Innate Immune Signals Modulate Antiviral and Polyreactive Antibody Responses during Severe Respiratory Syncytial Virus Infection

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Antiviral antibody production during respiratory syncytial virus (RSV) infection in infants is poorly understood. To characterize local B lymphocyte responses, lung tissue and secretions from infants with RSV bronchiolitis were analyzed for innate B cell–stimulating factors and antiviral antibodies. In lung tissues of infants with fatal RSV bronchiolitis, CD20+ lymphocytes and IgM-positive, IgG-positive, and IgA-positive plasma cells were prominent but CD4+ T lymphocytes were not. Type I interferon–induced proteins and B cell tropic factors, including B cell–activating factor (BAFF) and a proliferation-inducing ligand (APRIL), were colocalized in infected epithelium. In nasopharyngeal secretions from infants who survived RSV infection, class-switched antiviral and antinucleosomal antibodies were detected at presentation and correlated with BAFF and APRIL levels. Expression of APRIL and antiviral antibodies of IgA and IgM but not IgG isotype predicted better oxygen saturation. We conclude that B lymphocyte–stimulating factors derived from infected epithelium are primary determinants of the mucosal antibody response in infant RSV bronchiolitis.

Respiratory syncytial virus (RSV) is the most important cause of severe lower respiratory tract infection (LRI) in infants. Virtually all infants have encountered RSV before the age of 2 years, resulting in ~100,000 infant hospitalizations and 400 infant deaths in the United States each year and 1 million deaths worldwide annually [1–3]. RSV LRI in infancy has been associated with chronic wheezing and asthma later in childhood [4]. Because there is currently no vaccination strategy to pre-

vent infection in infants, RSV continues to pose a significant health problem.

B lymphocyte responses to RSV are thought to contribute to protection and immunopathologic activities associated with primary infection [5]. Low levels of serum IgM, IgG, and IgA are detected in most infants 1 month after exposure and persist for months after infection [6]. Antiviral antibodies are also found in nasopharyngeal secretions from patients with acute infection as early as 1 week after infection onset [7, 8]. The relationship between antiviral antibody responses and protection is unclear. Serum RSV-directed antibody titers are low in the youngest infants, possibly predisposing for more severe disease in this age group [9]. High levels of maternal antibody are associated with protection against acute infection, and boosted antibody levels with reexposure coincide with milder disease in older children [10, 11]. However, reinfections with RSV are common even in adults who have high anti-RSV antibody titers, suggesting that antiviral antibody is not completely protective [12]. In rodent models of RSV infection, T helper type 2 (Th2) lymphocyte responses, which

Table 1. Characteristics of 33 infants who survived lower respiratory tract infection (LRI) and 9 infants who died from bronchiolitis.

> This table is available in its entirety in the online edition of *The Journal of Infectious Diseases.*

favor antibody production over cytotoxic responses, were associated with RSV persistence and immunopathogenesis [13]. Clinical experience with a formalin-inactivated RSV vaccine also unfortunately demonstrated eosinophil and lymphocyte activation in blood and lung tissue after infection with wild-type RSV, consistent with a Th2-skewed response. Antibodies elicited with formalin-inactivated RSV vaccine were not protective and possibly contributed to immunopathogenesis when recipients were exposed to natural infection [14].

Recently, we had the opportunity to examine secretions from a cohort of infants with upper respiratory tract infection or LRI with RSV, in addition to lung tissue obtained from infants with acutely fatal RSV LRI [15]. Although our goal was to confirm T lymphocyte contributions to severe disease, we instead observed that both $CD4^+$ and $CD8^+$ lymphocytes and their associated

cytokines were nearly absent in RSV LRI. Our observations suggested that an inadequate adaptive immune response, rather than an exaggerated one, underlies severe RSV LRI in humans. However, weak T helper lymphocyte responses seemed at odds with reports of prominent mucosal antiviral antibody detection within days after initial RSV exposure [7, 8]. For this reason, we have extended our studies to characterize B lymphocyte responses during primary RSV disease in infants. Here, we report massive B lymphocyte recruitment to lung tissue that occurred immediately at the time of presentation of RSV infection. Marked expression of B cell activation and survival factors in infected epithelium occurs early after virus exposure and is correlated with mucosal production of RSV-directed immunoglobulin.

PATIENTS, MATERIALS, AND METHODS

Study populations. Postmortem lung tissues from 9 infants with RSV LRI were provided by Hospital Roberto del Rio (Santiago, Chile) (table 1, which appears only in the electronic edition of the *Journal*) [15]. Lung tissue from 3 age-matched infants who died from other causes were provided by the National In-
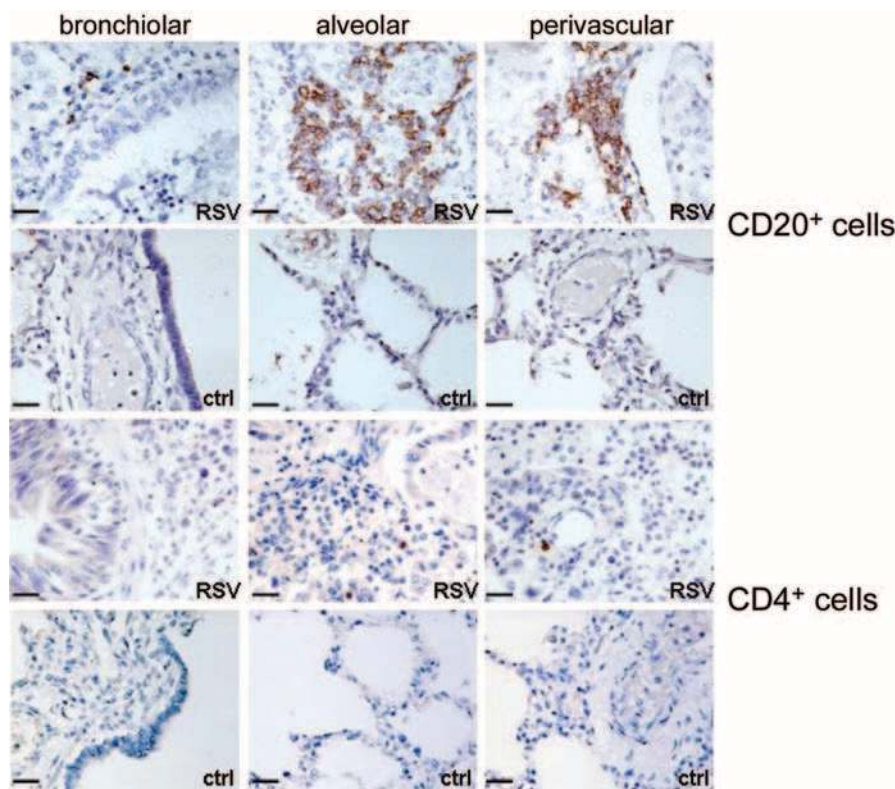

Figure 1. Detection of B lymphocytes in samples from persons with respiratory syncytial virus lower respiratory tract infection (RSV LRI). $CD20^+$ *(upper panels)* or $CD4^+$ *(lower panels)* lymphocytes were detected by immunohistochemistry analysis in formalin-fixed, paraffin-embedded lung tissue obtained at autopsy from infants who died of acute RSV LRI (RSV) or asphyxia (ctrl). Representative fields from bronchiolar *(left)*, alveolar *(middle)*, and perivascular *(right)* spaces are shown. Primary antibodies were detected with biotinylated secondary antibodies and streptavidin/HRP peroxidase conjugate, followed by the peroxidase substrate diaminobenzidine (original magnification, ×40). *Scale bar,* 15 μm.
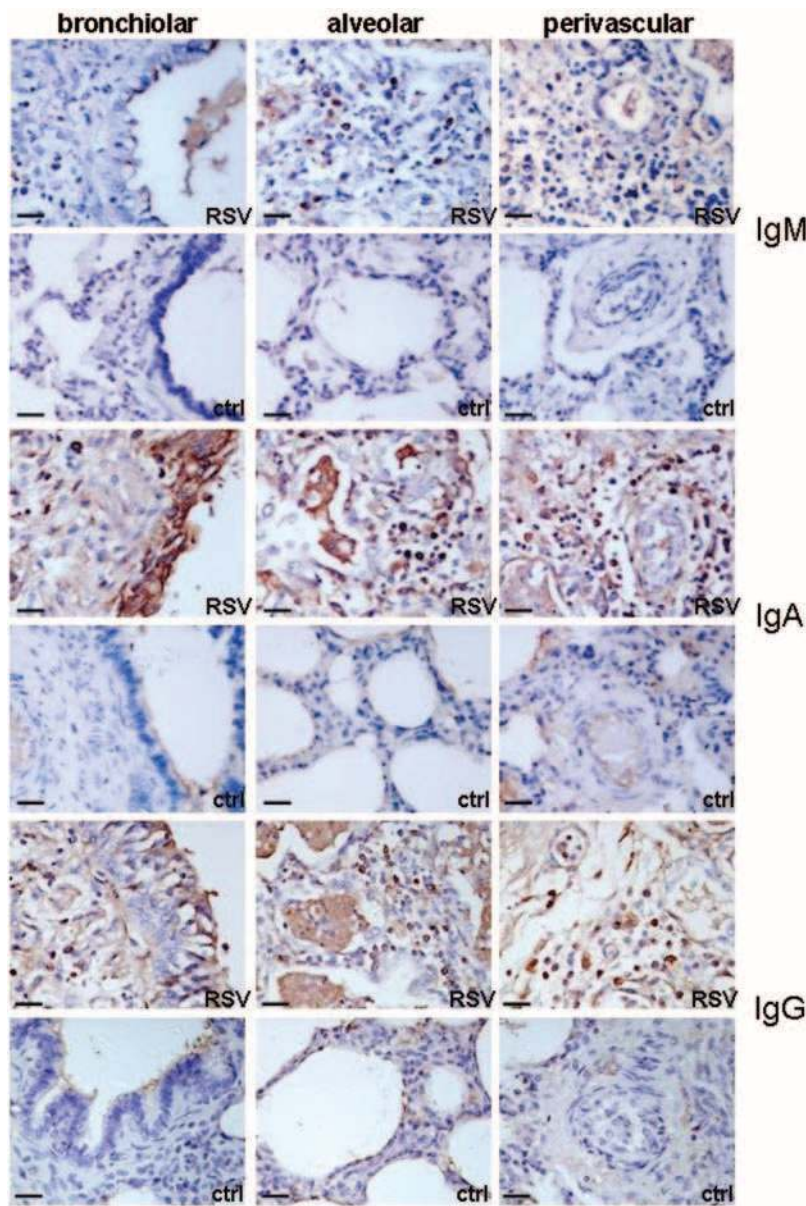
Figure 2. Detection of immunoglobulin isotypes in samples from persons with respiratory syncytial virus lower respiratory tract infection (RSV LRI). IgM *(upper panels),* IgA *(center panels),* and IgG *(lower panels)* were detected by immunohistochemistry analysis in formalin-fixed, paraffin-embedded lung tissue obtained at autopsy from infants who died of acute RSV LRI (RSV) or asphyxia (ctrl). Representative fields from bronchiolar *(left),* alveolar *(middle),* and perivascular *(right)* spaces are shown. Primary antibodies were detected with biotinylated secondary antibodies and streptavidin/HRP peroxidase conjugate, followed by the peroxidase substrate diaminobenzidine (original magnification, ×40). *Scale bar,* 15 μm.

stitute of Child Health and Development (Baltimore, MD). Nasopharyngeal secretions from surviving infants with acute bronchiolitis were collected at Women and Children's Hospital (Buffalo, NY), as previously described (table 1) [15]. No subject had received RSV antibody preparations, corticosteroids, or antiviral agents. Secretions were obtained ≤24 h after hospitalization and ≤5 days after the onset of respiratory symptoms in all cases. Values of oxygen saturation were determined by pulse oximetry at the time secretions were obtained.

Immunohistochemistry analysis. Formalin-fixed, paraffin-embedded lung tissues were sectioned and processed through xy-

lene to remove paraffin. Heat-induced epitope retrieval was performed before immunohistochemistry (IHC) analysis. Primary antibodies reactive with CD20 (Lab Vision), CD4, IgM, IgG, IgA (Millipore), myxovirus resistance protein A, OAS (Santa Cruz Biotechnology), vasoactive intestinal peptide (VIP [US Biological]), B cell–activating factor (BAFF), a proliferation-inducing ligand (APRIL), B cell maturation antigen (BCMA), transmembrane activator calcium modulator and cyclophylin ligand interactor (TACI), and BAFF-R (ProSci) were used according to the manufacturers' recommendations. Primary antibodies were detected with biotinylated secondary antibodies (Jackson Immunoresearch) and strep-
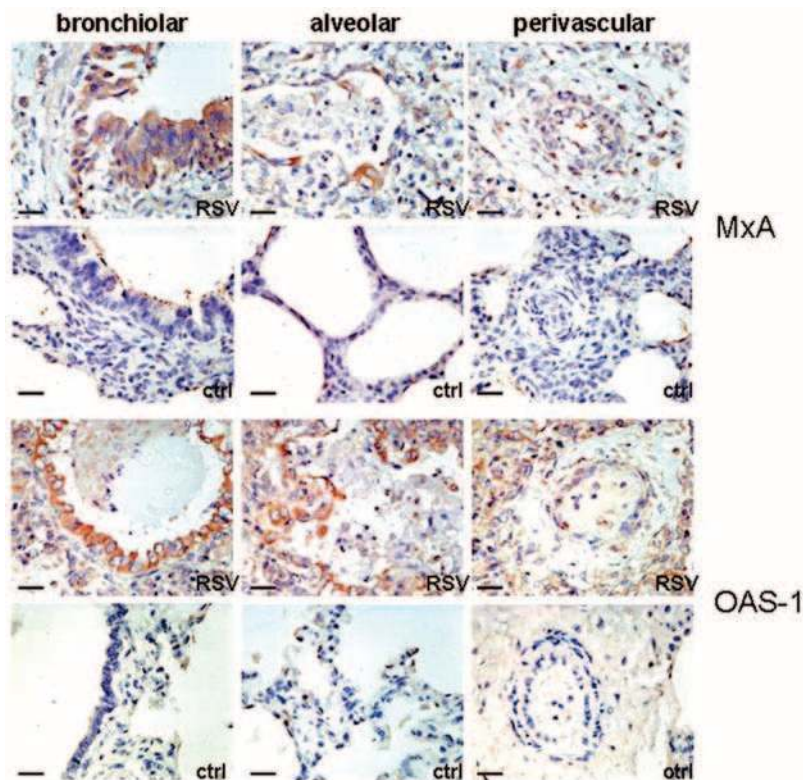
Figure 3. Interferon-induced protein expression in respiratory syncytial virus lower respiratory tract infection (RSV LRI). Myxovirus resistance protein A (MxA; *upper panels*) and 2',5'-oligoadenylate synthetase 1 (OAS1; *lower panels)* were detected by immunohistochemistry analysis in formalin-fixed, paraffin-embedded lung tissue obtained at autopsy from infants who died of acute RSV LRI (RSV) or asphyxia (ctrl). Representative fields from bronchiolar *(left),* alveolar *(middle),* and perivascular *(right)* spaces are shown. Primary antibodies were detected with biotinylated secondary antibodies and streptavidin/HRP peroxidase conjugate, followed by the peroxidase substrate diaminobenzidine (original magnification, ×40). *Scale bar,* 15 μm.

tavidin/horseradish peroxidase conjugate (GE Healthcare) with peroxidase substrate (Sigma Aldrich).

Nasopharyngeal secretions analysis. VIP, BAFF, and APRIL were measured in secretions by ELISA (Phoenix Pharmaceuticals; R&D Systems; Bender MedSystems) according to the manufacturers' instructions. Cytokines were assessed using the BioPlex human cytokine 17Plex panel (BioRad Laboratories). The lower limit of detection of mediators was 7.5 pg/mL. Total IgG, IgM, and IgA levels were measured using a capture-detect ELISA system (Bethyl Laboratories) according to the manufacturers' recommendations. For detection of antibody reactivity against RSV antigens, Hep-2 lysate, BSA, and nucleosomes, flat-well ELISA plates (Maxisorp [Nalge Nunc International]) were coated with RSV-A2 virus stock (10^4 pfu/well [MedImmune]), BSA (1 μg/mL), Hep-2 lysate (1 μg/mL), or a 1:500 dilution of nuclear extract prepared from campothecin-induced U937 cells (cell-death detection ELISA kit [Roche]). Nonspecific protein interactions were blocked by incubation in saline containing 3% BSA (Sigma). Nasopharyngeal secretions were added to wells at a 1:10 dilution in blocking buffer. Bound antibodies were detected with biotinylated, isotype-specific polyclonal antisera (Jackson Immunoresearch) and then streptavidin-HRP conjugate (GE Healthcare) and HRP substrate (Sure-

Blue [KPL]). The optical density was quantified at 410 nmol/L, using a 96-well plate reader (Bio-Tek Instruments).

B cell stimulation. Purified B cells were cultured for 7 days at 1×10^6 cells/mL in either 1 mL in 24-well culture plates or 100 μL in 96-well round-bottom culture plates. The cells were incubated with combinations of the Toll-like receptor 7 (TLR7) agonist imiquimod or the TLR9 agonist CpG-B(2006) (0.1–10 μg/mL [InvivoGen]), leukocyte-derived type I interferon (IFN; 100 IU/mL [PBL Interferon Source]), human IL-4 (100 ng/mL [R&D Systems]), BAFF (100 ng/mL [R&D Systems]), antihuman CD40 (1 μg/mL [R&D Systems]), and anti-IgM (5 μg/mL [Jackson ImmunoResearch]). Secreted IL-6 and IL-10 in the culture supernatant was measured using commercially available ELISA kits (R&D Systems) according to the manufacturer's instructions. Secreted immunoglobulin in the culture supernatant was quantitated by ELISA as previously described [16, 17].

The figure is available in its entirety in the online edition of the *Journal of Infectious Diseases.*

Figure 4. Detection of vasoactive intestinal peptide in respiratory syncytial virus lower respiratory tract infection.
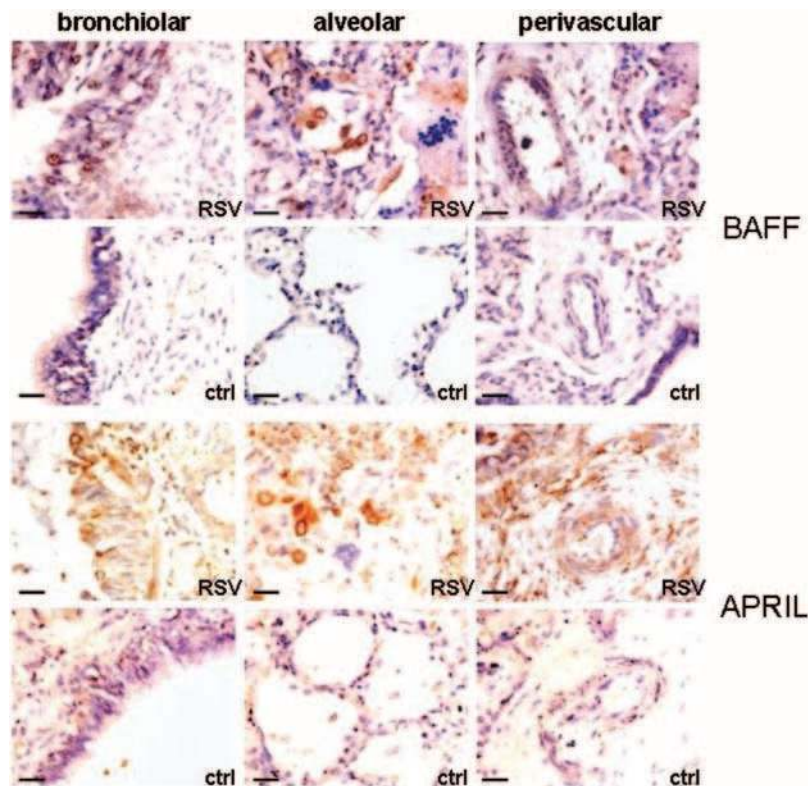
Figure 5. B cell–activating factor (BAFF) and a proliferation-inducing ligand (APRIL) expression in respiratory syncytial virus lower respiratory tract infection (RSV LRI). BAFF *(upper panels)* and APRIL *(lower panels)* were detected by immunohistochemistry analysis in formalin-fixed, paraffin-embedded lung tissue obtained at autopsy from infants who died of acute RSV LRI (RSV) or of asphyxia (ctrl). Representative fields from bronchiolar *(left)*, alveolar *(middle)*, and perivascular *(right)* spaces are shown. Primary antibodies were detected with biotinylated secondary antibodies and streptavidin/HRP peroxidase conjugate, followed by the peroxidase substrate diaminobenzidine (original magnification, ×40). *Scale bar,* 15 μm.

Statistical analysis. For analysis of nasopharyngeal secretions, data were log-transformed to obtain a normal distribution. Pearson analysis of covariation was performed using GraphPad Prism software. Statistical significance was defined as a P value of $\leq$.05.

RESULTS

B cells are prominent in lung tissue following primary RSV LRI. Mucosal antibodies may be transported from the serum to the lung through transudation and/or active transport [18, 19], or they are locally produced by B lymphocytes in tissue [20]. To determine the source of antibody in RSV-exposed infants, we identified B cells by IHC analysis in a panel of lung tissues obtained from 9 infants with acutely fatal RSV LRI. CD20$^+$ B lymphocytes were abundant in lung tissue of infants with RSV LRI and localized to

alveolar and perivascular spaces (figure 1). CD20$^+$ cells were nearly absent in control lung tissues obtained from 3 infants who died of asphyxia (figure 1). CD4$^+$ cells, in contrast, were rarely identified in lung tissue from patients with RSV LRI or from control patients (figure 1) [15]. In RSV LRI, the lung-associated B lymphocyte population included plasma cells, based on detection of substantial cytoplasmic levels of IgA, IgG, and IgM, whereas almost no IgA, IgG, or IgM was detected in lung tissue from age-matched control patients (figure 2). IgM-positive, IgG-positive, and IgA-positive cells were prominent in perivascular and alveolar spaces. IgA was also strongly deposited on bronchiolar epithelium in specimens from patients with RSV LRI.

Innate immune factors in RSV-infected lung tissue. Because a vigorous B lymphocyte response was apparent in lung tissue from infants with acute RSV LRI, whereas T lymphocytes were rare, we considered the possibility of T cell–independent B lymphocyte antibody production. Type I IFN has been implicated in early antiviral

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Figure 6. Detection of receptors for a proliferation-inducing ligand and B cell–activating factor in respiratory syncytial virus lower respiratory tract infection.

The figure is available in its entirety in the online edition of the *Journal of Infectious Diseases.*

Figure 7. Quantitation of total immunoglobulin levels in nasopharyngeal secretions.
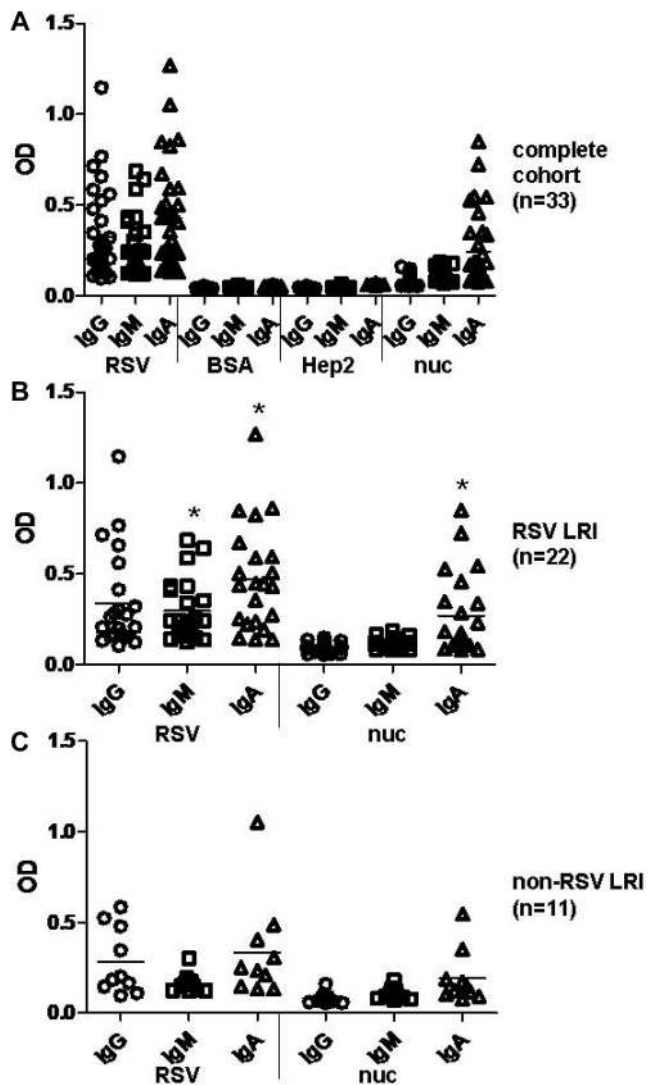
Figure 8. Immunoglobulin detection in nasopharyngeal secretions from patients with or without respiratory syncytial virus lower respiratory tract infection (RSV LRI). *A,* Antibodies reactive with RSV virions (RSV), bovine serum albumin (BSA), uninfected total cell lysate of Hep-2 epithelial cells (Hep-2), or nuclear extract of apoptotic U937 cells (nuc) were individually detected by ELISA (*n* = 33). IgG *(circles),* IgM *(squares),* and IgA *(triangles)* isotypes were separately evaluated. Individual absorbances at 405 nm plus means *(bars)* are shown. Antibodies reactive with RSV and with nuclear extract were also examined separately in aspirates from infants with *(B)* or without *(C)* RSV LRI. OD, optical density. *$P \le .05$.

B cell responses [21]. Reagents for detection of IFN-α or IFN-β by IHC analysis were not available. However, we did detect type I IFN–induced proteins, myxovirus resistance protein A, and 2′,5′-oligoadenylate synthetase 1 at high levels in bronchiolar and alveolar epithelium of lung tissue from patients with RSV LRI but not in tissue from control patients (figure 3). VIP promotes T cell–independent B cell activation [22], and the CD40L-related factors BAFF and APRIL have been more recently implicated in CD40-independent immuno-

globulin production and class-switch recombination [23, 24]. We were able to confirm strong expression of VIP (unpublished data) (figure 4, which appears only in the electronic edition of the *Journal*) and BAFF and APRIL (figure 5) in alveolar and bronchiolar epithelium of infants presenting with acutely fatal RSV infection. VIP and APRIL were also detected in perivascular immune cells. We detected minimal expression of VIP, BAFF, and APRIL in lung tissue from age-matched control patients (figures 4 and 5). Also, in RSV LRI tissues we detected expression of BAFF-R, TACI, and BCMA, the identified receptors for BAFF and APRIL [25], in a subset of perialveolar lymphocytes, some with paradigmatic plasma cell morphologic characteristics (figure 6, which appears only in the electronic edition of the *Journal*).

Antiviral and polyreactive antibodies in infant nasopharyngeal secretions. Next, we assessed immunoglobulin levels in nasopharyngeal secretions obtained from 33 infants with acute LRI (RSV was detected in 22, influenza virus in 10, and adenovirus in 1). The characteristics of this patient population are detailed in table 1. Total IgG, IgM, and IgA levels were similar in all nasopharyngeal secretions analyzed (figure 7, which appears only in the electronic edition of the *Journal*). As has been reported [26], IgA, IgG, and IgM antibodies reactive with RSV antigens were robustly detected in secretions obtained at the time of presentation from most infants with LRI (figure 8*A*) and were significantly correlated with the total immunoglobulin level (table 2, which appears only in the electronic edition of the *Journal*). Infants presenting with RSV LRI had significantly greater levels of RSV-directed IgM and RSV-directed IgA, compared with infants with non-RSV LRI (figure 8*B* and 8*C*). However, RSV-directed immunoglobulin was also detected in cases of influenza virus and adenovirus LRI (figure 8*C*). Because virtually all infants are exposed to RSV before the age of 2 years, the observation of anti-RSV antibodies in infants presenting with influenza virus or adenovirus infection could reflect previous RSV exposure. In fact, we did identify 1 infant presenting with influenza virus LRI who had received outpatient care for RSV LRI 1 week previously. Alternatively, respiratory infection may trigger secretion of poorly diversified, polyspecific antibodies reactive with pathogen-associated molecular patterns shared among highly conserved intracellular structures such as nucleic acids and nucleosomes [27, 28]. To address the question of polyspecific antibody production, we tested the nasopharyngeal aspirates for reactivity with a preparation of human nuclear and nucleosomal antigens, derived from campothecin treated U937

Table 2. Correlation of respiratory syncytial virus (RSV)–directed immunoglobulin (Ig) with total immunoglobulin (Ig) and anti-nuc Ig.

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Table 3. Correlates of local immunoglobulin (Ig) recovery from infants with lower respiratory tract infection (LRI).

Variable	IL-2		IL-4		IL-10		BAFF		APRIL		VIP	
	r	P	r	P	r	P	r	P	r	P	r	P
Total IgM	0.163	.167	0.126	.493	0.435	.013	0.711	<.001	0.193	.367	0.574	.001
Total IgG	0.250	.372	0.122	.507	0.418	.017	0.640	<.001	0.314	.136	0.438	.012
Total IgA	0.336	.060	0.214	.239	0.515	.003	0.624	<.001	0.144	.503	0.401	.023
RSV IgM	0.104	.568	0.109	.555	0.084	.648	0.540	.001	0.522	.009	0.412	.019
RSV IgG	0.301	.094	0.100	.592	0.365	.040	0.341	.056	0.169	.429	0.226	.214
RSV IgA	0.284	.114	0.460	.008	0.191	.296	0.333	.063	0.514	.010	0.386	.029
nuc IgM	0.067	.820	0.00063	.998	0.070	.813	0.296	.304	0.386	.270	0.019	.951
nuc IgG	0.124	.716	0.055	.872	0.324	.331	0.226	.503	0.051	.913	0.246	.466
nuc IgA	0.247	.172	0.498	.004	0.251	.166	0.351	.049	0.410	.047	0.314	.080
O_2 sat	0.212	.420	0.393	.120	0.041	.880	0.384	.117	0.698	.010	0.331	.190

NOTE. Nasopharyngeal secretions from 33 infants hospitalized with LRI were analyzed for Ig, Th2 cytokines, B cell–activating factor (BAFF), APRIL, and vasoactive intestinal peptide (VIP) content. Log-transformed values were assessed for correlation, using a Pearson 2-tailed test. O_2 sat, oxygen saturation.

cells. Immunoglobulin reactive with nuclear and nucleosomal antigens (anti-nuc) was present in aspirates, with detection of greater IgA levels, compared with IgG or IgM levels (figure 8A). However, no reactivity was observed against BSA or a lysate prepared from uninfected Hep-2 cells, the cell line in which the RSV antigens were prepared (figure 8A). Infants presenting with RSV LRI had significantly greater antinuclear and nucleosomal (anti-nuc) IgA than those presenting with infection due to influenza virus or adenovirus (figure 8B and 8C). Recovery of RSV-directed IgM and IgA was significantly correlated with recovery of anti-nuc IgM and IgA (table 2). Thus, the recovery of immunoglobulin reactive with apoptotic cell epitopes appeared to be linked to antiviral immunoglobulin recovery, raising the possibility that factors present in infected lung tissue might be responsible for promoting both immunoglobulin types.

Innate immune factors predict immunoglobulin response and oxygen saturation. We attempted to clarify a role for T cell–dependent versus T cell–independent processes in the generation of total, RSV-directed, and anti-nuc antibodies recovered from nasopharyngeal secretions from infants (table 3). T lymphocyte–associated cytokines, including IL-2, IL-4, and IL-10, have been implicated in CD40-dependent immunoglobulin secretion and class switch to the IgA isotype [29–31]. Low mean levels (±SD) of IL-2 (33.7 ± 7.2 pg/mL), IL-4 (59.4 ± 11.3 pg/mL), and IL-10 (50.5 ± 9.3 pg/mL) were detected in most secretions analyzed. IL-4 positively correlated with RSV-specific and anti-nuc IgA but not with other immunoglobulin measures (table 3). IL-2 levels also tracked with higher RSV-specific and anti-nuc IgA, although these relationships failed to reach statistical significance. On the other hand, IL-10 levels predicted higher levels of total immunoglobulin recovery. IL-10 was associated with higher RSV-specific IgG but did not correlate with RSV-specific IgM or IgA or with anti-nuc antibody levels. Interestingly, levels of T lymphocyte cytokines IL-2, IL-4, IL-5, and IL-13 strongly correlated with each other, but recovery of IL-10 was unrelated (figure 9A and 9B, which appears only in the electronic edition of the *Journal*), possibly implicating a non-T lymphocyte source for IL-10 during LRI. In contrast with the low levels of T lymphocyte–dependent cytokines observed in nasopharyngeal secretions, we recovered greater mean quantities (±SD) of VIP (5201.8 ± 1559.4 pg/mL), BAFF (108.0 ± 20.9 pg/mL), and APRIL (1518 ± 635.4 pg/mL) in most nasopharyngeal secretions. The strongest predictors of mucosal immunoglobulin recovery were VIP and BAFF, both of which also correlated with IL-10 recovery (table 3 and figure 9C). APRIL was strongly correlated with RSV-directed and anti-nuc IgA recovery and also predicted RSV-directed IgM recovery (table 3). Surprisingly, APRIL alone positively correlated with better oxygen saturation at the time of presentation (table 3), suggesting a protective role. RSV-directed IgM and IgA were associated with better oxygen saturation; however, RSV IgG was not (table 4, which appears only in the electronic edition of the *Journal*). We were not able to detect IFN-α or IFN-β consistently by ELISA but did observe that the IFN-induced protein CXCL10 (IP-10) was among the most expressed cytokines in nasopharyngeal secretions from infants with LRI (mean level [±SD], 2053.8 ± 297.5 pg/mL), perhaps consistent with type I IFN induction in lung tissue. The quantity of IFN-γ recovered was much less (mean level [±SD], 31.4 ± 6.0 pg/mL) and was not correlated with IP-10 (data not shown). Together, these data suggest that innate immune factors produced locally in large quantities shape the B cell response to primary RSV infection in concert with IL-4.

The figure is available in its entirety in the online edition of the *Journal of Infectious Diseases.*

Figure 9. Correlation of cytokines in nasopharyngeal secretions.

Table 4. Correlates of oxygen saturation (O$_2$ sat) in infants with lower respiratory tract infection (LRI).

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Synergy of T lymphocyte–dependent stimuli with innate immune factors. T lymphocyte–dependent and T lymphocyte–independent stimuli may influence the mucosal antibody repertoire in infants with RSV LRI. To determine how lung-localized B cells might integrate these disparate signals, we incubated human peripheral blood B cells with mixtures of type I IFN, the TLR7 agonist imiquimod, and BAFF, along with anti-IgM, anti-CD40, and IL-4. As has been previously observed [32, 33], B cells costimulated with type I IFN plus the TLR7 agonist imiquimod secreted IgM, IgG, and IgA, plus IL-6 and IL-10, in a dose-dependent manner (figure 10*A* and 10*B*). The addition of BAFF further enhanced both immunoglobulin secretion and cy-
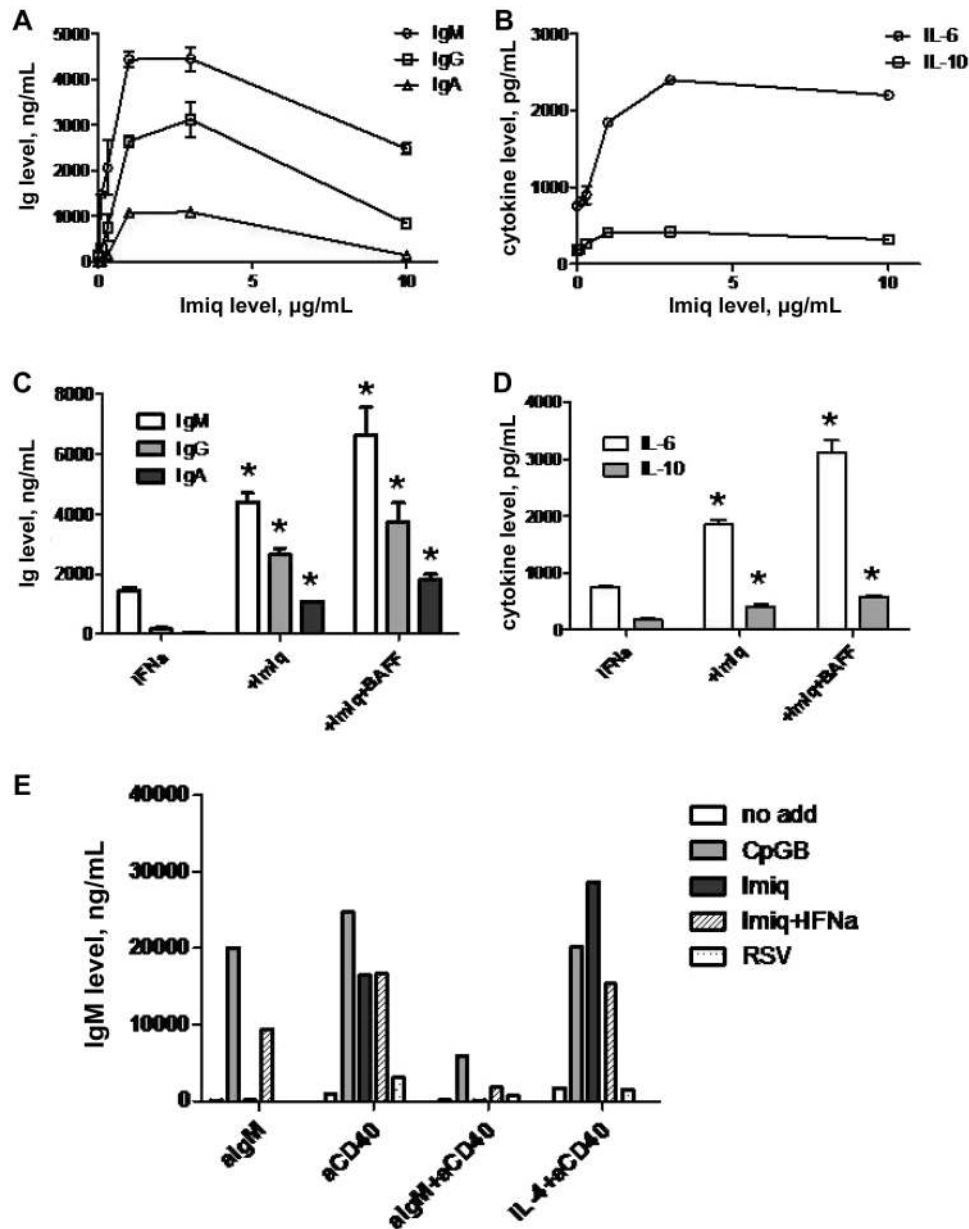

Figure 10. Combined Toll-like receptor 7 (TLR7) agonist, interferon (IFN), and B cell–activating factor (BAFF) promote immunoglobulin (Ig) secretion in vitro. Ig secretion (*A* and *C*) and cytokine release (*B* and *D*) in supernatants of peripheral blood B lymphocytes stimulated for 7 days with either graded concentrations of imiquimod (Imiq) plus leukocyte IFN (IFNa; *A* and *B*), or with an optimized combination of Imiq with leukocyte IFNa and BAFF (*C* and *D*). Representative data from 3 studies are shown. *E*, B cells were stimulated with anti-IgM, anti-CD40, anti-IgM with anti-CD40, or IL-4 with anti-CD40 plus CpG-B oligodeoxynucleotides, Imiq, Imiq with IFNa, or RSV virions. IgM secretion in supernatants was measured on day 7. *$P \le .05$.

Figure 11. Combined Toll-like receptor 7 agonist, interferon, and B cell–activating factor promote immunoglobulin secretion in vitro.

tokine release (figure 10*C* and 10*D*). We found that the combination of TLR7 agonist plus type I IFN could synergize with anti-IgM, anti-CD40, and IL-4 to augment secretion of IgM (figure 10*E*), IgG, and IgA in the absence of T cell help (figure 11*A* and 11*B*, which appears only in the electronic edition of the *Journal*). The magnitude of synergy was comparable to that observed with CpG-B oligodeoxynucleotides (figures 10*E,* 11*A,* and 11*B*), although CpG stimulated IgG and IgA release more efficiently. Immunoglobulin secretion in the presence of anti-CD40 or anti-CD40 plus IL-4 was augmented by TLR7 agonist, whereas type I IFN provided no synergy. As has been previously observed with influenza virus [34, 35], incubation of B lymphocytes with RSV virions, either alone or in combination with anti-IgM, anti-CD40, or IL-4, had minimal impact on immunoglobulin release (figures 10*E,* 11*A,* and 11*B*). These data indicate that innate stimuli associated with acute RSV infected cells, such as type I IFN, BAFF, and TLR7 ligand, may synergize to induce robust B cell responses in the absence of T cell help.

DISCUSSION

T lymphocyte–dependent antibody production is inefficient in infants. Neonatal T lymphocytes deficits include low expression of TCR, adhesion molecules, and CD40L, which result in blunted T cell help for B lymphocytes [36, 37]. Nevertheless, an antiviral antibody response in nasopharyngeal secretions of infants is present early during primary RSV LRI. In this study, we demonstrate that B lymphocytes, including plasma cells, are heavily recruited to infant lung at the peak of RSV illness, whereas CD4$^+$ T lymphocytes are not abundant. We also show that B cell detection in tissue coincides with local expression of B cell tropic factors, including BAFF, APRIL, and VIP, localized primarily to infected respiratory epithelium. Similarly, specific and total immunoglobulin levels in secretions from surviving infants with RSV LRI coincide and correlate with BAFF, APRIL, and VIP recovery, suggesting a causative relationship. Assuming similar disease pathogenesis in Chilean and US infants, these data implicate T lymphocyte–independent processes in shaping the primary antiviral antibody response in a setting where cognate T cell help is suboptimal.

Previous studies have focused on the dendritic cell (DC) as the prime regulator of antiviral immunoglobulin responses in adult systems, either indirectly through T cell stimulation or through IFN-dependent release of IL-6 and BAFF [38–40]. DCs obtained from infants exhibit multiple deficiencies [38], including limited responses to in vitro stimuli and low expression of major histocompatibility complex (MHC) class II and costimulatory

molecules. Thus, although mature DCs can clearly make enormous contributions to B cell activation and immunoglobulin production, it is unclear whether immature DCs in infants can play this role. Most studies that implicate DCs in antibody production measure serum levels of immunoglobulin. In fact, in infants <6 months old, serum IgM and IgA responses to RSV are almost undetectable during the first month [7, 40], whereas the antibody response in secretions appears within days and is similar qualitatively and quantitatively in infants and older children with acute RSV LRI [41]. This apparent dissociation between mucosal and systemic immunoglobulin responses in infants may highlight a differential role for DCs in these 2 anatomic sites. In the context of inefficient DC function, the local epithelium may provide an alternative source of B lymphocyte–directed stimuli to maintain mucosal antibody levels. In support of this point, recent in vitro studies show that RSV infection of respiratory epithelial cells results in TLR3 activation, IFN-β release, and IFN-β–dependent BAFF and APRIL expression in cultured respiratory epithelium [42, 43]. Our data confirm prominent epithelial expression of VIP, BAFF, and APRIL in vivo during RSV LRI. Colocalization of IFN-induced proteins such as MxA and OAS in RSV-infected alveolar epithelium and high levels of CXCL10 in nasopharyngeal secretions imply that type I IFN is probably also locally expressed. Together, these data point to RSV-infected epithelium as an important source of innate immune factors, particularly during uncontrolled respiratory infection, and suggest that these cytokines may be sufficient to induce T lymphocyte–independent antibody production if DC functions are immature.

BAFF and APRIL overexpression have been previously implicated in the generation of self-reactive antibodies in patients with autoimmune disease [44, 45]. Here, we report antibodies reactive with apoptotic cell epitopes in nasopharyngeal secretions during primary RSV LRI. Antinucleosomal antibodies in nasopharyngeal secretions were strongly correlated to BAFF and APRIL, suggesting that similar mechanisms of B lymphocyte activation could be present in systemic lupus erythematosus and infant respiratory infection. In acute RSV LRI, airways are occluded with dense apoptotic cellular debris arising from massive infection of respiratory epithelium [15]. Accumulation of apoptotic cellular debris is also a cardinal feature of systemic lupus erythematosus, in which a failure in macrophage clearance of debris is implicated in pathogenesis [46]. The generation of antibodies recognizing cellular debris may be a conserved, protective response. Autoreactive antibodies are proposed to have a beneficial role in removing immune-stimulating components of dying cells, thus avoiding inflammatory cell recruitment and tissue destruction [27]. Supporting this idea, APRIL and anti-nuc antibodies of IgA isotype were associated with better oxygen saturation values in this cohort of infants with bronchiolitis, suggesting a role for APRIL and/or APRIL-induced IgA in providing some degree of protection in air-exchange tissue. IgA is thought

to provide anti-inflammatory and tolerizing signals in the mucosa and may antagonize proinflammatory actions of IgG, such as DC maturation [47–49]. The ability to generate local polyspecific IgA may facilitate the clearance of cellular debris without augmenting inflammation, preserving lung tissue and respiratory function.

Sangster et al. [50] found that, in mice whose B lymphocytes lack MHC class II and CD40, $CD4^+$ T lymphocytes were still required for local production of influenza-neutralizing IgA. Thus, $CD4^+$ lymphocytes promoted antiviral immunity, even in the absence of cognate T cell–B cell collaboration. Although the nature of the help provided by $CD4^+$ T cells was not identified, our current studies suggest that bystander cytokine production may be a major contributor in the mucosal antibody response to virus infection. We have observed a direct correlation between IL-4 and local antiviral and anti-nuc IgA in vivo, whereas in vitro, IL-4 and anti-CD40 synergized with TLR7 ligand to enhance B cell activation and antibody secretion. Together, these data suggest that bystander IL-4 signaling, in synergy with innate signals, may support polyspecific and antiviral antibody secretion in the mucosa. When $CD4^+$ T cells are few in number, alternative sources of IL-4, such as mast cells, may be important contributors to mucosal antibody homeostasis.

Together, these data provide some insight into the challenges associated with an RSV vaccination approach for infants. In hospitalized infants, the T cell response to RSV is blunted, and mucosal antibody response to RSV appears linked to factors elaborated by infected epithelial cells. Thus, antiviral responses are initiating too late, after virus has already established a foothold in respiratory tissue. Novel adjuvant strategies, in which T cell–independent and T cell–dependent stimuli work in synergy to optimally stimulate lung-associated B lymphocytes, may enhance locally administered vaccines. Alternatively, attenuated live viruses may be used to stimulate sentinel macrophages and DCs in neonates and trigger more-prompt T and B lymphocyte collaboration after wild-type virus challenge. Engaging the unique contributions of both innate and adaptive antiviral responses may be required for a successful RSV vaccine in neonates.

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Table 1. Characteristics of 33 infants who survived lower respiratory tract infection (LRI) and 9 infants who died from bronchiolitis.

Characteristic	LRI group	Bronchiolitis group[a]
Age, months	4 (0.5–12)	3 (1–12)
Female sex	16	3
Infectious agent		
RSV	22	9
Influenza virus	10	0
Adenovirus	1	0
O_2 sat, %	93 (89–99)	. . .
Illness duration, days	3 (1–7)	<1 (<1–3)

NOTE. Data are no. of infants or median value (range). O_2 sat, oxygen saturation; RSV, respiratory syncytial virus.

[a] Infants with fatal RSV bronchiolitis demonstrated no evidence of immunodeficiency. There was no evidence of bacterial infection at autopsy, and no blood samples obtained before or after death yielded positive results on culture. The cause of death was identified by 2 pathologists and bronchiolitis. No record of antibiotic administration appears in autopsy reports. One of the 9 infants received mechanical ventilation for 3 days. Five had underlying cardiac disease. The features of bronchiolitis were not different between patients with or without cardiac disease or between patients who did or did not receive mechanical ventilation.
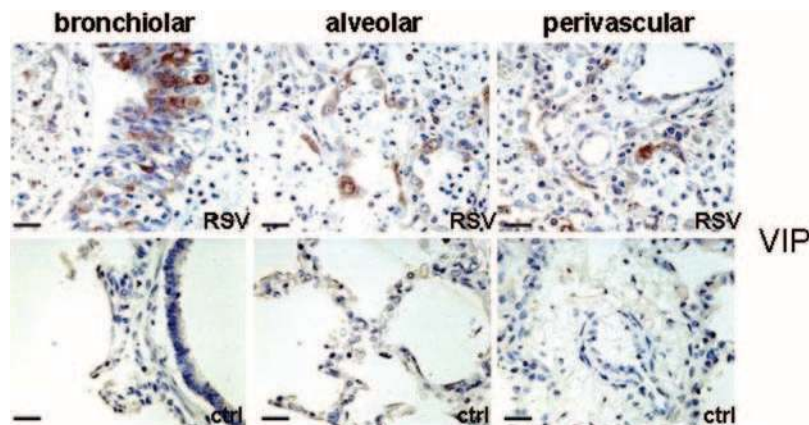
Figure 4. Detection of vasoactive intestinal peptide (VIP) in respiratory syncytial virus lower respiratory tract infection (RSV LRI). VIP was detected by immunohistochemistry analysis in formalin-fixed, paraffin-embedded lung tissue obtained at autopsy from infants who died of acute RSV LRI (RSV) or asphyxia (ctrl). Representative fields from bronchiolar *(left),* alveolar *(middle),* and perivascular *(right)* spaces are shown. Primary antibodies were detected with biotinylated secondary antibodies and streptavidin/HRP peroxidase conjugate, followed by the peroxidase substrate diaminobenzidine (original magnification, ×40). *Scale bar,* 15 μm.
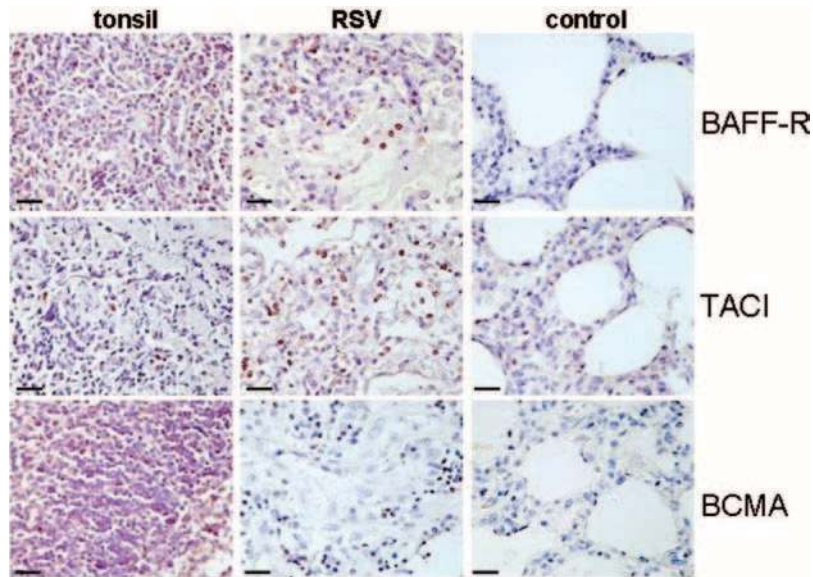
Figure 6. Detection of receptors for a proliferation-inducing ligand (APRIL) and B cell–activating factor (BAFF) in respiratory syncytial virus lower respiratory tract infection (RSV LRI). BAFF-R *(upper panels),* transmembrane activator calcium modulator and cyclophylin ligand interactor (TACI; *middle panels*), and B cell maturation antigen (BCMA; *lower panels*) were detected by immunohistochemistry analysis in formalin-fixed, paraffin-embedded tonsil tissue (positive control; *left panels*), lung tissue obtained at autopsy from infants who died of acute RSV LRI *(center panels),* or lung tissue obtained from age-matched control infants who died of asphyxia *(right panels).* Primary antibodies were detected with biotinylated secondary antibodies and streptavidin/HRP peroxidase conjugate, followed by the peroxidase substrate diaminobenzidine (original magnification, ×40). *Scale bar,* 15 μm.

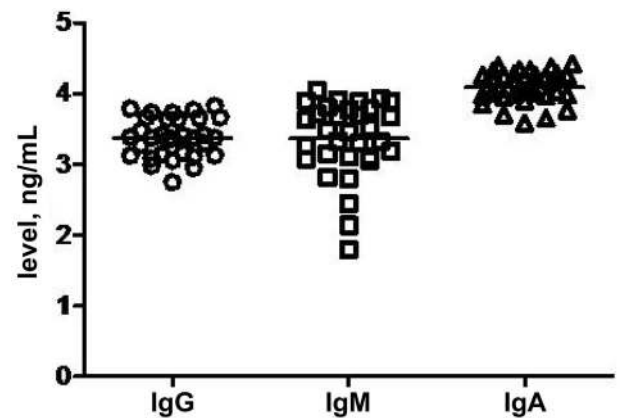**Figure 7.** Quantitation of total immunoglobulin levels in nasopharyngeal secretions. Total IgG *(circles),* total IgM *(squares),* and total IgA *(triangles)* levels were measured in nasopharyngeal secretions by ELISA. *Bars,* mean values.

Table 2. Correlation of respiratory syncytial virus (RSV)–directed immunoglobulin (Ig) with total immunoglobulin (Ig) and anti-nuc Ig.

RSV Ig	Total IgM		Total IgG		Total IgA		nuc IgM		nuc IgG		nuc IgA	
	r	P	r	P	r	P	r	P	r	P	r	P
IgM	0.625	<.001	0.314	.080	0.280	.120	0.324	.258	0.043	.900	0.349	.050
IgG	0.327	.068	0.447	.010	0.348	.050	0.546	.042	0.145	.672	0.012	.946
IgA	0.418	.017	0.278	.123	0.296	.100	0.253	.383	0.036	.916	0.705	<.001

NOTE. Nasopharyngeal secretions from 33 infants hospitalized with lower respiratory tract infection were analyzed for total Ig content and for Ig reactive with either RSV or nuclear/nucleosomal antigens. Log-transformed values were assessed for correlation, using a Pearson 2-tailed test.
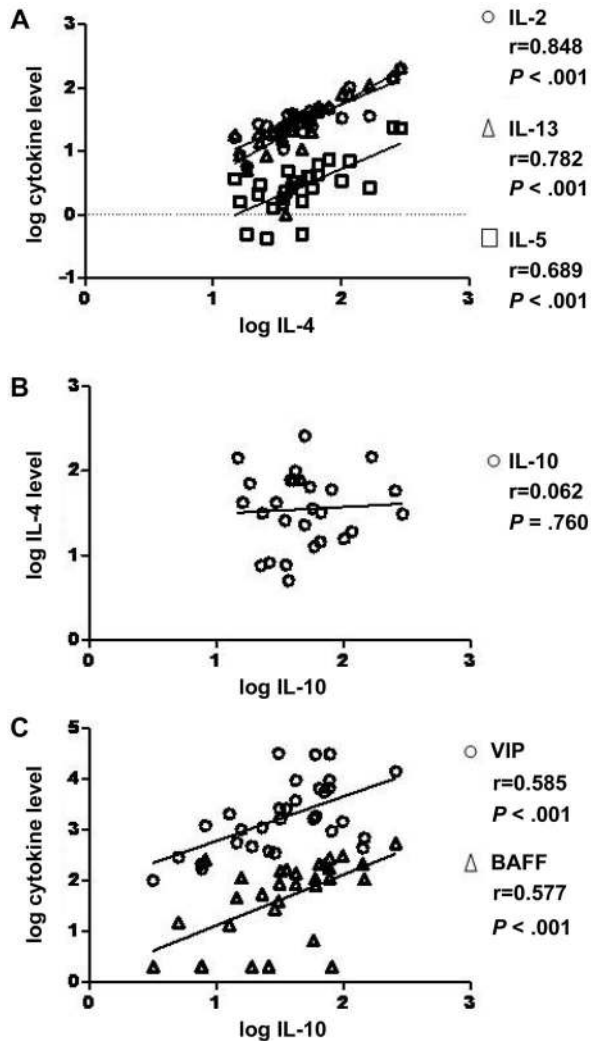

Figure 9. Correlation of cytokines in nasopharyngeal secretions. *A,* IL-2 *(circles),* IL-13 *(triangles),* and IL-5 *(squares)* measurements in each nasopharyngeal secretion were log transformed and plotted individually as a function of IL-4. *B,* Measurements of IL-10 and IL-4 in each nasopharyngeal secretion were log transformed and plotted individually. *C,* Vasoactive intestinal peptide and B cell–activating factor (BAFF) measurements in each nasopharyngeal secretion were log transformed and plotted as a function of IL-10. Correlation coefficients (r) were determined using Pearson analysis of covariance.

Table 4. Correlates of oxygen saturation (O_2 sat) in infants with lower respiratory tract infection (LRI).

Group	RSV IgM		RSV IgG		RSV IgA	
	r	P	r	P	r	P
Overall (*n* = 33)	0.503	.038	−0.261	.320	0.516	.033
RSV LRI (*n* = 22)	0.508	.030	−0.188	.461	0.552	.016
Non-RSV LRI (*n* = 11)	−0.306	.580	−0.591	.240	−0.332	.550

NOTE. Nasopharyngeal secretions from 33 infants hospitalized with LRI were analyzed for respiratory syncytial virus (RSV)–directed IgG, IgM, and IgA. O_2 sat was determined by pulse oximetry at the time of nasopharyngeal secretions collection. Log-transformed values were assessed for correlation, using a Pearson 2-tailed test. The entire cohort was analyzed together, then infants with and those without RSV LRI were considered separately.
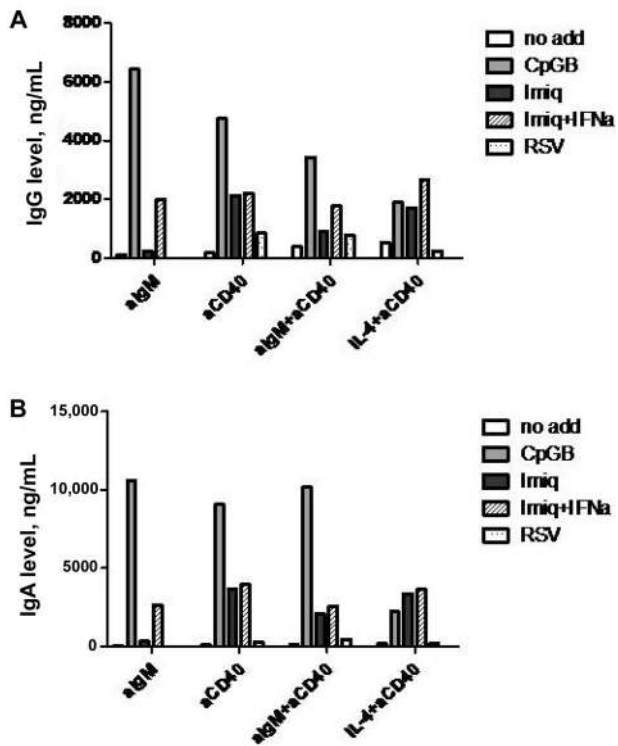

Figure 11. Combined Toll-like receptor 7 (TLR7) agonist, interferon (IFN), and B cell–activating factor (BAFF) promote immunoglobulin secretion in vitro. Peripheral blood B lymphocytes were stimulated with anti-IgM, CD40, anti-IgM with CD40, or IL-4 with CD40, plus CpG-B oligodeoxynucleotides, Imiq, Imiq with IFNa, or respiratory syncytial virus (RSV) virions. Secretion of IgG *(A)* and IgA *(B)* in supernatants was measured on day 7.