Leflunomide Prevents Alveolar Fluid Clearance Inhibition by Respiratory Syncytial Virus

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Rationale: **Previously, we demonstrated that intranasal infection of BALB/c mice with respiratory syncytial virus (RSV) resulted in an early 40% reduction in alveolar fluid clearance (AFC), an effect mediated via P2Y purinergic receptors.**

Objectives: **To confirm that RSV-induced inhibition of AFC is mediated by uridine triphosphate (UTP), and to demonstrate that inhibition of *de novo* pyrimidine synthesis with leflunomide prevents increased UTP release after RSV infection, and thereby also prevents inhibition of AFC by RSV.**

Methods: **BALB/c mice were infected intranasally with RSV strain A2. AFC was measured in anesthetized, ventilated mice by instillation of 5% bovine serum albumin into the dependent lung. Some mice were pretreated with leflunomide or 6-mercaptopurine.**

Measurements and Main Results: **RSV-mediated inhibition of AFC is associated temporally with a 20-nM increase in UTP and ATP content of bronchoalveolar lavage fluid, hypoxemia, and altered nasal potential difference. RSV-mediated nucleotide release, AFC inhibition, and physiologic sequelae thereof can be prevented by pretreatment of mice with the *de novo* pyrimidine synthesis inhibitor leflunomide, which is not toxic to the mice, and which does not affect RSV replication in the lungs. In contrast, pretreatment of mice with 6-mercaptopurine, an inhibitor of *de novo* purine synthesis, has no beneficial effect on AFC or other indicators of disease progression. Finally, RSV-mediated inhibition of AFC is prevented by volume-regulated anion channel inhibitors.**

Conclusion: **Pyrimidine synthesis or release pathways may provide novel therapeutic targets to counter the pathophysiologic sequelae of impaired AFC in RSV disease.**

Keywords: ion transport; paramyxovirus infections; pneumonia, viral; pulmonary edema

The dominant ion transport process of the alveolar epithelium is active movement of Na^+ ions from the airspace lining fluid to the interstitium (1). This creates an osmotic gradient, causing water to follow passively. This process of alveolar fluid clearance (AFC) is crucial to efficient gas exchange. Importantly, patients with acute lung injury with intact AFC have lower morbidity and mortality than those with compromised AFC (2).

Respiratory syncytial virus (RSV) is the most common cause of lower respiratory tract disease in infants and children worldwide (3), but its pathogenesis remains poorly understood. Previously, we demonstrated that infection of BALB/c mice with RSV results in reduced AFC at early time points after infection, and inferred that RSV-mediated inhibition of AFC 2 d after infection was mediated by uridine triphosphate (UTP), acting on P2Y receptors (P2YRs) on lung epithelial cells (4). The aim of the current study was to confirm the central role of UTP in mediating the inhibitory effects of RSV on AFC, and to demonstrate that pharmacologic inhibition of *de novo* pyrimidine synthesis with leflunomide prevents increased UTP release after RSV infection, and thereby also prevents inhibition of AFC by RSV. Furthermore, we demonstrated that blockage of RSV-mediated AFC inhibition with leflunomide would improve pulmonary edema and hypoxemia in RSV-infected mice. In addition, because RSV may infect epithelial cells throughout the entire respiratory tract, we determined whether RSV infection also alters upper respiratory tract epithelial ion transport, and to determine whether leflunomide treatment reverses any such effects. Finally, we demonstrated that UTP release into the alveolar space occurs through volume-regulated anion channels (VRACs), rather than being a consequence of low-level cell lysis in response to infection.

In this study, we show that RSV infection results in increased release of both UTP and adenosine triphosphate (ATP) on Day 2, and that systemic inhibition of *de novo* pyrimidine synthesis with leflunomide, which reduces bronchoalveolar lavage (BAL) nucleotide levels, reverses the impaired AFC, edema, hypoxemia, and diminution of nasal potential difference (NPD) in RSV-infected mice, despite having no effect on RSV replication. In contrast, systemic inhibition of *de novo* purine synthesis with 6-mercaptopurine (6-MP) has no detectable beneficial effects in RSV-infected mice. Finally, we show that RSV-mediated inhibition of AFC on Day 2 can be prevented by pharmacologic blockade of VRAC-like channels. These studies confirm our prior inference that UTP release is necessary for RSV-mediated inhibition of AFC, and demonstrate that therapeutic inhibition of the *de novo* pyrimidine synthesis and release pathway may alleviate the symptoms of RSV infection. Our study is also the first to successfully detect nucleotide release in the BAL fluid of mice, and to demonstrate that such release is increased after paramyxoviral infection of the respiratory tract. Some of the results of these studies have been previously reported in the form of abstracts (5, 6).

METHODS

Preparation of Viral Inocula and Infection of Mice

Preparation of viral stocks and intranasal infection of 8- to 12-wk-old (20–25 g) pathogen-free BALB/c mice of either sex with RSV strain A2 (10^6 plaque-forming units in 100 μl) were performed (4). Data for each experimental group were derived from a minimum of two independent infections. All mouse procedures were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham (Birmingham, AL).

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Mean Peripheral Blood Oxygen Saturation

Peripheral blood oxygen saturation was measured in conscious mice (7), using a Preemie OxyTip sensor connected to a TuffSat pulse oximeter (GE Medical Datex-Ohmeda, Inc., Madison, WI). Because of the rapid pulse rate of mice, oximetry values are presented as mean hemoglobin O_2 saturation (Sm_{O_2}) values from arterial and venous blood, and thus appear low relative to true arterial saturation values.

NPD

The potential difference across the nares of anesthetized mice (with the tail as reference) was recorded during perfusion of the nasal epithelium with lactated Ringer's solution (8). In some experiments, current pulses of ± 60 nA were applied across the epithelium by a 12-V battery in series with a 200-MΩ resistor. Changes in NPD in response to the current pulses (proportional to nasal transepithelial resistance, NR_{te}) were recorded (ΔNPD).

BAL

BAL fluid was collected (4), using 1 ml of sterile normal saline for cytokine ELISAs and cell counts, or 0.3 ml of sterile saline for nucleotide assays. Lavagates were centrifuged to remove cells and supernatants were stored at –80°C.

Nucleotide Measurement

Endogenous nucleotidases in BAL fluid were heat denatured (100°C, 3 min) and UTP/ATP content was measured in uridine diphosphate–glucose pyrophosphorylase and luciferin–luciferase assays, respectively (9).

Heme Measurement

BAL heme content was measured by Drabkin's cyanmethemoglobin assay (10).

Systemic Inhibition of *De Novo* Pyrimidine and Purine Synthesis

Leflunomide (35 mg/kg per mouse in 300 μl of distilled water containing 1% methylcellulose) was administered to conscious mice once daily by oral gavage for 8 d before infection, and then throughout the infection period (11). Vehicle controls were gavaged with an equivalent volume of 1% methylcellulose in distilled water. Uridine (1 g/kg in 100 μl of 0.9% NaCl [11]) or an equal volume of saline was administered to conscious mice by intraperitoneal injection every 12 h for 8 d before infection, and then throughout the infection period. 6-MP (35 mg/kg in 100 μl of 1 N NaOH, adjusted to pH 7.9 with 2 M Na_2HPO_4 [12]) was administered by intraperitoneal injection every 24 h, for 5 d before infection, and then throughout the infection period. All chemicals were from Sigma (St. Louis, MO).

Serum Chemistry

Serum was analyzed with a VetScan chemistry system (Abaxis, Inc., Union City, CA), using the Comprehensive Diagnostic Profile reagent rotor, in accordance with the manufacturer's instructions.

Other Methods

All other methods were performed as previously described (4, 13, 14).

Statistical Analyses

Descriptive statistics were calculated with InStat software (GraphPad, San Diego, CA). Differences between group means were analyzed by analysis of variance or Student *t* test (paired for conscious Sm_{O_2} studies only), with Tukey-Kramer multiple comparison post tests for analysis of variance. All data values are presented as means ± SEM.

RESULTS

Effect of RSV Infection and Leflunomide Treatment on BAL Nucleotide Levels

Previously, we demonstrated that intranasal infection of BALB/c mice with RSV results in a UTP-mediated decrease in AFC on Day 2, but not on Day 6, despite continued viral replication (4). In the current study, we found that BAL fluid from uninfected or mock-infected mice contained equivalent levels of ATP and UTP (Table 1). RSV infection resulted in a doubling of UTP and ATP levels on Day 2, but no change in nucleotide levels on Day 6, suggesting that the normalization of AFC on Day 6 results from a cessation of increased UTP release. Because lysis of contaminating erythrocytes in BAL fluid samples (which might be present at higher levels in infected mice as a result of lung inflammation) could result in significant release of nucleotides (particularly ATP), we measured BAL heme content. However, the heme content of BAL fluid samples on Day 2 (7.3 ± 1.4 μM) was not elevated compared with uninfected mice (7.3 ± 0.7 μM), demonstrating that elevated BAL nucleotide levels after RSV are not a consequence of contamination with lysed erythrocytes.

To directly confirm the causative relationship between increased UTP release and reduced AFC, we treated mice with leflunomide, an inhibitor of the *de novo* synthesis of pyrimidines. Leflunomide treatment to Day 2 after infection reduced BAL UTP and ATP content to levels below those in untreated, uninfected mice (Table 1). Uridine treatment, which stimulates pyrimidine salvage, resulted in elevated BAL UTP, but not ATP, on Day 2, whereas concomitant uridine and leflunomide treatment significantly increased BAL nucleotide content over that in untreated, RSV-infected mice, with a more pronounced effect on UTP than ATP. Interestingly, leflunomide treatment of normal mice was also associated with elevated BAL UTP levels, suggesting a possible compensatory activation of pyrimidine salvage in response to the block in *de novo* pyrimidine synthesis.

Effect of Nucleotide Synthesis Inhibition on Murine Metabolism

Leflunomide pretreatment was well tolerated by mice, and had no effect on body weight before infection. Mild hepatomegaly and mild, microvesicular, centrilobular hepatic fatty change was observed on Day 2 (data not shown). However, similar fatty change was also evident in liver tissue from methylcellulose-treated mice, suggesting that it might be a consequence of stress associated with repeated gavaging. Fatty change was somewhat more severe in leflunomide-treated mice killed on Day 8. Leflunomide therapy resulted in mild (< 50%) elevations of serum alkaline phosphatase and amylase on Day 2 and Day 8, consistent with mild liver dysfunction.

In contrast, 6-MP treatment to Day 2 appeared to be highly toxic, resulting in severe weight loss, and some deaths, both before and during the RSV infection period (overall mortality

TABLE 1. EFFECT OF RESPIRATORY SYNCYTIAL VIRUS INFECTION AND LEFLUNOMIDE TREATMENT ON BRONCHOALVEOLAR LAVAGE FLUID NUCLEOTIDE LEVELS

	n*	ATP†	UTP†
Uninfected	11	16 ± 2	16 ± 4
Mock infected	6	13 ± 4	10 ± 4
Uninfected, leflunomide treated	8	54 ± 9‡	61 ± 7‡
Day 2	14	38 ± 7‡	32 ± 4§
Day 6	9	17 ± 2	11 ± 4
Day 2, leflunomide treated	9	6 ± 1§	5 ± 2‡
Day 2, uridine treated	8	32 ± 4§	54 ± 9§
Day 2, leflunomide and uridine treated	7	69 ± 29	95 ± 29§

Definition of abbreviations: ATP = adenosine triphosphate; UTP = uridine triphosphate.

* Number of mice per group in which nucleotide levels were evaluated.

† Mean nucleotide concentration in bronchoalveolar lavage fluid ± SEM (nmol/L).

‡ $p < 0.0005$, compared with uninfected mice.

§ $p < 0.005$, compared with uninfected mice.

rate, 23%). Marked hepatomegaly and gross yellowing of liver tissue were observed on Day 2 in 6-MP–treated mice, reflected histologically by severe centrilobular fatty hepatic degeneration, neutrophil infiltration, and formation of scattered areas of frank coagulative necrosis, some involving entire liver lobules (data not shown). 6-MP therapy resulted in hypoproteinemia, hypoglycemia, significant elevations of serum amylase (80%), and a 16-fold increase in serum alanine aminotransferase on Day 2, indicative of severe hepatic degeneration. No other abnormalities of serum chemistry were detected in mice treated with either agent.

Effect of Nucleotide Synthesis Inhibition on RSV-mediated Inhibition of AFC

In previous studies, we found that AFC was significantly depressed (by 43% from mock-infected values) on Day 2, and that the remaining AFC was amiloride insensitive (4). Leflunomide pretreatment of RSV-infected mice blocked RSV-induced inhibition of AFC on Day 2 (Table 2). This effect was not mimicked by gavage with methylcellulose alone, and was reversed by concomitant uridine treatment. Uridine or saline vehicle treatment alone had no effect on AFC in RSV-infected mice. Leflunomide treatment also resulted in restoration of normal amiloride sensitivity to AFC: amiloride reduced AFC by 57% in leflunomide-treated mice on Day 2, and by 61% in uninfected mice, but had no significant effect on the remaining AFC in untreated mice on Day 2 (4). Finally, treatment of uninfected mice with leflunomide or uridine also resulted in significant AFC inhibition. This finding indicates that elevation of BAL nucleotides results in impairment of AFC, irrespective of the underlying cause: leflunomide therapy in normal mice or RSV infection for 2 d.

In contrast to the beneficial effect of leflunomide therapy, but consistent with our previous observations that ATP degradation has no effect on AFC (4), a similar regimen of systemic pretreatment with the *de novo* purine synthesis inhibitor 6-MP had no stimulatory effect on AFC on Day 2 (Table 2).

Effect of Nucleotide Synthesis Inhibition on Lung Water Content

Systemic therapy with leflunomide restored normal lung wet:dry weight ratios on Day 2, whereas concomitant uridine treatment prevented this effect (Figure 1). However, systemic therapy with 6-MP, which had no beneficial effect on AFC on Day 2, did not alter lung wet:dry weight ratios on Day 2. This finding further strengthens the correlation between UTP release, impaired AFC, and increased lung water content that we previously described in RSV-infected mice (4).

Effect of RSV Infection and Leflunomide Treatment on Lung Histopathology

Previously, we reported that RSV infection did not result in epithelial cell death or sloughing of epithelium at any time (4). Leflunomide treatment to Day 2 had no effect on pulmonary pathology either on Day 2 or Day 8 (Figures 2a–2c). However, continued leflunomide treatment to Day 8 did result in persistence of both pronounced lymphoid infiltration around major vessels, bronchi, and bronchioles, and parenchymal lymphoblast infiltration (Figure 2d).

Effect of Nucleotide Synthesis Inhibition on BAL Proinflammatory Cytokines

Leflunomide is used clinically as an immunosuppressive agent. To verify the efficacy of our treatment regimen, we analyzed its effect on BAL proinflammatory cytokines. Small amounts of interleukin 1β (IL-1β) and keratinocyte cytokine (KC) (the murine homolog of human IL-8) were detected in BAL fluid from mock-infected mice (Table 3). Significant amounts of IL-1β, KC, and tumor necrosis factor α (TNF-α) were present in BAL fluid on Day 2, but levels declined on Days 4 through 8. IFN-γ was found in significant quantities in BAL fluid only on Day 6 and Day 8. Neither IL-4 nor IL-10 was detectable at any point in time (data not shown).

Leflunomide therapy significantly reduced levels of IFN-α, IL-1β, KC, and TNF-α in BAL fluid on Day 2 (Table 3). Concomitant uridine administration had no effect on BAL cytokines, other than to increase IFN-α. 6-MP therapy resulted in a significant decline in BAL IFN-α, IL-1β, KC, and TNF-α levels comparable to that caused by leflunomide therapy (Table 3), but as noted above, had no effect on AFC.

Effect of Nucleotide Synthesis Inhibition on RSV Replication in Mouse Lungs

Leflunomide has been shown to prevent replication of HIV-1 (15) and herpesviruses (16, 17) *in vitro*. Because our previous

TABLE 2. EFFECT OF NUCLEOTIDE SYNTHESIS INHIBITION ON RESPIRATORY SYNCYTIAL VIRUS–MEDIATED INHIBITION OF ALVEOLAR FLUID CLEARANCE ON DAY 2

	n†	AFC‡
Uninfected	7	34.89 ± 2.49§
Uninfected + 1.5 mM amiloride in instillate*	7	14.65 ± 1.59‖
Uninfected, leflunomide treated	17	29.98 ± 1.5¶
Uninfected, uridine treated	8	18.18 ± 2.67
Day 2*	25	22.01 ± 1.04
Day 2 + 1.5 mM amiloride in instillate*	7	22.82 ± 1.92
Day 2, methylcellulose treated	11	22.89 ± 1.27
Day 2, leflunomide treated	14	34.52 ± 2.1§
Day 2, leflunomide treated, + 1.5 mM amiloride in instillate	11	14.92 ± 2.3‖
Day 2, saline treated	10	19.55 ± 1.4
Day 2, uridine treated	19	22.89 ± 2.22
Day 2, leflunomide and uridine treated	10	21.9 ± 2.69
Day 2, 6-mercaptopurine treated	11	16.52 ± 2.51

Definition of abbreviation: AFC = alveolar fluid clearance.
* Dataset includes previously published data (4).
† Number of mice in which AFC was evaluated.
‡ Mean percentage of AFC ± SEM.
§ $p < 0.0005$, compared with AFC on Day 2.
‖ $p < 0.0005$, compared with AFC on Day 2 + 1.5 mM amiloride in instillate.
¶ $p < 0.005$, compared with AFC on Day 2.

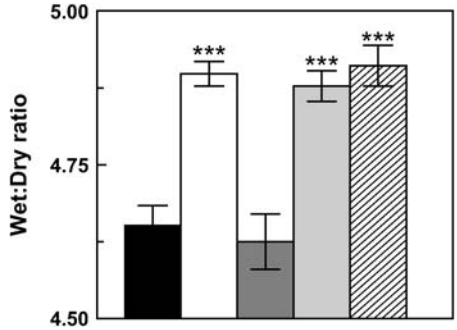

Figure 1. Effect of nucleotide synthesis inhibition on lung water content after respiratory syncytial virus (RSV) infection. Shown are the effects of leflunomide, uridine, and 6-mercaptopurine (6-MP) treatment on lung water content on Day 2 (n = 7 or 8 for all groups, except n = 15 for 6-MP–treated mice). Lung water content was measured as the wet:dry weight ratio. *Black bar*, mock; *white bar*, untreated; *dark gray bar*, leflunomide; *light gray bar*, leflunomide + uridine; *hatched bar*, 6-MP. *** $p < 0.0005$, compared with wet:dry weight ratio in uninfected mice.

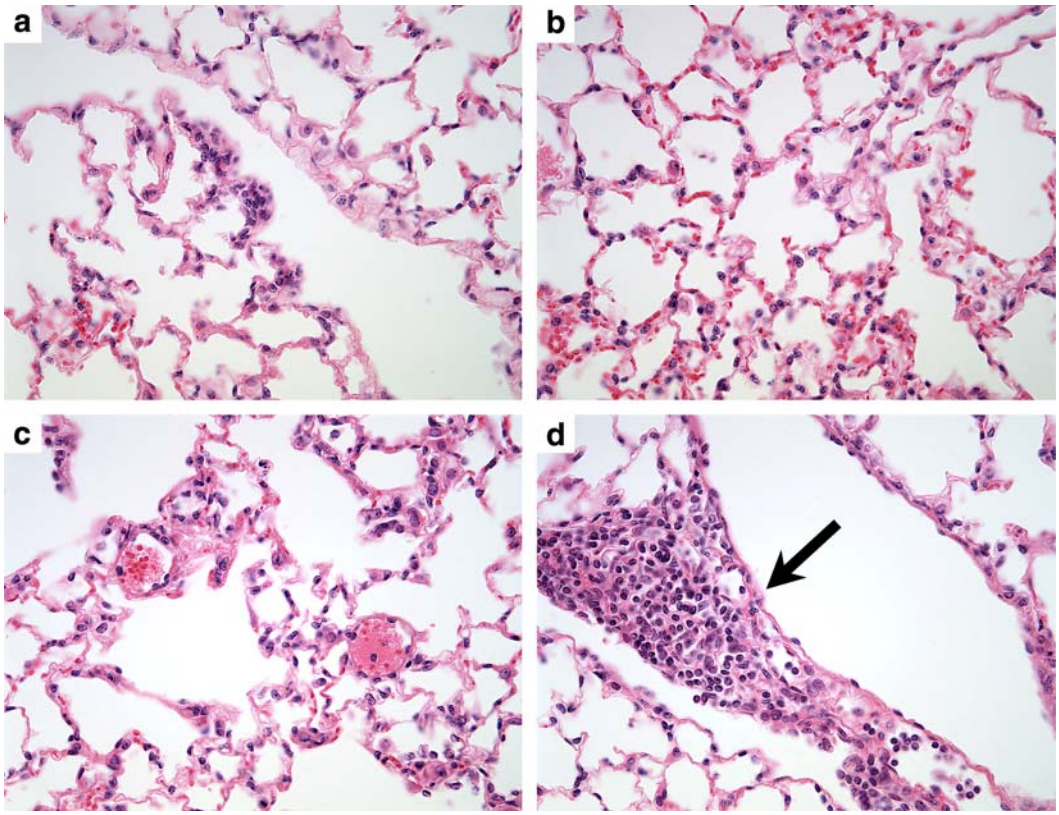

Figure 2. Effect of leflunomide treatment on lung histopathology after RSV infection. (*a*) Day 2, leflunomide-treated mice. (*b*) Day 8, untreated mice. (*c*) Day 8, mice treated with leflunomide to Day 2. (*d*) Day 8, mice treated with leflunomide to Day 8; *arrow* denotes pronounced lymphoid infiltrate around bronchus. Representative images from hematoxylin- and eosin-stained sections of formalin-fixed, paraffin-embedded tissues (n = 8/group). Original magnification, ×40 for all images.

studies had demonstrated that the inhibitory effect of RSV on AFC requires active viral replication (4), it was possible that the beneficial effects of leflunomide might simply be a consequence of inhibition of replication by this drug. However, RSV replication on Day 2 was unaffected by leflunomide, uridine, or 6-MP treatment (Figure 3a).

Replication of RSV declines to undetectable levels on Day 8 (4, 18). Because this decline may be due to an influx of immune cells into the lung (19), and because leflunomide, being an immunosuppressive agent, might prevent this immune response, we evaluated RSV replication on Day 8 in mice treated with leflunomide. When leflunomide treatment was continued throughout the 8-d infection period, RSV replication persisted at high levels on Day 8 (Figure 3b). However, when leflunomide treatment was discontinued after Day 2, RSV replication was only minimally increased on Day 8. A similar experiment using 6-MP unfortunately proved impossible: treatment for such a prolonged period resulted in extremely high mortality levels among the mice (more than 90% by Day 6), and so had to be discontinued. However, our findings with leflunomide support the notion that

TABLE 3. EFFECT OF RESPIRATORY SYNCYTIAL VIRUS INFECTION AND NUCLEOTIDE SYNTHESIS INHIBITION ON BRONCHOALVEOLAR LAVAGE FLUID PROINFLAMMATORY CYTOKINES

	n[§]	IFN-α[‖]	IFN-γ[‖]	IL-1β[‖]	KC[‖]	TNF-α[‖]
Mock	8	0[¶]	0	10 ± 6[¶]	80 ± 21[¶]	0[¶]
Day 2	13	151 ± 12	2 ± 1	136 ± 24	913 ± 36	81 ± 16
Day 4	8	ND	30 ± 16	6 ± 1[¶]	106 ± 27[¶]	0[¶]
Day 6	8	ND	912 ± 116[¶]	20 ± 3[¶]	72 ± 9[¶]	1 ± 1[¶]
Day 8	6	ND	195 ± 25[¶]	8 ± 2[¶]	88 ± 16[¶]	0[¶]
Day 2, LEF*	12	72 ± 12[¶]	ND	20 ± 8[¶]	425 ± 58[¶]	0[¶]
Day 2, LEF + U[†]	10	246 ± 68	ND	20 ± 6[¶]	334 ± 62[¶]	0[¶]
Day 2, 6-MP[‡]	8	105 ± 14**	ND	9 ± 3[¶]	329 ± 63[¶]	0[¶]

Definition of abbreviations: 6-MP = 6-mercaptopurine; KC = keratinocyte cytokine (murine homolog of human interleukin 8); LEF = leflunomide; ND = not done; TNF-α = tumor necrosis factor α; U = uridine.

*Leflunomide-treated mice.

[†] Leflunomide- and uridine-treated mice.

[‡] 6-MP–treated mice.

[§] Number of mice in which bronchoalveolar lavage (BAL) cytokine levels were measured.

[‖] Mean cytokine concentration in BAL fluid ± SEM (pg/ml).

[¶] $p < 0.0005$, compared with cytokine concentration on Day 2.

** $p < 0.05$, compared with cytokine concentration on Day 2.

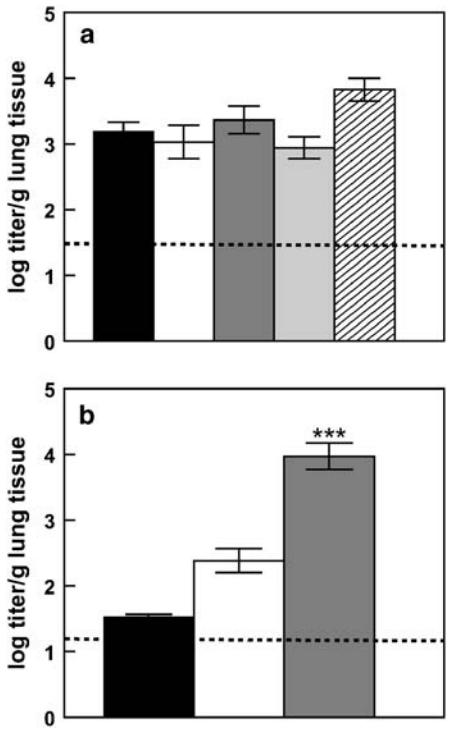

Figure 3. Effect of nucleotide synthesis inhibition on RSV replication in mouse lungs. (*a*) Effects of leflunomide, uridine, and 6-MP treatment on virus replication on Day 2 (n = 6 for untreated, uridine-treated, and leflunomide- and uridine-treated mice; n = 12 for leflunomide-treated mice and 6-MP–treated mice). *Black bar*, untreated; *white bar*, leflunomide; *dark gray bar*, uridine; *light gray bar*, leflunomide + uridine; *hatched bar*, 6-MP. (*b*) Effects of cessation of leflunomide treatment on Day 2 versus continued leflunomide treatment to Day 8 on virus replication on Day 8 (n = 6 for untreated mice; n = 12 for mice treated with leflunomide to Day 8; n = 6 for mice treated with leflunomide to Day 2). *Black bar,* untreated; *white bar,* leflunomide-treated to Day 2; *gray bar,* leflunomide-treated to Day 8. *Dashed lines* indicate limits of detection of assay. ***$p < 0.0005$, compared with Day 8 viral titer in untreated mice.

an intact inflammatory and immune response is essential to RSV clearance from the lung.

Effect of Leflunomide Treatment on BAL and Peripheral Blood Cell Counts

Infection with RSV for 8 d resulted in a significant increase in BAL total cell count and mononuclear cells (alveolar macrophages and lymphocytes), compared with uninfected or mock-infected mice (Figures 4a–4d). When leflunomide therapy was continued to Day 8 after infection, BAL total cell, lymphocyte, and neutrophil counts were significantly reduced, as compared with untreated, infected mice, but peripheral blood lymphocyte and neutrophil counts were elevated (data not shown). Interestingly, when leflunomide treatment was discontinued on Day 2, BAL total cell and neutrophil counts on Day 8 were normal, although BAL lymphocyte counts remained low and peripheral blood neutrophil counts remained elevated. Leflunomide treatment had no effect on BAL macrophage or blood monocyte counts at any time point, and treatment of normal mice resulted in only mild lymphopenia and neutrophilia (data not shown).

Effect of RSV Infection and Leflunomide Treatment on Peripheral Blood Oxygenation

Impairment of AFC on Day 2 was temporally associated with a small but significant reduction in peripheral blood Sm_{O_2} in conscious RSV-infected mice, compared with matched preinfection Sm_{O_2} values (Figure 5a). No decline in Sm_{O_2} was found at other times after infection. Smee and coworkers (7) reported Sm_{O_2} values (85%) for mock-infected mice similar to those reported in the current study. Leflunomide therapy had no detrimental effect on preinfection Sm_{O_2} levels (data not shown) and prevented the decline in matched Sm_{O_2} readings seen in untreated, RSV-infected, conscious mice on Day 2 (mean Sm_{O_2} on Day 2, 84 ± 1%; n = 26).

We have shown previously that normal mice (with intact AFC) are normoxic even at the end of the 30-min AFC period (20). However, we found that the inability to clear alveolar fluid normally on Day 2 after RSV infection was associated with significant depression of Sm_{O_2} at the end of the 30-min AFC period (Figure 5b). This depression in Sm_{O_2} was prevented by leflunomide treatment, but was restored by concomitant uridine treatment. To confirm these findings, we measured Sm_{O_2} in uridine-treated, uninfected mice: the depressed AFC in these animals was also associated with a significant and comparable drop in Sm_{O_2} at the end of the AFC assay period. There was no difference in duration of anesthesia for the AFC procedure between any groups (data not shown). Taken together, these findings support our hypothesis that defective AFC contributes to development of hypoxemia in RSV disease.

Effects of RSV Infection and Leflunomide Treatment on NPD

NPD values in normal BALB/c mice in our study were comparable to those previously reported for C57BL/6 mice (8, 21), and were not altered by infection with RSV for 2 d. However, NPD was significantly reduced on Day 4 and Day 8 (Figure 6a).

To estimate NR_{te} after RSV infection, we measured the change in NPD (ΔNPD) elicited by applying a ± 60-nA pulse to the nasal epithelium. ΔNPD was significantly greater on Days 4 and 8 than in mock-infected controls (Figure 6b), indicating that NR_{te} increases after RSV infection, presumably as a result of inhibition of active ion transport by the virus. ΔNPD was also significantly higher in normal mice after addition of 100 μM amiloride to the perfusate, indicating that an increase in ΔNPD truly reflects a reduction in current flow. Finally, treatment with leflunomide throughout the infection period completely prevented RSV-induced declines in NPD (Figure 6c) and NR_{te} (Figure 6d).

Effect of Anion Channel Blockade on RSV-mediated Inhibition of AFC

VRAC-like channels have been proposed as candidates for nucleotide release, and have been shown to mediate ATP release from respiratory epithelial cells *in vitro* (22). Although no specific VRAC inhibitors exist, these channels can be blocked by a variety of pharmacologic agents that, individually, have pleiotropic effects on other cellular systems (reviewed in Nilius and coworkers [23]). RSV-mediated inhibition of AFC on Day 2 was blocked by addition to the AFC instillate of each of several structurally unrelated VRAC inhibitors, but not by inhibitors of cystic fibrosis transmembrane regulator (24) or Ca^{2+}-activated Cl^- channel (25) activity (Table 4). Although studies using such nonspecific inhibitors must be interpreted with due caution, the differential blockade of RSV-mediated suppression of AFC by a broad spectrum of VRAC inhibitors provides strong evidence that VRAC inhibition prevents the detrimental effects of RSV on AFC.

Addition of 500 nM UTP to the AFC instillate completely reversed the effect of VRAC inhibition (Table 4), indicating that reduced AFC in RSV infection is not a consequence of increased Cl^- secretion through VRACs (if it were, UTP would have no effect on AFC in the presence of a VRAC inhibitor) but is instead a result of UTP release through these channels.

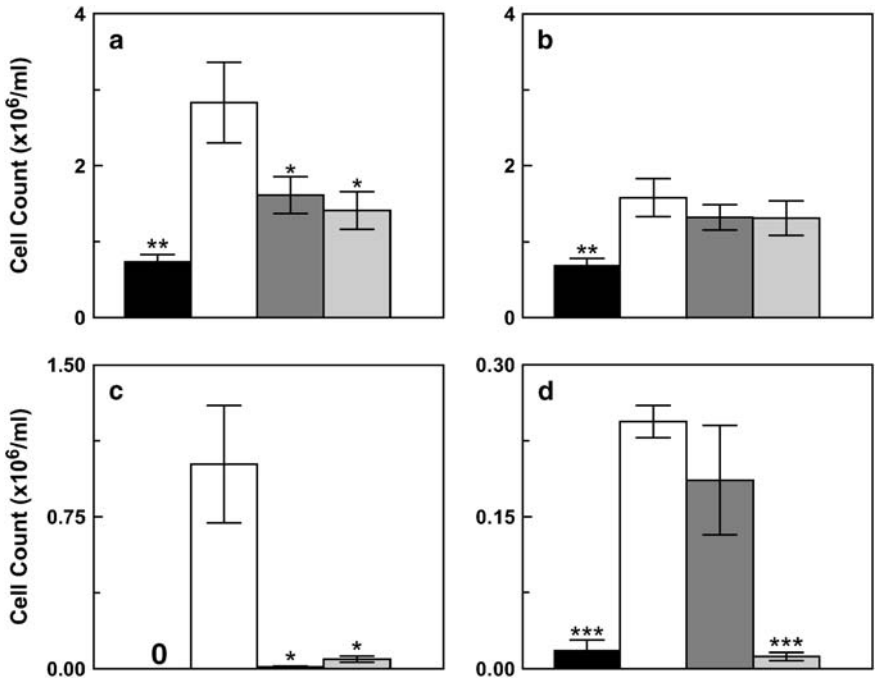

Figure 4. Effect of leflunomide treatment on bronchoalveolar lavage (BAL) cell counts on Day 8 after RSV infection. (*a*) Total cell counts. (*b*) Alveolar macrophage counts. (*c*) Lymphocyte counts. (*d*) Neutrophil counts. (n = 9 for uninfected mice and for untreated, RSV-infected mice; n = 6 for mice treated with leflunomide to Day 2 or to Day 8). *Black bars*, uninfected; *white bars*, untreated; *dark-gray bars*, leflunomide to Day 2; *light-gray bars*, leflunomide to Day 8. *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, compared with untreated, RSV-infected mice on Day 8.

DISCUSSION

Previously, we demonstrated that RSV, but not mock infection, inhibits AFC in BALB/c mice, and inferred that inhibition of AFC by RSV is mediated by UTP, released into the airspace lining fluid and acting in a paracrine fashion on epithelial P2YRs to inhibit active Na^+ transport (4). Subsequently, we have been able to perform the technically challenging task of measuring nucleotide triphosphate levels in mouse BAL fluid samples, which has not previously been reported. This has allowed us to confirm the temporal and functional association between elevated BAL nucleotide levels and impaired AFC.

Effect of RSV Infection and Leflunomide Treatment on BAL Nucleotides

Levels of ATP and UTP in BAL fluid from normal mice appear to be extremely low (10–15 nM). However, BAL fluid samples are by their nature diluted and local nucleotide concentrations at the epithelial surface may be considerably higher. There is also growing evidence that within the airspace lining fluid, a network of ectonucleotidases and ectonucleoside diphosphokinases may catalyze breakdown and interconversion of released nucleotides to locally modulate P2YR ligand profiles (reviewed in Reference 26). Indeed, previous studies have shown that nucleotide measurements may underestimate true nucleotide levels by up to 10-fold, because almost 90% of ATP is rapidly hydrolyzed on release from cells (27, 28). Donaldson and coworkers (29) reported steady state UTP concentrations in normal human nasal aspirates to be about 40 nM, although they found significantly higher ATP levels (470 nM). It is unclear whether this discrepancy reflects differences in sampling site, species, sampling technique, or dilution factor.

Infection with RSV results in only a 20 nM increase in UTP release on Day 2, but this is sufficient to inhibit AFC by more than 40%. This finding supports our previous inference that UTP levels are elevated after RSV infection, but that this elevation need not be large (4). Surprisingly, given that our previous studies had shown that RSV-mediated inhibition of AFC was a consequence of P2YR activation, but not by ATP (4), RSV infection increased BAL ATP levels. Our findings are consistent with a previous study by Lazarowski and coworkers (30), which demonstrated parallel release of UTP and ATP from human astrocytoma cells in response to mechanical stimulation. Mouse lung expresses at least two subtypes of P2YR: $P2Y_2R$ and $P2Y_6R$ (31). Because ATP and UTP are equivalent ligands for $P2Y_2R$, the inhibitory effect of UTP on AFC in RSV infection may instead be mediated by the UTP metabolite UDP, acting via $P2Y_6R$, for which it is the sole ligand. Unfortunately, no subtype-specific P2YR antagonists currently exist.

Given the normal respiratory epithelial intracellular ATP level ($\sim$ 20 nmol/10^6 cells [22]), a 20 nM increase in ATP content in 300 μl of BAL fluid is equivalent to the ATP content of only 300 cells, and so could result from a low level of viral cytopathicity that is undetectable histologically. Even if, as in T cells (32), intracellular UTP levels are 10-fold lower, a similar increase in BAL UTP content would require lysis of only 3,000 cells. However, the blocking effect of VRAC inhibitors on RSV-mediated AFC inhibition indicates that BAL nucleotide levels are elevated because of increased release from cells, rather than cell lysis. Moreover, lysed cells are unlikely to be a significant source of BAL nucleotides, because BAL nucleotide levels are increased on Day 2, when no lactate dehydrogenase is detectable in BAL fluid (4), but are not increased on Day 6, when BAL lactate dehydrogenase levels are elevated.

It remains unclear why RSV replication triggers UTP release on Day 2, but not on Day 6, but this difference may reflect temporal variations in either viral gene product expression or cell tropism. Alternatively, cessation of UTP release may be secondary to the onset of immune-mediated viral clearance from the lungs, and a concomitant alteration in the cytokine milieu. However, although nucleotides can modulate cytokine release by inflammatory cells (reviewed in Reference 33), there is no evidence that cytokines can modulate nucleotide release (26). Nevertheless, there is a clear temporal association in our studies between elevated BAL nucleotide levels and impaired AFC after RSV infection. An *in vitro* study demonstrated that another paramyxovirus, Sendai virus, also inhibits active Na^+ transport

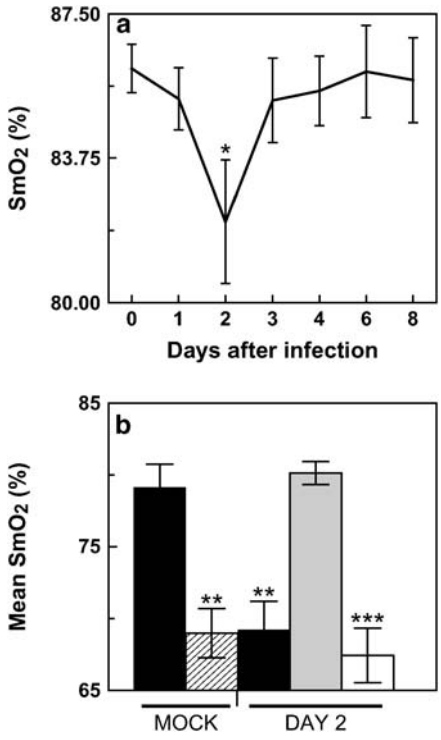

Figure 5. Effect of RSV infection and leflunomide treatment on peripheral oxygenation. (*a*) Time course of effect of RSV infection on Sm_{O_2} in conscious BALB/c mice (n = 10–36/d, paired data). *$p < 0.05$, compared with preinfection values. (*b*) Effect of leflunomide and uridine treatment on peripheral Sm_{O_2} in anesthetized, uninfected mice, or on mice infected with RSV for 2 d, at the conclusion of the 30-min AFC assay (n = 8 or 9/group). *Black bars*, untreated, *hatched bar*, uridine; *gray bar*, leflunomide; *white bar*, leflunomide + uridine. **$p < 0.005$, ***$p < 0.005$, compared with uninfected, untreated mice.

and stimulates Cl^- secretion by an ATP/P2YR-mediated mechanism (34). However, nucleotide release was not formally demonstrated, and the kinetics of the effect differ significantly from our studies.

Inhibition of *de novo* pyrimidine synthesis with leflunomide had a suppressive effect on BAL UTP and ATP levels. *De novo* purine and pyrimidine synthesis pathways are usually concordantly regulated (reviewed in Reference 35), and these findings are consistent with that observation. Uridine treatment not only reversed the decrease in BAL UTP and ATP levels induced by leflunomide therapy during RSV infection, but also resulted in a significant increase in both UTP and ATP levels above those in infected, untreated mice. Interestingly, BAL nucleotide levels were also elevated in leflunomide-treated normal mice, suggesting that inhibition of *de novo* UTP synthesis normally results in a compensatory increase in salvage pathway synthesis. Taken together, these data indicate that UTP release is limited by UTP availability in cells: the pool of UTP that is normally available for release may be small, and is primarily derived from *de novo* pyrimidine synthesis, but UTP release can be significantly increased by exogenous or compensatory activation of the pyrimidine salvage pathway. However, it appears that actively replicating RSV depletes the free nucleotide pool, so that salvage pathway activity can no longer compensate for the inhibitory effects of leflunomide on *de novo* pyrimidine synthesis, and UTP release into the BAL fluid therefore effectively ceases.

Effect of *De Novo* Nucleotide Synthesis Inhibition on AFC

To directly confirm the causative relationship between increased nucleotide release and reduced AFC, we treated mice with inhibitors of *de novo* synthesis of pyrimidines (leflunomide, which inhibits dihydroorotate dehydrogenase [36]) or purines (6-MP, which inhibits inosine monophosphate dehydrogenase). Leflunomide has additional nonspecific inhibitory effects on tyrosine kinases, but these are not reversible on administration of exogenous uridine, which allows pyrimidine synthesis via a salvage pathway, bypassing the leflunomide block. To our knowledge, leflunomide and 6-MP have previously been used in mice solely as immunosuppressive agents; this is the first occasion on which they have been used to directly modulate epithelial cell function. Despite differential toxicities, both agents produced a similar degree of immune suppression in RSV-infected mice, as assessed by BAL proinflammatory cytokine and immune cell levels, confirming that therapy with both agents was successfully administered. However, unlike leflunomide, 6-MP had no beneficial effect on AFC on Day 2. Finally, treatment of uninfected mice with leflunomide also resulted in inhibition of AFC. However, whereas BAL nucleotide levels in uninfected, leflunomide-treated mice were comparable to, or slightly higher than, levels in untreated, RSV-infected mice on Day 2, the degree of AFC inhibition was significantly less. This finding suggests that, unlike in RSV-infected mice, AFC in normal mice may be regulated by multiple factors.

The finding that 6-MP induces a degree of immunosuppression comparable to that of leflunomide, but has no effect on AFC, argues that the beneficial effects of leflunomide pretreatment on AFC in RSV infection are independent of its immunosuppressive effects. Indeed, even without considering the absence of effect of 6-MP on AFC, there is little evidence that the decline in AFC on Day 2 is a consequence of the immune response to the virus. Although certain proinflammatory cytokines have been shown to modulate Na^+ transport *in vitro* or *in vivo* (37–40), analysis of BAL fluid from RSV-infected mice reveals no temporal correlation between pulmonary inflammation and inhibition of AFC. For example, whereas IFN-γ has been shown to inhibit Na^+ transport *in vitro* (38), no IFN-γ is detectable in BAL fluid on Day 2, when AFC is most depressed, and AFC values are normal on Day 6, when BAL IFN-γ levels are highest. Moreover, the effects of leflunomide on RSV-induced AFC inhibition, increased lung water content, and increased BAL UTP levels are all reversed by uridine, but, with the exception of IFN-α, its effects on BAL proinflammatory cytokines are not. Thus, the beneficial effect of leflunomide on AFC results from its inhibitory effect on *de novo* pyrimidine synthesis, whereas its immunosuppressive effect results chiefly from nonspecific inhibition of tyrosine kinases, which is consistent with previous studies (11, 41). IFN-α cannot be the mediator of impaired AFC, because BAL IFN-α is reduced in 6-MP–treated mice, despite persistence of impaired AFC on Day 2 in these animals.

Our previous studies had demonstrated that the inhibitory effect of RSV on AFC requires active viral replication (4), and the beneficial effect of leflunomide on AFC on Day 2 might therefore simply be a consequence of inhibition of RSV replication. This is clearly not the case; if anything, leflunomide treatment tends to prolong viral replication in mouse lungs. Nevertheless, prolonged viral replication on Day 8 was curtailed if leflunomide treatment was discontinued on Day 2, despite clear suppression of BAL lymphocyte responses, suggesting that components of the innate immune response may play a significant role in viral clearance. Our histopathologic and cell count findings support such a role: continuation of leflunomide treatment to Day 8 after infection resulted in an apparent impairment in

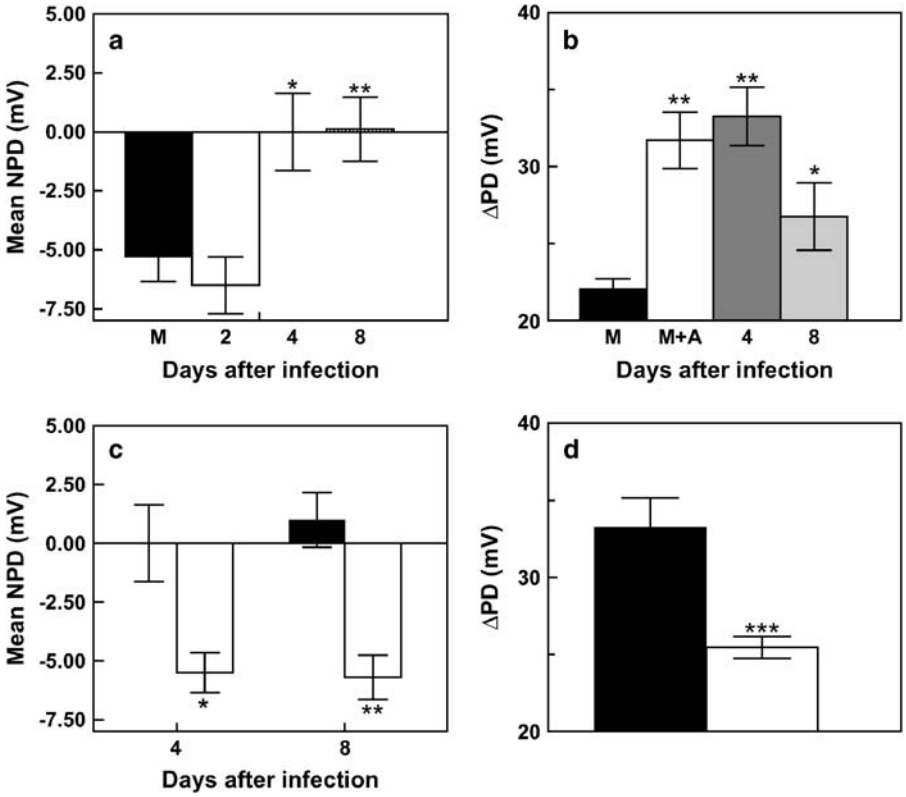

Figure 6. Effect of RSV infection and leflunomide treatment on nasal potential difference (NPD) in BALB/c mice. (*a*) Effect of RSV infection on NPD. M (*black bar*) = mock-infected mice; *white bar,* RSV-infected mice. (*b*) Effect of amiloride or RSV infection on change in NPD (ΔNPD) after application of ± 60-nA pulses to nasal epithelium. M (*black bar*) = mock-infected mice; M + A (*white bar*) = mock-infected mice, 100 μM amiloride in perfusate; 4, 8 = days after RSV infection (shading added for emphasis). (*c*) Effect of leflunomide treatment on NPD in RSV-infected mice. *Black bar,* vehicle; *white bars,* leflunomide. (*d*) Effect of leflunomide treatment on ΔNPD on Day 4 after RSV infection. *Black bars*, untreated; *white bars*, leflunomide. *$p < 0.05$, **$p < 0.005$, compared with mock-infected animals on Day 4, ***$p < 0.0005$. n = 5–9 for all groups.

leukocyte recruitment to the airspaces (trapping of leukocytes around major blood vessels, with reduced BAL and elevated peripheral blood leukocyte counts), whereas cessation of leflunomide treatment on Day 2 resulted only in a reduction in BAL neutrophil levels and peripheral blood lymphocytosis. However, the mechanism underlying impaired leukocyte recruitment is currently unknown. Finally, and despite having a mechanism of action similar to that of ribavirin (both are inhibitors of inosine monophosphate dehydrogenase), 6-MP treatment also has no inhibitory effect on RSV replication.

TABLE 4. EFFECT OF ANION CHANNEL INHIBITORS ON RESPIRATORY SYNCYTIAL VIRUS–MEDIATED INHIBITION OF ALVEOLAR FLUID CLEARANCE ON DAY 2

Inhibitor	Target	Concentration (μ*M*)	n†	AFC‡
None*	—	—	25	22.01 ± 1.04
Fluoxetine	VRAC	10	16	34.54 ± 0.79§
Fluoxetine + UTP	VRAC	10/0.5	8	23.64 ± 2.42
Tamoxifen	VRAC	25	9	34.50 ± 0.94§
Clomiphene	VRAC	20	8	31.05 ± 2.65§
Verapamil	VRAC	10	6	33.04 ± 1.49‖
NPPB	VRAC	100	9	32.70 ± 2.18‖
R(+)-IAA 94	VRAC	100	5	34.25 ± 1.98§
Glibenclamide	CFTR	100	9	24.24 ± 4.24
Niflumic acid	CaCC	100	10	20.28 ± 1.53

Definition of abbreviations: AFC = alveolar fluid clearance; CaCC = Ca^{2+}-activated Cl^- channel; CFTR = cystic fibrosis transmembrane regulator; NPPB = 5-nitro-2-(3-phenylpropylamino) benzoic acid; *R*(+)-IAA 94 = *R*(+)-([6,7-dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-1-oxo-1*H*-inden-5-yl]-oxy) acetic acid 94; UTP = uridine triphosphate; VRAC = volume-regulated anion channel.

*Dataset includes previously published data (4).

† Number of mice in which AFC was evaluated.

‡ Mean percentage of AFC ± SEM.

§ $p < 0.0005$, compared with AFC on Day 2

‖ $p < 0.005$, compared with AFC on Day 2.

Effect of Leflunomide on Hypoxemia in RSV Infection

Hypoxemia is a primary indicator of disease in children with RSV (42). We previously hypothesized that hypoxemia could result from impaired AFC in RSV infection (4), and in this study we found that infection of mice with RSV resulted in mild hypoxemia in conscious mice on Day 2, when compared with matched preinfection Sm_{O_2} values. Although the reduction in Sm_{O_2} at this time point was small (3%), this may in reality reflect a significant reduction in arterial Po_2. Indeed, a comparable decline in Sm_{O_2} was reported in BALB/c mice infected with vaccinia virus on Day 5, just before the onset of mortality (7). Because RSV induces only mild pulmonary disease in the mouse, a greater change in Sm_{O_2} would have been surprising. Moreover, when infected mice were subjected to AFC studies and the resultant stress of additional fluid loading of the lungs, their impaired AFC capacity did lead to development of severe hypoxemia. Both the hypoxemia observed in conscious mice and that seen at the end of the AFC procedure could be prevented by leflunomide administration, suggesting that RSV-induced hypoxemia and pulmonary edema may be at least partially the result of impaired AFC. Although no decline in Sm_{O_2} was observed on Day 4, this may be a consequence of both the inherent variability and insensitivity in Sm_{O_2} measurements in conscious mice, and the relatively reduced level of hypoxemia that might be predicted to result from a smaller impairment of AFC at this time point.

Effect of Leflunomide on NPD in RSV-infected Mice

We found that NPD was essentially absent in RSV-infected mice on Day 4 and Day 8, but was normal on Day 2 when AFC was most impaired. The reason for this discrepancy in timing of ion transport abnormalities between the upper and lower respiratory tract is unclear, but may reflect differences in kinetics of viral replication between the two sites. Nevertheless, our finding of

altered NPD in response to RSV infection is consistent with previous studies. NPD values are abnormally low in normal adult volunteers after nasal instillation of rhinovirus (43), and in children with cystic fibrosis who have rhinitis (44, 45). These reductions in NPD were assumed to be a consequence of nonspecific inflammation of the nasal mucosa, resulting in a leaky epithelium and dissipation of the normal potential gradient via paracellular pathways (46, 47). However, our data indicate that NR_{te} does not decrease after RSV infection, indicating that reductions in NPD are a result of reduced Na^+ conductance across the nasal epithelium. Again, leflunomide treatment prevents the dysregulation of NPD and NR_{te} seen in untreated, RSV-infected mice, demonstrating that the beneficial effects of systemic leflunomide therapy extend throughout the respiratory tract.

RSV Triggers Nucleotide Release via VRACs

Several pathways have been proposed for nucleotide release from cells: exit via nucleotide or VRAC-like channels, facilitated diffusion, and exocytosis of nucleotide-filled granules (reviewed in Reference 48). We found that the inhibitory effects of RSV on AFC on Day 2 could be prevented by the addition of VRAC inhibitors to the AFC instillate, but not by blockers of other anion channels. We should note, however, that our findings provide no information regarding nucleotide release pathways in uninfected lung, and demonstrate only that VRACs are the pathway of nucleotide release during RSV infection. Moreover, we cannot exclude the possibility that, rather than being the release pathway, VRACs merely facilitate nucleotide release via another mechanism, or modulate UTP levels by altering synthesis or degradation mechanisms.

Conclusions

Taken together, our data suggest that activation of the UTP VRAC release pathway may be a component of an epithelial paracrine signaling system that is activated by RSV infection. In this model, release of UTP (and perhaps ATP) in response to RSV infection may be viewed as a double-edged sword for the host, resulting in impairment of AFC and perhaps hypoxemia, but also aiding clearance of the virus from the lung, by promoting surfactant release and mucociliary clearance (26). Indeed, Huang and coworkers (49) have proposed that local physical stimulation of airway surfaces, by activating apical nucleotide release and P2YR signaling, may trigger mechanisms that flush noxious stimuli away. We propose that viral infections promote a similar response. Moreover, UTP, by its effects on P2YR on immune cells (50, 51), may also stimulate the proinflammatory cytokine cascade that initiates viral clearance from the lungs. However, this inflammatory process may itself be responsible for lung injury: several studies have demonstrated that depletion of T cells leads to markedly attenuated illness and pulmonary pathology, despite viral persistence in the lungs for up to 4 wk after infection (52, 53).

In conclusion, our studies indicate that RSV infection results in increased nucleotide release into the airspace lining fluid, and that these nucleotides, by impairing AFC, ultimately engender significant physiologic impairment of the host. Moreover, we show that systemic or local therapy with inhibitors of dihydroorotate dehydrogenase or UTP release may be of therapeutic benefit in RSV disease, by promoting normal AFC and thereby helping to counter the development of hypoxemia. Although impaired AFC has been described after a variety of physical insults to the lung (reviewed in Reference 1), previous attempts to reverse such defects in AFC have focused on the use of β-adrenergic agonists to restore normal AFC (54). To our knowledge, we are the first to successfully apply an entirely different approach—that is, to directly block the mediator responsible for impaired AFC in the injured lung.

Conflict of Interest Statement: I.C.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. E.R.L. is a coinventor of a patent with the University of North Carolina. As part of the license agreement between the University of North Carolina and Inspire Pharmaceuticals, all coinventors received $300 in 2002 and $300 in 2004 in royalties and 1,900 stock shares from Inspire Pharmaceuticals. E.R.L. has received $300 in 2005 for speaking in a seminar at Inspire Pharmaceuticals in Durham, North Carolina. He also has been reimbursed by the Novartis Foundation for travel expenses for attending Novartis Foundation Symposium 276, in London, UK, in 2005. J.M.H.-D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.A.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. F.-P.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. X.Z. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. E.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. L.M.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. W.M.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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References

1. Sartori C, Matthay MA. Alveolar epithelial fluid transport in acute lung injury: new insights. *Eur Respir J* 2002;20:1299–1313.
2. Ware LB, Matthay MA. Alveolar fluid clearance is impaired in the majority of patients with acute lung injury and the acute respiratory distress syndrome. *Am J Respir Crit Care Med* 2001;163:1376–1383.
3. Black CP. Systematic review of the biology and medical management of respiratory syncytial virus infection. *Respir Care* 2003;48:209–233.
4. Davis IC, Sullender WM, Hickman-Davis JM, Lindsey JR, Matalon S. Nucleotide-mediated inhibition of alveolar fluid clearance in BALB/c mice after respiratory syncytial virus infection. *Am J Physiol Lung Cell Mol Physiol* 2004;286:L112–L120.
5. Davis IC, Sullender WM, Graves LM, Zhao X, Matalon S. Respiratory syncytial virus inhibits alveolar fluid clearance by inducing release of *de novo*–synthesized UTP [abstract 684.3]. *FASEB J* 2004;18.
6. Davis IC, Sullender WM, Graves LM, Zhao X, Matalon S. Pyrimidine biosynthesis and release mechanism in respiratory syncytial virus–induced inhibition of alveolar fluid clearance [abstract]. *Am J Respir Crit Care Med* 2004;169:A669.
7. Smee DF, Bailey KW, Wong MH, Sidwell RW. Effects of cidofovir on the pathogenesis of a lethal vaccinia virus respiratory infection in mice. *Antiviral Res* 2001;52:55–62.
8. Grubb BR, Vick RN, Boucher RC. Hyperabsorption of Na^+ and raised Ca^{2+}-mediated Cl^- secretion in nasal epithelia of CF mice. *Am J Physiol* 1994;266:C1478–C1483.
9. Lazarowski ER, Harden TK. Quantitation of extracellular UTP using a sensitive enzymatic assay. *Br J Pharmacol* 1999;127:1272–1278.
10. Drabkin DL. Heme binding and transport: a spectrophotometric study of plasma glycoglobulin hemochromogens. *Proc Natl Acad Sci USA* 1971;68:609–613.
11. Pinschewer DD, Ochsenbein AF, Fehr T, Zinkernagel RM. Leflunomide-mediated suppression of antiviral antibody and T cell responses: differential restoration by uridine. *Transplantation* 2001;72:712–719.
12. Krynetskaia NF, Brenner TL, Krynetski EY, Du W, Panetta JC, Ching-Hon P, Evans WE. Msh2 deficiency attenuates but does not abolish thiopurine hematopoietic toxicity in *msh2*$^{-/-}$ mice. *Mol Pharmacol* 2003; 64:456–465.
13. Hickman-Davis JM, Lindsey JR, Matalon S. Cyclophosphamide decreases nitrotyrosine formation and inhibits nitric oxide production by alveolar macrophages in mycoplasmosis. *Infect Immun* 2001;69: 6401–6410.
14. Hickman-Davis JM, Gibbs-Erwin J, Lindsey JR, Matalon S. Role of surfactant protein-A in nitric oxide production and mycoplasma killing in congenic C57BL/6 mice. *Am J Respir Cell Mol Biol* 2004;30:319–325.
15. Schlapfer E, Fischer M, Ott P, Speck RF. Anti-HIV-1 activity of leflunomide: a comparison with mycophenolic acid and hydroxyurea. *AIDS* 2003;17:1613–1620.
16. Knight DA, Hejmanowski AQ, Dierksheide JE, Williams JW, Chong AS, Waldman WJ. Inhibition of herpes simplex virus type 1 by the

experimental immunosuppressive agent leflunomide. *Transplantation* 2001;71:170–174.
17. Waldman WJ, Knight DA, Lurain NS, Miller DM, Sedmak DD, Williams JW, Chong AS. Novel mechanism of inhibition of cytomegalovirus by the experimental immunosuppressive agent leflunomide. *Transplantation* 1999;68:814–825.
18. Graham BS, Perkins MD, Wright PF, Karzon DT. Primary respiratory syncytial virus infection in mice. *J Med Virol* 1988;26:153–162.
19. Rutigliano JA, Johnson TR, Hollinger TN, Fischer JE, Aung S, Graham BS. Treatment with anti-LFA-1 delays the $CD8^+$ cytotoxic-T-lymphocyte response and viral clearance in mice with primary respiratory syncytial virus infection. *J Virol* 2004;78:3014–3023.
20. Hardiman KM, Lindsey JR, Matalon S. Modulation of active ion transport by nitric oxide in hyperoxia. *Am J Physiol Lung Cell Mol Physiol* 2001;281:L722–L731.
21. Hardiman KM, McNicholas-Bevensee CM, Fortenberry J, Myles CT, Malik B, Eaton DC, Matalon S. Regulation of amiloride-sensitive Na^+ transport by basal nitric oxide. *Am J Respir Cell Mol Biol* 2004;30:720–728.
22. Okada SF, O'Neal WK, Huang P, Nicholas RA, Ostrowski LE, Craigen WJ, Lazarowski ER, Boucher RC. Voltage-dependent anion channel-1 (VDAC-1) contributes to ATP release and cell volume regulation in murine cells. *J Gen Physiol* 2004;124:513–526.
23. Nilius B, Eggermont J, Droogmans G. The endothelial volume-regulated anion channel, VRAC. *Cell Physiol Biochem* 2000;10:313–320.
24. Sheppard DN, Robinson KA. Mechanism of glibenclamide inhibition of cystic fibrosis transmembrane conductance regulator Cl^- channels expressed in a murine cell line. *J Physiol* 1997;503:333–346.
25. White MM, Aylwin M. Niflumic and flufenamic acids are potent reversible blockers of Ca^{2+}-activated Cl^- channels in *Xenopus* oocytes. *Mol Pharmacol* 1990;37:720–724.
26. Lazarowski ER, Boucher RC, Harden TK. Mechanisms of release of nucleotides and integration of their action as P2X- and P2Y-receptor activating molecules. *Mol Pharmacol* 2003;64:785–795.
27. Joseph SM, Buchakjian MR, Dubyak GR. Colocalization of ATP release sites and ecto-ATPase activity at the extracellular surface of human astrocytes. *J Biol Chem* 2003;278:23331–23342.
28. Lazarowski ER, Tarran R, Grubb BR, Van Heusden CA, Okada S, Boucher RC. Nucleotide release provides a mechanism for airway surface liquid homeostasis. *J Biol Chem* 2004;279:36855–36864.
29. Donaldson SH, Lazarowski ER, Picher M, Knowles MR, Stutts MJ, Boucher RC. Basal nucleotide levels, release, and metabolism in normal and cystic fibrosis airways. *Mol Med* 2000;6:969–982.
30. Lazarowski ER, Homolya L, Boucher RC, Harden TK. Direct demonstration of mechanically induced release of cellular UTP and its implication for uridine nucleotide receptor activation. *J Biol Chem* 1997;272:24348–24354.
31. Cressman VL, Lazarowski E, Homolya L, Boucher RC, Koller BH, Grubb BR. Effect of loss of $P2Y_2$ receptor gene expression on nucleotide regulation of murine epithelial Cl^- transport. *J Biol Chem* 1999;274:26461–26468.
32. Fairbanks LD, Bofill M, Ruckemann K, Simmonds HA. Importance of ribonucleotide availability to proliferating T-lymphocytes from healthy humans: disproportionate expansion of pyrimidine pools and contrasting effects of *de novo* synthesis inhibitors. *J Biol Chem* 1995;270:29682–29689.
33. Di Virgilio F, Chiozzi P, Ferrari D, Falzoni S, Sanz JM, Morelli A, Torboli M, Bolognesi G, Baricordi OR. Nucleotide receptors: an emerging family of regulatory molecules in blood cells. *Blood* 2001;97:587–600.
34. Kunzelmann K, Konig J, Sun J, Markovich D, King NJ, Karupiah G, Young JA, Cook DI. Acute effects of parainfluenza virus on epithelial electrolyte transport. *J Biol Chem* 2004;279:48760–48766.
35. Huang M, Graves LM. *De novo* synthesis of pyrimidine nucleotides: emerging interfaces with signal transduction pathways. *Cell Mol Life Sci* 2003;60:321–336.
36. Davis JP, Cain GA, Pitts WJ, Magolda RL, Copeland RA. The immunosuppressive metabolite of leflunomide is a potent inhibitor of human dihydroorotate dehydrogenase. *Biochemistry* 1996;35:1270–1273.
37. Rezaiguia S, Garat C, Delclaux C, Meignan M, Fleury J, Legrand P, Matthay MA, Jayr C. Acute bacterial pneumonia in rats increases alveolar epithelial fluid clearance by a tumor necrosis factor-α-dependent mechanism. *J Clin Invest* 1997;99:325–335.
38. Galietta LJ, Folli C, Marchetti C, Romano L, Carpani D, Conese M, Zegarra-Moran O. Modification of transepithelial ion transport in human cultured bronchial epithelial cells by interferon-γ. *Am J Physiol Lung Cell Mol Physiol* 2000;278:L1186–L1194.
39. Fukuda N, Jayr C, Lazrak A, Wang Y, Lucas R, Matalon S, Matthay MA. Mechanisms of TNF-α stimulation of amiloride-sensitive sodium transport across alveolar epithelium. *Am J Physiol Lung Cell Mol Physiol* 2001;280:L1258–L1265.
40. Galietta LJ, Pagesy P, Folli C, Caci E, Romio L, Costes B, Nicolis E, Cabrini G, Goossens M, Ravazzolo R, *et al.* IL-4 is a potent modulator of ion transport in the human bronchial epithelium *in vitro. J Immunol* 2002;168:839–845.
41. Xu X, Shen J, Mall JW, Myers JA, Huang W, Blinder L, Saclarides TJ, Williams JW, Chong AS. *In vitro* and *in vivo* antitumor activity of a novel immunomodulatory drug, leflunomide: mechanisms of action. *Biochem Pharmacol* 1999;58:1405–1413.
42. Brooks AM, McBride JT, McConnochie KM, Aviram M, Long C, Hall CB. Predicting deterioration in previously healthy infants hospitalized with respiratory syncytial virus infection. *Pediatrics* 1999;104:463–467.
43. Knowles MR, Paradiso AM, Boucher RC. *In vivo* nasal potential difference: techniques and protocols for assessing efficacy of gene transfer in cystic fibrosis. *Hum Gene Ther* 1995;6:445–455.
44. Alton EW, Currie D, Logan-Sinclair R, Warner JO, Hodson ME, Geddes DM. Nasal potential difference: a clinical diagnostic test for cystic fibrosis. *Eur Respir J* 1990;3:922–926.
45. Hofmann T, Bohmer O, Huls G, Terbrack HG, Bittner P, Klingmuller V, Heerd E, Lindemann H. Conventional and modified nasal potential-difference measurement in cystic fibrosis. *Am J Respir Crit Care Med* 1997;155:1908–1913.
46. Knowles MR, Carson JL, Collier AM, Gatzy JT, Boucher RC. Measurements of nasal transepithelial electric potential differences in normal human subjects *in vivo. Am Rev Respir Dis* 1981;124:484–490.
47. Wilson R, Alton E, Rutman A, Higgins P, Al Nakib W, Geddes DM, Tyrrell DA, Cole PJ. Upper respiratory tract viral infection and mucociliary clearance. *Eur J Respir Dis* 1987;70:272–279.
48. Schwiebert EM. ATP release mechanisms, ATP receptors and purinergic signalling along the nephron. *Clin Exp Pharmacol Physiol* 2001;28:340–350.
49. Huang P, Lazarowski ER, Tarran R, Milgram SL, Boucher RC, Stutts MJ. Compartmentalized autocrine signaling to cystic fibrosis transmembrane conductance regulator at the apical membrane of airway epithelial cells. *Proc Natl Acad Sci USA* 2001;98:14120–14125.
50. Marriott I, Inscho EW, Bost KL. Extracellular uridine nucleotides initiate cytokine production by murine dendritic cells. *Cell Immunol* 1999;195:147–156.
51. Idzko M, Panther E, Bremer HC, Sorichter S, Luttmann W, Virchow CJ Jr, Di Virgilio F, Herouy Y, Norgauer J, Ferrari D. Stimulation of P2 purinergic receptors induces the release of eosinophil cationic protein and interleukin-8 from human eosinophils. *Br J Pharmacol* 2003;138:1244–1250.
52. Varga SM, Wang X, Welsh RM, Braciale TJ. Immunopathology in RSV infection is mediated by a discrete oligoclonal subset of antigen-specific $CD4^+$ T cells. *Immunity* 2001;15:637–646.
53. Schwarze J, Cieslewicz G, Joetham A, Ikemura T, Hamelmann E, Gelfand EW. CD8 T cells are essential in the development of respiratory syncytial virus–induced lung eosinophilia and airway hyperresponsiveness. *J Immunol* 1999;162:4207–4211.
54. Mutlu GM, Koch WJ, Factor P. Alveolar epithelial β_2-adrenergic receptors: their role in regulation of alveolar active sodium transport. *Am J Respir Crit Care Med* 2004;170:1270–1275.