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Inflammatory Response to Oxygen and Endotoxin in Newborn Rat Lung Ventilated With Low Tidal Volume

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ABSTRACT: Herein, we determined the contribution of mechanical ventilation, hyperoxia and inflammation, individually or combined, to the cytokine/chemokine response of the neonatal lung. Eight-day-old rats were ventilated for 8 h with low (~3.5 mL/kg), moderate (~12.5 mL/kg), or high (~25 mL/kg) tidal volumes (V_T) and the cytokine/chemokine response was measured. Next, we tested whether low-V_T ventilation with 50% oxygen or a preexisting inflammation induced by lipopolysaccharide (LPS) would modify this response. High-, moderate-, and low-V_T ventilation significantly elevated CXCL-2 and IL-6 mRNA levels. Low-V_T ventilation with 50% oxygen significantly increased IL-6 and CXCL-2 expression *versus* low-V_T ventilation alone. LPS pretreatment combined with low-V_T ventilation with 50% oxygen amplified IL-6 mRNA expression when compared with low V_T alone or low V_T + 50% O_2 treatment. In contrast, low V_T up-regulated CXCL-2 levels were reduced to nonventilated levels when LPS-treated newborn rats were ventilated with 50% oxygen. Thus, low-V_T ventilation triggers the expression of acute phase cytokines and CXC chemokines in newborn rat lung, which is amplified by oxygen but not by a preexisting inflammation. Depending on the individual cytokine or chemokine, the combination of both oxygen and inflammation intensifies or abrogates the low V_T-induced inflammatory response. **(*Pediatr Res* 68: 63–69, 2010)**

Bronchopulmonary dysplasia (BPD) remains the most important cause of respiratory morbidity in very low birth weight infants. Mechanical ventilation (MV), intra-uterine infections and oxidative stress up-regulate proinflammatory cytokines/chemokines including IL-1β, IL-6, and IL-8 (1). Elevated concentrations of these cytokines/chemokines in amniotic fluid and bronchoalveolar lavage fluid (BALF) have been associated with BPD (2,3). The contribution of each risk factor, alone or combined, to the inflammatory response remains to be determined.

Ample animal studies have suggested that high frequency oscillatory ventilation (HFOV) is less injurious compared with conventional ventilation (CMV) (4,5). However, in the baboon model of BPD impaired alveolarization and capillary development occurred in spite of appropriate oxygenation and use of HFOV (4). MV with moderate and high tidal volumes increased lung cytokine/chemokine response to systemic endotoxin in rabbits (6) and newborn rats (7). Oxidant injury alone can produce the pathologic features of BPD (8). Inflammatory cells such as monocytes and neutrophils are primary contributors to the oxygen-induced lung injury (9,10). Other animal studies have investigated the contributions of oxygen exposure and MV alone or in combination. In term ventilated piglets hyperoxia caused less lung damage than hyperoxia combined with hyperventilation but more than hyperventilation alone (11). Premature baboons ventilated with the minimum necessary supplemental oxygen had significant less damage than those ventilated with 100% oxygen (12), but alveolarization and capillary development was still impaired (4).

To our knowledge, no previous study has evaluated the combination of MV, hyperoxia, and inflammation. Therefore, we first assessed the effect of low, moderate, and high tidal volume (V_T) ventilation on cytokine/chemokine production. To mimic the clinical situation we used a newborn rat model (7). Rat lungs at birth have a saccular appearance, similar to the preterm neonate, and alveolarization in rats occurs postnatally between P4 and P21. High-V_T ventilation has been reported to cause injury in newborn rat lung (13,14) and was included as positive control. We hypothesized that continuous cyclic (over)stretching of the primitive air sacs would adversely affect cytokine/chemokine production and the adverse effect would be stretch-amplitude dependent. Second, we assessed the effect of low-V_T ventilation with controlled oxygen superimposed on a systemic inflammation on cytokine/chemokine production. To induce a mild systemic inflammation, we pretreated the newborn rats with lipopolysaccharide (LPS) (7). We hypothesized that low-V_T ventilation with 50% oxygen superimposed on a relatively mild systemic inflammation would enhance the adverse inflammatory mediator production by low tidal volume alone.

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Abbreviations: **V_T,** tidal volume; **BPD,** bronchopulmonary dysplasia; **BALF,** bronchoalveolar lavage; **HFOV,** high frequency oscillatory ventilation; **LPS,** lipopolysaccharide; **NV,** non-ventilated; **MV,** mechanical ventilation; **LV_T,** MV with low tidal volume; **MV_T,** MV with moderate tidal volume; **HV_T,** MV with high tidal volume; **LV_T + LPS,** LV_T after exposure to LPS; **LV_T + O_2,** LV_T and 50% oxygen; **LV_T + LPS/O_2,** LV_T and 50% oxygen after exposure to LPS; **PEEP,** positive end expiratory pressure; **MPO,** myeloperoxidase; **CXCL,** chemokine (C-X-C motif) ligand

METHODS

Animals. In two series of experiments, newborn (postnatal d 8) Spraque-Dawley rats (average weight 16.7 ± 1.0 g) were ventilated for 8 h using rodent ventilators (FlexiVent Scireq, Montreal, PQ). After rats were anesthetized by *i.p.* injection of 30 mg/kg pentobarbital, a tracheotomy was performed. The trachea was cannulated with a 1-cm 22G cannula. Dynamic compliance was estimated from data obtained during a single-frequency forced oscillation maneuver, using a mathematical model-fitting technique according to the specifications of Scireq Inc. (Montreal, PQ). To determine ventilator settings, we started with the normal breathing frequency of a 8-d-old rat [~160/min (15)] and adjusted V_T and positive end expiratory pressure (PEEP) to achieve normal blood gases. The V_T and PEEP values for this frequency were ~12.5 mL.kg^{-1}and 2 cm H_2O, respectively. Next, we choose a lower and higher V_T and adjusted the ventilator frequency accordingly. Increasing the PEEP in the low tidal volume (LV_T) group led to increase of CO_2 and early death, most likely due to inadvertent PEEP. Animals were monitored by ECG. Rectal temperature was maintained around 37°C by using a thermal blanket, lamp and plastic wrap. To prevent spontaneous respiratory efforts 5 mg/kg pancuronium was administered *i.p.* Every 2 h 0.1 mL saline was administered to prevent dehydration. At the end of the ventilation period, a blood sample from the carotid artery was taken for blood gas analysis before euthanasia. Lung tissues were processed for histology or fresh frozen for molecular/protein analyses. The study was conducted according to the guidelines of the Canadian Council for Animal Care and with approval of the Animal Care Review Committee of the Hospital for Sick Children.

Series I: different V_T. Animals were randomly assigned to one of the following four groups: 1) nonventilated (NV) controls; 2) low V_T (V_T ~3.5 mL/kg, frequency 600/min, PEEP 0 cm H_2O); 3) moderate V_T (V_T ~12.5 mL/kg, frequency 160/min, PEEP 2 cm H_2O); 4) high V_T (V_T ~25 mL/kg, frequency 20/min, PEEP 2 cm H_2O).

Series II: preexposure to LPS and low-V_T ventilation with oxygen. Rats were randomly assigned to injection (*i.p.*) of either 3 mg/kg body weight of LPS from *E. coli* serotype 026:B6 or the same volume of 0.9% NaCl (7). Twenty-four hours after treatment animals were randomly assigned to one of the following six groups: 1) NV after NaCl injection; 2) NV after LPS injection (NV + LPS); 3) low V_T (V_T ~3.5 mL/kg, freq. 600/min, PEEP 0 cm H_2O) with room air after NaCl injection (LV_T): 4) low V_T with room air after LPS injection (LV_T + LPS); 5) low V_T with 50% oxygen after NaCl injection (LV_T + O_2); 6) Low V_T with 50% oxygen after LPS injection (LV_T + LPS/O_2).

Immunohistochemistry. After flushing lungs were infused *in situ* with 4% (vol/vol) paraformaldehyde (PFA) in PBS with a constant pressure of 20 cm H_2O to equalize filling pressure over the entire lung. Under these constant pressure conditions the cannula was removed and the trachea immediately ligated. The excised lung tissue was immersed in 4% (vol/vol) PFA in PBS overnight and then dehydrated in an ethanol/xylene series and embedded in paraffin. Five micron sections were deparaffinized, rehydrated in a graded series of ethanol. After antigen retrieval by heating in 10 mM sodium citrate pH 6.0, endogenous peroxidase quenching and blocking with NGS/BSA, sections were stained with 1:200 diluted mouse anti-CD68 (Serotec, Raleigh, NC) and 1: 100 diluted rabbit anti-myeloperoxidase (MPO) antibodies (Lab Vision Corporation, Fremont, Canada), using the avidin-biotin (ABC) immunoperoxidase method. Biotinylated rabbit anti-mouse IgG or goat anti-rabbit IgG were used as secondary antibodies, respectively. All sections were counterstained with hematoxylin.

Quantitative RT-PCR. Total RNA was extracted from lung tissues and reverse transcribed. cDNA was amplified for our target genes (IL-1β'IL-6, IL-10, CXCL-2 GRO2/MIP-2: macrophage inflammatory protein-2, a functional rodent homolog of human IL-8) and 18S as previously described (7,10). For relative quantification, polymerase chain reaction signals were compared between groups after normalization using 18S as an internal reference. Fold change was calculated.

Cytokine protein measurement in BALF. Lungs were infused with 0.5 mL of saline, followed by withdrawal and re-infusion two more times (7). Total protein was determined and IL-1β, IL-6 and CXCL-1 (also known as GRO1/KC) were measured in BALF using multiplex immunoassays for Luminex technology (7). CXCL-1 was measured because of lack of CXCL-2 detection kit for the Luminex system.

Statistical analysis. Stated otherwise all data are presented as mean ± SD. Data were analyzed using SPSS software version 15 (SPSS Inc, Chicago, IL). Depending on the distribution and the homogeneity of variation within the groups, statistical significance ($p < 0.05$) was determined by using either one-way ANOVA, or Kruskal-Wallis test. *Posthoc* analysis was performed using Duncan's multiple-range test (data presented as mean ± SD) or Mann-Whitney test (data presented as median and interquartile range). Because data of NV and LV_T groups of series I and II were similar, they were combined in the analysis.

RESULTS

Series I: different V_T

Physiologic data. Blood gases were in the normal range after 8 h of ventilation with different V_T (Table 1). Ventilator set V_T differed from inspired V_T, namely 6, 16, and 40 mL/kg for low, moderate, and high V_T, respectively. Dynamic compliance of the respiratory system is shown in Figure 1. Dynamic compliance of animals ventilated with high V_T significantly increased within minutes of ventilation and then remained stable for the rest of the experiment, indicative of larger airspaces and loss of tissue recoil due to hyperinflation (Fig. 2). Overall mortality during ventilation was 16.4% with no differences between V_T groups. No autopsy was performed, and electrolytes were not measured.

Inflammatory cells in lung. High-V_T ventilation was associated with a significant increase of MPO-positive neutrophils in comparison with NV, LV_T, and MV_T. To a lesser extent, moderate-V_T ventilation also increased the number of MPO-positive neutrophils (mainly in the alveolar space) in comparison with LV_T (Table 2). HV_T increased the number of neutrophils in both lung parenchyma and alveolar space. The number of macrophages (CD-68 antigen) did not alter among the ventilation groups (Table 2).

Cytokine mRNA expression. The effect of ventilation with different V_T on IL-1β, IL-6, CXCL-2, and IL-10 mRNA expression is shown in Figure 3. Low-V_T ventilation increased

Table 1. *Blood gas analysis after 8 hours of mechanical ventilation of 8-d newborn rats with different tidal volumes*

	Tidal volume		
	LV_T	MV_T	HV_T
pH	7.39 ± 0.08	7.39 ± 0.02	7.39 ± 0.04
$PaCO_2$ (mm Hg)	45 ± 4.4	44 ± 6.4	42 ± 2.2
PaO_2 (mm Hg)	72 ± 13.8	83 ± 8.1	91 ± 9.8
Saturation (%)	95 ± 3.2	96 ± 0.8	97 ± 1.2

Data are mean ± SD, $n = 4$ animals per group.

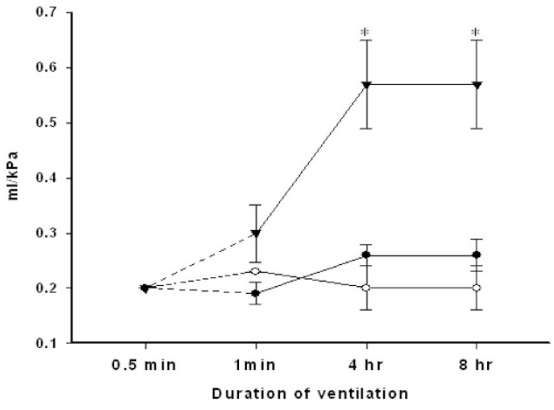

Figure 1. Dynamic compliance of 8-d newborn rats ventilated with room air and low V_T (○), moderate V_T (●), and high V_T (▼). Analysis of variance of parameters was assessed using 1-way ANOVA. Data are mean ± SD, $n = 8$ animals per group. *$p < 0.05$ *vs* ventilation for 1 min. Dash lines are extrapolated.

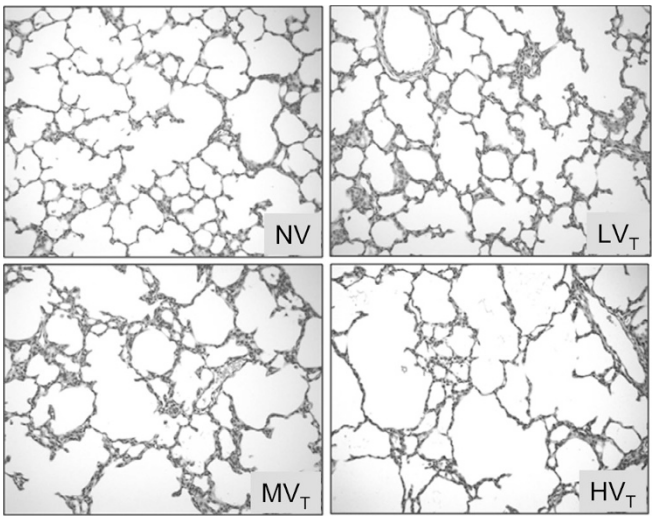

Figure 2. Representative sections of ventilated lungs stained with hematoxylin and eosin. Low-, moderate-, and high-V_T ventilation of 8-d neonatal rats do not show any areas of atelectasis, indicating no differences in lung recruitment. Air spaces are larger after high-V_T ventilation due to hyperinflation.

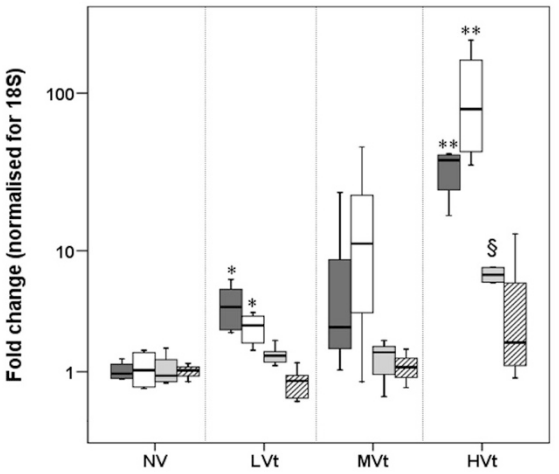

Figure 3. Effect of low-, moderate- and high-V_T ventilation on proinflammatory gene expression in lungs of 8-d newborn rats. ■ = CXCL-2, □ = IL-6, □ = IL-1β, ▨ = IL-10. Medians with 25th and 75th quartiles are shown; bars are 5th and 95th percentiles. NV and LV_T: $n = 8$; MV_T: $n = 6$; HV_T: $n = 4$. *$p < 0.05$ *vs* NV group, **$p < 0.05$ *vs* NV and LV_T group, §$p < 0.05$ *vs* other groups.

Table 2. *Effect of low, moderate, and high V_T ventilation on number of myeloperoxidase- and CD68-positive inflammatory cells in lungs of 8-d newborn rats*

	Tidal volume			
	NV	LV_T	MV_T	HV_T
MPO total	19.6 ± 8.4	14.0 ± 5.6	28.0 ± 3.0*	58.3 ± 4.2§
MPO tissue	9.7 ± 3.5	10.8 ± 6.3	8.3 ± 0.3	32.3 ± 13.1§
MPO air	10.0 ± 5.6	3.3 ± 2.8	19.7 ± 3.3*	26.0 ± 11.1*
CD 68	4.9 ± 0.9	6.1 ± 0.88	3.7 ± 0.4	4.4 ± 1.0

Number of immunopositive cells per unit area (40× high power field) are mean ± SD, $n = 5$ fields per slide, three slides per animal and four animals per group.

*$p < 0.05$ *vs* LV_T and HV_T.

†$p < 0.05$ *vs* NV and LV_T.

‡$p < 0.05$ *vs* other groups.

Table 3. *Effect of low, moderate, and high V_T ventilation on BALF cytokine protein content of 8-d newborn rats*

Cytokine	NV	LV_T	MV_T	HV_T
IL-6	0.7 (0.6–1.1)	2.2 (1.1–4.9)	8.0 (3.3–11.7)	5.2 (1.7–21.0)
IL-1β	0.13 (0.1–0.2)	0.2 (0.17–0.4)	0.33 (0.24–0.43)	1.0 (0.94–1.5)*
CXCL-1	10.3 (7.5–12.3)	9 (8.5–9.8)	7.9 (3.7–12.0)	14.9 (12.3–20.7)†

Concentrations are expressed as pg cytokine/100 pg of total BALF protein. Data are expressed as medians with 25th and 75th quartiles, $n = 8$ for groups NV and LV_T, $n = 4$ animals for other groups.

*$p < 0.05$ *vs* NV, LV_T, and MV_T.

†$p < 0.05$ *vs* LV_T and MV_T.

Table 4. *Blood gas analysis after 8 h of mechanical ventilation of 8-d newborn rats after exposure to endotoxin and/or 50% oxygen*

	Tidal volume			
	LV_T	LV_T + LPS	LV_T + O_2	LV_T + LPS/O_2
pH	7.39 ± 0.08	7.40 ± 0.04	7.32 ± 0.18	7.30 ± 0.08
$PaCO_2$ (mm Hg)	45 ± 4.4	36.0 ± 3.7	58.3 ± 14.4	54.3 ± 16.6
PaO_2 (mm Hg)	72 ± 13.8	87.7 ± 6.6*	131.0 ± 42.5*	167.0 ± 40.4†
Saturation (%)	95 ± 3.2	96.5 ± 1.2	96.4 ± 3.6	98.4 ± 1.6

Data are mean ± SD, $n = 4$ animals per group.

*$p < 0.05$ *vs* animals ventilated with LV_T.

†$p < 0.05$ *vs* animals ventilated with LV_T and LV_T + LPS.

CXCL-2 and IL-6 mRNA levels *versus* NV animals ($p < 0.05$), whereas those of IL-1β and IL-10 were not altered. Moderate-V_T ventilation seemed to further increase the expression of CXCL-2 *versus* NV animals and that of IL-6 *versus* NV and low-V_T rat pups, but the differences were not significant. Message levels of IL-1β and IL-10 were also not altered by MV_T. High-V_T ventilation significantly increased mRNA expression of IL-1β, CXCL-2, and IL-6, but not IL-10, *versus* all other groups ($p < 0.05$).

Cytokines in BALF. Table 3 shows the amount of IL-1β, IL-6, and CXCL-1 in BALF after 8 h of ventilation. The volume of lavaged material recovered from each animal (0.29 ± 0.08 mL) and BALF total protein content (0.22 ± 0.08 μg/μL) did not differ significantly between treatment groups There was a trend toward an increase of IL-1β and IL-6 with increasing V_T. However, only HV_T ventilation significantly increased IL-1β and CXCL-1 levels.

Series II: pre-exposure to LPS and low-V_T ventilation with oxygen

Physiologic data. Ventilation for 8 h with low V_T with room air after exposure to LPS (LV_T + LPS), ventilation with 50% oxygen (LV_T + O_2), and ventilation with oxygen after exposure to LPS (LV_T + LPS/O_2) resulted in normal pH and $PaCO_2$ (Table 4). Low-V_T ventilation with room air after exposure to LPS (LV_T + LPS) and ventilation with 50% oxygen (LV_T + O_2) significantly increased the PaO_2 when compared with ventilation with room air (LV_T). The combination of ventilation with oxygen and exposure to LPS (LV_T + LPS/O_2) further increased PaO_2 *versus* LV_T and LV_T + LPS groups ($p < 0.05$). Mean airway pressures and peak pressures remained stable during the ventilation period and were not different between groups. Dynamic compliance of the respiratory system is shown in Figure 4. Ventilation with room air after exposure to LPS (LV_T + LPS), ventilation with 50% oxygen (LV_T + O_2), and ventilation with oxygen after exposure to LPS (LV_T + LPS/O_2) significantly decreased the

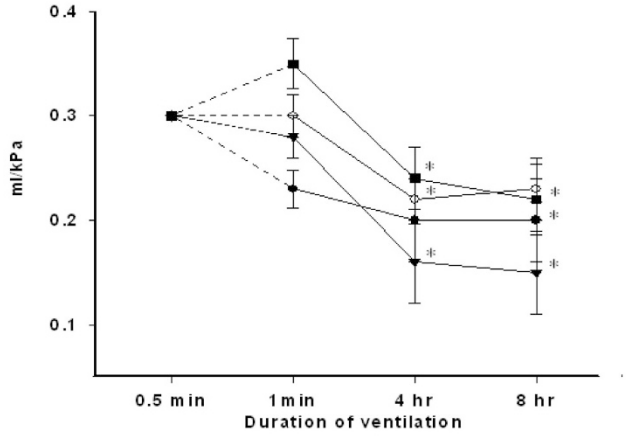

Figure 4. Dynamic compliance of 8-d newborn rats ventilated with low V_T and room air (●), low V_T and room air after exposure to LPS (○), low V_T and 50% oxygen (▼) and low V_T with 50% oxygen after exposure to LPS (▽). Data are mean ± SD, $n = 8$ animals per group. The dash lines are extrapolated. *$p < 0.05$ *vs* ventilation for 1 min.

Table 5. *Combined effect of endotoxin, oxygen, and low V_T ventilation on number of myeloperoxidase- and CD68-positive inflammatory cells in lungs of 8-d newborn rats*

	Tidal volume		
	NV	NV + LPS	LV_T
MPO total	19.6 ± 8.4	211.0 ± 74.0*	14.0 ± 5.6
MPO tissue	9.7 ± 3.5	187.3 ± 73.3*	10.8 ± 6.3
MPO air	10.0 ± 5.6	23.8 ± 2.2†	3.3 ± 2.8
CD 68	4.9 ± 0.9	11.8 ± 0.33*	6.1 ± 0.88
	LV_T + LPS	$LV_T + O_2$	LV_T + LPS/O_2
MPO total	186.0 ± 117.5*	12.0 ± 1.4	188.7 ± 257.9§
MPO tissue	169.3 ± 114.3*	8.3 ± 2.9	176.8 ± 259.3§
MPO air	16.7 ± 9.7‡	3.7 ± 3.5	11.8 ± 3.4*
CD 68	2.5 ± 2.8*	7.4 ± 0.6	13.4 ± 2.0*

Number of immunopositive cells per unit area (40× high power field) are mean ± SD, $n = 5$ fields per slide, three slides per animal and four animals per group. $LV_T + O_2$ group was not compared with NV + LPS and LV_T + LPS groups.

*$p < 0.05$ *vs* NV and LV_T.

†$p < 0.05$ *vs* NV, LV_T, and LVT + LPS/O_2.

‡$p < 0.05$ *vs* LV_T.

§$p < 0.05$ *vs* NV, LV_T, and $LV_T + O_2$.

dynamic compliance after 4 h of ventilation. Loss of compliance can be explained by increase of stiffness of the lung as a result of lung injury. No further worsening of dynamic compliance was seen during the last 4 h of ventilation. Overall mortality during ventilation was 8.1% with no differences between the four groups. No autopsy was performed.

Inflammatory cells in lung. Mean values and ranges for the number of macrophages (CD68-antigen) and MPO-positive neutrophils per unit area are shown in Table 5. Exposure to LPS, independent of ventilation with or without oxygen, was associated with a significant increase of MPO-positive neutrophils as well as CD-68 positive macrophages. The number of neutrophils was profoundly increased in the parenchyma and to a lesser extent in the alveolar space.

Cytokine mRNA expression. The effect of LPS, LV_T ventilation and LV_T ventilation after exposure to LPS (LV_T + LPS) on IL-1β, IL-6, CXCL-2, and IL-10 mRNA expression is shown in Figure 5*A*. LPS significantly increased the expression of IL-6 and IL-1β even 24 h after administration when compared with saline treated animals (LPS > NV, $p < 0.05$). However, mRNA expression of CXCL-2 and IL-10 was not altered by the LPS pretreatment. The combination of LV_T and LPS pretreatment did not increase the expression of IL-6 and CXCL-2 above that observed in animals ventilated with LV_T ($LV_T \approx LV_T$ + LPS > NV; $p < 0.05$) and decreased IL-6 mRNA levels compared with LPS treatment alone (LPS > LV_T + LPS, $p < 0.05$). LV_T ventilation after exposure to LPS significantly decreased the expression of IL-10 mRNA ($p < 0.05$). Figure 5*B* shows the effect of ventilation with room air or 50% oxygen on IL-1β, IL-6, CXCL-2, and IL-10 mRNA expression. As shown in Series I, low V_T ventilation with room air increased the expression of IL-6 and CXCL-2 *versus* NV controls. Ventilation with 50% oxygen further increased the message levels of both cytokines ($LV_T + O_2 > LV_T$, $p < 0.05$). The expression of IL-1β and IL-10 mRNA was not altered by ventilation with room air or oxygen. Ventilation with 50% O_2 after LPS pretreatment (Fig. 5*C*) resulted in the greatest increase in IL-6 mRNA levels (LV_T + LPS/O_2 > NV + LPS > LV_T + LPS ≈ $LV_T + O_2 > LV_T$ > NV) (Fig.

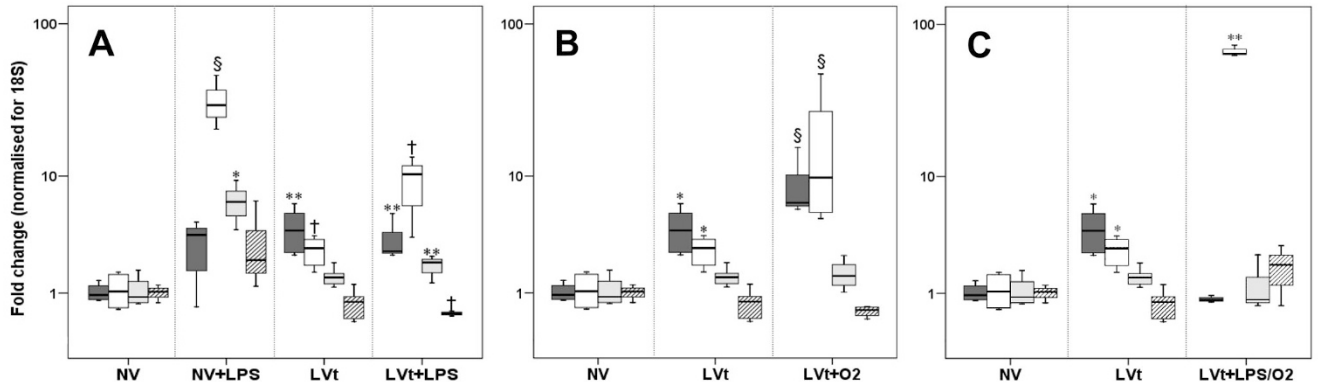

Figure 5. Effect of endotoxin and oxygen on low-V_T ventilation triggered pro-inflammatory gene expression in lungs of 8-d newborn rats. ■ = CXCL-2, □ = IL-6, □ = IL-1β, ▨ = IL-10. Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles. NV and LV_T: $n = 8$; NV + LPS, $LV_T + O_2$ and LV_T + LPS: $n = 5$; LV_T + LPS/O_2: $n = 6$. *A*, Low V_T after exposure to LPS. *$p < 0.05$ *vs* NV and LV_T, **$p < 0.05$ *vs* NV, §$p < 0.05$ *vs* NV, LV_T, and LV_T + LPS, †$p < 0.05$ *vs* NV and NV + LPS. *B*, Low-V_T ventilation with 50% oxygen. *$p < 0.05$ *vs* NV and $LV_T + O_2$, §$p < 0.05$ *vs* NV and V_T. *C*, Low-V_T ventilation with 50% oxygen after LPS exposure. *$p < 0.05$ *vs* NV and LV_T + LPS/O_2, **$p < 0.05$ *vs* NV and LV_T.

Table 6. *Combined effect of endotoxin, oxygen, and low V_T ventilation on BALF cytokine protein content of 8-d newborn rats*

Cytokine	NV	NV + LPS	LV_T
IL-6	0.7 (0.6–1.1)	0.7 (0.5–1.1)	2.2 (1.1.–4.9)
IL-1β	0.13 (0.1–0.2)	0.07 (0.06–0.08)*	0.2 (0.17–0.4)
GRO/KC	10.3 (7.5–12.3)	22.2 (18.3–23.4)†	9 (8.5–9.8)
	LV_T + LPS	LV_T + O_2	LV_T + LPS/O_2
IL-6	3.5 (2.6–3.8)‡	2.1 (1.3–2.9)§	3.0 ± 0.68‡
IL-1β	0.22 (0.18–0.23)	0.45 (0.38–0.55)§	0.29 (0.25–0.45)‖
GRO/KC	15.7 (10–21.4)¶	7.6 (7.0–9.2)	9.6 (7.4–12.2)

Concentrations are expressed as pg cytokine/100 pg of total BALF protein. Data are expressed as medians with 25th and 75th quartiles, $n = 8$ for groups NV and LV_T, $n = 4$ animals for other groups. LV_T + O_2 group was not compared with NV + LPS and LV_T + LPS groups.

*$p < 0.05$ *vs* NV, LV_T, LV_T + LPS, and LV_T + LPS/O_2.

†$p < 0.05$ *vs* NV, LV_T.

¶$p < 0.05$ *vs* NV, LV_T, and LV_T + LPS/O_2.

‖$p < 0.05$ *vs* NV, NV + LPS, and LV_T + LPS.

§$p < 0.05$ *vs* NV.

‡$p < 0.05$ *vs* NV and NV + LPS.

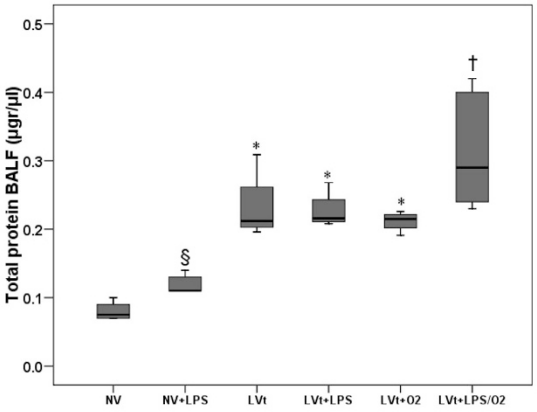

Figure 6. Effects of low-V_T ventilation, endotoxin, and oxygen on BALF total protein of 8-d newborn rats. Medians with 25th and 75th quartiles are shown, bars are 5th and 95th percentiles. NV and LV_T: $n = 8$; NV + LPS: $n = 5$; LV_T + LPS and LV_T + O_2: $n = 4$; LV_T + LPS/O_2: $n = 6$. LV_T + O_2 group was not compared with NV + LPS and LV_T + LPS groups. *$p < 0.05$ *vs* NV; §$p < 0.05$ *vs* LV_T, LV_T + LPS, and LV_T + LPS/O_2; †$p < 0.05$ *vs* LV_T + LPS and LV_T + O_2.

5*A-C*). Interestingly, the combination of ventilation with oxygen and preexposure to LPS decreased the expression of CXCL-2 *versus* ventilation with room air (LV_T + LPS/$O_2 \approx$ NV $< LV_T$, $p < 0.05$) (Fig. 5c).

Cytokines in BALF. Table 6 shows the amount of IL-1β, IL-6, and CXCL-1 protein in BALF after exposure to either LPS, ventilation with room air or oxygen, ventilation after exposure to LPS or the combination of ventilation with oxygen after exposure to LPS. The amount of BALF total protein was significantly increased after exposure to LPS and further increased after ventilation with room air or oxygen and after ventilation with oxygen of a LPS-exposed lung (LV_T + LPSLO2 $\approx LV_T + O_2 \approx LV_T$ + LPS $\approx LV_T >$ LPS $>$ NV, $p < 0.05$), consistent with lung injury (Fig. 6). The volume of lavaged material recovered from each animal (0.31 ± 0.06 mL) did not differ significantly between the groups. IL-6 content of BALF increased after ventilation with oxygen ($LV_T + O_2 >$ NV, $p < 0.05$). An increase was also noted after ventilation of a LPS-exposed lung (LV_T + LPS $>$ NV $\approx$ NV + LPS, $p < 0.05$). Ventilation of a LPS-exposed lung with oxygen did not further increase the BALF IL-6 content (LV_T + LPS/$O_2 \approx LV_T + O_2 \approx LV_T$ + LPS, $p > 0.05$). The concentration of IL-1β in BALF only increased after ventilation with oxygen and after ventilation with oxygen after LPS exposure (LV_T + LPS/$O_2 \approx LV_T + O_2 > LV_T$ + LPS $\approx LV_T \approx$ NV, $p < 0.05$). Independent of ventilation and oxygen, the concentration of CXCL-1 was increased after exposure to LPS (NV + LPS $\approx LV_T$ + LPS $> LV_T \approx LV_T + O_2 \approx LV_T$ + LPS/O_2, $p > 0.05$).

DISCUSSION

MV, (intrauterine) infection, and oxygen are well-recognized risk factors for BPD and known to trigger a proinflammatory response. In this study, we demonstrate that low-V_T ventilation—presumed to be a less injurious form of ventilation—triggers a proinflammatory cytokine/chemokine response in neonatal rats, which is amplified by ventilation with oxygen, but not endotoxin pretreatment. The combination of ventilation with oxygen and endotoxin pretreatment either intensifies or abrogates the low V_T response, depending on individual cytokine or chemokine.

In the first series of experiments, we assessed the effect of low-, moderate-, and high-V_T ventilation on pro and anti-inflammatory cytokine/chemokine production. Clinical data and experimental studies using premature animal models have compared HFOV (HFOV: low V_T) and CMV (CMV: high V_T) with respect to pro-inflammatory cytokine/chemokine production and release into the alveolar and/or vascular compartment. Some studies reported findings favoring low-V_T ventilation (4,16), whereas others did not find any significant differences in cytokine/chemokine production/release between CMV and HFOV ventilation (17,18). In this study, we found a significant increase of mRNA expression of CXCL-2 and IL-6 after 8 h of low-V_T ventilation. Further increases were noted with increasing V_T as reported previously (14). Although there was a tendency to higher concentrations of IL-6 and IL-1β protein in the BAL fluid, we did not find significant increases in total number of inflammatory cells after low V_T ventilation. In contrast, moderate and to a higher extent high-V_T ventilation increased the inflammatory response as shown by increases in IL-6, IL-1β, and CXCL-1 content in BALF and number of inflammatory cells in the lung. In addition, high V_T altered the dynamic compliance due to hyperinflation as shown previously (7). The difference between set and inspired V_T suggests tube leakage and/or expansion of tubing of the ventilator circuit which may influence the compliance measurement. No clinical signs for pneumothorax were observed. Thus, low-V_T ventilation with room air for 8 h results in a mild inflammatory response in the neonatal lung that is not overtly injurious (no change in dynamic compliance but an increase in the amount of protein in BALF). However, it is plausible that longer durations of low-V_T ventilation increase the levels of pro-inflammatory cytokines sufficiently to cause lung injury. Although the low-V_T ventilation strategy (~3.5 mL/kg) cannot be compared with clinical

applied HFOV (V_T 0.5–2.0 mL/kg), our finding of an inflammatory response may explain why randomized controlled trials did not show any beneficial effect of protective HFOV in preventing BPD in premature infants. This explanation is supported by several studies in which MV elevates pulmonary cytokines without cellular injury (19–21).

LPS triggers a network of inflammatory responses by activation of macrophages and recruitment of neutrophils, which was also observed in this study. Activated macrophages release different proinflammatory cytokines and neutrophil activation causes the production of oxygen radicals and the release of granular enzymes, which are associated with injurious processes in the lung (10,22,23). Especially, CXC chemokines and IL-8 activate and attract neutrophils into interstitial and alveolar spaces of the lung. Blocking neutrophils by blocking the CXCL-2 receptor led to increased alveolar formation and CXCL-2 null mice exhibited less ventilator-induced lung injury (10,24). Several studies have shown that high-V_T ventilation combined with another lung injury amplifies the inflammatory response in adult lungs (25,26). A significant increase of CXCL-2 was measured in BALF of adult rats when high-V_T ventilation (40 mL/kg) was superimposed on a systemic inflammatory process (25). Ventilation of adult mice with smaller V_T (6 mL/kg) after induction of lung injury with hydrochloric acid showed a significant increase of IL-6 content in lung tissue *versus* ventilation alone (26). High-V_T ventilation of neonatal rat lungs superimposed on a systemic inflammation induced by LPS (7) significantly increased IL-6 mRNA expression compared with high-V_T ventilation alone. In this study, we found an additive effect of 50% oxygen on LV_T-induced expression of IL-6, in agreement with our previous study using 100% oxygen and high V_T (14). Whether the additive effect of oxygen on IL-6 expression is harmful remains a matter of speculation. IL-6 has long been considered a pro-inflammatory cytokine but adult transgenic mice that over-express IL-6 are more resistant to oxidative injury (27), whereas newborn IL-6 transgenic mice demonstrated more cell death after 100% oxygen exposure (28). These data suggest that high levels of IL-6 in the lung may actually be beneficial in adult mice but harmful in newborn mice. Surprisingly, we found that preexposure to LPS did not further increase IL-6 message levels when compared with LV_T ventilation alone. In contrast, the combination of low-V_T ventilation with 50% oxygen after exposure to LPS further amplified the mRNA expression of IL-6. The increased expression of IL-6 mRNA after LV_T ventilation with and without oxygen, which was reflected in increased concentrations of this cytokine in BALF. LPS preexposure did not further increase BALF levels of IL-6. LV_T ventilation with 50% oxygen also increased the mRNA expression of CXCL-2 *versus* ventilation alone. Pretreatment with LPS had no significant additive effect on CXCL-2 expression, in contrast to our previous findings with high-V_T ventilation (7). No correlation between CXCL-2 mRNA expression and CXCL-1 protein content in BALF was observed, suggesting that CXCL-2 and CXCL-1 are not interchangeable. Together, these findings suggest that low V_T ventilation avoids the synergistic effect of ventilation and systemic inflammation on cytokine/chemokine expression seen with high V_T (7,10). In contrast, oxygen has an additive effect on ventilation-induced cytokine/chemokine expression which is V_T independent. Interestingly, upregulated CXCL-2 message was reduced to NV control levels when LPS-treated newborn rats were ventilated with 50% oxygen. This complex immunomodulatory regulation of CXCL-2 and IL-6 resembles that seen in LPS tolerant mice (29). Pulmonary IL-6 levels were significantly increased in the tolerant mice on further LPS challenge, whereas CXCL-2 levels were significantly reduced. However, it is unlikely that a single exposure to LPS induced tolerance in our model.

We conclude that even low tidal volume ventilation can mount an inflammatory response in the newborn rat, which is amplified by a clinically relevant concentration of inspired oxygen.

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