Halothane Modulates the Type I Interferon Response to Influenza and Minimizes the Risk of Secondary Bacterial Pneumonia through Maintenance of Neutrophil Recruitment in an Animal Model

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ABSTRACT

Background: To minimize the risk of pneumonia, many anesthesiologists delay anesthesia-requiring procedures when patients exhibit signs of viral upper respiratory tract infection. Postinfluenza secondary bacterial pneumonias (SBPs) are a major cause of morbidity and mortality. An increased host susceptibility to SBP postinfluenza has been attributed to physical damage to the pulmonary epithelium, but flu-induced effects on the immune system are being shown to also play an important role. The authors demonstrate that halothane mitigates the risk of SBP postflu through modulation of the effects of type I interferon (IFN).

Methods: Mice (n = 6 to 15) were exposed to halothane or ketamine and treated with influenza and Streptococcus pneumoniae. Bronchoalveolar lavage and lung homogenate were procured for the measurement of inflammatory cells, cytokines, chemokines, albumin, myeloperoxidase, and bacterial load.

Results: Halothane exposure resulted in decreased bacterial burden (7.9 ± 3.9 × 10^5 vs. 3.4 ± 1.6 × 10^6 colony-forming units, P < 0.01), clinical score (0.6 ± 0.2 vs. 2.3 ± 0.2, P < 0.0001), and lung injury (as measured by bronchoalveolar lavage albumin, 1.5 ± 0.7 vs. 6.8 ± 1.6 mg/ml, P < 0.01) in CD-1 mice infected with flu for 7 days and challenged with S. pneumoniae on day 6 postflu. IFN receptor A1 knockout mice similarly infected with flu and S. pneumoniae, but not exposed to halothane, demonstrated a reduction of lung bacterial burden equivalent to that achieved in halothane-exposed wild-type mice.

Conclusion: These findings indicate that the use of halogenated volatile anesthetics modulates the type I IFN response to influenza and enhance postinfection antibacterial immunity. (Anesthesiology 2015; 123:590-602)

What We Already Know about This Topic

- To minimize the risk of pneumonia, many anesthesiologists delay anesthesia-requiring procedures when patients exhibit signs of viral upper respiratory tract infection
- Whether anesthetics modulate the risk of postflu secondary bacterial pneumonia by modulating immunity is unknown

What This Article Tells Us That Is New

- Using mice exposed to halothane or ketamine and infected with influenza and Streptococcus pneumoniae, the authors demonstrated that the use of halogenated volatile anesthetics modulates the type I interferon response to influenza and enhance postinfection antibacterial immunity

A major complication of postviral morbidity and mortality is secondary bacterial pneumonia (SBP). In a previous prospective blinded cohort study, we observed that pediatric patients exposed to halothane who had viral upper respiratory tract infections had significantly fewer new clinical symptoms, and their symptoms were of a shorter duration compared with patients who did not receive halothane. Although not isolated from patients in this study, one of the most common and potentially deadly viral pathogens is influenza, because of the unique relationship it has with pneumococcal SBP.

Influenza predisposes the host to pneumococcal infection in several ways, including hindrance of the mucociliary escalator, denuding the epithelial barrier allowing bacterial adherence, and impairing antibacterial immunity because of factors of the host antiviral response. Before the 2009 H1N1 pandemic, 40,000 to 200,000 hospitalizations occurred because of influenza or its complications, and 25% of mortality was attributable to SBP. These numbers vary with pandemics and flu season duration. The major cause of death with the 1918 to 1919 Spanish flu was SBP, before the
advent of antimicrobial compounds. However, given the rapid rate of gain-of-resistance by pathogens to these agents, novel therapies that do not target the pathogen life cycle, but modulate host immunity, are urgently needed. The 2009 H1N1 pandemic was fortunately milder than predicted, but seasonal flu with SBP is a perennial killer, and the next pandemic always looms on the horizon.

Volatile anesthetics have been in use for decades, yet their anesthetic mechanism of action is far from completely understood as is their capacity to modulate immune responses. We hypothesized that the beneficial effect(s) of volatile anesthetics observed in our previous study is because of immune modulation, as exposure to volatile anesthetics does not alter peak viral titers during influenza. We have also shown that halothane and isoflurane modulate the influence of type I interferons (IFNs) on immune cells and completely abrogate type I IFN-induced cytotoxic action of natural killer lymphocytes. From our previous work, we hypothesized that halothane could decrease type I IFN-mediated host susceptibility to SBP postinfluenza by minimizing impairment of the host antibacterial immune response postflu.

To test this hypothesis, we used our H1N1 murine model of influenza-associated pneumococcal infection, whereby we challenged mice with Streptococcus pneumoniae 6 days postflu. Our goals of this study were to determine the parameters of the host antibacterial immune response postinfluenza that are beneficially altered by halothane, whether these alterations enhance resistance to SBP, and whether modulation of the type I IFN signaling axis is central to the ability of halothane to minimize the risk of SBP postflu. Findings from this study may lead to novel immune-modulating therapies to combat SBP postflu, such as low-dose halogenated volatile anesthetic usage to enhance pulmonary antibacterial immunity in patients suffering from flu. In addition, this study may contribute to the creation of novel therapeutics that modulate the immune response through mechanisms similar to halothane.

Materials and Methods

Use of Animals
All procedures involving the mice in these studies were approved by the Institutional Animal Care and Use Committee of the Veterans Administration Western New York Healthcare System (Buffalo, New York) and conformed to the guidelines described in the Guide for the Care and Use of Laboratory Animals from the National Academy of Sciences.

Mouse Models
Male, 3 weeks old, CD-1 (outbred strain) mice were obtained from Charles River Laboratories (USA) and housed for 1 week to acclimate before initiating experiments. Male, 4 to 6 weeks old, IFNAR-1 receptor knockout (IFNAR KO; Ifnar) mice on a C57BL/6 background were obtained from the Mutant Mouse Regional Resource Center (c/o The Jackson Laboratory, USA). They were bred, along with C57BL/6 wild-type (WT) control mice, in the laboratory animal facility of the Veterans Administration Western New York Healthcare System. The knockout strain has no overt phenotypic defects. The number of animals used in this study was based on our previous work with influenza in mice. As a general rule, a specific experiment was repeated three to four times with a minimum of three mice in each group to account for interexperiment and intraexperiment variability and potential experimental or assay failures. Our flu model has been established as a nonlethal challenge, wherein no more than 10% of any group succumbs to illness. Increasing the number of insults (i.e., flu and bacteria) increases the risk of animal mortality, and so additional animals were typically allocated to such groups. For humane reasons, in the interest of using as few animals as possible, fewer animals were used in subsequent experiments once it was determined that the double infectious insult was a nonlethal model.

Halothane Exposure
Mice were exposed to 1.5% halothane in 100% oxygen for 2 h in a 37°C chamber infused at 1.5 l/min just before influenza infection (day 0) and again exposed for 2 h on day 4 postinfluenza infection. An experimental timeline explaining groups and treatment/infection schedule is provided (fig. 1). Chamber concentrations of halothane, O2, and CO2 were continuously monitored with a Rascal II Ramen gas analyzer (Ohmeda Medical Equipment, USA). Our previous work demonstrated that day 0 exposure to anesthetic exerted the most clinical benefit (mortality, morbidity, lung damage, and clinical score), and day 4 postinfluenza was the last day postinfluenza infection before killing at which clinical benefit could be observed. The goal of this study was to identify the immune-modulating effects of halothane. To maximize the likelihood of identifying the specific parameters altered by this anesthetic, we wished to maximize the beneficial effects of the agent. At the end, we exposed all mice to halothane twice, as described in Halothane Exposure, on both day 0 (day of influenza infection) and day 4 (4 days postinfluenza).

Influenza Virus Strain and Infection Procedure
The A/PR/8/34, mouse-adapted H1N1 strain of influenza virus was the generous gift of Dr. Hunein Massab (University of Michigan, USA). This strain was originally isolated from a patient in Puerto Rico in 1934. At the end of the halothane exposure, mice were inoculated with 40 plaque-forming units of influenza (determined by plaque formation in Madin-Darby canine kidney epithelial cell monolayers), intranasally. Nonhalothane exposed control mice were sedated by intraperitoneal injection of 100 mg/kg ketamine in normal saline to facilitate virus inoculation.
Fig. 1. Experimental groups, and model timeline of anesthesia, treatment, and infections. (A) CD-1 mouse experimental group names used in the text and their respective treatments. In the case of type I interferon knockout mice and their wild-type controls, all mice were challenged with secondary bacterial pneumonia, and the only variable was ±HAL exposure. (B) Timeline of events in the experimental model. Influenza inoculation (or mock infection) occurred on day 0 PVI. Mice in the HAL groups were exposed for 2 h on both days 0 and 4 PVI. EF3030 infection occurred on day 6 PVI. Mice were killed on day 6 PVI, 3 h post-EF3030, or day 7 PVI, 24 h post-EF3030. In neutropenia studies, day 6 PVI cont = control; EF3030 = Streptococcus pneumoniae; HAL = halothane; SBP = secondary bacterial pneumonia.

Streptococcus pneumoniae Strain and Infection Procedure
The EF3030 strain of S. pneumoniae, a clinical otitis media isolate with a 19F capsular serotype, was grown to mid-log phase in Todd-Hewitt broth with 0.2% yeast extract media, aliquoted, and stored at −80°C. At the time of bacterial infection (6 days postviral infection [PVI]), an aliquot was thawed and diluted in phosphate-buffered saline, and either 2.5 × 10⁶ or 120 colony-forming units (CFUs, μl aliquots before being diluted 1:10) was delivered to the lungs by oropharyngeal aspiration in a 50-μl volume during ketamine sedation.

Morbidity/Clinical Scoring
Mice were randomly assigned to the various groups and weighed daily throughout the virus infection period. Just before killing (24 h postbacterial infection [PBI], i.e., 7 days PVI), the symptoms of illness were scored (0 to 6) daily by the same blinded researcher by awarding 1 point each for the following: piloerection, hunched posture, impaired gait, labored breathing, lethargy, and weight loss (≥10% of the body weight at the time of infection).¹⁹

Sacrifice, Tissue Harvest, and Bronchoalveolar Lavage Procedures
At 7 days PVI (with or without 24-h bacterial exposure), the mice were anesthetized with 2.75% isoflurane and exsanguinated by transecting the vena cava/abdominal aorta through a peritoneal incision. The diaphragm and rib cage were cut away to facilitate injection of 5-ml Hank balanced salt solution with Ca²⁺ and Mg²⁺ (37°C) into the right ventricle of the heart to flush the pulmonary vasculature of residual blood. After exposure of the trachea a 22-ga stainless steel cannula was inserted into the trachea and secured with a suture. A four-way stopcock with two 5-ml syringes attached was connected to the cannula, and bronchoalveolar lavage (BAL) was performed by instilling 1-ml Hank balanced salt solution with Ca²⁺ and Mg²⁺ (37°C) into the lungs with one syringe and collecting the volume with the other. This procedure was repeated for a total of five instillations with the collected volume stored on ice. Finally, the lungs were excised and stored on ice.

Sample Processing
Collected BAL fluid was centrifuged at 1,500g for 3 min at 4°C, and the supernatant was stored at −80°C. The recovered cells were enumerated with a MultiSizer III Coulter counter (Beckman Coulter, USA); a cytospin was prepared with a Cytospin 3 cytospinifuge (Shandon Lipshaw, USA) and stained with Diff-Quik (Dade Behring, USA) for differential counting by light microscopy. The lungs were homogenized in buffer containing 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, and pH 7.4 with protease inhibitor cocktail (Calbiochem, USA) containing 500 μM 4-(2-aminoethyl)benzenesulfonyl fluoride, 150 mM aprotinin, 1 μM E-64, 0.5 mM EDTA, and 1 μM leupeptin (total weight of buffer + lungs = 3 g) using a Polytron PT-2000 tissue homogenizer (Brinkman Instruments, USA). Myeloperoxidase (MPO) was extracted from the homogenate, as described previously.²⁰

Determination of Lung Bacterial Load
EF3030 bacterial titer was determined in BAL fluid and lung homogenate by removing 100 μl aliquots before being centrifuged and plating 10-fold serial dilutions in phosphate-buffered saline on tryptic soy with 5% sheep blood agar plates (VWR, USA), incubating at 37°C for 24 h, and counting colonies, each representing a CFU. Lung bacterial load was calculated as (BAL fluid CFU/ml × recovered BAL volume, ml) + (lung homogenate CFU/ml × 3 ml).

Cytokine/Chemokine/Albumin Analysis by Enzyme-linked Immunosorbent Assay
BAL fluid cytokine, chemokine, and albumin concentrations were determined by ELISA, as described previously.²¹ Capture and detection antibodies and recombinant protein standards were acquired from R&D Systems (USA) except for albumin (Bethyl Laboratories, USA).²²

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**Myeloperoxidase Activity Assay**

MPO activity was assessed by adding 10 μl of lung homogenate extract to 300 μl of buffer containing 50 mM KH₂PO₄, 176 mM H₂O₂, 52.5 mM o-dianisidine dihydrochloride, and pH 6.0 in a 96-well flat-bottom plate. Absorbance readings at 460 nm were taken for 90 s every 2 s using a SpectraMax 190 microplate reader with SoftMax Pro v.4.0 software (MDS Analytical Technologies, USA). MPO activity was expressed in arbitrary units as absorbance change per minute (abs/min) over the linear portion of the curve.²⁰

**In Vivo Polymorphonuclear Cell (Neutrophil) Depletion**

Vinblastine (Sigma-Aldrich, USA), 5 mg/kg, was administered to mice intravenously on day 2 PVI by retroorbital injection.²² Successful depletion of polymorphonuclear cells (PMNs; neutrophils) 4 days after administration of vinblastine (day 6 PVI), at the time of secondary pneumococcal challenge, was confirmed by obtaining a drop of blood from a tail snip, producing a blood smear slide, and staining with Diff-Quik. A leukocyte differential count was determined by light microscopy.

**Statistical Analysis**

A two-way ANOVA was performed with influenza and halothane exposure as the factors to determine whether there was a main effect because of halothane exposure (P < 0.05 considered to be significant). A two-tailed Bonferroni post hoc test (correcting for multiple comparisons) was performed to determine the source of that variability. Analyses were performed using Prism 5 for Mac OS X (GraphPad Software, USA). Some data were lost during the study because of animal mortality (minor contributor to loss because of use of nonlethal influenza inoculum), cytokine ELISA assay failure because of occasional hyperreactive wells of assay plates, and contamination of blood agar plates that prevented bacterial quantification.

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**Results**

**Halothane Maintained Antibacterial Immune Function Postflu and Decreased Morbidity and Lung Injury**

At day 6 PVI, halothane exposure significantly minimized clinical symptoms after influenza (flu) infection (fig. 2A, P < 0.05 by two-way ANOVA with Bonferroni post hoc test). On pulmonary challenge with 2.5 × 10⁶ CFU of the EF3030 strain of pneumococcus, mock-flu–infected mice (mock-control [cont] and mock-halothane [HAL] groups; fig. 1) exhibited an equivalent level of clinical symptoms 24 h PBI, regardless of anesthetic exposure (fig. 2B). However, mice infected with influenza 6 days before the EF3030 challenge, our SBP groups (SBP-cont and SBP-HAL) had significantly less symptoms of illness 24 h PBI if anesthetized with halothane. SBP-HAL mice exhibited symptoms comparable with mice that had never been infected with flu, indicating diminution of host morbidity on SBP challenge because of halothane exposure (fig. 2B). Importantly, airway albumin levels, a reliable marker of pulmonary damage, were significantly decreased by more than fourfold in SBP-HAL mice compared with the SBP-cont group (fig. 2C). Assessment of bacterial burden at 24 h PBI revealed that mock-cont and mock-HAL mice challenged only with EF3030 displayed no difference in bacterial burden because of halothane exposure, indicating that halothane does not fundamentally enhance the antibacterial capacity of the host in the absence of previous flu infection (fig. 2D). Of note, given the initial 2.5 × 10⁶ CFU inoculum of EF3030, most of the SBP-HAL mice had bacterial burdens less than this initial inoculum at 24 h PBI, indicating effective bacterial clearance, whereas SBP-cont mice harbored burdens in the 10⁸ CFU range, indicating pneumococcal outgrowth far larger than the...
Halothane Enhances Bacterial Immunity Postflu

Halothane Altered the Expression of Cytokines and Chemokines that Affect Immune Cell Recruitment during an Influenza Infection and 24-h Secondary Pneumococcal Challenge

SBP-HAL mice exhibited significantly decreased levels of total airway infiltrates, airway macrophages, and parenchymal PMN compared with SBP-cont mice at 24 h PBI (data not shown). In accordance with this, keratinocyte-derived chemokine (KC) and macrophage inflammatory protein-2 (MIP-2) (CXC neutrophilic chemokines) and monocyte chemotactic protein-1 (MCP-1) (a CC monocyte chemokine) were also diminished in the SBP-HAL group compared with SBP-cont mice (data not shown). However, the significant differences of bacterial burden and lung injury between the SBP-cont and SBP-HAL mice at the 24 h PBI time point could significantly affect these immunologic parameters, so definitive conclusions regarding halothane-mediated immune-modulation postflu cannot be made (fig. 2, C and D). Therefore, we sought to determine how halothane might beneficially alter the ability of the host to immediately respond to a secondary bacterial challenge postflu at 3 h PBI, when bacterial burden and lung injury are equivalent for all groups.

Mice were again challenged with $2.5 \times 10^6$ CFU EF3030 6 days PVI (or after mock flu infection) and killed at 3 h PBI to identify differences in their immediate immunologic response to pneumococcal challenge. At 3 h PBI, mice in all groups harbored more pneumococci than the original inoculum, indicating that all groups experience an initial and equivalent outgrowth of bacteria in the first few hours PBI, irrespective of previous flu infection or anesthetic exposure (fig. 3A). Importantly, tissue damage was also equivalent between the two mock flu–infected groups and between the two SBP groups (fig. 3B). This eliminated the concern that differences

Fig. 3. In a mouse model of secondary bacterial pneumonia (SBP), at 3 h postbacterial infection (PBI), EF3030 burden and lung damage were equivalent, but halothane (HAL)-induced alterations in cytokine expression were observed in flu-infected mice. CD-1 mice were inoculated intranasally with 40 plaque-forming units A/PR/8/34 influenza virus (SBP) or mock infected (mock). Mock-HAL and SBP-HAL mice (white bars) were exposed to 2% HAL for 2 h just before infection (day 0) and again for 2 h on day 4 postviral infection (PVI). Mock-cont and SBP-cont mice (black bars) received ketamine sedation. On day 6 PVI, mice were inoculated with $2.5 \times 10^6$ CFU EF3030, and 3 h later (3 h PBI), mice were killed, BAL was performed, and lungs were harvested. (A) Nonclarified recovered BAL fluid and lung homogenates were titered for EF3030 to determine the total EF3030 lung burden. Cell-free BAL fluid was assessed for (B) albumin, (C) interleukin (IL)-1β, (D) IL-6, (E) tumor necrosis factor (TNF)-α, (F) MCP-1, (G) MIP-2, (H) KC, and (I) interferon (IFN)-γ. Sample sizes are displayed above groups. *P < 0.05, **P < 0.01 SBP-HAL compared with SBP-cont. Data are expressed as mean ± SEM. BAL = bronchoalveolar lavage; CFU = colony-forming units; cont = control; EF3030 = Streptococcus pneumoniae.
in pneumococcal burden or tissue damage could affect the host immune response and allowed for adequate assessment of the immunologic parameters altered by halothane at this early time point postpneumococcal challenge.

Amongst SBP groups, halothane demonstrated the ability to minimize levels of the proximal inflammatory cytokine, interleukin-1β (IL-1β) during SBP (fig. 3C). In contrast, interleukin-6 was significantly increased in the SBP-HAL group compared with the SBP-cont group (fig. 3D). However, tumor necrosis factor-α (TNF-α) levels were unaffected by halothane in SBP-HAL mice (fig. 3E). This demonstrates that in the context of SBP postflu, halothane exposure uncouples the immediate expression levels of these three proximal inflammatory cytokines that are believed to be coregulated with one another by the p50 and p65 nuclear factor-κB heterodimer. Expression levels of these cytokines were unaffected by halothane in mock flu–infected mice (fig. 3F). These results provide additional evidence that halothane exposure alters the host immune response to a secondary bacterial challenge after a previous flu infection, but not in the mock flu–infected animals challenged only with pneumococci.

Next, we assessed the levels of antiviral cytokines (type I IFNs) and chemokines known to be critical for leukocyte recruitment to the lung environment. Flu infection with SBP (SBP-cont and SBP-HAL groups) resulted in increased MCP-1 levels compared with the mock flu–infected mice challenged only with pneumococcus (mock-cont and mock-HAL). Halothane exposure had no effect on the production of this chemokine (fig. 3F). There was no significant difference in MIP-2 levels between the two mock flu groups or between the two SBP groups (fig. 3G). However, KC (the neutrophilic chemokine) was significantly increased in the SBP-HAL mice compared with the SBP-cont group at this early 3 h PBI time point despite the equivalent bacterial burden and lung injury (fig. 3H).

Type I and II IFNs are essential elements that protect the host during virus infection, and both have been shown to inhibit antibacterial immunity in the lung postflu through impaired PMN recruitment and alteration of macrophage phenotype.5–7 Given their potential to inhibit pulmonary antibacterial immunity, pulmonary expression levels of IFN-α (type I IFN) and IFN-γ (type II IFN) were investigated. On day 6 postflu, IFN-α was low to undetectable in all groups of mice (data not shown), but the level of IFN-γ was increased in the flu-infected SBP-cont mice compared with SBP-HAL. Halothane exposure reduced the IFN-γ levels to nearly undetectable levels (fig. 3I). From these findings, we postulated that the antibacterial immune function of alveolar macrophages may be compromised because of increased type II IFN levels and that halothane may confer protection by minimizing the type II IFN production. In addition, halothane may cause an increase in KC-mediated PMN recruitment to compensate for the altered macrophage phenotype, thus increasing antibacterial defense and decreasing pulmonary injury. To begin to test this hypothesis, we assessed the levels of various immune cell infiltrates.

### Halothane Fosters Rapid Neutrophil Recruitment Postinfluenza in Response to a Secondary Pneumococcal Challenge

In SBP-cont mice, levels of total immune infiltrates were significantly enhanced as early as 3 h PBI compared with all other groups (fig. 4A). Mock flu–infected control mice had 4.5 ± 0.8 × 10^5 macrophages recovered from the BAL that is within the range of 2 to 5 × 10^5 airway macrophages expected to be isolated from a naïve mouse unchallenged with any pathogen from the past experience of our laboratory (fig. 4B and data not shown). In addition, although PMNs were the predominant leukocyte in all groups at 24 h PBI (data not shown), mock flu–infected controls did not recruit significant numbers of PMN at 3 h PBI, constituting less than 20% of total infiltrates (fig. 4, A and C). These mock flu–infected mice received ketamine sedation. On day 6 PVI, mice were inoculated with 2.5 × 10^6 CFU EF3030, and 3 h later (3 h PBI), mice were exposed to 2% HAL for 2 h just before infection (day 0) and again for 2 h on day 4 PVI. Mock-cont and SBP-cont mice (black bars) received ketamine sedation. On day 6 PVI, mice were inoculated with 2.5 × 10^6 CFU EF3030, and 3 h later (3 h PBI), mice were killed, BAL was performed, and lungs were harvested. The cells recovered from the pulmonary airspaces by BAL were analyzed:

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<th>Group</th>
<th>Total Leukocytes</th>
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Fig. 4. In a mouse model of SBP, at 3 h PBI, HAL exposure resulted in a decrease in the number of alveolar macrophages and an increase in the number of neutrophils (PMNs) in the pulmonary airspaces in flu-infected mice. CD-1 mice were inoculated intranasally with 40 plaque-forming units A/PR/8/34 influenza virus (SBP) or mock infected (mock). Mock-HAL and SBP-HAL mice (white bars) were exposed to 2% HAL for 2 h just before infection (day 0) and again for 2 h on day 4 PVI. Mock-cont and SBP-cont mice (black bars) received ketamine sedation. On day 6 PVI, mice were inoculated with 2.5 × 10^6 CFU EF3030, and 3 h later (3 h PBI), mice were killed, BAL was performed, and lungs were harvested. The cells recovered from the pulmonary airspaces by BAL were analyzed:

A: Total number of leukocytes
B: Total number of macrophages
C: Total number of PMNs

The harvest lungs were homogenized, and MPO activity was determined as a measure of PMN infiltration into the lung parenchyma. Sample sizes are displayed above groups. *P < 0.05; **P < 0.01.
mice also had the lowest bacterial burdens at 24 h PBI, indicating that the macrophage population in these mice was more efficacious at controlling the bacterial challenge. By comparison, both SBP groups that had been challenged with flu 6 days before had macrophage numbers greater than or equal to those of mock flu–infected controls (fig. 4B). Despite having the highest macrophage numbers, the SBP-cont group was the least capable of mounting an antibacterial response. This finding supports our hypothesis that impairment of macrophage antibacterial function because of host immune responses to flu infection (i.e., enhanced type II IFN levels in the SBP-cont group; fig. 3I) is mitigated by halothane. Also in agreement with our hypothesis, halothane enhanced the KC levels in SBP-HAL mice (fig. 3H) that correlated with an increase in immediate airway PMN recruitment (fig. 4C). Levels of parenchymal PMN were also increased in the SBP-HAL group (fig. 4D), as indicated by lung homogenate MPO activity. The ratio and overall number of PMNs recruited relative to macrophage recruitment in SBP-cont compared with SBP-HAL mice was markedly different. Despite significantly less total immune infiltrates in the SBP-HAL group, these mice have significantly more total PMN than the SBP-cont mice (fig. 4, A and C). It was found that 65% of all airway infiltrates are PMN in SBP-HAL mice, compared with only 27% in SBP-cont animals. Halothane reversed the PMN:macrophage recruitment ratio of 1:2 in SBP-cont mice to 2:1 in the SBP-HAL group. This finding indicates that the diminished expression of KC because of previous influenza infection is likely biologically significant and results in less PMN recruitment in SBP-cont mice that may be necessary to compensate for the impaired antibacterial immune state in the lungs that we observe. The enhanced PMN recruitment mediated by halothane exposure may be required to compensate for this, resulting in approximately 3-log less pneumococcal burden and less lung damage at 24 h PBI.

To test whether early recruitment of PMN facilitated by halothane was central to this agent’s ability to minimize the risk and severity of SBP, we predicted neutropenic mice challenged with SBP would no longer exhibit enhanced antibacterial immune function in the lungs after halothane exposure.

**Neutropenic Mice Challenged with Pneumococci Demonstrate PMN Recruitment Is Essential to Halothane Maintenance of Antibacterial Immunity Postinfluenza**

To test whether the early PMN recruitment facilitated by halothane exposure is central to this agent’s ability to benefit the host and minimize the risk and severity of SBP postflu, we administered vinblastine on day 2 PVI to make the mice neutropenic by day 6 PVI, at the time of secondary pneumococcal challenge. Selective depletion of PMN by vinblastine, a chemotherapeutic agent, is accomplished because of the rapid generation time of these cells and their short lifespan (1 to 3 days), which is far shorter than other cells of the immune system. We predicted that halothane’s beneficial enhancement of the host antibacterial immune response postinfluenza would not be exhibited in neutropenic mice. At the time of bacterial challenge (6 days PVI and 4 days after administration of vinblastine), circulating PMNs were reduced to approximately 1% of circulating leukocytes, as assessed by blood smear differential counts (data not shown). Also indicating successful PMN depletion by vinblastine (including the intravascular PMN margination pool), cell recovery from the BAL of the halothane SBP group at 3 h PBI was 7.7% of the PMN recovery in the same group in nonneutropenic mice (4.7 ± 1.4 × 10^3 PMNs; fig. 5A) compared with 6.1 ± 0.5 × 10^4 PMNs (fig. 4C, P < 0.05). This level of PMN recruitment was similar to that observed in the mock flu–infected groups (fig. 5A). Macrophage levels in the lungs at the time of pneumococcal challenge were not decreased because of our vinblastine regimen (data not shown). In neutropenic mice, halothane failed to minimize clinical symptoms (fig. 5B), lung injury (fig. 5C), and pneumococcal burden (fig. 5D) at 24 h PBI in SBP mice. At day 7 PVI/24 h PBI (5 days after administration of vinblastine), there was an increase in PMNs recovered in the BAL in both flu-infected groups (fig. 5E). These findings indicate that early and immediate PMN recruitment mediated by halothane in the SBP-HAL group is essential for ability of this agent to mitigate SBP.

We assessed the immediate (3 h PBI) inflammatory response in neutropenic animals on day 6 PVI. SBP-HAL mice had reduced levels of MCP-1 in the BAL (fig. 6A) with an associated decrease in numbers of macrophages recovered by BAL (fig. 6B) compared with SBP-cont mice. However, despite the early increase in macrophage recruitment, SBP-cont mice were not protected from SBP, given the equivalent burdens in both SBP groups at 24 h PBI (fig. 5D), potentially indicating that these macrophages were inefficient at limiting pneumococcal infection because of previous flu infection.

The CXC neutrophilic chemokine MIP-2, but not KC, was increased in SBP-HAL mice compared with the SBP-cont group (fig. 6, C and D). This is in contrast to our previous observations in PMN-replete mice of increased KC levels (which was associated with rapid PMN recruitment), but there were no differences in MIP-2 levels in SBP-HAL mice compared with SBP-cont mice (fig. 3, G and H). However, this increase of MIP-2 was to no avail given PMN depletion, and SBP-HAL mice had even less airway and interstitial PMN than SBP-cont mice, and yet these two groups harbored equivalent E3030 burden at 3 and 24 h PBI, indicating that despite enhanced PMN levels in SBP-cont mice, this was insufficient for enhanced protection from pneumococcal infection postflu (figs. 5E and 6, C–E). Of note, IFN-γ levels in the lungs of mice in the SBP-HAL group were far less than that assessed in SBP-cont mice as we observed previously (fig. 6F). This indicated that halothane maintains its ability to minimize the IFN-γ levels in PMN-depleted mice as it has done in PMN-replete mice.
In our PMN-depletion studies, halothane decreased MCP-1, macrophage recruitment, and IFN-γ in SBP-HAL compared with SBP-cont mice, and despite significantly more macrophages in the SBP-cont group, these mice harbored the same pneumococcal burden at 24 h PBI similar to the SBP-HAL group. These findings imply that the antibacterial function of alveolar macrophages postflu is superior in SBP-HAL mice compared with the SBP-cont group. To assess the ability of halothane to mitigate the dysfunction of alveolar macrophages postflu, we challenged neutropenic mice postflu with a small inoculum of EF3030 that does not require PMN recruitment for clearance and assessed the ability of the lungs to clear the bacteria.

**A Minor Insult of Pneumococci in Neutropenic Mice Is Potentially Infectious, Indicating Macrophage Dysfunction Postflu That Is Mitigated by Halothane**

In both neutropenic and PMN-replete animals, IFN-γ levels were increased in SBP-cont mice, but halothane exposure reduced those levels (Figs. 3I and 6F). This cytokine has been shown to inhibit macrophage antibacterial function postflu. To assess whether halothane maintains macrophage antibacterial function, we used our same model, but challenged neutropenic mice with only 120 CFU as opposed to the 2.5 × 10⁶ CFU of EF3030 used previously. Although lung injury was not improved (Fig. 7A), morbidity (Fig. 7B) and pulmonary burden of EF3030 (Fig. 7C) were reduced in neutropenic SBP-HAL mice compared with SBP-cont mice. Although mock flu controls and SBP-HAL mice successfully managed to curtail pneumococcal outgrowth in the absence of PMN assistance, the SBP-cont mice failed to do so. The SBP-cont mice exhibited outgrowth of bacteria from the initial inoculum. This indicated that halothane not only fosters antibacterial competence of macrophages in the lung postflu.

**In the Absence of Type I IFN Influence, Halothane Fails to Protect the Host from SBP**

Our previous work has demonstrated that volatile anesthetics (i.e., halothane, isoflurane, sevoflurane) minimize the effects of type I IFNs on immune cell activation and clinical symptoms of patients afflicted with respiratory tract infections. Type I IFNs enhance the Th1 adaptive immune response.
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Fig. 6. CD-1 mice were inoculated intranasally with 40 plaque-forming units A/PR/8/34 influenza virus (SBP) or mock infected (mock). Mock-HAL and SBP-HAL mice (white bars) were exposed to 2% HAL for 2 h just before infection (day 0) and again for 2h on day 4 PVI. Mock-cont and SBP-cont mice (black bars) received ketamine sedation. On day 2 PVI, mice were administered 5 mg/kg vinblastine to induce neutropenia. On day 6 PVI, mice were inoculated with 2.5×10^6 CFU EF3030, and at 3 h PBI, mice were killed, BAL was performed, and lungs were harvested. (A) BAL (MCP-1), (B) total number of macrophages recovered in the BAL, (C) BAL (MIP-2), (D) BAL (KC), (E) lung homogenate MPO activity, and (F) BAL IFN-γ were determined. Sample sizes are displayed above groups. *P < 0.05, **P < 0.01 SBP-HAL compared with SBP-cont. Data are expressed as mean ± SEM. BAL = bronchoalveolar lavage; CFU = colony-forming units; cont = control; EF3030 = Streptococcus pneumoniae; HAL = halothane; IFN = interferon; MPO = myeloperoxidase; PBI = postbacterial infection; PVI = postviral infection; SBP = secondary bacterial pneumonia.

Fig. 7. CD-1 mice were inoculated intranasally with 40 plaque-forming units A/PR/8/34 influenza virus (SBP) or mock infected (mock). Mock-HAL and SBP-HAL mice (white bars) were exposed to 2% HAL for 2 h just before infection (day 0) and again for 2h on day 4 PVI. Mock-cont and SBP-cont mice (black bars) received ketamine sedation. On day 2 PVI, mice were administered 5 mg/kg vinblastine to induce neutropenia. On day 6 PVI, mice were inoculated with 120 CFU EF3030, and at 24 h PBI, mice were killed, BAL was performed, and lungs were harvested. (A) BAL (albumin), (B) mouse clinical score, and (C) total lung EF3030 burden were determined. Sample sizes are displayed above groups. **P < 0.01 SBP-HAL compared with SBP-cont. Data are expressed as mean ± SEM. BAL = bronchoalveolar lavage; CFU = colony-forming units; cont = control; EF3030 = Streptococcus pneumoniae; HAL = halothane; PBI = postbacterial infection; PVI = postviral infection; SBP = secondary bacterial pneumonia.

after influenza infection, which enhances IFN-γ expression and inhibits both PMN recruitment and macrophage antibacterial capacity rendering the host highly susceptible to SBP. We hypothesized that halothane protection from SBP vulnerability would be dependent on the type I IFN elaboration. To test this hypothesis, we used Ifnar1<sup>tm1Agt</sup>/Ifnar1<sup>Ftm</sup> mice devoid of the type I IFN receptor/IFN-α receptor (IFNAR) and assessed whether these knockout mice had any benefit from halothane compared with WT controls.

WT (C57BL/6) and IFNAR KO mice were infected with flu and challenged with 2.5×10^6 CFU of EF3030 on day 6 PVI. At 24 h PBI, mice were killed, and lung injury and EF3030 bacterial burden were assessed. Similar to the previous experiment in CD-1 mice (fig. 2, C and D), halothane-exposed WT mice exhibited decreases in lung injury and bacterial burden compared with the WT control (not exposed to halothane) mice (fig. 8, A and B). The levels of lung injury and bacterial burden in the IFNAR KO mice, with or without halothane exposure, were similar to the levels attained in the WT mice that were exposed to halothane. Halothane did not produce any additional benefit in regards to both lung injury and bacterial burden in the IFNAR KO mice than what was afforded by the loss of an intact type I IFN response. This result supports the hypothesis that...
halothane protection from SBP is conferred by halothane modulation of the type I IFN response to flu.

Discussion

Previously, we demonstrated that pediatric patients exposed to halothane for minor procedures, who had viral upper respiratory tract infections, had significantly fewer symptoms and shorter duration of symptoms compared with patients who did not receive halothane. However, this study was not powered to address the risk of viral-associated SBP, a major cause of morbidity and mortality worldwide. In the case of the 1918 “Spanish Flu”, SBP was the major cause of death, and S. pneumoniae was a major pathogen responsible for this. It is interesting that this commonly commensal bacterium of the nasopharynx can be so devastating. Yet it is evident that after influenza infection, this bacterium possesses the capacity to wreak havoc on the lungs, compromising pulmonary function and in severe circumstances lead to death. Despite the advent of antibiotics, this pathogen is still a major cause of SBP postflu.

Halothane did not enhance baseline antibacterial immune function nor was the bactericidal agent as evidenced from our mock flu–infected controls (fig. 2D). Instead, halothane maintained baseline antibacterial immunity in previously flu-infected SBP mice, equivalent to that of mock flu–infected controls. This led us to our hypothesis that halothane alters aspects of the host antiviral response such that antibacterial immunity is enhanced and the risk and severity of SBP is diminished.

We demonstrate that rapid PMN recruitment to the lungs is critical following SBP challenge, and halothane facilitates this, likely through enhanced production of neutrophilic chemokines such as KC and MIP-2. When PMN are ablated, halothane no longer benefits the host, and SBP severity is equivalent to that observed in controls. Mice challenged with SBP exhibited a significant increase in pulmonary IFN-γ levels, and this was minimized by halothane (fig. 3). This cytokine has been shown to inhibit antibacterial functions of macrophages, rendering the host at an increased risk of SBP. Minimization of this cytokine by halothane was associated with an increased PMN recruitment and a decreased macrophage recruitment and ultimately decreased bacterial infection and lung injury. We postulated that enhanced recruitment of PMN on SBP challenge that we observed because of halothane was essential to compensate for impaired alveolar macrophage antibacterial function. Impaired macrophage antibacterial function postflu was indeed observed in our PMN-depletion studies using a minor (120 CFU) EF3030 inoculum that does not require PMN recruitment, and halothane ameliorated this as well, whereas the SBP-cont group demonstrated bacterial burden greater than the inoculum (outgrowth). These findings indicate that halothane allows the host to combat bacterial infection postflu through decreased IFN-γ expression, by minimizing the impairment of macrophage antibacterial function and by fostering compensatory PMN recruitment while diminishing newly recruited, potentially damaging macrophages, to the pulmonary environment (fig. 9).

Other studies have shown that the detrimental roles of type I IFN postflu can leave the host more susceptible to SBP. These effects of type I IFN include impaired expression of neutrophilic chemokines, alteration of bone marrow monocyte precursors to a phenotype that preferentially recruits more inflammatory macrophages instead of PMN, and enhancement of numerous aspects of the adaptive Th1 response (of which IFN-γ is the quintessential cytokine) that results in increased pulmonary injury and diminished macrophage antibacterial activity. All our biochemical and cytologic studies indicate that halothane protects the host from these known responses to type I IFN postflu, without altering type I IFN expression levels. These findings coupled with our previous work demonstrating that the effects of type I IFN on immune cells are significantly diminished by halothane led us to the hypothesis that modulation of the effects of type I IFN by this agent in the context of SBP is most likely responsible for…
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We tested this through the use of mice devoid of the type I IFN receptor and found that when the type I IFN signaling axis is absent, halothane no longer benefited animals. Regardless of halothane exposure status, IFNAR KO mice had low levels of lung injury and bacterial burden equivalent to that of WT SBP mice receiving halothane, and WT SBP mice that did not receive halothane had far more lung injury and pneumococcal infection. The lack of benefit from halothane in IFNAR KO mice can be explained by the fact that their genetic status provides protection from improperly delayed type I IFN expression postflu, and therefore, halothane serves no additional benefit, unlike in WT controls. This confirmed that alteration of the effects of type I IFN by halothane was the major mechanism responsible for minimizing the risk of SBP.

Type I IFN evolved to protect organisms from viral infections, and this is why all nucleated cells in the human body can express type I IFN. Clearly type I IFN did not evolve over

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**Fig. 9.** Proposed mechanism of halothane (HAL) type I interferon (IFN) modulation during flu and secondary bacterial pneumonia. The influenza virulence factor, NS1, delays epithelial cell (white rhomboid, brown nucleus) type I IFN production in response to viral nucleic acids until day 5 postviral infection (PVI). (A) In the absence of HAL exposure, the full effects of this delayed type I IFN (IFN-α) expression on both innate (phagocytic alveolar macrophages, yellow stars) and adaptive cells (lymphocytes, purple) are unopposed. (B) After HAL exposure, the effects of delayed type I IFN are effectively blocked, without altering the type I IFN expression levels. This renders cells of the immune system less susceptible to phenotypic alteration because of this cytokine. (C) At day 6 PVI, 3 h postbacterial infection (PBI), immune cells exhibit altered phenotypes as a result of type I IFN and lack of HAL exposure that would dampen these effects of type I IFN. This results in enhanced lymphocyte production of IFN-γ, decreased phagocytic activity of macrophages, and enhanced recruitment of inflammatory monocytes (red) through MCP-1 production. All these factors result in increased bacterial infection (blue ovals) and pulmonary damage. (D) HAL-mediated blockade of the effects of type I IFN results in reduced IFN-γ production, maintenance of phagocytic macrophage activity, decreased inflammatory monocyte recruitment, and preferential recruitment of neutrophils (green) through KC/MIP-2 expression after bacterial challenge postflu. This results in bacterial clearance and decreased pulmonary damage. KC = keratinocyte-derived chemokine; MCP-1 = monocytic chemotactic protein-1; MIP-2 = macrophage inflammatory protein-2.
ons to serve as an Achilles heel to the host postflu. However, influenza perturbs the proper expression pattern of type I IFN (especially its timing), primarily through its virulence factor NS1, and this alteration is what we believe renders the host susceptible to catastrophic SBP after flu through both direct (inhibited PMN recruitment) and downstream actions (enhanced Th1 response; IFN-γ).28,29 Instead of peak type I IFN expression occurring before peak viral titers, peak expression of type I IFN occurs at day 5 PVI in our model, well past peak viral titers that occur on day 3 PVI. Altered type I IFN expression is found in patients suffering from flu also.30 At this time, type I IFN cannot serve its appropriate role of minimizing viral titers, yet it can dramatically enhance the Th1 response, impair antibacterial immunity, and increase immune-mediated damage to host tissue. This explains what we dub the “interferon paradox,” wherein type I IFN may be both beneficial and detrimental to the host. Essentially, inappropriately timed type I IFN fails to minimize the viral infection but enhances Th1 immune response and impairs antibacterial immunity. Halothane ameliorates this.

Halothane and ketamine were used in this study given our familiarity with these compounds in this model and our well-established evidence that halothane possesses the ability to alter host immune responses that we sought to further elucidate. Although ketamine has potential side effects, its use was necessary to sedate the live animals to ensure accurate delivery of inoculum to the lower airways. Through years of use with this model, we have not detected any affects of ketamine on any of the immunologic or clinical parameters assessed. We have also demonstrated that the commonly used clinical volatile anesthetics, isoflurane15 and sevoflurane (unpublished data in 2002 from Paul R. Knight, M.D., Ph.D., indicating that sevoflurane exposure reduced mortality in influenza-infected CD-1 mice), also possess the immunomodulatory abilities of halothane. It is possible that if these agents have the same efficacy in low concentrations, they might be used prophyllactically or therapeutically in patients with flu to mitigate the risk of SBP by modulating the errant type I IFN response. In addition, compounds that possess the same immunologic effects and lack anesthetic properties could be used clinically to enhance antibacterial immunity in the lungs postflu. Halothane facilitates rapid PMN recruitment and minimizes the impairment of macrophage antibacterial immune function, which is inhibited by host antiviral factors such as IFN-α (type I IFN) and IFN-γ (type II IFN), respectively. This immunomodulation greatly decreases the risk and severity of SBP. Microbial resistance to antibiotics is always increasing and is a major public health concern. Therefore, these studies have important scientific and clinical implications. Our animal model aims to explain the clinical observations as published by Tait and Knight.2 In the absence of airway manipulation and bronchospasm, our findings in the mouse model support that on exposure to volatile anesthetics, animals have less predisposition to SBP. These findings should be taken into consideration when assessing patients with underlying viral infection who are planning to undergo a surgical procedure. Second, this work challenges the belief that neutrophilic influx is always highly detrimental to the lungs. To be sure, PMN cause significant collateral damage that compromise pulmonary function, as evidenced in chronic illnesses such as emphysema. However, during SBP postflu, PMN recruitment to the lungs is essential. Finally, this work identifies a novel target that can be used to combat flu and SBP (modulation of the type I IFN response). This approach, unlike vaccines, antimicrobials, or antivirals, is not pathogen specific and therefore applicable to numerous diseases and has far less risk of organisms gaining resistance. Low concentration anesthetics, compounds with similar antitype I IFN effects, and novel therapies such as nanoparticle delivery of bioactive molecules targeting type I IFN are viable candidates to accomplish alterations in the host immune response.17,31

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Competing Interests
The authors declare no competing interests.

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