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*J Immunol* 2013; 191:302-311; Prepublished online 22 May 2013;
doi: 10.4049/jimmunol.1202824
http://www.jimmunol.org/content/191/1/302

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Influenza A Virus Infection Impairs Mycobacteria-Specific T Cell Responses and Mycobacterial Clearance in the Lung during Pulmonary Coinfection

Manuela Florido,* Michael A. Grima,* Caitlin M. Gillis,* Yingju Xia,† Stephen J. Turner,‡ James A. Triccas,§ John Stambas,† and Warwick J. Britton*§

Individuals infected with mycobacteria are likely to experience episodes of concurrent infections with unrelated respiratory pathogens, including the seasonal or pandemic circulating influenza A virus strains. We analyzed the impact of influenza A virus and mycobacterial respiratory coinfection on the development of CD8 T cell responses to each pathogen. Coinfected mice exhibited reduced frequency and numbers of CD8 T cells specific to Mycobacterium bovis bacille Calmette-Guérin (BCG) in the lungs, and the IFN-γ CD8 T cell response to BCG-encoded OVA was decreased in the lungs of coinfected mice, when compared with mice infected with BCG alone. Moreover, after 2 wk of infection, mice coinfected with both pathogens showed a significant increase in the number of mycobacteria present in the lung compared with mice infected with BCG only. Following adoptive transfer into coinfected mice, transgenic CD8 T cells specific for OVA257–264 failed to proliferate as extensively in the mediastinal lymph nodes as in mice infected only with BCG-OVA. Also noted was a reduction in the proliferation of BCG-specific CD4 transgenic T cells in mice coinfected with influenza compared with mice infected with BCG alone. Furthermore, phenotypic analysis of CD11c+ dendritic cells from mediastinal lymph nodes of the infected mice showed that coinfection was associated with decreased surface expression of MHC class II and class I. Thus, concurrent pulmonary infection with influenza A virus is associated with decreased MHC expression on dendritic cells, reduced activation of BCG-specific CD4 and CD8 T cells, and impaired clearance of mycobacteria. The Journal of Immunology, 2013, 191: 302–311.

Mycobacterium tuberculosis and influenza virus are two of the most important respiratory pathogens of humans. Tuberculosis (TB) is the major cause of death from a bacterial infection in adults; in 2010 alone there were 8.8 million new TB cases and 1.4 million deaths from TB (1). The burden of disease caused by influenza A viruses is also considerable because of high hospitalization and mortality rates, as highlighted during the 2009 H1N1 influenza pandemic, which affected healthy individuals as well as those with pre-existing respiratory diseases (2, 3). Occurrence of seasonal outbreaks of influenza means the pathogen is repeatedly encountered during one’s lifetime. Moreover, during sporadic pandemics of influenza A, the virus spreads rapidly worldwide, so that coexisting infection with mycobacteria is highly likely, particularly in areas where TB incidence is high (4, 5).

Mycobacteria and influenza virus infections induce different patterns of immune responses in the host. Following aerosol infection, mycobacteria infect alveolar macrophages and replicate slowly in the lungs before being detected in dendritic cells (DCs) in the mediastinal lymph nodes (MLNs) after 1 wk (6, 7). Specific T cells are activated in the MLNs and recruited back to the infected lungs, where they stimulate macrophages to inhibit mycobacterial growth (8). In mice infected with M. tuberculosis, the bacterial load plateaus at a level that is maintained for 9–12 mo, before progression of infection and death (9). Less virulent mycobacteria species, such as the attenuated M. bovis bacille Calmette-Guérin (BCG), are more easily controlled by the host immune response but nevertheless can persist for extended periods (7). Ag-specific CD4 and CD8 T cell responses are essential for the control of mycobacterial infections (10). The CD4 T cell response is critical for protection during the acute phase of infection (11, 12), whereas M. tuberculosis–specific CD8 T cells are essential for maintaining long-term control of TB infection (13, 14). In contrast to the chronic intracellular lung infection caused by mycobacteria, influenza produces a rapid, transient infection of the pulmonary track epithelia, and stimulates a potent and sustained CD8 T cell response that is essential for acute control of the primary influenza infection. Specific CD8 T cells develop 5–7 d post infection and clear the virus from infected mice by day 10 (15). Influenza-specific CD4 T cell populations are smaller and more diverse than those producing CD8 T cell responses, but also persist lifelong in mice and are essential for the development of the memory CD8 T cell response (15).

Concurrent or sequential infections with different pathogens can alter the response of the host to unrelated pathogens. One striking example is the infection with influenza virus in humans that often...
is associated with secondary bacterial pneumonia, most commonly caused by Streptococcus pneumoniae (16, 17). Multiple mechanisms contribute to the enhanced susceptibility to secondary bacterial infections following influenza infection (18). Influenza virus infects epithelial cells leading to tissue damage and increased viral neuraminidase-mediated exposure of cryptic receptors that enhance bacterial adherence and infectivity (19). In addition, cytokine and chemokine production during influenza infection affects leukocyte recruitment and function, in some cases with deleterious effects on the host response to secondary challenge with bacteria (20, 21). Type I IFNs are implicated, as concurrent influenza infection exacerbated lung S. pneumoniae through IFN-α/β-dependent inhibition of chemokine production and neutrophil recruitment (22). Recently, the induction of glucocorticoids during influenza infection was associated with suppression of the immune response against systemic secondary bacterial infection during co-infection with Listeria monocytogenes (23).

The interaction between influenza virus and mycobacterial infections has been less studied. Previous studies showed that concurrent influenza A infection exacerbated Mycobacterium tuberculosis loads in the lungs of infected mice (24), and that subsequent influenza infection suppressed tuberculin delayed-type hypersensitivity in mice previously infected with M. tuberculosis (25). However, the mechanisms for these effects were not defined.

We have developed a mouse model of pulmonary coinfection with M. bovis BCG expressing OVA (BCG-OVA) and an A/PR8/80-34 H1N1 influenza A virus expressing the CD8 T cell epitope of the M. tuberculosis protein 64 (MPT64) Ag (PR8-MPT64) to investigate the impact of coinfection on the generation of immune responses to each pathogen. Mice coinfected with influenza and BCG, compared with mice infected only with BCG-OVA, exhibited reduced levels of BCG-encoded OVA-specific CD8 T cells. This finding was associated with reduced proliferation of adoptively transferred BCG-specific CD8 T cells and CD4 T cells, reduced MHCII and MHCI expression on DCs, exacerbated disease, and delayed clearance of the mycobacteria from the lung. Therefore, pulmonary coinfection with influenza A virus caused impairment of the generation of effective CD8 and CD4 T cell response against concurrent mycobacterial infection.

Materials and Methods

Ethics statement

All animal experiments were performed in accordance with the Australian code of practice for the care and use of animals for scientific purposes and with approval of the University of Sydney Animal Care and Ethics Committee (K759-2009-3-S136).

Mice

Female 6- to 8-wk-old C57BL/6 and C57BL/6.J/Ptprca mice were purchased from the Animal Research Center (Perth, WA, Australia). OT-I transgenic (Tg) mice were a gift of Professor W. Heath (Walter and Eliza Hall Institute of Medical Research, Melbourne, VIC, Australia), and p25 CD4+ TCR Tg mice (specific for residues 240–254 of M. tuberculosis protein Ag85B [Ag85]) (26) were kindly provided by Professor K. Takatsu (Nihon University, Tokyo, Japan) and Dr. Joel Ernst (New York University, New York, NY) and were bred at the Centenary Institute Animal Facility (Newtown, NSW, Australia). Mice were maintained in specific pathogen-free conditions at the Centenary Institute Animal Facility.

Recombinant BCG and influenza A virus strains

Recombinant M. bovis BCG (Pasteur strain), engineered to express a truncated form of the OVA protein (27), or M. tuberculosis Ag85B under the control of the hspX promoter (28) was used. Recombinant influenza A virus strains were generated using an eight-plasmid reverse genetics system, as previously described (29–31). DNA encoding M. tuberculosis MPT64 (FAVTNDDGVI) peptide was inserted into the neuraminidase stalk of A/PR8/80/34 at amino acid position 44 by serial PCR. DNA plasmids containing all eight influenza genome segments, including the pHW2000 with the genetically manipulated neuraminidase segment, were transfected into a coculture of 293T and Madin–Darby canine kidney cells. Viruses were then amplified in 10-d-old embryonated eggs, and the presence of recombinant virus was determined using a hemagglutination assay. Insertion of the DNA encoding the MPT64 peptide was confirmed by sequencing.

BCG and influenza virus infection and quantification

Mice were anesthetized i.p. with ketamine/xylazine solution (125 mg and 8 mg/kg, respectively) and infected intranasally (i.n.) with 10^7 CFU BCG-OVA alone, 25 PFU PR8-MPT64 alone, or simultaneously with both pathogens at the same doses. In the model of sequential infection, mice were infected with 2 × 10^6 CFU BCG-OVA, and 3 wk later mice were treated or not treated with a combination of isoniazid (0.1 mg/ml) and rifampicin (0.1 mg/ml) in the drinking water for 4 wk. After antibiotic treatment, mice were infected i.n. with 25 PFU PR8-MPT64 or mock infected with 50 µl sterile PBS. Bacterial quantification was performed by plating serial dilutions of lung homogenates onto Middlebroek 7H11 supplemented with 10% oleic acid–albumin enrichment (Difco) agar plates.

Preparation of cell suspensions

Lungs were digested in RPMI 1640/10% FCS containing collagenase 1 at 100 U/ml and DNase II at 25 µg/ml for 30 min at 37°C. Lymph nodes and spleens were disrupted by passage through 70-µm cell strainers and washed with RPMI 1640/10% FCS. Spleen and lung cell suspensions were incubated with ACK lysis buffer for 3 min to remove RBCs, then washed and resuspended in the appropriate volume of RPMI 1640/10% FCS.

Flow cytometry for surface markers and tetramer staining

The following Abs, which were purchased from BD Pharmingen unless stated otherwise, were used: Pacific blue anti-CD45.2 (BioLegend), PerCP anti-CD3, Alexa Fluor 700 anti-CD4, APC-Cy7 anti-CD8, FITC anti-CD44, PECy7 anti-CD62L, PerCP anti-CD45.1 (BioLegend), PE anti-CD19, PE anti-CD3, PE/Cy7 anti-CD11c, biotin MHC I (1-H2D^b, Bioscience), APC anti-MHCII (I-A/E, Bioscience), and Alexa 700 anti-CD11b.

Two million cells were incubated with 1.25 µg/ml anti-CD3/CD2/C16 (eBioscience) in FACS wash buffer (PBS/2% FCS/0.1% NaN3) for 30 min to block Fc receptors, then washed and incubated for 30 min with a mix of fluorescent Abs. Fixable Blue Dead Cell Stain (Invitrogen) was added to the Ab mix at the dilution recommended by the manufacturer to allow dead cell discrimination.

In addition, APC-conjugated MHC I Kb OVA257–264 and PE-conjugated MHC I D^b MPT64 (164-198) Tetramers (both supplied by the Tetramer Unit, Department of Microbiology and Immunology, University of Melbourne) were added to the Ab mix, and the cells were stained for 45 min on ice. Stained samples were then washed, resuspended in FACS wash buffer, and run on an LSRII Flow cytometer. Analysis was performed using FlowJo software (TreeStar, Ashland, OR).

IFN-γ ELISPOT

Cells were cultured at a density of <2 × 10^6 cells per milliliter of RPMI 1640/10% FCS in an ELISPOT plate precoated with 15 µg/ml anti-mouse IFN-γ mAb (clone A18) in the presence of pathogen-specific peptides NP366–374, MPT64 (164-198), and OVA257–264 (Genscript) at a final concentration of 10 µg/ml or with BCG-OVA lysate, at a final concentration of 5 µg/ml. As controls, cells were incubated with media alone or with 3 µg/ml of Con A (Sigma). After 18 h of incubation, plates were thoroughly washed with PBS/0.01% Tween 20 and incubated with biotinylated anti-mouse IFN-γ mAb (clone XMG1.2) at a final concentration of 6 µg/ml for at least 2 h at 37°C. Development was achieved by incubation with avidin-conjugated alkaline phosphatase (Sigma-Aldrich) followed by the addition of AP-conjugate substrate (Bio-Rad). The numbers of spots in the wells were determined using an AID ELISPOT Reader.

Adaptive experiments

Splenocytes from OT-I or p25 Tg mice were labeled with 3 µM CFSE (27). A total of 5 × 10^6 Tg cells were transferred by IVI into the recipient mice either 1 d prior to infection with BCG-OVA or BCG-OVA plus PR8-MPT64 (OT-I Tg cells) or 6 d post infection with BCG-Ag85 or BCG-Ag85 plus PR8-MPT64 (p25 Tg cells). At different time-points post infection, the proliferation of Tg cells in the lungs, lymph nodes, and spleen of the infected mice was analyzed by CFSE dilution by flow cytometry.
Histology
Right lobes from the lungs of each infected mouse were perfused with a 10% buffered formalin solution and processed for light microscopy. Tissue sections were stained with H&E.

Statistical analysis
Data were analyzed using the Student t test, when comparing two experimental groups, or ANOVA followed by the Tukey posttest for more than two groups. Differences with $p < 0.05$ were considered statistically significant.

Results
Pulmonary BCG-specific CD8 T responses were impaired in coinfected mice
We first addressed how coinfection affected the frequency and number of specific T cells, by quantifying at different time-points the CD8 T cells in the lungs of mice infected with BCG-OVA alone, with PR8-MPT64 alone, or with both pathogens (Fig. 1A). K$^b$-OVA$^{257–264}$ tetramer$^+$ CD8 T cells were detected in the lungs of mice infected with BCG-OVA, and D$^b$-MPT64$^{190–198}$ tetramer$^+$ CD8 T cells were detected in mice infected with PR8-MPT64, whereas both populations were present in the lungs of coinfected mice (Fig. 1B). BCG-OVA–specific CD8 T cells were detected in the lungs of mice infected with BCG-OVA from 7 d post infection, and the frequency increased with time to > 10% of CD8 T cells at day 21. Remarkably, the frequencies of BCG-OVA–specific CD8 T cells in the lungs of coinfected mice were significantly lower than those in mice infected with BCG-OVA alone, at all time-points tested (Fig. 1C). This finding contrasts with the frequencies of CD8 T cell specific for the MPT64 epitope.

FIGURE 1. Coinfected mice have decreased OVA-specific CD8 T cells in the lungs. (A) Experimental plan. (B) Representative flow cytometry plots after staining with K$^b$OVA$^{257–264}$ and with D$^b$MPT64$^{190–198}$ tetramers the lung cells from mice infected i.n. with 10$^6$ CFU of BCG-OVA, with 25 PFU of PR8-MPT64, or with both pathogens at the same doses. Dot plots were generated after gating on CD3+CD8+ events. At 7, 14, and 21 d post infection, the frequency of K$^b$OVA$^{257–264}$ tetramer$^+$ CD8 T cells (C) and the frequency of D$^b$MPT64$^{190–198}$ tetramer$^+$ CD8 T cells (D) were determined for each individual mouse, and the respective numbers of cells in the lung were calculated (E, F, G). (E) Number of total leukocytes (left), CD4 T cells (center), and CD8 T cells (right) during the course of infection in the lungs of mice infected with BCG-OVA (•), BCG-OVA + PR8-MPT64 (▴), or PR8-MPT64 alone (○). (H) Number of activated (CD44$^{high}$CD62L$^{low}$) CD8 T cells in the lungs of infected mice at 21 d post infection. In panels (E)–(H), data are shown as means ± SEM (n = 4) and are representative of two experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ANOVA followed by Tukey posttest.
which were similar in mice infected with PR8-MPT64 or with PR8-MPT64 and BCG-OVA during the first 2 wk of infection. By day 21, the percentage of MPT64-specific CD8 T cells in the lungs of mice infected with PR8-MPT64 alone was higher than that in coinfected mice (Fig. 1D).

The total number of leukocytes in the lungs of PR8-MPT64–infected mice decreased following clearance of the viral infection, which occurred before day 10 post infection, whereas the number of cells in the lungs of BCG-OVA–infected mice increased over the time-course examined (Fig. 1E, left). From day 14 post infection, the number of cells recovered from the lungs of coinfected mice was significantly higher than that recovered from the lungs of mice infected with BCG-OVA alone. Most importantly, the number of total OVA-specific CD8 T cells present at day 21 in the lungs of coinfected mice was significantly reduced compared with the number in mice infected with BCG-OVA alone, showing that coinfection with influenza A virus leads to impaired development of CD8 T cell responses to OVA expressed by BCG (Fig. 1F). The CD8 T cell response to OVA was decreased in the lungs of coinfectected mice at a time when a higher number of activated CD8 T cells were present in the lungs of coinfectected mice than in mice infected with the individual pathogens (Fig. 1H). Coinfection did not influence pulmonary viral-specific responses, as the number of MPT64-specific CD8 T cells did not differ between the lungs of mice coinfected with both pathogens and those infected with PR8-MPT64 alone (Fig. 1G). Thus, coinfection reduced BCG-OVA–specific CD8 T cell responses and had no impact on influenza-specific CD8 T cell responses in the lung.

**Coinfection affects T cell IFN-γ responses to pathogen-specific Ags**

We then examined how coinfection impacted T cell function by determining the frequency of cells producing IFN-γ in response to BCG-OVA or PR8-MPT64–specific Ags. As expected, responses to virus-specific peptides (endogenous NP366–374 and engineered MPT64190–198) were detected earlier (from day 7) in both groups of mice infected with PR8-MPT64, whereas responses to BCG-OVA Ag (OVA257–264) were detected only in mice infected with BCG-OVA at days 14 and 21 post infection. IFN-γ responses of lung T cells to the viral-encoded NP366–374 and MPT64190–198 peptides were not significantly affected by coinfection at all the time-points tested (Fig. 2A). The frequency of IFN-γ–producing lung cells in response to OVA257–264 peptide was significantly reduced in coinfected mice compared with mice infected with BCG-OVA alone at days 14 and 21 post infection (Fig. 2A). This result is consistent with the previous observation of decreased percentage of OVA tetramer+ cells in the lungs of coinfectected mice (Fig. 1C). At day 21, the IFN-γ responses to pathogen-specific recall Ags were also analyzed in the MLNs and spleens of mice coinfected with BCG-OVA and PR8-MPT64 or mice infected with each pathogen alone. The CD8 T cell IFN-γ responses to virus-specific Ags were reduced in the MLNs (to NP366–374 peptide) and in the spleen (to NP366–374 and MPT64190–198 peptides) of coinfected mice compared with mice infected with PR8-MPT64 alone (Fig. 2B). Thus, coinfection was associated with reduced induction of IFN-γ–secreting BCG-OVA–specific CD8 T cell responses in the lung and influenza-specific CD8 T cell responses in the MLNs and spleen.

**Influenza A virus infection reduces BCG-specific CD8 T cell response during chronic BCG infection**

To address whether infection with influenza A virus had any impact on the CD8 T cell responses during a chronic infection with mycobacteria, a similar analysis was done in mice infected with influenza A virus 7 wk post infection with BCG-OVA. To determine if the presence of active coinfection was required for this effect,
cohort of mice infected with BCG-OVA were treated or not treated with antibiotics for 4 wk prior to infection with PR8-MPT64 (Fig. 3A). Treatment with antibiotics cleared the BCG infection, whereas the groups of mice not treated with antibiotics still had detectable mycobacteria in the spleen and lung prior to influenza infection (data not shown). As observed in mice infected concurrently with both pathogens, sequential infection with influenza A of mice with continuous BCG infection resulted in reduced IFN-γ T cell response to BCG-encoded OVA257–264 peptide. Importantly, the reduction of BCG-OVA–specific CD8 T cell response did not occur in mice following clearance of BCG infection (Fig. 3B). Thus, sequential infection with influenza A virus reduces BCG-OVA–specific CD8 T cell response, but only when the mycobacteria are still present in the lungs of the mice.

Coinfection impairs the proliferation of BCG-specific CD8 T cells

To determine the mechanism responsible for the observed reduction of the BCG-specific CD8 T cell response in the lungs of coinfected mice, we examined the initial priming of CFSE-labeled OVA257–264–specific OT-I CD8 Tg cells in mice infected with BCG-OVA or with BCG-OVA and PR8-MPT64. At 6 d post infection, adoptively transferred OT-I cells had already undergone division in the draining lymph nodes (DLNs) of both groups of mice; however, more cells had entered the fourth round of division in mice infected with BCG-OVA alone than in coinfecte mice. By 9 d post infection, >70% of the OT-I cells in the MLNs of mice infected with BCG-OVA alone had undergone more than four rounds of division, compared with an average of only 30% in coinfecte mice (Fig. 4A). Furthermore, a significant reduction in the proliferation index was found in the MLNs of coinfecte mice at all time-points tested (Fig. 4B). Decreased frequencies of divided OT-I cells were found at days 6 and 9 in the spleen and non-DLNs of coinfecte mice compared with mice infected with BCG-OVA alone (Fig. 4A).

Consistent with these results, analysis of the OVA tetramer+ CD8 T cells present at the MLNs of infected mice showed that at day 7 a significantly higher number of OVA-specific CD8 T cells were noted in the MLNs of mice infected with BCG-OVA alone than in mice coinfecte with both pathogens (Fig. 4C, left). No difference was found in the number of MPT64 tetramer+ CD8 T cells at day 7 (Fig. 4C, right), and by 2 wk post infection, the frequency of virus-specific T cells had reduced in both groups infected with PR8-MPT64 (<0.2% of total CD8 T cells, data not shown).

The quantity of Ag present at the lymph node is crucial in determining the magnitude of the T cell response generated (27). To determine if the reduction of BCG-specific CD8 T cell response observed in coinfecte mice was due to Ag limitation in the MLNs of coinfecte mice, we quantified the number of viable mycobacteria present in the MLNs of mice infected with BCG-OVA alone or with both BCG-OVA and PR8-MPT64 at 5, 15, and 21 d post infection. We found no significant difference in the number of viable mycobacteria present in the MLNs of mice from the two experimental groups (data not shown). Therefore, coinfection impaired BCG-encoded OVA-specific CD8 T cell proliferation despite the availability of Ag at the MLN.

**Pulmonary BCG-specific CD4 T cell response is impaired during coinfection**

Having observed that coinfection with influenza A virus reduced the CD8 T cell response to BCG, we examined the CD4 T cell response to BCG, using a recombinant strain of BCG overexpressing Ag85B [BCG-Ag85; (26)]. This procedure permitted analysis of Ag85B–specific CD4 T cell responses using the Ag85240–254–specific CD4 Tg T cells in an adoptive transfer model (26). Mice were infected with BCG-Ag85 alone or coinfecte with BCG-Ag85 and PR8-MPT64, and recall responses of lung cells to the Ags were quantified by IFN-γ ELISPOT. After 21 d of infection, fewer cells from the lungs of coinfecte mice responded to the CD4 T cell epitope, Ag85240–254, compared with cells from the lungs of mice infected only with BCG-Ag85 (Fig. 5A). CFSE-labeled p25 Tg cells were transferred at day 6 post infection of mice with either BCG-Ag85 or BCG-Ag85 plus PR8-MPT64, and their activation was analyzed at day 9. As observed for CD8 OT-I cells, the transferred p25 Tg CD4 T cells failed to proliferate as extensively in the MLNs of coinfecte mice as in the MLNs of mice infected with BCG-Ag85 alone (Fig. 5B). Therefore, coinfection also has an impact on BCG-specific CD4 T cell proliferation and cytokine production.

**Reduced MHC class I and II expression on DCs during coinfection**

Effective priming of T cells is highly dependent on presentation of Ag by DCs in the DLNs. Therefore, we analyzed by flow cytometry the activation phenotype of DCs present in the draining MLNs of mice infected for 10 d with BCG-OVA or with both BCG-OVA and PR8-MPT64. DCs were identified as two distinct populations of CD11c+MHCIhigh (gate I) and CD11c+MHCIIintermediate (gate II)
cells (Fig. 6A). The frequencies of these populations and their levels of expression of MHCII and MHCI molecules were analyzed (Fig. 6B, 6C). The MLNs of coinfected mice had significantly lower frequencies of CD11c+MHCIIhigh cells and higher frequencies of CD11c+MHCIIintermediate, compared with mice infected with BCG-OVA alone. In addition, both populations of DCs from coinfected mice expressed lower levels of MHCII than did cells from the MLNs of BCG-OVA–infected mice, as evidenced by the significant differences in the respective mean fluorescence intensity (Fig. 6B). Moreover, CD11c+MHCIIintermediate cells from the MLNs of coinfected mice also expressed lower levels of MHCI molecules (Fig. 6C).

Thus, coinfection with influenza and BCG results in a higher proportion of DCs that express lower levels of MHCI and MHCII molecules in the MLNs.
Mycobacterial clearance is delayed and disease exacerbated in the lungs of coinfected mice

Finally, we addressed whether coinfection with BCG and influenza A virus had any impact on the disease and on control of the pathogens in the lung. Histological analysis of the lungs of mice infected with PR8-MPT64 revealed that at 21 d post infection only very limited areas of the lung showed accumulation of leukocytes, usually in the vicinity of airways (Fig. 7A, right). The lungs of mice infected with BCG-OVA alone revealed numerous small foci of macrophages and lymphocytes scattered throughout the lung tissue, often in perivascular localizations (Fig. 7A, left). Mice infected with both BCG-OVA and influenza A PR8-MPT64 virus had scattered small lesions, similar to those in mice infected with BCG-OVA alone, but also displayed extensive areas of leukocyte accumulation surrounding the airways (Fig. 7A, center). This extensive accumulation of cells was found in both PR8-MPT64–infected and coinfected mice at day 7 and day 10 (data not shown), but had resolved by day 21 in the mice infected with PR8-MPT64 alone, whereas it persisted and increased in area in the lungs of coinfected mice.

To determine the effect of coinfection on mycobacterial and viral clearance, the number of viable mycobacteria and the viral titers present in the lungs of infected mice were quantified. Productive infection with influenza A virus resulted in weight loss and recovery; however, coinfection did not increase the weight loss associated with PR8-MPT64 infection (Fig. 7B). The number of viable BCG-OVA in the lungs of coinfected mice was significantly higher at day 14 and day 21 compared with mice infected with BCG-OVA alone (Fig. 7C). No difference in the bacterial loads present in the spleen was found between experimental groups (data not shown). There were no significant differences in the pulmonary viral loads between mice infected with BCG-OVA and PR8-MPT64 and those infected with PR8-MPT64 alone at days 3 and 5 post infection, and by day 10 no viruses were detected in either experimental group (Fig. 7D). When mice were infected with PR8-MPT64 3 wk post infection with BCG-OVA, coinfected mice had decreased pulmonary viral loads (Fig. 7E). Thus, coinfection with influenza and BCG results in increased pulmonary disease and a delay in mycobacterial clearance from the lungs of the infected mice, without reducing host control of the viral infection.

Discussion

Several studies addressing the interaction between influenza virus and S. pneumoniae have attributed the increased susceptibility to secondary bacterial infections to deleterious effects on innate immunity (21, 33, 34). This study demonstrates the novel finding that pulmonary coinfection with influenza A virus impairs the development of acquired immunity against an intracellular bacterial pathogen, BCG, resulting in decreased protection against mycobacterial infection. Coinfection with influenza A virus reduced the frequency and number of BCG-OVA–specific CD8 T cells in the lung and the magnitude of BCG-specific CD4 and CD8 T cell IFN-γ–secreting responses. This effect occurred regardless of the fact that the lungs of coinfected mice harbored a larger total number of T cells than did those of mice infected with the individual pathogens, indicating that the decreased magnitude of BCG-specific response was not due to failure of recruitment of T cells to the lung.

The T cell responses elicited by influenza A virus and BCG infections are characterized by different kinetics. CD8 T cell responses to influenza infection are generated early post infection, peaking at day 10, and with clearance of the virus, the T cell population contracts rapidly owing to programmed cell death triggered by the decline in Ag levels and inflammatory cytokines (35). The T cell response specific to BCG, however, is only detected in the lung 2 wk post infection at a time when, in coinfected mice, contraction...
Coinfection resulted in downregulation of surface expression of infected with BCG alone (Fig. 6). This finding suggests that was decreased on DCs of coinfected mice compared with mice the MLN (Fig. 6). Moreover, expression of MHCII and MHCI increased frequency of DC cells expressing high levels of MHCII in tested, suggesting that trafficking of cells containing BCG from enza virus or with BCG alone were similar at all the time-points present in the MLNs of mice infected with both BCG and influ- optimal T cell activation (36). We found that the bacterial loads molecules, such as CD86, CD80, and CD40, which are required for priming of CD4 T cells is partially responsible for the decreased IFN- effect of coinfection is not limited to CD8 T cell responses, as the deleterious impact on the control of pulmonary viral loads. In fact, mice infected with mycobacteria and influenza virus. Of interest, altered peptide presentation to CD4 T cells (40). It will be relevant to determine in the future if a similar effect occurs in the DCs of mice coinfected simultaneously with PR8-MPT64 and BCG-OVA (▴) at days 5, 14, and 21 post infection. Results are representative of two experiments. CD4 T cell responses in the lungs of coinfected mice.

Activated DCs express high levels of MHC and costimulatory molecules, such as CD86, CD80, and CD40, which are required for optimal T cell activation (36). We found that the bacterial loads present in the MLNs of mice infected with both BCG and influenza virus or with BCG alone were similar at the time-points tested, suggesting that trafficking of cells containing BCG from the lung to the MLN was not impaired in coinfected mice, and that availability of Ag in the MLN was not reduced by coinfection. Coinfection with influenza was associated, however, with decreased frequency of DCs expressing high levels of MHCII in MLN DCs and/or increased recruitment of cells expressing lower levels of MHCII molecules to the lymph node, and this led to impaired Ag presentation and generation of BCG-specific T cell responses. These results contrast with previous reports that lung DCs from influenza-infected mice have higher expression of costimulatory molecules and enhanced capacity to promote CD4 T cell proliferation (37) and that DCs from the lymph nodes of mice infected with influenza underwent maturation and elicited enhanced T cell responses toward an unrelated protein Ag (38). These differences may relate to the site of the DCs analyzed, the time of analysis post infection, or the strain of virus, as none of the previous studies used the highly virulent PR8 strain. In our model of heterologous coinfection, a different inflammatory environment may alter influenza-induced effects. Influenza virus can infect DCs and other APCs, and alter the cell Ag-processing machinery—for instance, by elevating active cathepsin B levels (39). In this regard, influenza A virus infection of epithelial cells inhibited the IFN-γ-induced CIITA, resulting in decreased processing of Ags through the MHC II pathway and altered peptide presentation to CD4 T cells (40). It will be relevant to determine in the future if a similar effect occurs in the DCs of mice infected with mycobacteria and influenza virus. Of interest, mycobacteria themselves use a similar strategy to evade the host immune response, through the effect of cell wall mannosylated lipo-arabinomannan and lipoproteins suppressing IFN-γ–induced upregulation of MHCII molecules (41, 42).

Simultaneous infection with influenza and BCG had no deleterious impact on the control of pulmonary viral loads. In fact, when infection with influenza virus occurred when BCG-induced inflammation was at its peak, coinfected mice were able to control the viral infection more efficiently than mice infected with the virus...
alone. Nevertheless, coinfection with influenza virus and BCG did influence maintenance of the late CD8 T cell response to influenza, although this had no significant impact on viral replication in the lungs and the early virus-specific T cell response in the lungs. At 3 wk post infection, the frequency of IFN-γ-producing influenza-specific CD8 T cells was significantly reduced in the spleen and MLNs of coinfected mice. As the magnitude of a secondary response to influenza virus is largely determined by the frequency of available memory T cells prior to challenge (31), these results suggest that chronic infections with mycobacteria may have an impact on the generation of secondary immune responses to unrelated viral respiratory infections. Indeed, pre-existing memory CD8 T cells specific to L. monocytogenes were eroded by subsequent BCG infection, and this effect was found to be dependent on IFN-γ induced by the mycobacterial infection (43). Similar mechanisms may be involved in the decrease in frequency of virus-specific memory CD8 T cells in the lymphoid organs of coinfected mice. The degree of attrition of previously established memory CD8 T cells pools correlated with the virulence of the pathogen used for the experimental heterologous infection (43), and a similar effect may occur during chronic infections with M. tuberculosis that induce high levels of IFN-γ expression. Therefore, it will be important to extend these studies to use M. tuberculosis in a coinfection model with influenza. Improved understanding of the effects of chronic mycobacterial infection on the pre-existing memory T cell pools against influenza virus may influence vaccination strategies, as well as the management of influenza outbreaks in areas where TB is endemic.

Coinfection with influenza and BCG in the lung led to exacerbation of the pulmonary disease and, in contrast to models in which influenza and BCG infection occurred in separate organs (44), resulted in delayed clearance of BCG. Although influenza-mediated mechanisms affecting innate immunity may influence mycobacterial clearance from the lungs, both CD4 and CD8 T cells are required for effective control of BCG infections (45). Therefore, the reduction in CD4 and CD8 T cell responses observed during coinfection is likely to contribute to increased mycobacterial burden.

In summary, this study demonstrates that pulmonary coinfection with influenza reduces the generation of protective T cell responses against intracellular mycobacteria. In addition, as the decrease of mycobacteria-specific CD8 T cell responses occurs both in concurrent influenza and mycobacterial infection and upon infection with influenza A virus during chronic mycobacterial infection, the possibility is raised that sporadic or recurrent infections with influenza A virus may contribute to exacerbation of active and/or reactivation of latent TB infection. In experimental models, early studies demonstrated that coinfection with influenza A virus is associated with increased susceptibility to M. tuberculosis (24) and with suppression of tuberculin hypersensitivity (25). This deleterious impact of influenza A virus infection on the host response to established mycobacterial infection may only be evident clinically a long period after the coinfection occurred or following recurrent episodes of influenza, so that the cause–effect association is less apparent. The present study highlights the need for further investigation into the interaction between influenza A virus and M. tuberculosis infections in both experimental and clinical settings.

Acknowledgments

We thank St. Jude Children’s Research Hospital and Dr. Richard Webby for provision of influenza reverse genetics plasmids, Dr. L. Cavanagh for advice, and P. Reynolds for technical support.

Disclosures

The authors have no financial conflicts of interest.


