Exogenous Activation of Invariant Natural Killer T Cells by α-Galactosylceramide Reduces Pneumococcal Outgrowth and Dissemination Postinfluenza

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ABSTRACT Influenza A virus infection can predispose to potentially devastating secondary bacterial infections. Invariant natural killer T (iNKT) cells are unconventional, lipid-reactive T lymphocytes that exert potent immunostimulatory functions. Using a mouse model of postinfluenza invasive secondary pneumococcal infection, we sought to establish whether α-galactosylceramide (α-GalCer [a potent iNKT cell agonist that is currently in clinical development]) could limit bacterial superinfection. Our results highlighted the presence of a critical time window during which α-GalCer treatment can trigger iNKT cell activation and influence resistance to postinfluenza secondary pneumococcal infection. Intranasal treatment with α-GalCer during the acute phase (on day 7) of influenza virus H3N2 and H1N1 infection failed to activate (gamma interferon [IFN-γ] and interleukin-17A [IL-17A]) iNKT cells; this effect was associated with a strongly reduced number of conventional CD103+ dendritic cells in the respiratory tract. In contrast, α-GalCer treatment during the early phase (on day 4) or during the resolution phase (day 14) of influenza was associated with lower pneumococcal outgrowth and dissemination. Less intense viral-bacterial pneumonia and a lower morbidity rate were observed in superinfected mice treated with both α-GalCer (day 14) and the corticosteroid dexamethasone. Our results open the way to alternative (nonantiviral/nonantibiotic) iNKT-cell-based approaches for limiting postinfluenza secondary bacterial infections.

IMPORTANCE Despite the application of vaccination programs and antiviral drugs, influenza A virus (IAV) infection is responsible for widespread morbidity and mortality (500,000 deaths/year). Influenza infections can also result in sporadic pandemics that can be devastating: the 1918 pandemic led to the death of 50 million people. Severe bacterial infections are commonly associated with influenza and are significant contributors to the excess morbidity and mortality of influenza. Today’s treatments of secondary bacterial (pneumococcal) infections are still not effective enough, and antibiotic resistance is a major issue. Hence, there is an urgent need for novel therapies. In the present study, we set out to evaluate the efficacy of α-galactosylceramide (α-GalCer)—a potent agonist of invariant NKT cells that is currently in clinical development—in a mouse model of postinfluenza, highly invasive pneumococcal pneumonia. Our data indicate that treatment with α-GalCer reduces susceptibility to superinfections and, when combined with the corticosteroid dexamethasone, reduces viral-bacterial pneumonia.
disruption of the pulmonary (epithelial) barrier, exposure of new attachment sites, and impaired mucociliary function also contribute to postinfluenza bacterial superinfection (4, 9, 18–20).

Invariant natural killer T (iNKT) cells constitute a highly conserved subset of innate-like T lymphocytes with potent immunostimulatory properties. These cells recognize lipid antigens presented by the monomorphic major histocompatibility complex (MHC) class I-like homologue CD1d expressed by antigen-presenting cells (21–24). Among the latter, conventional dendritic cells (DCs) are particularly well equipped to efficiently activate iNKT cells (25, 26). In response to T cell receptor triggering, iNKT cells rapidly release large amounts of Th1, Th2, and/or Th17 cytokines, which transactivate cells from the innate and adaptive immune systems (21–24). In view of this unique property, iNKT cells are critical for the regulation of innate and adaptive immune responses.

α-Galactosylceramide (α-GalCer) is a glycosphingolipid originally isolated from a marine sponge during a screen for antitumor agents (27). This molecule holds great promise as a cancer drug, and ongoing clinical trials are under way with the goal of optimizing antitumor properties mediated by iNKT cells (28–30). Prophylactic treatment with α-GalCer can also protect against many infectious diseases—including pneumococcal infections (21–24, 31–33). In the latter setting, the production of gamma interferon (IFN-γ) and interleukin-17A (IL-17A) by iNKT cells has a crucial role in bacterial clearance (32, 33). In the present study, we set out to evaluate the efficacy of α-GalCer and to determine whether it could minimize the harmful effect of combined IAV and S. pneumoniae exposure.

RESULTS

Influenza A virus infection induces a long-lasting susceptibility to secondary invasive pneumococcal infection. To study the effects of exogenous iNKT cell activation on secondary bacterial infection, we established a mouse model of postinfluenza, highly invasive pneumococcal pneumonia. To this end, mice were intranasally (i.n.) infected with a sublethal dose (for the weight loss curve, see Fig. S1A in the supplemental material) of IAV/Scotland/20/74 (H3N2). At different time points thereafter, animals were challenged with a very low dose of the invasive S. pneumoniae serotype 1. While the dose of inoculated pneumococci was self-limiting as an infection in the absence of IAV, iNKT-experienced mice were susceptible to pneumococcal challenge—as reflected by the presence of bacteria in the lungs and spleen (indicative of systemic dissemination) (Fig. 1). When mice were challenged at 4 days postinfluenza (dpi), bacteria were detected in lungs—albeit to a lesser extent than at 7 dpi (Fig. 1A). It is noteworthy that mice challenged at 4 dpi were resistant to invasive pneumococcal infection; this finding is in line with maintenance of lung barrier functions at that time point (Fig. 1B and Fig. S1B). A time course analysis indicated that susceptibility to pneumococcal infection peaked at 7 dpi, corresponding (along with 4 dpi) to the peak in the viral load (Fig. 1A and Fig. S1C). Mice remained susceptible to pneumococcal infection at 14 dpi.

Early treatment with α-GalCer is associated with lower bacterial outgrowth in superinfected animals. We next looked at whether α-GalCer could limit bacterial outgrowth and systemic dissemination in superinfected animals at the peak in viral load. In mice infected 4 days earlier with IAV, i.n. inoculation of α-GalCer 16 h before the secondary bacterial challenge was associated with a marked reduction in the pneumococcal load in the lungs (Fig. 2A). In stark contrast, administration of α-GalCer at 7 dpi failed to lower the number of pneumococci in the lungs and spleen, although for the latter, a nonsignificant reduction was observed. Similar data (reduced bacteria at 4 dpi but not 7 dpi) were obtained in a model of high-pathogenicity H1N1-pneumococcal infection (see Fig. S2A in the supplemental material). We then looked at whether α-GalCer’s effects at 4 and 7 dpi were related to differences in iNKT cell activation. Inoculation of α-GalCer at 4 dpi resulted in production of IFN-γ and IL-17A (cytokines known to have antipneumococcal activities) by pulmonary iNKT cells (32–34) (Fig. 2B; see Fig. S2B for the gating strategy). In stark contrast, administration of α-GalCer at 7 dpi failed to trigger cytokine production by iNKT cells. This finding was confirmed in the H1N1 IAV-pneumococcal infection system, although the inhibitory effect was less intense for IFN-γ (Fig. S2C). Taken as a whole, our results show that α-GalCer activates iNKT cells and triggers antipneumococcal immune defenses in superinfected animals but only when inoculated soon after the influenza infection.

The lack of iNKT cell activation at 7 dpi is associated with a strongly reduced number of respiratory CD103+ cDCs. In order to establish whether or not the lack of iNKT cell activation at 7 dpi was cell intrinsic, pulmonary iNKT cells from IAV-infected mice
were exposed ex vivo to plate-bound α-GalCer-loaded CD1d. Influenza-experienced iNKT cells produced IFN-γ and IL-17A—indicating that they had retained their innate-like properties (Fig. 3A [data not shown]). This finding suggested that pulmonary factors have a role in the lack of in vivo cytokine production by iNKT cells in response to α-GalCer. One candidate factor is the immunosuppressive cytokine IL-10, which is known to be produced massively in the context of influenza infection (35, 36) (Fig. 3B). However, neutralizing IL-10 activities just before α-GalCer inoculation failed to rescue IFN-γ and IL-17A production by iNKT cells (Fig. 3C [data not shown]).

We have previously suggested that airway conventional DCs (cDCs [especially the CD103⁺ subset]) have a key role in the activation of pulmonary iNKT cells in response to i.n. delivery of α-GalCer (33). In agreement with this, pulmonary iNKT cells from mice deficient for the basic leucine zipper transcription factor 3 (Baf3⁻/⁻), which lack CD103⁺ conventional DCs (37, 38 [data not shown]) produced less IFN-γ than wild-type controls (see Fig. S3A in the supplemental material). At 7 dpi, the proportion and absolute count of conventional DCs (including CD103⁺ cells) were much lower than in mock-infected mice (Fig. 3D and Fig. S3B and S3C). This phenomenon was not due to a local cell death since the frequency of dying/dead conventional DCs was not increased in the lungs during the course of IAV infection (Fig. S3D). In parallel, a strong influx of monocyte-derived DCs and inflammatory monocytes was observed in the lungs of IAV-infected mice (Fig. 3D). These cell types expressed CD1d on their surface, although the level of expression on monocyte-derived DCs was low (Fig. 3E, left panel). When exposed to α-GalCer ex vivo, monocyte-derived DCs and inflammatory monocytes induced less IFN-γ and IL-17A release by iNKT cells, relative to α-GalCer-exposed conventional DCs isolated from naive animals (Fig. 3E, right panel [data not shown]). Taken as a whole, our results show that the reduced number of conventional (CD103⁺) DCs from the lungs of IAV-infected mice (7 dpi) is associated with a lack of iNKT cell activation upon in vivo stimulation with α-GalCer.

α-GalCer treatment during the resolution phase of influenza impairs pneumococcal outgrowth and dissemination in superinfected animals. We reasoned that the replenishment of respiratory conventional DCs in the lungs during the resolution phase of influenza might lead to enhanced resistance against superinfection upon α-GalCer treatment. The frequency/number of conventional DCs (both CD103⁺ and CD11b⁺) returned to basal levels at 14 dpi, while the proportion of monocyte-derived DCs and inflammatory monocytes fell (Fig. 4A, left panel).
We next wondered whether iNKT cell activation by α-GalCer is associated with enhanced antibacterial defenses. Inoculation of α-GalCer at 14 dpi lowered the pneumococcal count in the lungs (Fig. 4C and Fig. S4C, H1N1 IAV). More strikingly, α-GalCer almost fully abrogated the systemic dissemination of pneumococcii. Similar data were obtained when mice were treated at 21 dpi (a time point at which mice are still susceptible to superinfection) (Fig. S4D).

Combination treatment with dexamethasone and α-GalCer is associated with less intense pneumonia and a lower morbidity rate in IAV/pneumococcus-infected mice. Exacerbated lung-
jury, loss of respiratory function, and bacteremia are the main causes of mortality during postinfluenza superinfection (9, 39–42). We assessed the effect of α-GalCer treatment on the mortality rate in superinfected animals. Whatever the time point postinfluenza, α-GalCer treatment had no effect on the animals’ survival (Fig. 5A). This indicates that in our system, the size of the pneumococcal load is not correlated with the survival rate. Since the mortality rate in superinfected animals was lower at 14 dpi, we attempted to characterize the mechanisms leading to death at this time point. In line with the lower extent of bacterial dissemination in α-GalCer-treated superinfected mice (Fig. 4C), systemic inflammation (as measured by the serum concentration of IL-6) was lower in this group than in vehicle-treated counterparts (Fig. 5B). We therefore reasoned that excessive pneumonia (rather than sepsis) contributes strongly to mortality in α-GalCer-treated superinfected animals. A historical analysis of lung sections confirmed that severe pneumonia occurred in α-GalCer-treated superinfected animals to the same extent as in untreated superinfected animals—despite the lower bacterial load in the lungs (Fig. 5C). Similarly, α-GalCer did not improve the body weight loss after secondary pneumococcal infection (Fig. 5D). This prompted us to treat mice with the anti-inflammatory corticosteroid dexamethasone. It has been reported that combination treatment with α-GalCer and dexamethasone was not associated with a higher survival rate in superinfected animals (Fig. S5C). In summary, combined dexamethasone therapy and activation of iNKT cells is associated with lower bacterial outgrowth/dissemination, diminished pneumonia, and reduced morbidity (but no difference in survival) after IAV-pneumococcus infections.

**DISCUSSION**

Severe influenza can lead to secondary bacterial infections with devastating consequences for human health. In view of the threat of a highly pathogenic influenza pandemic and increased antibiotic resistance, the development of alternative options for controlling bacterial superinfections (along with antivirals, antibiotics, and vaccines) is crucial. Very few studies have focused on the impact of local innate immunostimulators (such as Toll-like receptor [TLR] agonists) on postinfluenza secondary bacterial infections. This might be due to the fact that IAV induces the sustained desensitization of some innate sensors (e.g., TLRs) in lung tissue (43). However, our recent data indicated that the combination of local inoculation of flagellin (a TLR5 agonist) with antibiotic treatment can reduce the pulmonary pneumococcal burden after influenza (44). Furthermore, local treatment with MALP-2 (a TLR2/TLR6 agonist) lowers the load of pneumococci in the lungs and increases the survival rate in superinfected mice (45). In the present study, we investigated whether another strategy (namely, the exogenous activation of iNKT cells) can trigger local immunostimulation in the lung in the context of influenza.

The lipid α-GalCer is a potent iNKT cell agonist that is currently in clinical development (21–24). Data from our group and
others indicate that α-GalCer provides prophylactic protection against lethal pneumococcal infections (an effect that depends on IFN-γ, IL-17A, and neutrophils) (31–33). We sought to establish whether this might also be the case in the context of prior influenza—even though the immunosuppressive environment imposed by IAV is known to contribute strongly to secondary bacterial infections (13–17) and to inhibition of iNKT cell activation in response to secondary pneumococcal challenge (36). Our results in H3N2 and H1N1 models showed that at 4 dpi (a time point when the viral load is high), treatment with α-GalCer is associated with a strong reduction of bacterial outgrowth in the lungs. The protective effect was associated with the activation of pulmonary iNKT cells (IFN-γ and IL-17A). This finding suggests that the effector cells (especially neutrophils) downstream of iNKT cells are functional at 4 dpi. In contrast, treatment with α-GalCer at 7 dpi (the peak in bacterial susceptibility) did not have an effect on secondary pneumococcal infection. Impairment of mucosal barrier function (which enables bacterial dissemination) and immune depression (as assessed by IL-10 synthesis, for example) also peak at 7 dpi. Functional changes in iNKT cells (including exhaustion) have been observed during cancer and infections (21, 46, 47). Under our experimental conditions, pulmonary iNKT cells from influenza-infected mice (7 dpi) became refractory in vivo to secondary stimulation with α-GalCer. An ex vivo analysis of iNKT cell functions showed that these cells retained their antigen-specific, innate-like properties in the context of IAV infection. Our data also rule out a dominant role for the immunosuppressive cytokine IL-10 in the inhibition of TCR-mediated iNKT cell activation. In fact, the strongly reduced number of conventional DCs (and especially CD103+ DCs) at 7 dpi might explain the lack of iNKT cell activation. Our data show that monocyte-derived DCs and inflammatory monocytes massively infiltrated the lungs during the acute phase of influenza but displayed low CD1d-based lipid-presenting capacities. Thus, at 7 dpi, IAV instigates mechanisms that affect lipid antigen-dependent activation of iNKT cells in vivo—thus hampering the potential therapeutic effect of α-GalCer. Among mechanisms leading to reduced frequency/number of pulmonary conventional DCs at 7 dpi, sustained emi-
Migration to the draining lymph nodes (48–50), rather than local cell death, is likely to be important.

We observed that IAV’s inhibitory effect was limited in time, since α-GalCer treatment at 14 dpi (during the viral recovery phase) and 21 dpi (data not shown) induced strong IFN-γ and IL-17A production by iNKT cells. This production was associated with repopulation of the lung tissue by CD1d-expressing (CD103+) conventional DCs. At 14 and 21 dpi, α-GalCer treatment in H3N2 and H1N1 models resulted in lower pneumococcal outgrowth in the lungs and reduced bacterial dissemination. The lack of a complete, effective antipneumococcal response following α-GalCer treatment in our system can be explained by the continuing depression of antibacterial functions in neutrophils and/or macrophages—i.e., cells known to act as downstream effectors of iNKT cells (21–24). The effect of α-GalCer at 14 dpi and thereafter was more prominent in terms of systemic bacterial translocation; this indicates a lower degree of immune depression in the periphery at these time points. The reduced local and systemic spread of pneumococci in α-GalCer-treated superinfected mice did not affect survival. Pneumonia, respiratory failure, and bacteremia are the major causes of death in IAV-superinfected patients (9, 39–42). Histological analysis of lung sections indicated the presence of severe postinfluenza bacterial pneumonia in vehicle-treated and α-GalCer-treated superinfected mice. This vigorous inflammatory response is usually associated with respiratory failure (9, 39–42) and is likely to have been the cause of death in our model of severe IAV-pneumococcal infection. Recent studies have shown that controlling bacterial outgrowth and exacerbated host inflammatory responses might be of particular value in reducing the negative outcomes of bacterial superinfections (17, 42). We took advantage of these findings and attempted to lower the morbidity and mortality rates of superinfected mice by treating mice with both α-GalCer and the anti-inflammatory corticoid dexamethasone. Combination treatment (at 14 dpi) resulted in a less intense IAV-pneumococcus-induced inflammatory response and a lower morbidity rate in the superinfected hosts. However, for reasons that have yet to be determined, this combination therapy did not have a significant effect on mouse survival. Our data therefore extend the results of animal studies (17, 42) in which strategies that lower the bacterial burden (e.g., antibiotics) do not impact mortality and thus must be combined with anti-inflammatory treatments that improve lung function. To the best of our knowledge, the present study is the first to show that the combination of an innate immunostimulator with an anti-inflammatory compound improves the outcome (bacterial outgrowth/dissemination, pneumonia, and morbidity) of postinfluenza bacterial superinfection. Targeting iNKT cells by combining specific agonists with other therapeutics (e.g., corticosteroids) might be of great value for the prevention and treatment of postinfluenza bacterial superinfections in humans. Consideration of the time window that effects are observed will be critical for future success in the human setting. Our study results also suggest that additional therapy is required to reduce mortality in superinfected hosts. The use of compounds that control lung injury and improve lung function (e.g., by reducing pulmonary edema) will be of value.

**MATERIALS AND METHODS**

Mice and ethics statement. Eight-week-old male wild-type (WT) C57BL/6 mice were purchased from Janvier (Le Genest-St.-Isle, France).

Mice were maintained in a biosafety level 2 facility in the Animal Resource Center at the Lille Pasteur Institute, All animal work conformed to the Lille Pasteur Institute animal care and use ethical guidelines (agreement no. AF 16/20090 and 00357.03). Mice lacking pulmonary CD103+ DCs (Batf3−/−) were described by Hildner and colleagues (37).

**Reagents and Abs.** α-GalCer was synthesized as described in reference 51, and dexamethasone was obtained from Sigma (St. Louis, MO). All labeled monoclonal antibodies (MAbs) were from BD Pharmingen (Le Pont de Clai, France). Neutralizing MAbs against IL-10 receptor (1B1.3A) and isotype controls were from BioXCell (West Lebanon, NH).

**Analysis of iNKT activation, purification, and culture.** Intracellular fluorescence-activated cell sorter (FACS) staining of iNKT cells was performed as previously described (50). Briefly, fixed cells were permeabilized and incubated with conjugated MAbs against IFN-γ or IL-17A or control rat IgG1 MAb. Cells were acquired and analyzed on an LSR Fortessa cytometer (Becton, Dickinson, Rungis, France) using the FACS- Diva and FlowJo software. To purify iNKT cells, lung mononuclear cells were labeled with phycoerythrin (PE)-conjugated PBS57-loaded CD1d tetramer and fluorescein isothiocyanate (FITC)-conjugated anti-TCR-β Ab. Cells were sorted using a FACSaria (BD Biosciences). For culture experiments, pulmonary iNKT cells (5 × 10^5 cells/well, purity of >98%) were stimulated with phorbol myristate acetate (PMA)/ionomycin or with plate-bound CD1d dimer loaded with α-GalCer.

**Infections and assessment of gene expression by quantitative RT-PCR.** Unless otherwise indicated, mice were intranasally (i.n. [50 µl]) infected with 30 PFU of the high-pathogenicity mouse-adapted H3N2 IAV strain Scotland/20/74 (50). In some cases, mice were infected with the high-pathogenicity H1N1 IAV strain WSN/33 (200 PFU). Assessment of gene expression by quantitative reverse transcription-PCR (RT-PCR) was performed as described previously (50). Superinfection was as follows. Mice were infected (or not) with IAV, and 4, 7, 14, or 21 days later, animals were i.n. inoculated with 1 × 10^3 CFU of S. pneumoniae serotype 1 (clinical isolate E1586) (33). To evaluate the effect of exogenous iNKT cell activation on superinfection, α-GalCer (2 µg/mouse) was i.n. inoculated 16 h before the secondary bacterial challenge. Enumeration of viable bacteria in lungs and spleen was determined 30 h after the S. pneumoniae challenge (33). Mouse survival and weight loss were measured daily.

**Analysis and purification of pulmonary conventional DCs, monocyte-derived DCs, and inflammatory monocytes.** To identify conventional DCs (cDCs) and monocyte-derived DCs, lung mononuclear cells were labeled with appropriate dilutions of FITC-conjugated anti-CD45, Brilliant Violet 421 anti-Siglec-F, allophycocyanin (APC)-H7-conjugated anti-Ly6G, phycoerythrin (PE)-Cy7-conjugated anti-CD11c, and IL-17A production was quantified by enzyme-linked immunosorbent assay (ELISA) 48 h later. Pulmonary histological analysis of superinfected mice and dexamethasone treatment. Scoring of lung sections was performed by an experienced veterinary pathologist who was blind regarding the composition of the groups. Broncho-interstitial pneumonia was scored from 1 to 5 as described in references 36 and 30. To lower lung inflammation, IAV-infected mice were treated daily intraperitoneally (i.p.) with dexametha-
sone (2.5 mg/kg) from day 12 to day 15 (histological analysis) or from day 12 to day 17 (measurement of weight loss).

**Statistical analyses.** A Mann-Whitney Student’s unpaired t test or a Wilcoxon signed-rank test was used to compare two groups unless otherwise specified. Comparisons of more than two groups with each other were analyzed by one-way ANOVA Kruskal-Wallis test (nonparametric), followed by Dunn’s posttest (PRISM v6 software; GraphPad). Survival of mice was compared using Kaplan-Meier analysis and log-rank test. Results are expressed as the mean ± standard deviation (SD) unless otherwise stated. A P value of <0.05 was considered significant.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01440-16/-/DCSupplemental.

Figure S1, TIF file, 0.1 MB.
Figure S2, TIF file, 0.1 MB.
Figure S3, TIF file, 0.1 MB.
Figure S4, TIF file, 0.1 MB.
Figure S5, TIF file, 0.1 MB.

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**REFERENCES**

1. Brundage JF. 2006. Interactions between influenza and bacterial respiratory pathogens: implications for pandemic preparedness. Lancet Infect Dis 6:303–312. http://dx.doi.org/10.1016/S1473-3099(06)70466-2.
2. Hartshorn KL. 2010. New look at an old problem: bacterial superinfection after influenza. Am J Pathol 176:536–539. http://dx.doi.org/10.2353/ajpath.2010.900880.
3. Brundage JF, Shenk TA, Hendel JD. 2008. Deaths from bacterial pneumonia during 1918–19 influenza pandemic. Emerg Infect Dis 14:1193–1199. http://dx.doi.org/10.3201/ eid1408.071131.
4. Morens DM, Taubenberger JK, Fauci AS. 2008. Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness. J Infect Dis 198:962–970. http://dx.doi.org/10.1086/591708.
5. File TM. 2003. Community-acquired pneumonia. Lancet 362:1991–2001. http://dx.doi.org/10.1016/S0140-6736(03)15021-0.
6. Kadioglu A, Weiser IN, Paton JC, Andrew PW. 2008. The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease. Nat Rev Microbiol 6:288–301. http://dx.doi.org/10.1038/nrmicro1871.
7. Pillai SK, Ewig S, Menéndez R, Ferrer M, Pulverino E, Reyes S, Gabarrús A, Marcos MA, Cordoba J, Mensa J, Torres A. 2012. Bacterial co-infection with H1N1 infection in patients admitted with community acquired pneumonia. J Infect 65:223–230. http://dx.doi.org/10.1016/j.jinf.2012.04.009.
8. McCullers JA, English BK. 2008. Improving therapeutic strategies for secondary bacterial pneumonia following influenza. Future Microbiol 3:303–314. http://dx.doi.org/10.2217/fmb.07.144.
9. McCullers JA. 2014. The co-pathogenesis of influenza viruses with bacteria in the lung. Nat Rev Microbiol 12:252–262. http://dx.doi.org/10.1038/nrmicro3231.
10. Lipscomb MF, Hutt J, Lovchik J, Wu T, Lyons CR. 2010. The pathogenesis of acute pulmonary viral and bacterial infections: investigations in animal models. Annu Rev Pathol 5:223–252. http://dx.doi.org/10.1146/annurev.patho.012808.150452.
11. Shresta S, Foxman B, Weinberger DM, Steiner C, Viboud C, Rohani P. 2013. Identifying the interaction between influenza and pneumococcal pneumonia using incidence data. Sci Transl Med 5:191ra184. http://dx.doi.org/10.1126/scitranslmed.3005982.
12. Rynda-Apple A, Robinson KM, Alcorn JF. 2015. Influenza and bacterial superinfection: illuminating the immunologic mechanisms of disease. Infect Immun 83:3764–3770. http://dx.doi.org/10.1128/IAI.00298-15.
13. McNamee LA, Harman-Attee LA. 2006. Influenza-induced neutrophil dysfunction and neutrophil-independent mechanisms contribute to increased susceptibility to a secondary Streptococcus pneumoniae infection. Infect Immun 74:6707–6721. http://dx.doi.org/10.1128/IAI.00789-06.
14. Sun K, Metzger DW. 2008. Inhibition of pulmonary antibacterial defense by interferon-gamma during recovery from influenza infection. Nat Med 14:558–564. http://dx.doi.org/10.1038/nm1765.
15. De Clippel A, Chou X, Wang J, Elasser Y, Raffi A, Liu SY, Belperio JA, Cheng G, Deng JC. 2009. Type I IFNs mediate development of postinfluenza bacterial pneumonia in mice. J Clin Invest 119:1910–1920. http://dx.doi.org/10.1172/JCI35412.
16. Sun K, Metzger DW. 2014. Influenza infection suppresses NADPH oxidase-dependent phagocytic bacterial clearance and enhances susceptibility to secondary methicillin-resistant Staphylococcus aureus infection. J Immunol 192:3301–3307. http://dx.doi.org/10.4049/jimmunol.1303049.
17. Ghoneim HE, McCullers JA. 2014. Adjunctive corticosteroid therapy improves lung immunopathology and survival during severe secondary pneumococcal pneumonia in mice. Infect Dis 209:1459–1468. http://dx.doi.org/10.1093/infdis/jit653.
18. Pittet LA, Hall-Stoodley L, Rutkowsi MR, Harmsen AG. 2010. Influenza virus infection decreases tracheal mucociliary velocity and clearance of Streptococcus pneumoniae. Am J Respir Cell Mol Biol 42:450–460. http://dx.doi.org/10.1165/rcmb.2007-0417OC.
19. Kash JC, Walters KA, Davis AS, Sandouk A, Schwartzman LM, Jagger BW, Chertow DS, Li Q, Kuestner RE, Ozinsky A, Taubenberger JK. 2011. Lethal synergism of 2009 pandemic H1N1 influenza virus and Staphylococcus aureus superinfection: illuminating the immunologic mechanisms of disease. Influenza and Other Respiratory Pathogens: Implications for Pandemic Preparedness. Lancet Infect Dis 11:865–874. http://dx.doi.org/10.1016/S1473-3099(09)70409-1.
20. Ellis GT, Davidson S, Crootta S, Branzek P, Papayannopoulos V, Vack A. 2015. TRAIL+ monocytes and monocyte-related cells cause lung damage and thereby increase susceptibility to influenza-Streptococcus pneumoniae coinfection. EMBO Rep 16:1203–1218. http://dx.doi.org/10.15252/embr.201540473.
21. Bendelac A, Savage PB, Teyton L. 2007. The biology of NKT cells. Annu Rev Immunol 25:297–336. http://dx.doi.org/10.1146/annurev.immunol.25.022106.141504.
22. Kroenenberg M, Kinjo Y. 2009. Innate-like recognition of microbes by invariant natural killer T cells. Curr Opin Immunol 21:391–396. http://dx.doi.org/10.1016/j.coi.2009.07.002.
23. Berzins SP, Smyth MJ, Baxter AG. 2011. Presumed guilty: natural killer T cell defects and human disease. Nat Rev Immunol 11:131–142. http://dx.doi.org/10.1038/nri2904.
24. Brennan PJ, Brilg M, Brenner MB. 2013. Invariant natural killer T cells:
an innate activation scheme linked to diverse effector functions. Nat Rev Immunol 13:101–117. http://dx.doi.org/10.1038/nri3369.

25. Bezbradica JS, Stanic AK, Matsuji N, Bour-Jordan H, Bluestone JA, Thomas JW, Unutma D, Van Kaer L, Joyce S. 2005. Distinct roles of dendritic cells and B cells in Val41Δ18 natural T cell activation in vivo. J Immunol 174:4769–4775. http://dx.doi.org/10.4049/jimmunol.174.8.4696.

26. Arora P, Baena A, Yu KO, Saini NK, Kharkwal SS, Goldberg MF, Kunannath-Velayudhan S, Carreño LJ, Venkataswamy MM, Kim J, Lazar-Molnar E, Lauvau G, Chang YT, Liu Z, Bittman R, Al-Shamkhani A, Cox LR, Jervis PJ, Veerapan N, Besra GS, Porcelli SA. 2014. A single subset of dendritic cells controls the cytokine bias of natural killer T cell responses to diverse glycolipid antigens. Immunity 40:105–116. http://dx.doi.org/10.1016/j.immuni.2013.12.004.

27. Kobayashi E, Tachibana M, Ikadai H, Kunieda T. 1995. Localization of a Na+,K+-ATPase alpha 2 subunit gene, Atpl2a, on rat chromosome 13. Mamm Genome 6:889. http://dx.doi.org/10.1007/BF02924424.

28. Cerundolo V, Salio M. 2007. Harnessing NKT cells for therapeutic applications. Curr Top Microbiol Immunol 34:235–340.

29. Motohashi S, Nakayama T. 2009. Invariant natural killer T cell-based immunotherapy for cancer. Immunoon 17:3–82. http://dx.doi.org/10.2217/1750743X.1.1.73.

30. Faveeuw C, Trottein F. 2014. Optimization of natural killer T cell-mediated immunotherapy in cancer using cell-based and nanovector vaccines. Cancer Res 74:1632–1638. http://dx.doi.org/10.1158/0008-5472.CAN-13-1304.

31. Kawakami K, Yamamoto N, Kinjo Y, Miyagi K, Nakasone C, Uezu K, Kinjo T, Nakayama T, Taniguchi M, Saito A. 2003. Critical role of Valpha14+ natural killer T cells in the innate phase of host protection against Streptococcus pneumoniae infection. Eur J Immunol 33:3322–3330. http://dx.doi.org/10.1002/eji.200324254.

32. Nakamatsu M, Yamamoto N, Hatta M, Nakasone C, Kinjo T, Miyagi K, Uezu K, Nakamura K, Nakayama T, Taniguchi M, Iwakura Y, Kakur M, Fujita J, Kawakami K. 2007. Role of interferon-gamma in Valpha14+ natural killer T cell-mediated host defense against Streptococcus pneumoniae infection in murine lungs. Microbes Infect 9:364–374. http://dx.doi.org/10.1016/j.micinf.2006.12.003.

33. Ivanov S, Fontaine J, Paget C, Macho Fernandez E, Van Maele L, Renssen J, Maillet I, Wolf NM, Rial A, Léger H, Byffel B, Frisch B, Chabaloigya JA, Sirard JC, Benecke A, Faveeuw C, Trottein F. 2012. Key role for respiratory CD103(+) dendritic cells, IFN-gamma, and IL-17 in protection against Streptococcus pneumoniae infection in response to alpha-galactosylceramide. J Infect Dis 206:723–734. http://dx.doi.org/10.1093/infdis/jir413.

34. Cao J, Wang D, Xu F, Gong Y, Wang H, Song Z, Li D, Zhang H, Li D, Zhang L, Xia Y, Li X, Lai X, Lin S, Zhang X, Rui G, Dai Y, Yin Y. 2014. Activation of IL-27 signaling promotes development of postinfluenza CD8(+) T cell responses to influenza. J Immunol 192:6564–6572. http://dx.doi.org/10.1121/11.I00498-15.

35. Beer S, Radunz P, Dietert K, Tschernig T, Wolff T, Hammer-schmidt S, Gruber AD, Sutter N, Witzenthurn M. 2015. Pulmonary immunostimulation with MALP-2 in influenza virus-infected mice increases survival after pneumococcal superinfection. Infect Immun 83: 4617–4629. http://dx.doi.org/10.1128/IAI.00948-15.

36. Kee SJ, Kwon YS, Park WY, Cho NY, Lee SJ, Kim TJ, Lee SS, Jang HC, Shin MG, Shin JH, Suh SP, Ryang DW. 2012. Dysfunction of natural killer T cells in patients with active Mycobacterium tuberculosis infection. Infect Immun 80:2100–2106. http://dx.doi.org/10.1128/IAI.00618-11.

37. Kim S, Lalani S, Parekh YV, Vincent TL, Wu L, Van Kaer L. 2008. Impact of bacteria on the phenotype, functions, and therapeutic activities of invariant NKT cells in mice. J Clin Invest 118:2301–2315. http://dx.doi.org/10.1172/JCI33071.

38. Ballesteros-Tato A, Léon B, Lund FE, Randall TD. 2010. Temporal changes in dendritic cell subsets, cross-priming and costimulation via CD70 control CD8(+) T cell responses to influenza. Nat Immunol 11:216–224. http://dx.doi.org/10.1038/ni.1838.

39. Helft J, Manicassamy B, Guernerez P, Hashimoto D, Silvin A, Agudo J, Brown BD, Schmolke M, Miller JC, Leboeuf M, Murphy KM, García-Sastre A, Merad M. 2012. Cross-presenting CD103(+) dendritic cells are protected from influenza virus infection. J Clin Invest 122:4037–4047. http://dx.doi.org/10.1172/JCI60659.

40. Paget C, Ivanov S, Fontaine J, Blanc F, Pichavant M, Renssen J, Bialecki E, Pothlichet J, Vendeville C, Barba-Spaeth G, Huerre MR, Faveeuw C, Trottein F. 2015. Influenza A virus-induced release of IL-27 from lung-derived dendritic cells promotes development of postinfluenza pneumococcal pneumonia. EMBO Mol Med 6:120–140. http://dx.doi.org/10.15252/emmm.201302890.

41. van der Sluijs KF, van Elden LJ, Nijhuis M, Schuurman R, Pater JM, Florquin S, Goldman M, Jansen HM, Lutter R, van der Poll T. 2004. IL-10 is an important mediator of the enhanced susceptibility to pneumococcal pneumonia after influenza infection. J Immunol 172:7603–7609. http://dx.doi.org/10.4049/jimmunol.172.12.7603.

42. Barthelmy A, Ivanov S, Fontaine J, Soulard D, Bouabe H, Paget C, Faveeuw C, Trottein F. 25 May 2016. Influenza A virus-induced release of interleukin-10 inhibits the anti-microbial activities of invariant natural killer T cells during invasive pneumococcal superinfection. Micuol Immunol http://dx.doi.org/10.1038/s41390-016-0049.

43. Hildner K, Edelson BT, Purtha WE, Diamond M, Matsushita H, Kohyama M, Calderon B, Schraml MU, Unanue ER, Diamond MS, Schreiber RD, Murphy TL, Murphy KM. 2008. Batf3 deficiency reveals a critical role during intramuscular immunization. J Immunol 180:5590–5602. http://dx.doi.org/10.4049/jimmunol.1002348.

44. Macho Fernandez E, Chang J, Fontaine J, Bialecki E, Rodriguez F, Werkeimer E, Krieger V, Ehret C, Heurtault B, Fournel S, Frisch B, Betbeder F, Faveeuw C, Trottein F. 2012. Activation of invariant natural killer T lymphocytes in response to the alpha-galactosylceramide analogue KRN7000 encapsulated in PLGA-based nanoparticles and microparticles. Int J Pharm 423:43–54. http://dx.doi.org/10.1016/j.ijpharm.2011.04.068.

45. Langlet C, Tamoutounour S, Henri S, Luche H, Aroudin L, Grégoire C, Malissen B, Guilliams M. 2012. CD64 expression distinguishes monocyte-derived and conventional dendritic cells and reveals their distinct role during intramuscular immunization. J Immunol 188:1751–1760. http://dx.doi.org/10.4049/jimmunol.1102744.