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Enhanced expression of murine β-defensins (MBD-1, -2,- 3, and -4) in upper and lower airway mucosa of influenza virus infected mice

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ABSTRACT

Although defensins are known to inhibit the replication of human influenza A virus (IAV) in vitro, their in vivo expression during IAV infection is not known. Here we investigated mRNA and protein expression of several β-defensins in the airways of IAV infected mice. Expression of murine β-defensin (MBD)-3 and -4 was enhanced (3 to 5-fold, $p<0.01$) in infected lungs, trachea and sinonasal mucosa. MBD-3 and -4 expressions were correlated with the time course of acute IAV infection suggesting their induction by IAV infection. Infected mice also showed increased levels of surfactant protein-D especially in the sinonasal mucosa. MBD-3 and -4 were localized to the conducting airway epithelial cells but not the alveolar tissue. We conclude that IAV infection upregulated the expression of inducible β-defensins in both the upper and lower airways. These novel findings suggest that β-defensins might contribute to innate and adaptive immune responses targeted against IAV infection.

Introduction

Human influenza A virus (IAV) is a major cause of morbidity and life-threatening respiratory tract disease worldwide. Although adaptive immunity against IAV has been extensively investigated, the components and interactions among the elements of innate immunity in IAV infection are less well studied (Wright et al., 2007). The main site of IAV infection is the respiratory mucosa including sinonasal, bronchiolar epithelium and the alveolar cells in which uncontrolled infection can often lead to significant lung pathology. After the initial exposure to IAV, virus infection advances rapidly reaching peak viral titers in the upper airway within 48 h after infection. This is readily demonstrated in experimentally infected people (Murphy et al., 1998) and in laboratory animals such as mice, ferrets, cotton rats and guinea pigs (Gubareva et al., 1998; Lowen et al., 2006; Ottolini et al., 2005; Zitzow et al., 2002). Thus, early virus–host interactions and innate immune defenses are likely to be important in the outcome and recovery from infection, and are crucial in immunologically naïve persons who lack specific adaptive defenses against IAV. Since the primary concern for IAV outbreaks is antigenic shift that greatly increases the non-immune population, there is clearly a need to better understand early innate responses to IAV.

In addition to protective mechanisms involving anatomic barriers and the mucociliary clearance apparatus, airway defenses include epithelial and inflammatory cells such as eosinophils, phagocytic neutrophils and macrophages. These cells express various pattern recognition receptors including toll-like receptors (TLRs), C-type lectin receptors, and scavenger receptors that are activated by microbial pathogens leading to the release of a myriad of defense molecules including surfactant proteins (SP-D) and antimicrobial peptides (Ganz 2003; Selsted and Quellettee, 2005; Wright 2005). In addition to directly killing microbes, certain antimicrobial peptides have been shown to trigger and facilitate the induction of host adaptive immune responses (Selsted and Quellettee, 2005). For instance, the epithelial derived β-defensins are chemotactic for various immune cells including memory T cells, monocytes and immature dendritic cells via specific chemokine receptors (Selsted and Quellettee, 2005; Yang et al., 1999).

β-defensin constitutes one of two major defensin subfamilies and are differentiated from the α-defensins by structural differences. Both of these defensins are small (2–6 kDa), cysteine-rich, cationic peptides that are known for their broad-spectrum antimicrobial activity against bacteria, fungi and viruses (Selsted and Quellettee, 2005; Lehrer 2004). In mammals, β-defensins are predominantly produced by barrier epithelial cells whereas α-defensins such as human neutrophil peptides (HNP1–3) are mainly stored in the azurophil granules of neutrophils (Selsted and Quellettee, 2005). Among human β-defensins (HBDs), HBD1 is expressed constitutively by keratinocytes whereas HBD2–4 are induced via activation of transcription factors

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such as NF-κB in keratinocytes and epithelial cells in response to microbial infection and proinflammatory stimuli such as interleukin-1, tumor necrosis factor, and lipopolysaccharide. Similarly, murine β-defensins (MBDs) including MBD-1, -2, -3, and -4 have also been detected in various mucosal tissues including the airways (Morrison et al., 1998; Moser et al., 2002; Yasin et al., 2004). These MBDs are antimicrobial and have been shown to play a role in resistance to infection (Moser et al., 2002; Morrison et al., 2002; Bals et al., 1999; Rivas-Santiago et al., 2006).

Recently, several defensins were shown to inhibit IAV replication in cell cultures and to exert their antiviral effect via a variety of mechanisms (Hartshorn et al., 2006; Leikina et al., 2005; Salvatore et al., 2007). For example, HBD-3 and a synthetic primate θ-defensin have been shown to block viral hemagglutinin induced membrane fusion (Leikina et al., 2005), whereas HNPs induce impairment of cellular pathways and increase neutrophil activity (Salvatore et al., 2007; Tecle et al., 2007). Similarly, these defensins are also inhibitory to several other enveloped viruses including human immunodeficiency and herpes simplex viruses (Chang et al., 2005; Hazrati et al., 2006; Quinones-Mateu et al., 2003; Sinha et al., 2003; Yasin et al., 2004; Zhang et al., 2002). In contrast, there have been few reports on the endogenous production of defensins during virus infection, and thus, little is known about the physiological role of defensins in virus infection. Here we investigated the in vivo mRNA expression and immunolocalization of defensins in the upper and lower airway of mice infected with two different IAV strains. In addition, we correlated defensin upregulation with the time course of IAV infection. We analyzed several MBDs, but not α-defensins because murine neutrophils express little or no defensins (Eisenhauer and Lehrer, 1992). Since SP-D have been shown to interact with defensins in vitro (Hartshorn et al., 2006), we also investigated SP-D expression in IAV infected mice in order to determine if defensins and SP-D are co-stimulated during IAV infection.

Results

Replication of IAV strains in upper and lower airways

We initially determined viral growth in airway tissues over a 7-day period so that we can characterize defensin expression in relation to time course of infection. To simulate natural infection that primarily targets the nasal tract, we inoculated mice with small droplets (10 μl) of either H1N1 or H3N2 influenza A. Although infected mice showed little respiratory symptoms, the rate of weight gain in these animals was lower than mock-infected mice throughout the duration of experiments. This reduction in body weight was especially prominent in H1N1 infected mice whose average weight was at 18.5% lower than control mice. In contrast, H3N2 infected animals showed only a 5.5% decrease in weight ($p<0.05$). Infected mice showed very high nasal titers 24 h after infection and viral levels persisted throughout the 7-day period (Fig. 1). Viral levels in the paranasal sinus mucosa were about 1.5 log lower ($p<0.01$) than nasal titer at 24 h post-infection, but their titers were more similar at later timepoints. H3N2 virus infected mice showed peak virus titers early in infection but viral levels declined rapidly beyond day 5 post-infection. Unlike the more mouse-virulent H1N1 virus, H3N2 virus showed lung titers of ~$1–4\times10^3$ pfu/g, which was 2–3 log lower compared to nasal, paranasal and trachea mucosa throughout the study duration ($p<0.001$). Mice infected with H1N1 strain showed significantly higher lung titers (~2×10^5 pfu/g) on day 1 and 3 post-infection ($p<0.01$) and more persistent viral levels in the trachea but viral titers in nasal and paranasal sinus mucosa were similar to H3N2 infection (Fig. 1B).

Expression of MBD-1, MBD-2, MBD-3, and MBD-4, SP-D mRNA in airway tissues

In preliminary analysis, we found that MBD-1 mRNA was readily detected in mock-infected animals, whereas MBD-3 and MBD-4

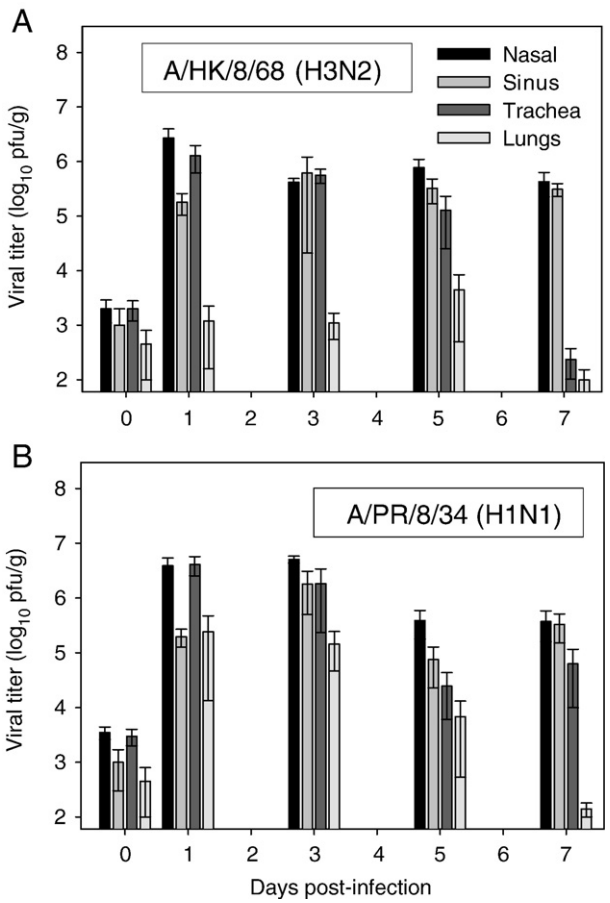

Fig. 1. Time course of virus replication in mice inoculated intranasally with (A) A/Hong Kong/8/68 (H3N2) and (B) A/Puerto Rico/8/34 (H1N1). Data point represents virus titer (mean ± SD) of the $\log_{10}$ pfu/g of tissue from individual animals (3 mice per group). The lower limit of virus detection was 50 pfu/g tissue.

mRNAs were expressed mainly in infected airway tissues. In contrast, MBD-2 was detectable only in IAV infected mouse lungs but not in the upper airway. Similar to MBD-1, SP-D mRNAs were readily detected in trachea, lungs and sinonasal specimens from both infected and mock-infected animals. Quantitative RT-PCR analysis were used to determine the extent of MBD and SP-D expression during early (day 1, 2 and 3) and late (day 6) IAV infection. Fig. 2 shows the levels of MBDs in infected tissues as expressed as fold change in mRNA expression relative to the respective mock-infected control airway mucosa. In sinonasal mucosa, MBD-1, MBD-3 and MBD-4 were modestly increased throughout the duration of infection except on day 3 post-infection when MBD-3 had a sharp increase of greater than 5-fold, which was significantly higher than the day 1 value ($p<0.01$). In lungs, the expression of all four MBDs were increased by 3 to 5-fold ($p<0.01$) on day 2 post-infection but only MBD-3 and MBD-4 were still modestly elevated by day 6 post-infection. In contrast, trachea showed little MBD expression during the first 2 days of infection but MBD-3 was enhanced by ~4 fold and MBD-4 was enhanced by >8 fold on days 3 and 6 post-infection. SP-D expression was also upregulated during IAV infection especially in the sinonasal mucosa at 2.5 to 5-fold increase during day 2 and 6 post-infection (Fig. 3). In contrast, there were a more modest increase in SP-D expression during most timepoints in the infected lungs although trachea showed 3-fold increase on day 6 post-infection.

Thus, SP-D, MBD-3 and MBD-4 were upregulated in both the upper and lower airway during IAV infection, whereas MBD-1 and MBD-2 were upregulated only in the lungs. In separate studies, we evaluated MBD expression using a more mouse-virulent viral strain (H1N1 PR8).

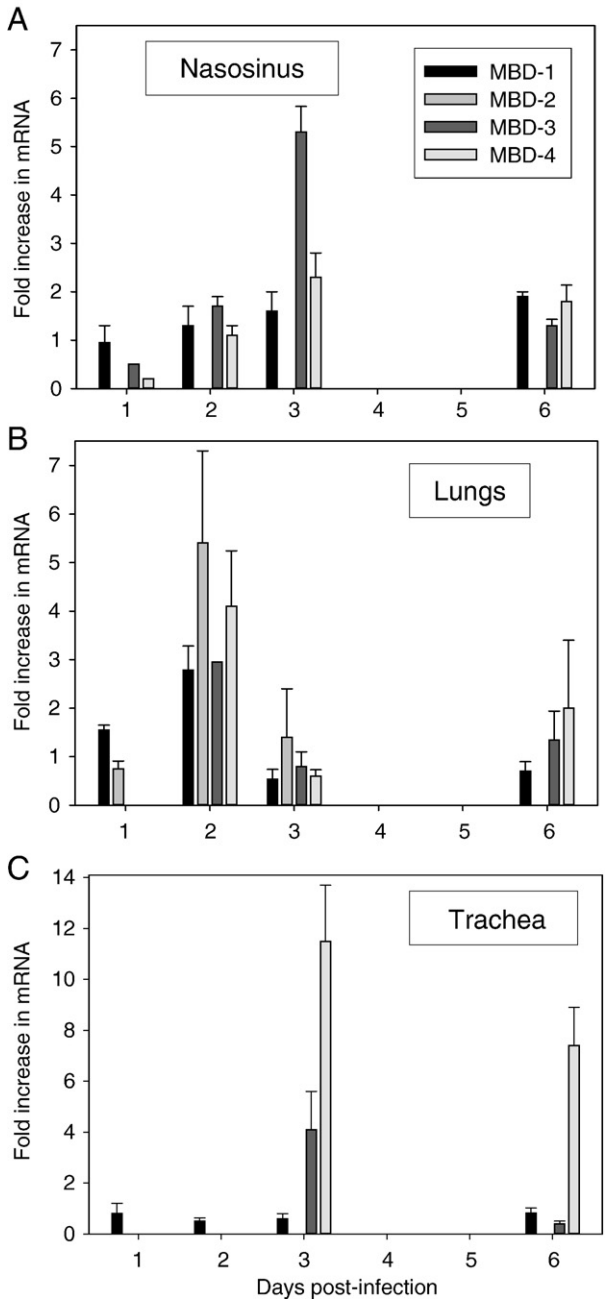

Fig. 2. RT-PCR analysis of murine β-defensins (MBDs) mRNA in sinonasal mucosa (A), lungs (B) and trachea (C) and at various times post-infection with A/Hong Kong/8/68 (H3N2). MBDs mRNA expression was analyzed by real-time RT-PCR. Bars represent defensins expression normalized to β-actin and relative to normal mucosa using the $2^{-\Delta\Delta CT}$ analysis as described in Materials and methods. Error bars represent the standard error of the mean of 2–3 analysis.

With H1N1 infection, we also noted enhancement in MBD-3 and MBD-4 expression in both lungs and the sinonasal mucosa at levels very similar to infection with H3N2 IAV strain except that there was a markedly greater increase in both MBD-3 and MBD-4 as early as day one post-infection in the sinonasal mucosa (Fig. 4).

Histology and immunolocalization of MBDs and SP-D in airway tissue sections

During the first three days of infection, the conducting airway tissues including trachea, nasal, paranasal, bronchus and bronchioles showed varying levels of epithelial cell desquamation and inflammation

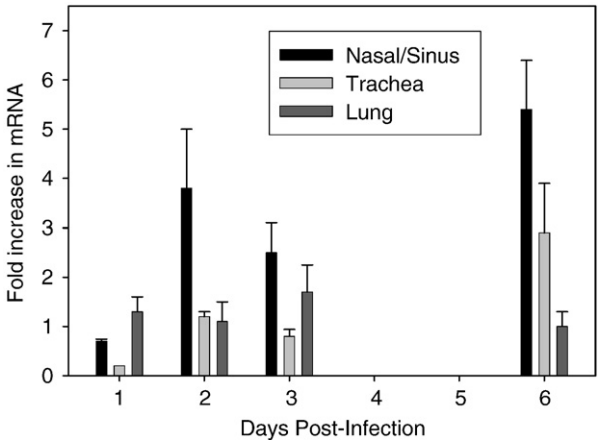

Fig. 3. Expression of surfactant protein-D (SP-D) mRNAs in airway mucosa 3 days post-infection with A/Hong Kong/8/68 (H3N2). Bars represent mRNA expression normalized to β-actin and relative to mock-infected mucosa using the $2^{-\Delta\Delta CT}$ analysis. Error bars represent the standard error of the mean of 2–3 analysis.

(Figs. 5A–C). Infected lungs showed evidence of acute bronchiolitis, bronchointerstitial pneumonia and alveolitis (Fig. 5A). Between days 5 and 7, most tissues showed much less pathology although virus antigen positive cells were still detectable in sinonasal mucosa. To determine the level of defensins and SP-D protein expression and localization

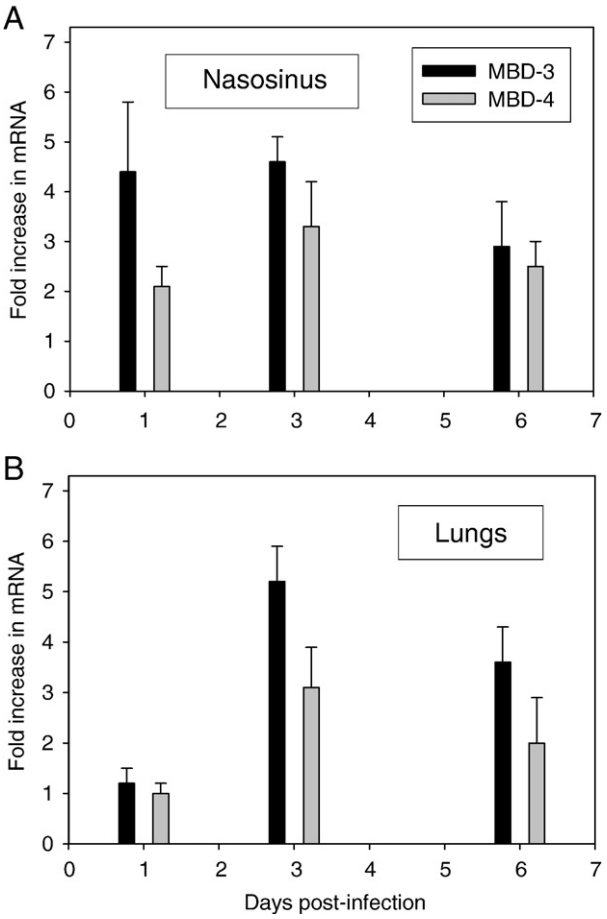

Fig. 4. RT-PCR analysis of murine β-defensins (MBDs) mRNA in sinonasal mucosa (A) and lungs (B) at various time post-infection with A/Puerto Rico/8/34 (H1N1). MBDs mRNA expression was analyzed by real-time RT-PCR. Bars represent defensins expression normalized to β-actin and relative to normal mucosa using the $2^{-\Delta\Delta CT}$ analysis as described in Materials and Methods. Error bars represent the standard error of the mean of 2–3 analysis.

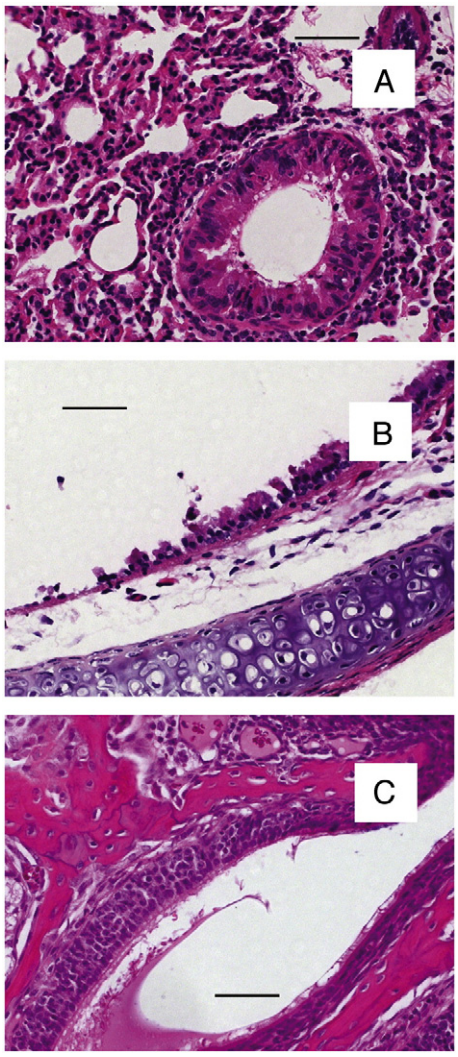

Fig. 5. Histological changes in hematoxylin and eosin stained tissues from mice at day 3 post-infection with A/Hong Kong/8/68 (H3N2). Sections shown were lungs (A), trachea (B) and nasosinus (C). The images shown are representative of multiple sections from each of three animals at this time point. Scale bar = 50 μm.

within the airway tissues, tissue sections were immunostained with specific antibody preparations. Immunostaining for MBD-1 and MBD-2 were not performed since IAV infection appeared to induce little enhancement in their expression. As shown in Figs. 6 (A, F, K), IAV viral proteins were readily detectable in epithelial cells on day 3 after infection in lungs, trachea and sinonasal mucosa. In mock-infected animals, only trace levels of MBD-3 and MBD-4 were visible but SP-D was more frequently detectable. In infected animals, MBD-3 and MBD-4 proteins were detectable in the conducting airway mucosa including the bronchi and bronchioles (Figs. 6C, D), trachea (H, I) and sinonasal mucosa (M, N) but not in the respiratory bronchioles and the alveoli. Some epithelial cells were stained for both MBD-3 and MBD-4, but MBD-4 staining appeared to be more widespread. In contrast, SP-D was more readily detectable in both the conducting and the respiratory mucosa including the alveoli (B, G, L). Staining was specific for MBDs since tissue sections exposed to serum preparations derived from preimmune or unrelated immunogens showed no reactivity (E, J, O).

Discussion

This report is the first to demonstrate upregulated expression of epithelial defensins (MBDs) in IAV infected murine upper and lower airways. Murine models of IAV infection are traditionally established by intranasal inoculation with a relatively large volume of virus (50–150 μl) to anesthetized mice (Bantia et al., 2006). Although this method yielded more consistent infection and disease severity, it also lead to an overwhelming infection associated with early and severe lung involvement, which however, is not characteristic of natural IAV infection. Studies using tracer dye have shown that intranasal administration of large fluid volume (>30 μl) in anesthetized mice will often lead to flooding of the respiratory tract leading to direct delivery to the lungs (Visweswaraiah et al., 2002). Since our objectives were to evaluate airway mucosal defensin expression during sublethal infection, we thus performed intranasal infection using small inoculation volume (~10 μl). As expected our mice showed infection progression more similar to upper airway infection of humans with later and lesser lung involvement. Unlike lung virus titers, infected mice still showed high nasal viral titers by day seven after infection. Nasal virus titers tend to persist for 7–9 days in animal models during sublethal infection with many IAV strains. However, the mechanism for this persistence is not known but could be associated with reinfection of regenerated airway epithelial cells or to more persistent infection of long-lived olfactory cells (unpublished data).

In mock-infected mice, MBD-1 was readily detectable in both upper and lower airways suggesting that its expression is constitutive, an observation that is consistent with previous reports on MBD-1 and HBD-1 (Selsted and Quellettee, 2005; Morrison et al., 1998; Moser et al., 2002; Chen and Fang 2004; Lee et al., 2002). However, we also noted that infected lungs showed 3-fold increase in MBD-1 expression two days after IAV infection. Although predominantly constitutive, HBD-1 is known to be upregulated in certain situations such as in monocytes exposed to interferon-γ, bacteria or endotoxin (Selsted and Quellettee, 2005), and in human epithelial cells exposed to poly (I:C), a Toll-Like-Receptor-3 agonist (Schaefer et al., 2005). In addition, β-defensin-1 has also been shown to be upregulated in lungs of sheep infected with parainfluenza virus (Gruboret al., 2004). Unlike MBD-1, mock-infected mice showed little or no MBD-3 and MBD-4 expression in either the upper or lower respiratory tracts. Importantly, both MBD-3 and MBD-4, which are the murine homologs of the inducible human β-defensin 2 and 3 (Ganz 2003; Bals et al., 1999; Quinones-Mateu et al., 2003), were significantly upregulated in IAV infected airways. These MBDs were localized predominantly in the conducting epithelial cells of both upper and lower airways. In the upper airway, we did not detect consistent immunostaining of either MBD-3 or MBD-4 in the submucosa glands although HBD-2 is known to be present in human submucosa gland epithelia (Singh et al., 1998). In *Pseudomonas aeruginosa* infected mice, Bals et al. (1999) showed that MBD-3 transcripts were present in both small and large airway conducting epithelial. For human airways, inducible β-defensins have been shown in nasal mucosa derived from chronic sinusitis patients (Chen and Fang 2004; Lee et al., 2002), and in cultured bronchial epithelial cells after infection with rhinovirus (Duits et al., 2003). Our results showed that MBD-1 was mainly constitutive whereas MBD-3 and MBD-4 were induced during acute infection with either H1N1 or H3N2 viral strains. These characteristics of MBD expression were also noted in mice infected with other microbes (Bals et al., 1999; Rivas-Santiago et al., 2006; Zaalouk et al., 2004). Of the two IAV strains used in this study, the PR8 H1N1 virus was more virulent and produced greater lung viral titers and more severe lung pathology. Although MBD-3 and MBD-4 expressions were greater in PR8 H1N1 virus infected mouse lungs and nasosinus mucosa, the difference was not statistically significant ($p>0.05$). Unlike the other MBDs, MBD-2 mRNA was detectable only in IAV infected lungs but not in the upper airway suggesting that the cellular source of MBD-2 might be associated with the infiltrating immune and inflammatory cells.

In uninfected lungs, SP-D is known to be widely expressed (Wright, 2005), and thus it was not unexpected that our mice with low grade lung infection showed only modest increase in SP-D expression. In this regard, our findings were in agreement with a

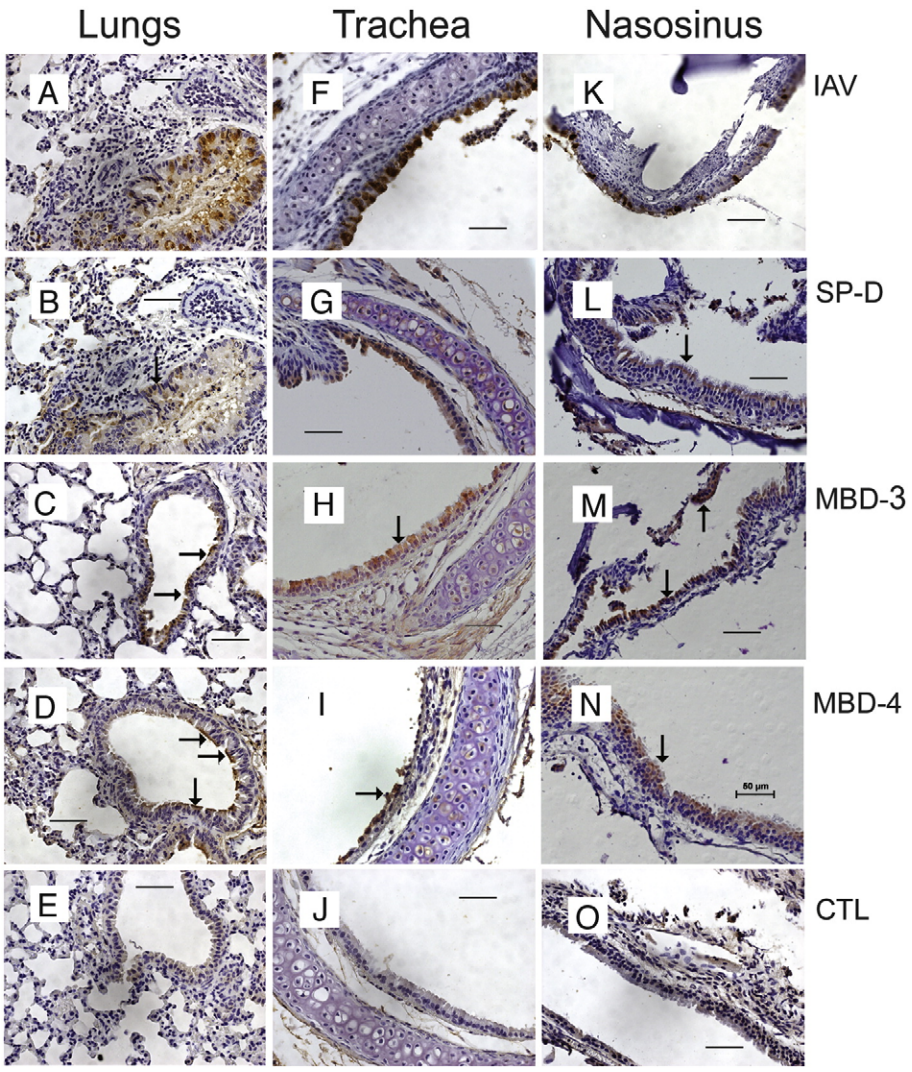

Fig. 6. Immunohistology of tissues from mice at day 3 post-infection with A/Hong Kong/8/68 (H3N2). Pictures shown are representative of the lungs (A–E), trachea (F–J) and nasosinus (K–O) from IAV-infected and mock infected (E, J, O) control mice (CTL). Immunostaining were performed using polyclonal antibody preparations against IAV (A, F, K) SP-D (B, G, L), MBD-3 (C, H, M) and MBD-4 (D, I, N). Tissue sections of lung (E), trachea (J), and sinonasal mucosa (O) exposed to serum preparations derived from preimmune or unrelated immunogens showed no reactivity. Arrows indicate positive staining (brown). The images shown are representative of multiple sections from each of three animals at this time point. Scale bar = 50 µm.

previous report that showed that SP-D level is enhanced by less than 2-fold in infected lung as measured by ELISA assay (LeVine et al., 2001). In contrast, infected mice showed a much higher increase (>4-fold) in SP-D levels in the sinonasal mucosa that persisted for at least six days after infection. Since the nasal sinus mucosa were heavily infected, this suggested that SP-D was induced by IAV infection. Although SP-D is believed to be protective against IAV infection, most evidence to date has come from in vitro studies (Wright, 2005). More recently, IAV infected SP-D knockout (SP-D−/−) mice were shown to develop more severe infection and inflammatory response (LeVine et al., 2001; Li et al., 2002). However, the tissue expression and cellular localization of SP-D during experimental IAV infection were not previously known. Thus, our demonstration of the upregulation of SP-D in the trachea and sinonasal mucosa provides evidence that SP-D plays a role in IAV infection of the upper airway in complement to its previously reported role in lung infection. Our finding is relevant to IAV infection in human since SP-D expression have recently been demonstrated in human sinonasal epithelia (Kim et al., 2007; Ooi et al., 2007).

In our study, the time course of both defensins and SP-D expression suggested that these mediators were induced by IAV infection in both the lower and upper airways. Previously, the extent of human rhinovirus growth has been shown to correlate with induction of HBD-2 mRNA and proteins in nasal epithelial cells both in vitro and in vivo (Proud et al., 2004). In our studies, levels of MBD-3 and MBD-4 increased during acute infection and decreased to near baseline levels late in infection suggesting that they likely play a role in recovery from infection. We did not observe a clear correlation between the extent of defensin upregulation and viral titers in specific organs. During acute infection, the release of proinflammatory cytokines and activation of transcription factors are important triggers of defensin upregulation. However, due to host variables in vivo, the production of proinflammatory cytokines is often not directly proportional to viral load. Since defensins are part of a first-line defense, it is perhaps not surprising that in our infected mice, defensin upregulation was not closely dependent on viral load. An alternate explanation for our not seeing a better correlation between viral loads and defensin expression is that our experimental approach failed to detect the correlation. Our data showed a rather large range of mRNA expression levels that might have resulted from inherent inconsistency in tissue sampling. For instance, as with most analysis of mRNA expression in tissues, we isolated total RNA from tissue fragments from large organs such as lungs, which were not evenly infected in vivo. Therefore, there likely will be differences in the property of infected

tissues from individual animals that were subjected to mRNA analysis. In contrast, viral assays were more representative of total lung infection since viral titers were determined in whole lung homogenates. In future studies, we could improve correlation analysis by the use of laser-capture microdissection procedure that can better define target tissue for mRNA analysis.

Our results show that mucosal defensins and SP-D were concurrently expressed in IAV infected organs, which confirmed that they are components of the innate defense repertoire. However, there were differences in the localization of SP-D and the inducible β-defensins. For example, SP-D was distributed widely within the lungs including both the conducting and respiratory epithelia whereas MBD-3 and MBD-4 were localized predominantly in the conducting airways including nasal, trachea, bronchi and bronchiole but not in the respiratory alveoli. Thus, our results suggested that defensins, due possibly to their highly lytic properties were not employed in the delicate alveolar tissue. Our mouse findings might be relevant to influenza virus infection in human since HBD-4 was recently identified in human lung tissues and importantly, HBD-4 was also shown to be present in bronchial and bronchiolar epithelium but not in the alveolar cells as evidenced by immunohistochemistry analysis (Yanagi et al., 2005). Early defensin induction allows them to serve as signaling molecules that facilitate the generation of adaptive immune responses. Although our study was not designed to analyze the molecular mechanisms of defensin induction, TLR-mediated events such as the production of proinflammatory cytokines are likely to be involved since their activations are known to induce β-defensins expression in various tissues (Selsted and Quellettee, 2005). In our infected mice, MBD induction was more pronounced beyond 2 days after infection, which suggested that MBDs might be induced by proinflammatory cytokines including tumor necrosis factor, interleukin-1α, interferon-α and γ that are known to be expressed very early during IAV infection (Ottolini et al., 2005).

In recent years, defensin peptides have attracted increasing interest as potential wide-spectrum antiviral peptide therapeutic agents. Accordingly, recombinant and synthetic defensin peptides have been evaluated in vitro against several viruses (Leikina et al., 2005; Hazrati et al., 2006; Quinones-Mateu et al., 2003; Sinha et al., 2003; Yasin et al., 2004; Zhang et al., 2002). Since defensins are often inactive in the presence of physiological concentration of salt and serum (Ganz 2003; Selsted and Quellettee, 2005; Dorschner et al., 2006), their in vivo applications have recently been studied (Brandt et al., 2007; Elahi et al., 2006). Currently, very little is known about the endogenous production of defensins during natural viral infection. In our study, we showed that several β-defensins were upregulated in both the upper and lower respiratory tracts. Our findings suggested that β-defensins might contribute to innate and adaptive immune responses targeted against IAV infection. In addition, our results provide a rationale for the development of defensin-based therapy for IAV using exogenous defensin preparations or by enhancing the production of de novo defensins in target epithelial surfaces. With current interest in using exogenous defensins to trigger adaptive immune responses and activate professional antigen-presenting cells via Toll-like receptors (Selsted and Quellettee, 2005; Yang et al., 1999; Schaefer et al., 2005; Claeys et al., 2003; Froy, 2005; Funderburg et al., 2007; Yang et al., 2000), our findings might also be important for IAV vaccine development.

Materials and methods

Animals

Specific-pathogen and respiratory disease free female C57BL/6 mice, 5–6 weeks old, (Jackson Laboratory, Bar Harbor, Maine) were used in all experiments. After 5–6 days quarantine, mice were randomly distributed into filter-top polycarbonate cages. Standard rodent chow and water were provided ad libitum, and all animals were maintained and handled in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee at the University Mississippi Medical Center.

Virus stocks and culture

Influenza viruses A/Puerto Rico/8/34 (H1N1) and A/Hong Kong/8/68 (H3N2) were obtained from ATCC and were grown in the allantoic cavity of 10-day-old embryonated hen eggs and stored as infectious allantoic fluid as previously described (Lowen et al., 2006; Zitzow et al., 2002). Virus titers were determined by plaque assay on Madin Darby canine kidney cells (MDCK) cells in medium overlays containing1–2 μg/ml TPCK-trypsin as previously reported (Gubareva et al., 1998). Culture plates were incubated for 72 h at 37 °C and plaques were counted under a dissecting scope and expressed as pfu/ml of fluid or pfu/g of tissue.

Infection of mice

For virus infection, groups of 12–15 mice were lightly anesthetized with isoflurane vapor and inoculated intranasally with 10^6 PFU of virus at 10-μl per nare in both nostrils. For tissue harvest, group of 3 mice were sacrificed at various times post-infection. Sinonasal compartments were microdissected as previously described (Fanucchi et al., 1999; Jacob and Chole, 2006). The head of each mouse was removed from the carcass, the lower jaw and skin were removed, and the head was split in half along the medial suture. Mucosa lining the septum, maxilloturbinates, nasoturbinates, and ethmoturbinates were removed by careful blunt dissection using fine ophthalmic surgical instruments and a Zeiss stereo zoom microscope. After collection, nasosinus mucosa, trachea and lung tissue were weighed, homogenized in culture media and supernatant were stored at −80 for virus plaque assays. In separate group of similarly infected mice, trachea, lung, and sinonasal mucosa were collected on day 1, 2, 3 and 6 days post-infection and stored immediately in RNAlater (Ambion) at −80 °C for subsequent isolation of total RNA. Mock-infected animals were used as normal controls.

RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted from tissue specimens using TRIzol Reagent as we previously reported (Chong et al., 2006). RNA samples were resuspended in DEPC-treated water and treated with DNase (DNA-Free, Ambion) to remove DNA contamination before cDNA synthesis. Total RNA was reverse transcribed with an iScript cDNA synthesis kit according to the manufacturer's instructions (Bio-Rad 170-8891). The synthesized cDNA was then amplified by PCR using specific sense and antisense primers for the genes of interest along with a housekeeping gene, β-actin, as control (see Table 1 for the primer sequences).

Real-time SYBR-green PCR assays were performed using an iCycler IQ™ Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA) as we previously described (Chong et al., 2006). Calculation of CT, standard curve preparation and quantification of mRNA in the samples were performed by the "post run data analysis" software provided with the iCycler system. Thermal cycling conditions for PCR reactions consisted of an initial denaturing step (96 °C, 5 min) followed by 40 cycles of denaturing (94 °C, 30 s), annealing (30 s at temperatures for specific primers), and extension (72 °C, 30 s), followed by final elongation for 3 min at 72 °C. Each sample was loaded in triplicate and run at 40 cycles under the conditions stated above. To confirm amplification of specific transcripts, agarose gel electrophoresis was performed initially to confirm that a single product of expected length was amplified, and melting curves were

Table 1
Primer sequence, annealing temperature and their predicted product sizes

Gene name	Primer	Sequences	Product size (bp)	Ta (°C)
B actin	Sense	5′-TGTGATGGTGGGAATGGGTCAGAA	140	67
	Antisense	5′-TGTGGTGCCAGATCTTCTCCATGT.		
MBD-1	Sense	5′-CCAGATGGAGCCAGGTGTTG-3′	98	64
	Antisense	AGCTGGAGCGGAGACAGAATCC-3′		
MBD-2	Sense	5′-AAGTATTGGATACGAAGCAG-3′	83	61
	Antisense	5′-TGGCAGAAGGAGGACAAATG-3′,		
MBD-3	Sense	5′-GCATTGGCAACACTCGTCAGA-3′	85	57
	Antisense	5′-CGGGATCTTGGTCTTCTCTA-3′		
MBD-4	Sense	5′-GCAGCCTTTACCCAAATTATC-3′	102	58
	Antisense	5′-ACAATTGCCAATCTGTCGAA-3′		
SP-D	Sense	5′-TGTGATGGTGGGAATGGGTCAGAA-3′	163	61
	Antisense	5′-TGTGGTGCCAGATCTTCTCCATGT-3′		

generated following each subsequent PCR run. To determine relative levels of gene expression, the comparative threshold cycle (ΔΔCT) method was employed (Livak and Schmittgen, 2001). CT was defined as the cycle number at which reporter fluorescence reached 10 times the standard deviation of the baseline fluorescence. For each sample, the mean CT value obtained for β-actin was subtracted from the mean CT value for the gene of interest to derive a ΔCT value. The ΔCT of test samples was then subtracted from ΔCT of the control sample to generate a ΔΔCT. The mean of these ΔΔCT measurements was then used to calculate target gene expression normalized to β-actin and relative to the control as: relative expression $= 2^{-\Delta\Delta CT}$.

Histology and immunohistochemistry

Tissue specimens were fixed in buffered formalin and processed for routine histological analysis. Tissue sections were subjected to heat mediated antigen retrieval steps in 0.01 M sodium citrate buffer (pH 6.0) and then pretreated to reduce non-specific staining according to standard protocols. Immunostaining was performed using commercial polyclonal primary antibody preparations against purified influenza virions (Virostat Inc. Portland, ME), MBD-3, MBD-4 (Santa Cruz Biotechnology Inc. Santa Cruz, CA) and SP-D (Millipore Corp. Billerica, MA). After incubation with primary antibody products, sections were incubated with the appropriate secondary antibody preparations (Jackson ImmunoResearch, West Grove, PA) and then stained using Vectastain ABC Kit for streptavidin-HRP and diaminobenzidine (DAB) chromogen substrate detection (Vector Laboratories, Burlingame, CA); sections were then counterstained with hematoxylin solution. Preimmune and irrelevant antibody preparations were used as negative staining controls.

Statistical analysis

Viral titers were expressed as means ± standard errors of the mean of 3 mice per time point. For PCR analysis, mRNA expression was determined from pooled tissues from 2–3 mice and shown as means ± standard errors of the mean of duplicate experiments. Differences in viral titers or mRNA fold change between groups as specified in the Results section were analyzed by the Student *t* test. A value of $p < 0.05$ was considered the minimal level of significance.

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