Several mechanisms have been proposed as the biological basis for the enhanced susceptibility to secondary bacterial infections following influenza, including viral destruction of the respiratory epithelium and upregulation of bacterial adhesion molecules facilitated by the viral neuraminidase (NA) enzyme (Peltola & McCullers, 2004), impaired innate response to bacterial infection due to the primary antiviral response (Shahangian et al., 2009; Sun & Metzger, 2008), desensitization to bacterial Toll-like receptors (TLR) (Didierlaurent et al., 2008) and suppression of neutrophil function (McNamee & Harmsen, 2006).

The increased mortality due to secondary bacterial infections during typical respiratory virus seasons may not be caused only by the interaction between influenza A viruses and *Streptococcus pneumoniae*. For instance, human metapneumovirus (hMPV), a member of the family Paramyxoviridae, has been associated with *S. pneumoniae* infections (Boivin et al., 2002; Hamelin et al., 2005a; Klein et al., 2010). Also, epidemiological studies conducted in South Africa, showed that the seven-valent pneumococcal-conjugated vaccine significantly reduced severe paramyxovirus infections in children (Madhi et al., 2004, 2006).

We showed that, similar to influenza A virus, hMPV predisposes mice to severe pneumococcal pneumonia and such interaction was driven by the presence of replicating virus (Kukavica-Ibrulj et al., 2009). On the other hand, Didierlaurent et al. (2008) showed that a prior influenza A infection desensitizes the bacterial TLR ligands for extended periods of time, i.e. long after viral replication has ceased, resulting in reduced recruitment of neutrophils to the site of bacterial infection.

In this study, we evaluated whether hMPV could predispose to long-term susceptibility to *S. pneumoniae* as described previously for influenza A virus by performing the bacterial challenge after lung viral clearance.

Four-to-six-week-old BALB/c mice (Charles Rivers) were infected intranasally on day 0 with either low doses of hMPV (1.5 × 10³ TCID₅₀ of clinical strain C-85473, group A), influenza A (10⁴ p.f.u. of A/WSN/33-H1N1) or PBS (control) and allowed to recover for 14 days, while being monitored for weight changes on a daily basis. On day 14 (at a time when there is no more replicating virus; Abed et al., 2006; Hamelin et al., 2005b), we challenged the mice with a non-lethal dose (10³ c.f.u.) of *S. pneumoniae* serotype 3 (Kukavica-Ibrulj et al., 2009), and then excised the lungs and determined the bacterial loads at three time points (i.e. at 6, 24 and 72 h post-bacterial challenge). hMPV-infected mice initially gained weight until day 5, followed by a slight weight loss on days 6 and 7 (3 %), and then a recovery after day 8. Mice infected with influenza A also experienced a 6 % weight loss on days 7 and 8 and regained their initial weight by day 10. Such weight loss followed by a recovery correlates with lung viral load kinetics as described previously (Abed et al., 2006; Hamelin...
Following *S. pneumoniae* challenge, we observed that only influenza-infected mice were susceptible to the secondary pneumococcal infection as they lost a mean of 8% of their body weight 72 h following secondary infection (Fig. 1). In contrast, hMPV-co-infected mice did not lose any weight following infection with *S. pneumoniae*.

Bacteria were only recovered from the lungs of previously influenza-infected mice at all time points with mean bacterial titres of 7 log10 c.f.u. per lung at 72 h post-challenge as reported in our previous study (Kukavica-Ibrulj et al., 2009). However, we did not recover bacteria from the lungs of mice that were initially infected with hMPV or with our PBS control (data not shown). These results suggest that BALB/c mice infected with hMPV and allowed to recover for a period of 2 weeks were able to mount an effective antibacterial response to *S. pneumoniae*, effectively clearing the pulmonary secondary infection. In contrast, mice infected with the influenza A virus had impaired bacterial lung clearance even after viral replication had ceased.

To investigate the difference in bacterial clearance between influenza-infected and hMPV-infected mice, we examined the cellular component of the host response by determining specific cell populations in bronchoalveolar lavage (BAL) samples using haematoxylin and eosin staining. Macrophages, which are essential in phagocytosis and in co-ordinating the innate immune response, were significantly increased at 6 and 24 h after bacterial infection in influenza-infected mice compared with the PBS control group (Fig. 2a). Overall, there were significantly more macrophages observed in BAL of mice with prior influenza infection than in BAL of mice previously infected with hMPV at both time points. On the other hand, the recruitment of neutrophils in BAL was not significantly increased in both virus–bacteria co-infection groups compared to control although there was a trend for increased neutrophil recruitment after influenza–pneumococcus than after hMPV–pneumococcus challenge (Fig. 2b). These results suggest that the reduced clearance of bacteria seen after a prior influenza infection is possibly not due to a depletion of macrophages or defective recruitment of neutrophils in the lungs although the function of these cells was not assessed.

There was no difference in the levels of the critical cytokines tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6 and IL-10 in BAL samples between the two virus–bacteria groups and the control group using the mouse cytokine 20-plex panel from Invitrogen (Fig. 3a). We also measured the local production of gamma interferon (IFN-γ) in BAL fluid of mice that were secondary challenged with pneumococcus. Mice that were previously infected with hMPV and then with pneumococcus cleared the infection with no increased production

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**Fig. 1.** Mean (%) weight loss (± SD) in groups of 18 BALB/c mice with dual virus–bacteria infections. Mice were infected with influenza A virus (Flu), human metapneumovirus (hMPV) or PBS on day 0 and then challenged with pneumococcus (Pn) on day 14. Monitoring was extended to 72 h after bacterial challenge. *P≤0.05; **P≤0.01; ***P≤0.001 between PBS and Flu groups. ††P≤0.01; †††P≤0.001 between hMPV and Flu groups (one-way analysis of variance and Tukey multiple comparisons test).

**Fig. 2.** Mean number (± SD) of macrophages (a) and neutrophils (b) in BAL in groups of mice with dual virus–bacteria infections. Eighteen mice per group were infected with influenza A virus (Flu) or human metapneumovirus (hMPV) or PBS on day 0 and then challenged with pneumococcus on day 14. BAL samples from six mice per group were collected 6 and 24 h post-bacterial challenge for cell counts after haematoxylin/eosin staining. ***P≤0.001 between PBS and Flu groups. †P≤0.05; ††P≤0.01; †††P≤0.001 between hMPV and Flu groups (one-way analysis of variance and Tukey multiple comparisons test).
of IFN-γ after bacterial challenge. In contrast, mice infected with influenza and then challenged with pneumococcus induced a high production of IFN-γ at 24 h and especially at 72 h post-pneumococcus infection (Fig. 3b), correlating with enhanced bacterial loads.

We described previously an animal model in which a prior hMPV infection predisposes the host to severe pneumococcal pneumonia (Kukavica-Ibrulj et al., 2009). In the present study, we show that such synergistic interaction is limited to the period of active hMPV replication in contrast to the long-term effects caused by the influenza A virus. The mechanisms whereby some respiratory viruses predispose the host to exacerbated bacterial disease are incompletely understood.

Recent studies have shown that influenza A virus can impair the innate immune response to bacterial infections. Didierlaurent et al. (2008) also showed that an influenza infection desensitizes the pathogen recognition receptors, which results in reduced neutrophil recruitment in the lungs and impaired cytokine production. Preliminary data from our laboratory showed that administration of TLR2 (lipoteichoic acid) and TLR4 (lipopolysaccharide) agonists to BALB/c mice previously infected with hMPV or influenza A did not suppress the production of critical cytokines such as TNF-α or IL-1β, which suggests that initial recognition of bacteria by their pathogen recognition receptors was still functional. It is possible that the effect observed by Didierlaurent et al. (2008) may be strain-dependent as they used the influenza X31 strain, a H3N2 virus with higher NA activity than the H1N1 virus (A/WSN/33) that we used (Peltola & McCullers, 2004).

In sharp contrast to hMPV initial infection, mice challenged with pneumococcus 14 days after influenza A infection did have enhanced pneumococcus growth in their lungs accompanied with a significant weight loss. We also found a significant increase of macrophages and a non-significant increase of neutrophils 24 h post-pneumococcus infection correlating with the enhanced bacterial growth. An important influx of neutrophils into the lungs has been suggested

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Fig. 3. Mean concentration of cytokines (± sd) in BAL in groups of mice with dual virus–bacteria infections. Eighteen mice per group were infected with influenza A virus (Flu) or human metapneumovirus (hMPV) or PBS on day 0 and then challenged with pneumococcus on day 14. BAL samples from six mice per group were collected 6 and 24–72 h post-bacterial challenge for cytokines quantification using the mouse 20-plex panel from Invitrogen. (a) TNF-α, IL-1β, IL-6 and IL-10. (b) IFN-γ. **P ≤ 0.001 between PBS and Flu groups. ***P ≤ 0.001 between hMPV and Flu groups (one-way analysis of variance and Tukey multiple comparisons test).
to contribute to the pathogenesis of synergistic viral–bacterial infections (Speshock et al., 2007), which might have also been the case in our influenza–pneumococcus synergy model. Also, the recruitment of neutrophils may not contribute to early clearance of the secondary bacterial infection as another study showed that neutrophils did not have a major role in initial bacterial clearance (Sun & Metzger, 2008). Indeed, Sun and Metzger showed that macrophages were essential for the clearance of bacteria and that the influenza virus may disrupt the immune response to secondary bacterial infection (Sun & Metzger, 2008). Their data also revealed that the production of IFN-γ following influenza virus challenge could suppress the innate response to bacterial infection by downregulating the expression of the MARCO receptor on alveolar macrophages (Sun & Metzger, 2008). Comparing with the latter study that showed a rapid increase in production of IFN-γ 7 days post-influenza and 4 h post-pneumococcus infections, we observed a delay in IFN-γ production (which was not detected at 6 h post-pneumococcus challenge) in our long-term co-infection model. However, there was a very good correlation between peak IFN-γ production and maximum bacterial titres in the two studies.

There are some limitations to our study including the use of a crude microscopic method to enumerate BAL cells instead of a more precise FACS analysis and the use of only one viral strain and a single inoculum for animal studies. In summary, we showed that immune recovery from a primary hMPV infection does not predispose to a secondary infection with pneumococcus. In fact, the host completely recovers after a primary hMPV infection and mounts an effective antibacterial response. In contrast, we observed that mice infected with influenza, despite a similar or more enhanced initial immune response to secondary bacterial challenge, are unable to clear the bacteria. We suggest that reduced clearance of pneumococcus from lungs of influenza-infected mice may be due to the presence of IFN-γ as demonstrated by Sun & Metzger (2008) since the local production of this cytokine paralleled an enhanced bacterial replication. In contrast, hMPV-infected mice did not produce increased levels of INF-γ compared with controls and effectively cleared the bacteria from the lungs. Additional experiments using exogenous IFN-γ as well as specific IFN-γ antibodies could be done to further confirm this differential mechanism of synergistic co-infections.

**Acknowledgements**

This study was supported by a research grant from the Canadian Institutes of Health Research to G.B. (MOP-151504). G.B. is a national scholar of the FRSQ and the holder of the Canada Research Chair on Emerging Viruses and Antiviral Resistance. H.P.L. is a postdoctoral scholar from the FRSQ-Respiratory Health Network.

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