DAP12 deficient mice are hyperactivated in response to mycobacterial infection, they not only express higher levels of MHC and co-stimulatory molecules but also pro-inflammatory cytokines including TNF-α and IL-12. (Chapter 2) (207). One of the theories to explain the slow T cell responses following mycobacterial infection is the lack of inflammation elicited by the bacterium. In line with this theory, we show there is little to no evidence of immune activation in the lungs of wt mice prior to 14 days post infection (Chapter 2) suggesting *M.tb* evades host detection by hiding out in the APC (182, 214) and suppresses immune activation (215,216). Several strategies to accelerate T cells priming have been tested which focus on increasing DC activation. One such approach was to administer an activating TLR ligand 14 days following infection but this approach had little effect on host immune responses. This lead the authors to conclude that T cell responses could not be accelerated by broad spectrum immune activation (215). Based on our observations we now know applying an immune adjuvant 14 days post infection is days too late to alter T cell priming because antigen presentation and T cell activation has already occurred in the LN. Here we provide evidence to contradict the belief that T cell activation cannot be potentiated by showing that enhanced DC activation as seen in DAP12 deficient APCs,

can indeed accelerate T cell priming. It is important to note that these alterations must be made immediately or in the first days following infection, rather than later. These findings highlight the need to better define the factors which limit T cell activation following *M.tb* infection including investigating the direct contribution of the bacterium itself in modulating the host response and developing experimental therapeutics which would accelerate T cell priming following a known exposure.

Another factor that may contribute to poor bacterial control is slow recruitment of effector T cells to the lung. We and others have shown that effector T cell responses are not recruited to the prior to 14 days post infection in wt mice. In contrast, cytokine secreting T cells are found it the lungs of DAP12 deficient mice in low number by 7 days post infection and robustly by 10 days post infection (Chapter 2). We may hypothesize that the increased recruitment of effector T cells into the lung and airway lumen of DAP12 deficient mice is due to enhanced chemokine production in the lung. Indeed lung derived APCs from DAP12 deficient mice are superior cytokine producers and are capable of greater antigen presentation during *M.tb* infection (Chapter 2). These data suggest that enhanced activation of lung resident APCs may also improve bacterial control by recruiting more activated T cells. We have not yet confirmed the increased chemokine levels observed in the BAL fluid contribute directly to increased activated T cell recruitment. This can be easily addressed by carrying out selective depletion of T cell chemokines IP-10, MIG and RANTES, alone and in combination to definitively show that heightened inflammatory responses in the lung during *M.tb* infection can improve survival. This has profound implications when designing vaccination strategies and therapeutic interventions. We and others have shown that vaccines that recruit effector T cells into the lung consistently provide better protection against pulmonary infections than parenterally administered vaccines because effector T cells are readily available at the lung mucosa to efficiently control bacteria upon infection. This lends itself to the idea that local activation of the immune response may stimulate the production of T cell recruiting chemokines, notably IP-10, MIG and RANTES, and result in increased T cells at the site of *M.tb* infection. This can be exploited in a therapeutic vaccination strategy to

recruit protective T cell responses into the lungs of previously vaccinated individuals. Several studies have shown that co-delivery of *M.tb* antigens and TLR ligands results in increased mycobacteria specific T cell responses to the lung and enhanced protection against virulent *M.tb* challenge. In reality, most individuals living in TB endemic countries contract *M.tb* before they reach adulthood making boost vaccine strategies more complicated. It is becoming increasing clear we must develop therapeutic vaccines that will allow us to boost pre-existing immunity and relocate activated T cells to the lung in order to ensure long term control of pulmonary bacteria. This approach will not only protect the individual but will also help to minimize bacterial spread to others. Some approaches are currently under investigation, including a therapeutic vaccination by delivering killed *M.tb* to the lung mucosa in latently infected patients in an attempt to increase the number of *M.tb* specific T cells in lung and boost protective immunity. This approach does boost adaptive immune responses in the lung and improve bacterial control although the mechanism leading to this enhanced control is not well defined (217). Indeed this approach may prove effective in boosting immune responses and relocating effector T cells to the lung mucosa, depositing a large bolus of *M.tb* antigen may also contribute to lung pathology in the long term.

CD4 T cell priming contributes lethal lung immunopathology

Robust T cell activation is generally considered beneficial during pulmonary infection with *M.tb* or influenza virus. On the flip side, excessive T cell responses can have deleterious effects on the host. In Chapter 3 we investigated the consequences of unchecked T cell activation following influenza infection. CD8 T cells are classically considered the major contributor to viral clearance due to their ability to produce cytokine and lyse infected cells. In recent years, we have come to appreciate that CD8 as well as CD4 T cells participate in viral clearance (73, 76, 218). Both CD4 and CD8 T cells produce potent cytotoxic cytokines IFN-γ and TNF-α to combat viral infection. These same cytokines have been linked to development of immunopathology (219-220). Here using DAP12 deficient mice we provide evidence in support of the idea that CD4 T cells are not only a potent source of anti-microbial cytokines but may also play a significant

role in lung injury. In hosts lacking DAP12 there is greater CD4 T cell priming, accumulation of effector T cells in the airways which is associated with severe destruction of the airway epithelial cells and the formation of pus, which untimely leads to death. In light current belief that patients with severe acute respiratory distress following pulmonary viral infection suffer from cytokine storm, we initially hypothesized that that elimination of cytotoxic cytokine IFN-γ would improve the outcome of infection. Surprisingly, cytokine neutralization did not improve survival at all. These findings are in line with the observation that immunosuppressive therapies do not dramatically improve severity of illness in virally infected patients. Instead, we found CD4 T cells from DAP12 deficient mice expressed greater FasL which has been implicated in lung injury in human pulmonary fibrosis. Indeed neutralization of FasL improved not only lung pathology but also overall survival. It is somewhat surprising that increased FasL expressed on activated T cells did not limit the amplitude of the T cell response. Fas mediated killing is well documented to preferentially eliminate Th1 cells. In our model, we still see increased Th1 cells in DAP12 deficient hosts. We have not examined the expression levels of Fas on T cell and as such cannot directly comment if this mechanism is impaired. However, at least some mechanisms that limit the size of the effector pool are intact based on the findings that beyond 9 days post infection DAP12 deficient hosts no longer succumb to infection and the Th1 T cell pool begins to decrease 18 days post infection. In summary, the work in Chapter 3 highlight that CD4 T cells have the potential to cause lung pathology and identify a previously unknown mechanism of lung pathology during influenza infection. These data provide rationale for further investigation into the role of CD4 T cells in human disease and help identify a novel therapeutic option for combating severe respiratory virus illness.

DAP12 regulates APC maturation and T cell priming

APCs play a critical role in initiating an immune response. APCs sense infection through innate pattern recognition receptors. Early experimentation indicated DAP12 amplified inflammation in APCs though it is now clear that DAP12 also plays a critical role in

regulating inflammatory responses. In both live *M.tb* and influenza infection we observed accelerated and heightened T cell responses in DAP12 deficient hosts, and in both models found heightened APC maturation in response to infection. Here we showed that DAP12 deficient APCs are highly sensitive to live replicating pathogenic challenge and are able to secrete pro-inflammatory cytokines in response to lower concentrations of pathogen derived stimuli. We also found that DAP12 deficient APCs responses most strongly to live replicating pathogen. Killed pathogen or TLR ligands alone still stimulated a heightened pro-inflammatory cytokines response from DAP12 deficient APCs however it is not as strikingly elevated compared to live pathogen challenge. These findings suggest DAP12 signaling controls the inflammatory response by raising the threshold necessary to respond to a pathogen. We further show that this enhanced level of APC activation translates into more APCs expressing MHC class II and B7 co-stimulatory molecules in response to both mycobacteria and influenza. DAP12 deficient APCs present more antigen and prime greater T cell responses suggesting not only are DAP12 deficient APCs more activated in response to intracellular infections, but present antigen better than DAP12 competent APCs. The reason behind this increased T cell activation is not yet understood although others have suggested that DAP12 signaling promotes immunoregulatory IL-10 production by APCs, thus limiting T cell activation (213). Furthermore, it has been reported that regulatory T cells are impaired in DAP12 deficient hosts (221). To date, we have not observed any defect in IL-10 production in APC populations or in the number of regulatory T cells nor in immuno-modulatory cytokines. We believe the enhanced T cell priming can be attributed to increased pro-inflammatory cytokine production, most notably IL-12 and TNF-α. IL-12 plays a dual role in DC functionality. IL-12 is required for migration of DCs to the local draining LN as well as T cell priming. Indeed IL-12 deficient hosts have impaired effector T cell responses and mounting evidence suggests that IL-12 levels strongly correlate with enhanced Th1 polarization (222,223). Gene array analysis is immanently required to fully identify genes regulated by DAP12 signaling including receptors and molecules such as integrins, cell

adhesion molecules, cytokine/chemokine receptors and survival genes that may direct affect T cell priming.

Our findings show that abrogation of DAP12 signaling results in accelerated and increased T cell priming and activation in both *M.tb* and influenza infection as a result of increased activation by APCs. It is important to note that T cells primed in the absence of DAP12 become fundamentally overactivated in response to APCs and this level of activation is maintained even when transferred to a DAP12 proficient environment (Chapter 3). This led us to conclude that T cells are fundamentally altered as a result of priming. These findings confirm our previous observation that T cell activation is increased following infection with a live replicating pathogen. This is in direct contrast to other publications showing that T cell priming is impaired in DAP12 deficient hosts. The reason for this discrepancy is not understood. Based on the observations made over the course of experimentation, it is obvious that DAP12 deficient APCs are highly sensitive to live replicating pathogen. Models showing impaired T cell priming in KO animals used parenteral inoculation of killed/fragmented adjuvants in conjunction with peptide antigen. While we do not currently understand why, we believe live replicating pathogen is responsible for these differences.

DC can be manipulated ex vivo to present “your favourite antigen”, express T cell co-stimulatory molecules and even secrete cytokines necessary to polarize T cell responses. This allows us to tailor a vaccine strategy to prime the most appropriate immune response, be it Th1 or Th2 polarized, NK cell biased or Ab mediated, to combat a wide variety of diseases including cancer and infectious disease. A lot of effort has gone into increasing the immunogenicity of DC based vaccines with optimal T cell priming in mind. Viral transduction of DCs results in greater antigen presentation, cytokine production and T cell priming that antigen pulsed DCs (224). Parenteral vaccination with DC based vaccines primed robust T cells in the systemic compartment but fail to elicit protective T cell responses at the mucosa (224). In Chapter 4, using a model of TB infection we explored the feasibility of using ex vivo manipulated DCs to prime T cell responses at the lung mucosa. Delivery of vaccine DCs to the lung primed both CD4 and

CD8 T cell response which provided protection against virulent TB. Migration of vaccine DCs to the local draining LN occurred with 24 hours of delivery. This migration is independent of host factors such as IL-12. Furthermore, vaccine DCs were primarily responsible for T cell priming as opposed to transferring antigen to endogenous DCs. These findings are important because they confirm DC based vaccine strategies can prime T cell responses independently of the host immune response. This is direct contrast to most vaccine strategies where the host must sense foreign antigen, initiate inflammation, acquire and present antigen to T cells. These processes may be severely impaired in some populations including the elderly, the immunocompormized, and those with HIV infection. These individuals have a marked decline in the number and functionality of DCs (225,226). Developing vaccine strategies to stimulate protective memory responses without relying on host APCs is especially important when attempting to boost immune responses in those individuals who are most susceptible to infection.

Virally transduced DC vaccines serve not only as the antigen presenting cell but also provide immune activating cytokines giving them the ability to prime T cell responses. Virally transduced DCs will sense and respond to the viral infection through innate PRRs resulting in the production of T cell polarizing cytokines such as IL-12 in order to preferentially prime Th1 responses. This is in contrast to the mechanism traditional vaccines use to elicit immune responses whereby proteins administered with an adjuvant resulting predominantly in humoral or Ab responses. The most common vaccine adjuvant is alum, which along with the vaccinating antigen, is phagocytosed by macrophages. Because alum conjugated proteins lack the necessary PAMPS to mimic intracellular infection, antigen is almost exclusively presented on MHC class II to CD4 T cells. In the absence of Th1 polarizing PAMPS, T cell responses are very weak or become Th2 polarized. Indeed current vaccine strategies fail to elicit robust T cell responses and instead provide protection through neutralizing antibodies. Here we have shown the potential of DC based vaccines in priming Th1 dominated protective immune responses.

In clinical trials, DC vaccines have been variably effective. Primarily used as a cancer immunotherapy, DC based vaccines are typically pulsed with tumour antigen and can

stimulate modest T cell response and is some instances limit tumour growth (reviewed in ref 227). In many of the studies that report efficacy with DC based vaccines, extensive manipulation of vaccine DCs is required. Lapteva et al. show that introduction of a molecular switch that continually signals through the CD40 in conjunction with TLR4 stimulation generates vaccine APCs that produce greater amounts of IL-12 and were highly effective at priming T cell responses (228,229). This vaccine formulation has successfully stimulated immune response in human patients with metastatic prostate and liver cancer and even stimulated tumour regression in patients with metastatic liver cancer (unpublished data). Similarly, Turnis et al. show that silencing of IRAK-M, a negative regulatory of TLR signaling, results in greater migration of vaccine DCs to the LN, increased IL-12 production and T cell priming leading to enhanced tumour control in mice (230). These approaches to targeted overactivation of vaccine DC may prove beneficial in optimizing T cell priming and enhance the efficacy of therapeutic DC vaccination. In Chapters 2 and 3 we showed that DAP12 negatively regulates T cell priming by limiting the amplitude of APC maturation, controlling the threshold of activation and limiting migration to the LN. Our findings suggest that DAP12 can potentially be targeted to increase the effectiveness of DC based vaccines aimed at priming anti-tumour T cell responses. Experiments carried out in mouse DCs indicate that DAP12 signaling can be effectively inhibited by small interfering RNA technology prior to adoptive transfer into patients (213). This approach is much less labour intensive, more cost effective and safer than other DC based vaccine strategies currently in clinical trial. Based on the collective findings presented in this thesis, we unexpectedly identified DAP12 singling is an under-appreciated negative regulatory pathway that limits APC maturation and subsequent T cell priming. Further investigation into the therapeutic potential of DAP12 in immunotherapy is required.

DC based vaccines also hold promise as a therapeutic option for treating patients with chronic infectious disease such as MDR and XDR TB, Hepatitis and HIV. Effective long term management of all three diseases rely heavily on T cell responses to control the pathogen and prevent pathogen mediated destruction of vital organs. Boosting T cell

responses may increase mean survival as well as limit pathogen replication. Indeed there is evidence that DC based vaccines may be able to boost protective immunity during chronic infection. Vaccination of humans with chronic Hepatitis B infection with antigen pulsed DCs resulted in decreased viral burden (231). The goal of DC vaccination in TB infected individuals is 2-fold. First, mucosal vaccination will recruit *M.tb* specific T cells to the lung and second, vaccination may boost T cell responses, together improving bacterial control. DC based vaccines are more effective than subunit vaccines and are believed to be safer than live vaccines in those with a pre-existing infection.

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Appendix I:

Review article: Pulmonary mucosal dendritic cells in T cell activation: implications for TB therapy

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Perspective

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EXPERT REVIEWS

Pulmonary mucosal dendritic cells in T-cell activation: implications for TB therapy

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Mycobacterium tuberculosis, the causative agent of pulmonary TB, causes chronic intracellular infection of lung-resident antigen-presenting cells, including macrophages and dendritic cells (DCs). Life-long bacterial control requires robust T-cell immune responses. Lung DCs are critical for initiating and co ordinating adaptive immune responses against TB. The recent description of previously uncharacterized DC subsets has prompted the re-examination of lung DCs and their role in priming antimycobacterial T-cell responses. While there is some data on these new DCs in their naive state, very little is known about how these DCs respond to pulmonary mycobacterial infection. In this article, we attempt to identify the major antigen-presenting cell subsets that may be critical to lymph node homing, T-cell priming and controlling mycobacterial infection. We further examine the areas of DC heterogeneity that may relate to differential susceptibility between mouse strains. Furthermore, we discuss how DCs may be manipulated and exploited as a cell-based prophylactic TB vaccine and the prospect of using this strategy for post-M. tuberculosis exposure settings.

KEYWORDS: alveolar macrophages • DC vaccines • dendritic cells • ex vivo manipulated DC • lung • migration • Mycobacterium tuberculosis • T cells • tuberculosis

Mycobacterium tuberculosis infects one-third of the world's population, which leads to an estimated 9 million new clinical cases of TB and 2 million deaths every year. *M. tuberculosis* lodges in the lung mucosa via aerosols. Once in the lung, *M. tuberculosis* establishes persistent intracellular infection where it evades degradation by innate immune cells. Robust $CD4^+$, and to a lesser extent $CD8^+$, T-cell responses are essential for limiting bacterial replication and spread by secreting IFN-γ required to drive macrophage activation and nitric oxide production. Lung dendritic cells (DCs) are critical to T-cell priming and effective adaptive immune responses in the tissue. While most individuals are vaccinated at infancy against TB, the current vaccine is only effective in preventing disseminated forms of childhood disease, and often an individual living in a TB-endemic area is latently infected by adulthood. In the face of increasing challenges, such as drug-resistant strains of TB and the HIV–TB co-epidemic, new vaccine and therapeutic strategies are needed for effective control of the TB epidemic. Research to date has been more focused on effector T-cell responses and much remains to be understood about the pulmonary DC subsets that drive T-cell activation and mediate local immune responses against TB. Expanding our understanding of lung DCs and their function is critical for improving anti-TB vaccine efficacy and, one day, therapeutic intervention strategies.

Pulmonary DC subsets in the naive lung

Our understanding of lung DCs is limited by the thinking that DCs are only one type of cells possessing uniform phenotypes and functions. Our knowledge of DC biology is primarily derived from studying DCs in the secondary lymphoid organs, such as the spleen, which are phenotypically and functionally different from tissue DCs. The respiratory mucosa is home to a complex network of DCs and macrophages that balance immune activation and tolerance. Splenic DCs are derived from blood-borne precursor cells that differentiate in the lymphoid tissues into a uniform population of resident, nonmigrating cells and such DCs can be divided into two groups based on their ability to cross-prime T cells [1,2]. However, such a simplistic classification is not suitable for the DCs in the lung owing to the variety of functions the lung must perform.

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To appreciate the spectrum of lung antigen-presenting cells (APCs), we must first consider the anatomy of the lung. The lung mucosa can be divided into two compartments: the airway lumen and the lung parenchyma. Airway luminal cells are the first to encounter the inhaled antigen or a pathogen. Two major types of cells can be found in the naive airway lumen: the alveolar macrophage (AM) and the alveolar DC (ADC). The naive airway is predominantly populated by the AM, comprising more than 95% of all cells recovered in the bronchoalveolar lavage (BAL). AMs in the naive steady state are myeloid-derived cells identified as $CD11c^{high}CD11b^{-}MHCII^{-/low}F4/80^{+/low}$ [3–6]. The AM is a front-line 'sentry' cell, acquiring antigens and/or pathogens in the airway lumen rapidly, generally within hours of infection, causing the AM to upregulate expression of CD11b and secrete pro-inflammatory cytokines such as TNF-α, effectively sounding the immunological alarm [7–9]. AMs are poor at priming naive T cells *in vivo*, although they are adept at activating antigen-experienced T cells [7]. Despite being a minor population in the naive BAL (<5% of total cells), ADCs are important for initiating adaptive T-cell responses in the lung. Naive ADCs have an immature phenotype of $CD11c^{high}CD11b^{-}MHCII^{high}$, only distinguishable from the AMs by MHC expression levels, and do not emit autofluorescence (as measured in the FL1 channel by flow cytometry) (Table 1) [4,6,10,11]. ADCs have the ability to acquire antigen in the airway lumen, although they do not take up antigen as readily as AMs. Upon exposure to inflammatory stimuli, ADCs will rapidly upregulate MHCII, costimulatory molecules, lymph node (LN) homing receptors and integrins, acquiring the ability to migrate to the T-cell zones in the local LN. ADCs are superior to AMs at naive T-cell priming in part due to the level of MHC expression and costimulatory molecules [10]. AMs are thought to be incapable of migrating to the LN [9], although in recent years this dogma is being challenged [12]. The exact mechanism through which the ADC manages to migrate out of the airway lumen to the draining LN remains poorly understood.

Airway APC populations are the first to encounter inhaled antigen and are critical to initiate early defences, however, many pathogens such as *M. tuberculosis* have evolved mechanisms to breach the epithelial barrier and invade the parenchyma. In this case, tissue APCs become equally important in initiating an adaptive immune response. Parenchymal DCs are more heterogeneous and can be divided into two categories based upon their basic function: conventional DCs (cDCs) are primarily involved in priming and co-ordinating T-cell responses, and plasmacytoid DCs, which are major sources of type 1 interferons during viral infections (Table 1). The old nomenclature of 'myeloid' and 'lymphoid' DCs are no longer appropriate when describing tissue DCs. cDCs are the most abundant in the lung parenchyma, which comprise the intraepithelial DC (iDC) located within the airway epithelium. This type of cDC was initially described in bronchial tissues back in the mid-1980s [13,14] and more recently identified to be interdigitated with the epithelial layer of the conducting airways. iDCs can be isolated from lung tissues by enzymatic digestion and have been broadly described as $CD11c^{high}CD11b^{-}MHCII^{high}CD103^{+}$ (Table 1). Sung *et al.* describe that these cells have the unique feature of expressing langerins and claudins, the molecules that form the tight junction between cells. The location and expression of tight junction proteins allow iDCs to open mucosal epithelial cell tight junctions, and extend dendrites into the airway lumen to continually sample the airway luminal environment [3,15]. Owing to the vast epithelial surface in the lung, these cells make up 40–60% of all pulmonary DCs. iDCs are adept at acquiring antigen and express pathogen-sensing Toll-like receptors (TLRs). Furthermore, these DCs are potent at inducing transgenic T-cell proliferation and secrete high levels of IL-12, making them highly efficient at Th1 and Th17 priming [3,15–17]. In light of their phenotype, most notably CD103 (integrin $\alpha_E\beta_7$) expression, which is believed to be involved in LN homing, iDCs are the most likely parenchymal DCs to migrate to the local LN upon sensing pathogenic infection. The chemokine receptor expression status of iDCs following pulmonary infection is as yet undefined. Immediately beneath the mucosal layer of the major conducting airways is a population of submucosal $CD11c^{high}CD11b^{high}CD103^{-}$ cDCs, now termed $CD11b^{+}$ DCs (Table 1). In lieu of CC chemokine receptor (CCR)7, these DCs express SIRPα, a molecule that binds CD47 to drive migration [18,19]. $CD11b^{+}$ DCs are less efficient at antigen acquisition, although

Table 1. Summary of major lung antigen-presenting subsets.

Cell type	Phenotype	Migration	T-cell priming	T-cell activation	Antimycobacterial function
Alveolar					
AM	$CD11c^{high}CD11b^{-}MHC^{-/low}F4/80^{+/low}AF^{high}$	+/?	±	++	++
ADC	$CD11c^{high}CD11b^{-}MHC^{high}AF^{-}$	?	+++	+++	±
Parenchymal					
iDC	$CD11c^{high}CD11b^{-}MHCII^{high}CD103^{+}$	+++ (CCR7)	++	++	±
$CD11b^{+}$ DC	$CD11c^{high}CD11b^{high}CD103^{-/low}$	+ (SIRPα)	?	+++	?
PM	$CD11c^{high}CD11b^{int}MHC^{low}CD103^{low}AF^{high}$	?	?	?	+++
pDC	$CD11c^{low}CD11b^{-}Gr1^{+}B220^{+}PDCA\text{-}1^{+}$	+	-	-	?

+/?: Low levels of activities or controversial data; ±: Weak activities; ++: Medium activities; +++: High activities; -: Lack of such activities; ?: No data available.
ADC: Alveolar dendritic cell; AF: Autofluorescence; AM: Alveolar macrophage; CCR: CC chemokine receptor; iDC: Intraepithelial dendritic cell; pDC: Plasmacytoid dendritic cell; PM: Pulmonary macrophage.

they are potent sources of proinflammatory cytokine production and are superior at driving transgenic T-cell proliferation, although interestingly enough are poor at priming *in vivo* T-cell responses. Sköld *et al.* report that cells with a similar phenotype are a critical source of antimicrobial molecules following pulmonary mycobacterial infection [20]. These characteristics and their anatomical location, directly adjacent to iDCs in the mucosa, suggest that $CD11b^+$ DCs may in fact be a population of tissue-resident DCs that are an important source of proinflammatory cytokines and chemokines important in co-ordinating T-cell responses in the tissues as well as activating newly recruited monocytes and lymphocytes [16,21,22].

Interspersed in the lung parenchyma and around smaller airways are a long-lived population of $CD11c^{high}CD11b$ with or without cDCs, which are accessible by enzymatic digestion of the lung. These DCs are found in the distal areas of the lung, not associated with the conducting airways. These DCs possess phagocytic and T-cell priming qualities like other DC populations but are less well-characterized, largely due to their limited numbers in the naive lung [8,23].

Not to be overlooked as an important APC is the pulmonary macrophage (PM). Classically considered to be functionally distinct from pulmonary DCs, the PM expresses many of the same cell surface molecules as DCs being $CD11c^{high}CD11b^{int}MHC^{low}CD103^{low}$ and highly autofluorescent. Indeed these macrophages are excellent at phagocytosis and capable of inducing *ex vivo* T-cell proliferation. Two questions still remain: do these PMs differ functionally from the pulmonary DCs or do they in fact serve as precursors to other APC populations in the airway and parenchyma that have yet to mature and/or migrate?

Several groups have noted a population of $CD11c^-CD11b^+$ cells (some report them to express the monocyte marker Ly6G/Gr1) in the lung parenchyma that expands in response to pathogenic challenge. These cells are suggested to be either newly recruited blood monocytes that have yet to fully differentiate or granulocytes (in particular neutrophil precursors) [11]. Blood monocyte precursors can differentiate in the lung into both DC and macrophage-like cells in both the noninflammatory and inflammatory lung, suggesting these cells are critical for replenishing the lung DC and macrophage populations [24]. Indeed, there is evidence from multiple sources suggesting blood monocytes recruited to the lung develop into PMs, which serve as precursors to AMs [24–26]. While the same blood monocytes can either become DC-like or macrophage-like [20], tissue-specific factors that determine the fate of newly recruited monocytes are now becoming an important area of investigation that has many implications in modulating lung biology, particularly in the context of allergy, infection and pulmonary fibrosis.

Plasmacytoid DCs make up a smaller fraction of total lung DCs, accounting for only 1% in humans and mice [27,28]. In the murine lung, these cells can be identified as $CD11c^{low}CD11b^-Gr1^+B220^+$ $PDCA\text{-}1^+$ and have the unique ability to produce type I interferons upon CpG stimulation. Initially identified as critical sources of antiviral type 1 interferons [29], plasmacytoid DCs also possess immune-regulatory properties, involved in preventing allergic airway T-cell responses to harmless inhaled antigen [27].

Pulmonary DCs in primary mycobacterial infection

It was assumed that the primary target of *M. tuberculosis* infection was the AM. Several groups have shown that all airway luminal APCs are susceptible to mycobacterial infection. Reljic *et al.* demonstrate that following intranasal instillation of fluorescent attenuated mycobacteria, 5% of total lung $CD11c^+$ cells are infected, including many parenchymal APCs [30]. Greater numbers of AMs are initially infected, partly due to their abundance in the conducting airways and partly due to their strong phagocytic ability, although DCs are equally susceptible to infection [10,18]. Mycobacteria utilize a number of receptors, including CD14, complement receptors, mannose receptor, scavenger receptors and DC-SIGN to gain entry into airway and lung APCs [31]. Naive AMs express a number of receptors for *M. tuberculosis* binding including CD14, mannose receptor and scavenger receptors, while DCs are believed to express a less diverse array of phagocytic receptors, perhaps delaying the initial uptake of mycobacteria into the cells. Binding of mycobacteria to these receptors on AMs leads to phagocytosis of the bacteria, ushering it into the phagosome where it can slowly grow and express the genes necessary to subvert antimicrobial host defences. A similar process is believed to take place in the DC, although DC-SIGN is believed to be the primary receptor used by *M. tuberculosis* to gain access to DCs. The contribution of DC-SIGN was largely overlooked until it was discovered that DC-SIGN is upregulated on lung DCs in response to mycobacterial infection. Data from human TB patient samples supports the idea that DC-SIGN is involved in bacterial dissemination during the chronic phase of the infection in light of the finding that DC-SIGN is expressed on $CD11b^+$ airway derived cells in patients with chronic TB infection and is used by *M. tuberculosis* to gain entry to cells expressing the molecule [32,33]. It is important to note that there is a lack of understanding of the receptors used by *M. tuberculosis* to infect different APC populations, and little or no data exists regarding the susceptibility of newly identified airway lumenal and parenchymal DC populations. We do not yet appreciate the unique dynamics that occur over the course of mycobacterial infection as phagocytic receptors are differentially modulated on both resident and newly recruited cells and how that may contribute to bacterial dissemination. Chronic, life-long infection is attributed to the ability of *M. tuberculosis* to evade destruction/degradation in the host cell by a variety of strategies, including preventing fusion of the phagosome with the lysosome, escaping into the cytoplasm by directly inhibiting macrophage maturation and nitric oxide production, and delaying adaptive immunity by blocking presentation of mycobacterial antigens on MHC [34–36]. At this point, the fate and function of macrophages and DCs diverge. *In vitro* analysis using *ex vivo*-derived macrophages and DCs revealed macrophages control intracellular infection better than DCs by producing greater levels of antimicrobial molecules, including nitric oxide [36]. By contrast, mycobacterially infected DCs are better equipped to upregulate costimulatory molecules, MHCII and endothelial adhesion molecules such as ICAM-1, generating a phenotype that enables them to migrate and prime T-cell responses [36]. As a consequence, infected DCs become a 'Trojan

horse' by transporting viable bacilli to systemic sites, including the LN. Despite this, presentation of mycobacterial-derived antigens by DCs is essential in initiating protective T-cell responses. Our understanding of the fate of *M. tuberculosis* within APCs is limited to the studies carried out using *ex vivo* differentiated bone marrow-derived cells, which may not represent what is occurring *in vivo*. In light of the unique nature of airway cells, there is a need to separate the analysis of airway luminal cells from bulk lung-derived cells in order to appreciate their roles in host defence against *M. tuberculosis* infection.

Histological examination of lung infected with *M. tuberculosis* reveals viable bacteria in APCs in the airway lumen as well as in the granulomas clustered around major airways in the lung parenchyma. It was believed that following infection, the AMs are responsible for establishing local tissue inflammation in an attempt to control bacterial replication and spread. The AM associates with the lung epithelium where it releases potent pro-inflammatory mediators in response to growing bacterial burden, recruiting innate leukocytes and initiating the formation of an early 'innate' granuloma [10,37,38]. Bacterial components such as phosphatidylinositol mannan, 19 kDa lipoprotein, lipoarabinomannan and bacterial-derived DNA are recognized by cell-associated pathogen-associated molecular patterns which trigger proinflammatory responses through TLR-2, -4 and -9 expressed on many lung APCs including AMs, iDCs and parenchymal CD11b$^+$ DCs [39]. This results in secretion of TNF-α and IL-1β as well as chemokines that recruit additional effector cells. Recruitment of blood monocytes early in the infection is essential for initiating and co-ordinating the granuloma as well as priming and activating T-cell responses. The robust recruitment of activated T cells in the lung does not occur until 21 days postinfection. Recruitment of APCs is a continuous process such that by approximately 19 days postinfection, 80–90% of all DCs and macrophages in the lung are newly recruited [10,40]. It is interesting to note that the foamy macrophage, a hallmark of the *M. tuberculosis* granuloma, in fact resembles a DC, being CD11c^{high}CD11b$^+$MHC^{high}, and expresses costimulatory molecules as well as anti-apoptosis molecules [37], raising the question as to whether the foamy macrophage is indeed a macrophage or actually a DC unable to control infection.

The primary goal of a DC is simple: process antigen and prime effector T cells. On the other hand, the contribution of AM to co-ordinating antimycobacterial responses may be different. In line with their function as a sentry cell, the number of AMs does not increase following infection and they primarily function to co-ordinate inflammatory responses and recruit innate cells to the site of infection. Indeed, macrophages exposed to mycobacteria produce monocyte chemoattractant protein (MCP)-1, the ligand for CCR2 [41] and RANTES (CC chemokine ligand [CCL]5) [40], which are critical for early resistance against pulmonary TB challenge. In both cases, efficient bacterial control was dependent on efficient recruitment of DC and macrophage precursors to the site of infection. As a result of continual monocyte recruitment in response to mycobacterial infection, the number of CD11c^{high}CD11b^{high} DCs increase steadily and bear the majority of the bacterial burden [10]. These cDCs express higher levels of MHC and costimulatory molecules (CD80, CD86 and CD40), and drive T-cell proliferation and T-cell cytokine production better than other DC subsets. Wolf *et al.* report that the majority of mycobacterially infected DCs that migrate to the LN are CD11c^{high}CD11b^{high}, do not significantly accumulate in the LN prior to 14 days postinfection and increase dramatically between 14 and 21 days postinfection [42]. It is not known what naive lung DC subset these cells are derived from or how their phenotype may have been altered in response to mycobacterial infection. This mycobacterial-specific finding may account for the delayed appearance of T cells in the infected lung. However, it is also possible that delayed T-cell priming is a result of pathogen-mediated subversion of antigen presentation by MHC. Further phenotypic and functional characterization of these migrating and nonmigrating DC populations, including CD103, MHC, TLR and chemokine receptor levels, has not yet been carried out in a mycobacterial infection model.

Migration of DCs to the LN & T-cell priming

It has long been known that CD11c$^+$ cells are essential for T-cell priming during pulmonary infection such that depletion of CD11c$^+$ cells delays T-cell priming and protective anti-TB immune responses [43,44]. The requirement of the LN in priming T-cell responses during pulmonary infection has long been a point of debate amongst immunologists. Recently, two papers by Day *et al.* and Kashino *et al.* indicate that LNs are dispensable for T-cell priming [45,46]. In these studies, areas of inducible bronchus-associated lymphoid tissues (iBALT) developed prior to day 14 postinfection and persisted throughout the course of infection. It is noteworthy that as these studies all used LN-deficient mouse strains, the role of iBALT in T-cell priming is likely artificially exaggerated. In the immune-competent host, T-cell priming occurs in the mediastinal LN. Indeed, in human cases of TB, swelling of the mediastinal LN helps to diagnose and indentify disease severity. Live bacteria can be recovered from the local draining LN and bacterial antigen in the local draining LN was found to be required for CD4$^+$ T-cell priming and subsequent development of protective immune responses [42].

Dendritic cell migration to the mediastinal LN, the lung draining LN, is CCR7-dependent. CCR7 is upregulated along with costimulatory molecules upon DC maturation [47–49]. The maturing DC homes to the T-cell zones of the LN in response to stromal cells expressing CCL19 and CCL21, the ligands for CCR7. Not surprisingly, mice deficient in CCR7 are more susceptible to *M. tuberculosis* infection and succumb to infection more rapidly than wild-type mice owing to increased bacterial loads [50]. Interestingly, in spite of impaired DC migration, CD4$^+$ T cells can still be primed in CCR7$^{-/-}$ mice in the lungs, rather than the draining LNs, a phenomenon unique to *M. tuberculosis* infection and not seen during viral infection [50]. Although it is not known to which extent T-cell priming occurs outside of the LN in a wild-type host, these findings provide us with a novel biomarker to help identify pulmonary DC subsets involved in T-cell priming. Following infection, we may be able to predict which cells will

migrate by their CCR7 expression, helping to identify how T-cell priming by DCs may be improved to accelerate T-cell responses in the infected lung.

In addition to adhesion molecules and chemokines receptors, cytokines are an essential component involved in DC migration from the *M. tuberculosis* infected lung to the LN. It has long been known that IL-12 is essential for optimal priming of Th1 and $CD8^+$ T-cell responses, and autocrine IL-12p40 signaling is necessary for DC maturation and migration and even CCR7 responsiveness to CCL19 [51,52]. This finding breathes new life into an old strategy of using cytokines as an adjuvant to enhance DC maturation and migration.

In the spleen and LN, $CD8\alpha^+$ DCs have been identified to be superior to $CD8\alpha^-$ DC subsets in T-cell priming and cross-priming although no $CD8\alpha^+$ DCs have been identified in the mouse lung [3,53]. Interestingly, Edelson *et al.* made the observation that $CD8\alpha^+$ knockout mice also lack the subset of pulmonary $CD11c^+CD11b^-CD103^+$, suggesting a common development pathway for both $CD8\alpha^+$ DCs and CD103 lung DCs [16,54]. These lung $CD103^+$ DCs are thought to be the migratory DCs which home from nonlymphoid tissues to the local draining LN to prime $CD4^+$ T-cell responses. $CD103^-$ DCs can also be found in the draining LN and appear to preferentially prime CD8 T cells. Found in the lung, gut and skin, these $CD103^+$ DCs migrate in a CCR7- dependent manner and are specialized in $CD4^+$ and $CD8^+$ T-cell priming as well as cross-priming [16]. Although $CD8\alpha^-$ DCs are not found in naive lung tissues, $CD8\alpha^+$ DCs may be recruited from the blood or systemic lymphoid organs such as the spleen. However, $CD8\alpha^+$ DCs and their functionality have yet to be examined in a TB model.

Robust T-cell responses are essential to activating infected cells in the lung to control intracellular bacteria. Effector $CD4^+$ and, to a lesser extent, $CD8^+$ T cells found in the lung interstitium and airway lumen of mycobacterially infected mice secrete IFN-γ and TNF-α, and express other macrophage-activating molecules such as CD40L and cytotoxic molecules FasL and perforin/granzyme. T-cell priming following pulmonary mycobacterial infection is delayed when compared with acute viral infections, such that no marked mycobacterial-specific T-cell responses can be detected in the lung prior to 15 days postinfection [10]. The delay in T-cell priming is hypothesized to be due to delayed DC migration because there is no appreciable accumulation of the DCs bearing mycobacteria that express green fluorescent protein in the LN prior to 14 days postinfection [10]. Along the same lines, using a transgenic T-cell model, Reiley *et al.* show that T-cell activation is indeed delayed to 10 days postinfection [55]. These findings have led some to believe that T-cell activation is delayed due to decreased DC migration. In fact, the delay in T-cell priming may be due to delayed presentation of antigen rather than decreased DC migration. Mycobacterial infection is known to suppress DC activation and proinflammatory responses, which may cause a decrease in DC migration. Furthermore, the infected DCs may be broadly immunosupressed due to the production of immune-dampening IL-10 [34,56]. In support of this idea, Divangahi *et al.* showed that virulent mycobacteria can dramatically alter the fate of an infected APC by tipping the scales in favor of cell necrosis, leading to increased bacterial load by way of subverting the innate immune system [57]. They also reported that virulent mycobacteria block apoptosis of DCs and cross-presentation of mycobacterial antigens, suggesting that this is responsible for delayed/reduced T-cell priming [58]. This strongly indicates that the pathogen itself has a huge impact on how the host senses and deals with infection, a factor that has been largely overlooked in the past. The question remains how the DCs that migrated to the LN manage to overcome the *Mycobacterium*-imposed deactivating signals. It is reasonable to believe that any strategies designed to counter such DC-suppressive signals shall facilitate DC migration and thus speed up T-cell priming in the LN.

DCs as a determinant for susceptibility/resistance to *M. tuberculosis* infection

Different strains of mice are known to have varying degrees of susceptibility to *M. tuberculosis* infection. There is no question that lower levels of type 1 immunity (TNF-α, IL-17 and IFN-γ) observed in BALB/c mice significantly contributes to weakened antibacterial host defence in the long term [59,60]. Along the same lines, Chackerian *et al.* report that delayed dissemination of mycobacteria in susceptible C3H mice leads to delayed T-cell priming and subsequently reduced host defence [61]. Cardona *et al.* report higher bacterial burdens in DBA/2 mice in spite of increased inducible nitric oxide synthase, macrophage inflammatory protein-2 and MCP-1 levels [62]. Upon closer consideration we can hypothesize that the increased susceptibility reported in these mouse strains could be attributed to the dysregulation of early control of bacterial replication in the infected AM, DC migration and T-cell priming, and the extent of infection of newly recruited monocytes in the lung. Different strains of mice with different susceptibility to pulmonary mycobacterial infection provide us with an opportunity to investigate the potential role of DC migration and T-cell priming in genetically determined host resistance to pulmonary *M. tuberculosis* infection.

Role of DAP12 in modulating DC functions & T-cell responses

We and others have reported that the signaling adaptor molecule DNAX-activating protein of 12 kDa (DAP12) expressed on APCs negatively regulates DC activation following exposure to pathogen-associated molecular patterns and whole live pathogens [63,64]. DAP12KO DCs express higher levels of type 1 immune polarizing proinflammatory cytokines TNF-α and IL-12 as well as increased MHC and costimulatory molecule expression. In human monocytes, DAP12 signaling leads to increased CCR7 expression and decreased apoptosis [65]. Increased DC activation translates into more rapid T-cell priming by DAP12KO DCs and heightened T-cell responses, leading to enhanced protection against virulent and avirulent mycobacterial challenge [63,66]. To date, it is not known whether DAP12KO-derived DCs possess enhanced migratory properties for expedited T-cell priming or are better equipped to prime T cells and to control intracellular bacterial infection compared with wild-type DCs. The DAP12KO mouse provides

a model for examining the contribution of DC and macrophage responses to antimycobacterial T-cell responses and may provide essential clues into effective host resistance against pulmonary TB.

Outstanding questions in lung DC biology in mycobacterial infection

The current practice of enzymatic digestion of the whole lung following mycobacterial infection does not allow us to accurately appreciate the difference between front-line airway luminal DCs and distal parenchymal DC populations. With the discovery of new DC subsets, our current understanding of the early immune events following pulmonary mycobacterial infection must be re-examined. In particular, there is a need to use freshly isolated airway luminal and lung-derived DCs and macrophages to clarify the pattern of receptor expression which dictate the extent of bacterial phagocytosis. Furthermore, there is a lack of information on *in vivo* data addressing the fate of both infected AMs and ADCs following infection, how these two cell types diverge functionally and their roles in early and chronic bacterial control. Historically, AMs were thought to be restricted to the airways, but in a model of Gram-positive bacteria, AMs were found to migrate into the LN [12], challenging our understanding. Newly identified parenchymal iDCs have been shown to sample airway luminal antigen in allergic airway disease models and sense inflammatory TLR stimuli, although their role in *M. tuberculosis* infection has not yet been examined. Similarly, based on results from other models, the role of the $CD11b^+$ DC may be critical in antimycobacterial immunity by initiating granuloma formation and polarizing newly recruited monocytes, greatly influencing the success of all aspects of the immune response.

The role of early innate responses in the control of mycobacterial infection may have been overlooked. There is an urgent need to re-examine early responses following *M. tuberculosis* infection (prior to 7 days). *In vitro* data show DC activation occurs soon after infection although we cannot reconcile these *in vitro* observations with the delayed appearance of *M. tuberculosis* antigen-specific T cells in the infected lung. While there is data to suggest that DCs do not immediately migrate *en masse* to the LN, it is possible that the delayed appearance of activated T cells is due to the impaired ability of DCs to present mycobacterial antigens when infected with *M. tuberculosis* [10] instead of impaired migration. Overcoming this impaired DC function and speeding up migration of DCs to the LN and presentation will be important when targeting DCs to improve vaccination strategies.

Targeting DCs for enhanced T-cell activation & protection

Many new vaccine formulations can prime robust T-cell responses in the spleen and even the lung parenchyma but most of these vaccines fail to provide appreciable protection against virulent *M. tuberculosis* infection in the lung. There appears to be a strong correlation between the number of airway luminal T cells and the ability of a vaccinated host to control mycobacterial burden in the lung as well as at systemic sites. It is therefore essential to design vaccination strategies that draw the peripheral T cells into the airway lumen for rapid activation of infected AMs in the early phase of infection [67]. In spite of the improvements made to BCG, it cannot be used in a homologous prime–boost regimen and cannot be safely delivered to the lung because it is a live replicating organism [68,69]. Taking into consideration what we have learned in the past years about lung DC biology, we can begin to better target lung DC populations that will prime T-cell responses at the site of pathogen entry.

One strategy to improve the protection against *M. tuberculosis* challenge is to deliver antigens to DCs directly at the lung mucosa. Mucosal vaccines against TB have taken on three major forms in recent years: whole protein-, DNA- and viral vector-based vaccines. Delivery of soluble *M. tuberculosis* antigens was once considered a potential approach to priming T-cell responses because they are highly safe, contain TLR ligands and are easy to produce and distribute. In practice, delivery of soluble *M. tuberculosis* antigens to the lung such as secreted proteins Ag85, ESAT-6 and TB10.4 fail to elicit protective immunity unless conjugated to a robust immune adjuvants such as concentrated TLR ligands. Although ineffective as a T-cell priming strategy, soluble antigen delivery has been shown to help decrease bacterial burden in the lung and improve lung pathology due to increased immune responses [70]. This approach is currently being tested in a Phase II clinical trial in latently infected patients. In an attempt to enhance antigen uptake and presentation, targeting to the DC by recombinant monoclonal antibodies has been used as an approach to expand antigen-specific T cells. A recombinant antibody against DEC-205 bound to antigen has been shown to enhance antigen uptake by DCs and drive T-cell responses [71,72]. While this approach has not been tested in a TB model, this type of selective antigen delivery may enhance the number of polyfunctional effector T cells and promote effective bacterial control.

DNA-based vaccines are safe and have even been used effectively in veterinary medicine to prevent West Nile virus infection, replacing the previous attenuated virus vaccine. DNA-based vaccines encoding *M. tuberculosis* antigens have also been tested for efficacy against *M. tuberculosis* infection and were once thought to be highly promising because they were able to directly transduce *M. tuberculosis* antigens into the host cell for prolonged periods of time. In reality, DNA-based vaccines have been ineffective in providing protection against pulmonary *M. tuberculosis* challenge, even when delivered mucosally. This is at least in part due to the fact that DNA administration to the airway does not readily lead to antigen presentation by lung APCs and further fails to activate T cells. Coadministration of cationic lipids with DNA vaccines directly activated airway APCs leading to enhanced T-cell responses, although the enhanced immunogenicity was modest at best [73,74]. Although adjuvant and formulation improvements have led to increased immune responses and provided some level of protection against *M. tuberculosis* challenge, DNA vaccines are always limited by their poor ability to transduce DCs. It is important to note that, no anti-TB DNA vaccines are currently moving into clinical trials.

Recombinant viral vaccines are one of the most promising vaccine platforms because they play a dual role by delivering antigens to lung APCs as well as acting as an adjuvant. Viruses with a

natural tropism for the respiratory mucosa, such as adenoviruses and vesicular stomatitis virus [75], are ideal candidates as mucosal vaccines because they can directly infect AMs and ADCs. Infection of DCs and macrophages results in upregulation of MHCII, costimulatory molecules and type 1 polarizing cytokine IL-12 [76,77]. Viral-based vaccines also have the advantage of being able to prime protective $CD4^+$ and $CD8^+$ T-cell responses. In primate models, this adenovirus-based vaccine primed robust $CD4^+$ and $CD8^+$ T-cell responses in the airway lumen, providing protection against virulent *M. tuberculosis* challenge. $CD8^+$ cell activation can be attributed to direct transduction of local APC populations at the site of injection, which leads to processing and presenting of virus-encoded antigens by MHCI. $CD4^+$ T-cell activation is thought to be achieved by two possible mechanisms: cross-presentation of vaccine antigens by infected tissue-resident APCs or acquisition of vaccine antigens from infected cells that have undergone apoptosis. Naturally, viral-vectored vaccines will also infect nonimmune cells at the site of injection and these nonimmune cells may in fact play an essential role as depots of antigen, which persist long beyond viral infection to maintain the number of memory T cells. Efficacy of recombinant viral vaccines will always be limited by pre-existing host immunity in humans to the virus, forcing vaccinologists to search out new viral strains and explore zoonotic viruses.

Caution must be taken when developing respiratory vaccines. Striking a balance between the minimum level of immune activation for T-cell priming and overactivation is critical. Indeed, from studies using direct application of TLR ligands to the lung, we can appreciate that an overactivated immune system will have adverse effects on the well-being of patients [78]. While transient inflammation may be beneficial and even necessary for efficient immune activation, it will have an effect on lung function including oxygen transport, the function of epithelial cells as well as airway remodeling in response to inflammatory insult.

Ex vivo manipulation of DCs as a vaccine platform

In vitro/*ex vivo* manipulation of DCs and subsequent *in vivo* administration has been an important strategy in immunization against cancer and infectious diseases. This approach to T-cell priming can be advantageous because it reliably delivers antigen directly to the DC, and does not rely on the host DCs to acquire antigen. A variety of vaccine formulations have been examined including pulsing *in vitro* differentiated DCs with whole protein antigen, peptide antigen, attenuated mycobacteria and viral transduction with a variety of attenuated viruses. In the field of vaccine immunology, parenteral intramuscular immunization with adenovirus-transduced DCs leads to higher T-cell activation and protection against pulmonary *M. tuberculosis* challenge than protein- or peptide-loaded DCs [76]. In keeping with the potency of direct intranasal delivery of viral-vectored anti-TB vaccine, intranasal delivery of *in vitro* viral TB vaccine-transduced DCs results in activation and recruitment of airway luminal T cells, which correlates with increased protection against *M. tuberculosis* challenge [51,79]. Viral transduction results in increased MHC and costimulatory molecule expression as well as type 1 polarizing T-cell cytokines, such as IL-12, and stimulates DC migration to the mediastinal LN. In comparison, whole *M. tuberculosis* protein- or peptide-loaded DCs fail to upregulate the necessary inflammatory signals required for efficient T-cell priming.

Taken together, we and others have demonstrated the virally transduced DCs to be the best immune activators with the potential for therapeutic application.

Virally transduced DCs are advantageous in that they do not rely on the host immune system to prime T cells [51]. This is especially important in the case of an individual that is infected with HIV, where DCs and T-cell functionality is impaired. DC-based vaccines are a safe and effective means of activating the immune system. However, this approach will be costly. In cancer vaccine trials for each patient it is estimated to cost in excess of US$93,000 for a series of three immunizations [80]. As such, DC-based vaccination is not likely to be a first-line therapy for all but rather it may be considered as a therapeutic intervention in those patients where conventional antimicrobial treatment fails. The therapeutic potential of DC-based vaccines in TB has not yet been evaluated. Animal models, such as the Cornell model of latent TB infection, will prove to be essential for the initial evaluation of its therapeutic potential. In cancer immunotherapy trials, variable levels of immune activation have been reported with little or no improvement in survival.

The future of in vitro/ex vivo-manipulated DCs for anti-TB therapy

Dendritic cell-based vaccination is an expensive endeavour requiring sterile culture facilities, costly recombinant cytokines to drive DC maturation *in vitro* and extensive quality control steps. As such, DC vaccination will not be considered as a first-line choice. Rather, it may be used in selected situations where safety and overcoming the host's DC impairment are the primary concerns. In the face of the TB–HIV twin epidemics, DC-based vaccination may represent an important adjunct therapy to current chemotherapeutic protocols. Using the archival RUTI vaccination regime as a template, short-term antibiotic vaccination followed by intranasal vaccination with *ex vivo*-manipulated DCs may be a viable option for treating HIV-positive patients at high risk for TB reactivation.

Dendritic cell-based vaccines also have the advantage that the same formulation can be delivered repeatedly without worry of developing immunity against the vector, as is the concern with viral-based vaccines. In the situation of a patient with multidrug-resistant or extensive drug-resistant TB where all chemotherapeutic intervention fails, DC vaccination could be investigated as a means to boost T-cell immunity and re-establish bacterial control.

With the recent discovery of the contribution of CCR7 in DC migration to the LN, we now have a novel target to improve DC-based vaccine efficacy. Cloning a gene encoding IL-12p40 in addition to CCR7 into the viral vector may improve the efficacy of vaccine migration to the LN and T-cell zone and enhance T-cell priming. Another approach may include prolonging DC survival by blocking Bcl-x_L [81] to allow vaccine DCs to persist both in the local lung environment as well as local draining LN for enhanced vaccine efficacy.

Expert commentary

Dendritic cells are undoubtedly the most important immune cell bridging the innate immunity with the adaptive resistance against infections. While our understanding of pulmonary DCs is expanding rapidly, the precise role of different pulmonary DC subsets in anti-TB T-cell activation and tissue responses still largely remains to be elucidated. In comparison, there is more information coming from the field of DC-based TB vaccines and its further clinical investigation may be fostered by the initial promising results from the clinical study testing a cell-based hepatitis therapeutic strategy.

Five-year view

In the coming years, we can hope to see the full spectrum of DC and macrophage function analyzed in the course of pulmonary mycobacterial infection. A better phenotypic analysis of optimal T-cell priming DCs will shed light on molecules currently used as phenotypic biomarkers that will enhance T-cell priming. One such molecule that needs in-depth analysis is CD103. Some believe that this integrin dictates DC migration and can be used as a predictive marker of T-cell-activating DCs versus other nonstimulatory DCs. The development of CD103 gene knockout mice will provide us a useful tool to better investigate its function.

There is a great deal of phenotypic and functional overlap between DCs and macrophages. The question still remains whether they represent discrete APC populations or a continuum of a single cell population at varying stages of mycobacterial infection and immune activation. Furthermore, the mechanisms that drive the emergence of different pulmonary DC subsets remain to be investigated. Not to be overlooked is the contribution of the pathogen in modulating DC and macrophage function early in infection as well as during the chronic phase. Further enhanced understanding on all of these fronts will help improve DC-based vaccination strategies for prophylactic and therapeutic applications.

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Key issues

- Our understanding of pulmonary dendritic cell (DC) biology in the context of TB has not grown with the rest of the field in the past 5 years.
- The type of pulmonary DCs that migrate to the LN to activate mycobacterial antigen-specific T cells and their relationship with the delayed T-cell priming in the LN following pulmonary mycobacterial infection remain to be determined.
- Emerging evidence supports that mycobacterial granuloma is immune-suppressed. Sophisticated experimental techniques are now available to identify the relationship of the DCs with mycobacteria within the granuloma and develop DC-based strategy to overcome such immune suppression.
- There is a need to test DC-based vaccines in appropriate latent TB models for their therapeutic potential.

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Appendix II.

Review article: Manipulation if dendritic cells for host defence against intracellular infections

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Manipulation of dendritic cells for host defence against intracellular infections

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Abstract

Dendritic cells (DCs) are an important innate immune cell type which is the bridge between innate and adaptive immunity. Mounting experimental evidence suggests that manipulating DC represents a powerful means to enhance host defence against intracellular infectious diseases. We have developed several strategies to manipulate DC either *in vivo* or *in vitro* for the purpose of enhancing the effect of vaccination or immunotherapeutics. *In vivo* delivery of transgene encoding GM-CSF (granulocyte/macrophage colony-stimulating factor), a DC-activating cytokine, increases the number and activation status of DC at various tissue sites and enhances antimicrobial immune responses in murine models. Co-expression or co-delivery of GM-CSF gene transfer vector with an antimicrobial vaccine enhances microbial antigen-specific T-cell responses and immune protection. Murine bone marrow-derived DCs are being manipulated *in vitro* and exploited as a vaccine delivery system. Transduction of DC with a virus-vectored tuberculosis vaccine is a powerful way to activate T-cells *in vivo*. Such genetically modified DC vaccines can be administered either parenterally or mucosally via the respiratory tract.

Introduction

Many diseases for which there remains no effective vaccine are caused by intracellular pathogens such as HIV, hepatitis, chlamydia and TB (tuberculosis). Current vaccine strategies have been successful in preventing infections by generating a strong humoral response leading to the generation of antibodies directed against the invading pathogen. However, these vaccines have failed to elicit a strong cellular immune response which is required to deal with intracellular infections. The failure of the current vaccine strategies has led to a great deal of research into the mechanism of generating an effective immune response against intracellular pathogens with the hopes of designing better vaccines. These efforts have discovered that a type 1 cellular immune response characterized by IFN-γ (interferon γ)-secreting $CD4^+$ and $CD8^+$ T-cells is required to combat intracellular infections. Generation of an efficient T-cell-mediated response relies on the antigen-presenting cell.

The most potent antigen-presenting cell is the DC (dendritic cell). DCs are found in a variety of bodily tissues, including lymphoid tissues such as the spleen and lymph nodes and the mucosal tissue of the lung, gut, genital tract and skin, where many pathogens gain entry to the body. DCs originate from bone marrow progenitors and mature with the aid of growth factors such as GM-CSF (granulocyte/macrophage colony-stimulating factor), IL-4 (interleukin-4), Flt3 (Fms-like tyrosine kinase 3) ligand and IL-6. DCs differentiate along myeloid or lymphoid pathways into several populations of $CD11c^+$ MHCII (MHC class II)$^+$ $CD11b^{\pm}$ $CD8^{\pm}$ with discrete phenotypes and functions [1]. The most important function of the DC is to bridge innate and adaptive immune responses by processing scavenged pathogenic material into peptide fragments and presenting them to T-cells on MHCI and MHCII [2]. DCs are the most potent professional antigen-presenting cells, capable of 10–100-fold greater formation of peptide MHC complexes on their surfaces than other antigen-presenting cells [3], leading to greater T-cell activation, the hallmark of cellular immunity.

DCs act as innate immune cells, recognizing pathogen-associated molecular patterns through germline encoded receptors such as Toll-like receptors and mannose receptors leading to release of alarm cytokines [IL-6 and TNFα (tumour necrosis factor α)] and chemokines [4,5], thereby generating a pro-inflammatory microenvironment suitable for further influx of immune cells. DCs are also capable of pathogen scavenging and degradation by a variety of mechanisms including receptor-mediated endocytosis, macropinocytosis or phagocytosis [2]. Upon antigen encounter, DCs migrate into lymphoid organs, usually the local draining lymph node. While migrating, DCs undergo a maturation process from an immature phenotype to a mature phenotype, whereby they express an enhanced level of MHCII, co-stimulatory molecules CD80 (B7-1), CD86 (B7-2) and CD40 and continue the secretion of cytokines, IL-12, IL-4 or IL-10 [2,5]. In the local draining lymph nodes, DCs activate T-cells, initiating an adaptive immune response.

Key words: cellular immunity, cytokine adjuvant, dendritic cell, host defence, intracellular infection, tuberculosis (TB).

Abbreviations used: Ad, adenovirus; Add, empty adenovirus vector; Ag85A, antigen 85A; BCG, Bacille Calmette-Guérin; DC, dendritic cell; DCAdAg85A, DCs virally transduced with AdAg85A; Flt3, Fms-like tyrosine kinase 3; GM-CSF, granulocyte/macrophage colony-stimulating factor; IFN-γ, interferon γ; IL, interleukin; MHCII, MHC class II; TB, tuberculosis; TNFα, tumour necrosis factor α.

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Many intracellular infections require strong type 1 immune responses characterized by activation of IFN-γ-secreting $CD4^+$ and $CD8^+$ T-cells. Since DCs are localized at all key mucosal surfaces where many intracellular pathogens enter the body and have been shown to be the key in co-coordinating immune responses, one of the vaccine strategies has focused on targeting DC activation and expansion to generate long-lasting cellular immunity. The polarization of the cellular immune response is determined by the way the DC is activated during pathogen encounter and antigen processing, which is in turn determined by the nature of pathogen. In order for a type 1 immune response to be initiated, the activated DC must secrete large amounts of IL-12 [6,7]. However, pathogen encounter does not always result in IL-12 secretion. For instance, when DCs encounter *Mycobacterium tuberculosis*, they secrete IL-10 and IL-6 [4,5].

Therefore generating a strong type 1 immune response by means of vaccination has been the goal of many vaccinologists who seek a safe and effective vaccine against such intracellular infectious diseases as pulmonary TB. TB remains to be a significant cause of illness in many countries around the world in spite of the introduction of BCG (Bacille Calmette–Guérin) vaccination during infancy 85 years ago (http://www.who.int/tb/en/). The failure of BCG in controlling adult TB appears to be due to a lack of long-lasting T-cell memory. In search for improved TB vaccines, vaccinologists have begun to target DC both *in vivo* and *ex vivo* in order to generate potent T-cell activation leading to stable memory formation.

Expanding *in vivo* DC populations by GM-CSF cytokine adjuvant

Because DCs play such a pivotal role in the initiation of adaptive immune responses, targeting endogenous DC populations *in vivo* with cytokine adjuvants may amplify the level of immune activation. Moreover, the state of maturation of the DC is critical as a DC that is not fully matured can lead to T-cell tolerance. Therefore a cytokine adjuvant that recruits and matures DC could potentiate an immune response. We have taken a novel approach by using GM-CSF as a cytokine adjuvant to enhance the immunogenicity of a vaccine against intracellular infections.

We generated a replication-deficient AdGM (adenovirus expressing a murine GM-CSF transgene) which can be delivered to a variety of tissues, resulting in a high level but self-limited expression of GM-CSF. When delivered intravenously (i.v.), intramuscularly (i.m.) or subcutaneously (s.c.), GM-CSF levels reach a peak in the serum at 3 days post-inoculation [9]. This systemic expression of the GM-CSF transgene leads to significant expansion of endogenous DC populations in the spleen, reaching a peak 200–260-fold increase at 1 week post-gene transfer [9]. Interestingly, while AdGM expands DCs in the spleen, AdGM does not appear to expand the T-cell, NK (natural killer) cell and macrophage population. The expansion of the DC population in the spleen is long lived; with DC numbers remaining increased 15-fold at day 75 post-AdGM [9]. In order to determine whether the DCs expanded by AdGM injection could result in increased immune activation, the functionality of the recruited DCs was evaluated. It was found that these DCs produce large amounts of TNFα and IL-6, and have enhanced antigen capture and presentation capabilities. Most importantly, AdGM-expanded DCs were capable of far superior T-cell activation, resulting in potent cytotoxic T-lymphocyte generation [9]. These results suggest that AdGM expands a population of DCs that are functionally superior to the DCs that exist in the spleen in the absence of exogenous GM-CSF.

It has been postulated that one of the shortcomings of BCG as a vaccine is its poor ability to induce memory $CD8^+$ T-cell responses. Since it has been demonstrated that AdGM enhances DC recruitment and subsequent T-cell activation following systemic or local administration, we next sought to determine whether GM-CSF could enhance the immunogenicity of BCG. When AdGM was co-administered subcutaneously with BCG, the immunogenicity of BCG was markedly enhanced up to 12 weeks post-immunization [10]. T-cells isolated from lymph nodes and spleen of BCG/AdGM-immunized mice secreted more IFN-γ than mice immunized with BCG alone or with BCG and Addl (the empty adenovirus) [10]. When AdGM was injected subcutaneously or intradermally, most of the GM-CSF transgene product was detected in the skin of the animal, leading to locally enhanced accumulation of cutaneous DCs and other MHCII-expressing antigen-presenting cells [11]. Such increased DCs at the site of vaccination and subsequently enhanced immune activation led to improved protection against secondary mycobacterial or *M. tuberculosis* challenge in the lung [10]. Similarly, intramuscular immunization with a plasmid DNA vaccine co-expressing both GM-CSF and an *M. tuberculosis* antigen Ag85A (antigen 85A) led to a much higher level of type 1 T-cell activation *in vivo* than the vaccine that expressed only Ag85A (unpublished work). Q1

Since AdGM acts to expand endogenous DC populations, delivering AdGM to a naturally DC-rich area, such as a mucosal surface, may prove to be a far superior strategy to generate efficient immune protection against a pathogen that enters via the mucosa. To this end, we delivered AdGM intranasally (i.n.) to the airway of mice. Intranasal administration of AdGM resulted in an 8 times greater influx of immune cells into the airway lumen compared with Addl [12]. The predominant cell types infiltrating the airway lumen as a result of GM-CSF was the monocyte/macrophage and lymphocytes which persisted for up to 19 days [12]. Of importance, AdGM delivery expanded and activated a myeloid DC population locally in the lung, which appeared to be macrophage-derived [12]. Upon *in vitro* re-stimulation with adenoviral antigens, both $CD4^+$ and $CD8^+$ T-cells from AdGM-treated mice produced significantly more IFN-γ than $CD4^+$ and $CD8^+$ T-cells from Addl-infected mice, suggesting that lung GM-CSF transgene expression enhanced viral antigen-specific type 1 immune responses via its effect on myeloid DCs [12]. It is important to note that the level

of DC recruitment and activation demonstrated using the recombinant AdGM could not be duplicated by injection of poly(ethylene glycol)-conjugated GM-CSF, unmodified GM-CSF or AdFlt3 (adenovirus expressing Flt3) [9,12].

AdGM can also be used as an immune adjuvant for an organism-based chlamydial vaccine to potentiate the immune activation against pulmonary *Chlamydia trachomatis* infection. Thus co-administration of inactivated chlamydial organisms with AdGM resulted in significantly enhanced numbers of DCs recruited to the airway and potent systemic type 1 immune activation which was accompanied by reduced *Chlamydia* bacterial burden upon secondary challenge [13]. Interestingly, not only did AdGM increase the cellular immunity, but also significantly increased the amount of lung mucosal IgA [13].

Ex vivo manipulation of DCs

While targeting DCs *in vivo* by using adenoviral-mediated GM-CSF gene expression is an effective way to expand and activate endogenous DCs, its direct *in vivo* application may cause undesired inflammatory responses and trigger anti-adenoviral antibody responses which may limit the second administration. Since it is becoming increasingly difficult to treat TB infections caused by multidrug-resistant strains of *M. tuberculosis* and many patients who complete drug therapy are at increased risk of re-infection, there is now an urgent need to develop a therapeutic vaccine for these individuals. In this regard, DC-based TB vaccines possess the power of antigen presentation and do not trigger an overwhelming acute inflammatory response as a 'naked' viral vaccine does *in vivo*, thus serving as an ideal candidate of therapeutic TB vaccines.

DC-based TB vaccines have been examined to a limited extent in murine models. One of the first approaches to manipulating DCs *ex vivo* to induce immunity against pulmonary *M. tuberculosis* challenge was carried out by Demangel et al. [14]. They report that *ex vivo* generated DCs infected with BCG delivered intratracheally induced only a short-lived protective immune response against pulmonary *M. tuberculosis* challenge. However, it was also reported that the *ex vivo* DCs infected with BCG were able to harbour viable bacilli [14,15]. Delivering viable bacilli to the respiratory tract is not a safe approach to inducing TB immunity in TB patients, particularly those with immune-compromised conditions. Other strategies to using DC-based vaccines in more recent years have focused on pulsing *ex vivo* derived DCs with whole proteins or immunodominant peptides [16,17]. Ag85A is a major secreted protein found in all clinically isolated strains of *M. tuberculosis*. DCs pulsed with whole Ag85A protein delivered intranasally gave rise to increased numbers of IFN-γ-secreting $CD4^+$ and $CD8^+$ T-cells in the lung [17]. However, these cells were not able to confer protection against pulmonary *M. tuberculosis* challenge [17]. We have demonstrated that DCs pulsed with Ag85A immunodominant CD4 and CD8 peptides induced slightly better immune responses than DCs pulsed with whole protein when delivered intramuscularly or intravenously [18].

We have developed a novel approach to using DC-based vaccines in anti-TB vaccinology. We infected bone marrow-derived DCs with a recombinant adenovirus expressing Ag85A, which is capable of expressing Ag85A fused to a signal peptide sequence, so that Ag85A can be secreted from infected mammalian cells. We found that DCAdAg85A (DCs virally transduced with AdAg85A) induced significantly greater $CD4^+$ and $CD8^+$ IFN-γ^+ responses than peptide- or protein-pulsed DCs when delivered intramuscularly. This enhanced immune activation is probably due to the increased antigen presentation, by the infected DC on MHCI as well as antigen presentation of secreted Ag85A by endogenous DCs on MHCII. Upon infection with AdAg85A, DCs expressed enhanced co-stimulatory molecules CD80, CD86 and enhanced production of IL-6 and IL-12 [18], thus having greater type 1 immune-activating capabilities. In order to better target lung-specific immunity, DCAdAg85A was given intranasally. Mucosally administered DCAdAg85A resulted in recruitment of antigen-specific $CD4^+$ IFN-γ^+ and $CD8^+$ IFN-γ^+ T-cells to the airway lumen, which conferred protection against pulmonary *M. tuberculosis* infection (unpublished work). Such respiratory mucosal immunization with DCAdAg85A may represent an effective way to trigger anti-TB memory T-cell responses without causing an unwanted inflammatory response. We are planning to use this strategy to treat pulmonary TB in mouse models.

To enhance further the antigen-presenting power of the DC, our preliminary results suggest that DCs transduced with AdAg85A co-expressing GM-CSF can further potentiate the antigen-specific T-cell responses. This is probably due to GM-CSF secreted by the virally transduced DCs recruiting endogenous DCs to the vaccination location and site of antigen deposition. The secreted GM-CSF may act on the infected DCs, causing full maturation as well as acting on the endogenous DCs to provide a maturation signal to recruited DCs, which can then pick up the secreted Ag85A from the vaccine DCs to potentiate the number of DCs activated by a single immunization event. Thus DCs manipulated *ex vivo* to express an antigen of interest may recruit and expand the endogenous DCs, thus leading to a robust anti-TB immune response.

Conclusions

DCs are critical players in the initiation of an effective immune response against intracellular infections such as *M. tuberculosis* Efforts that have focused on expanding endogenous populations of DCs *in vivo* have proven to be very effective in increasing the immunogenicity of vaccines and such cytokine adjuvants as GM-CSF would be prime candidates to be applied in conjunction with current BCG vaccination strategies. More recent efforts have focused on manipulating DCs *ex vivo* to generate cell-based vaccines that can be used *in vivo* to trigger type 1 immunity against intracellular infections. While *ex vivo* derived DCs pulsed

with antigenic peptides or proteins have resulted in mediocre induction of protective immunity, virally transduced DCs are capable of much greater protective immunity. Such virally transduced DC vaccines may also serve as therapeutic vaccines to treat intracellular infections without causing tissue damage and virus-neutralizing antibodies.

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