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Effect of serial-day exposure to nitrogen dioxide on airway and blood leukocytes and lymphocyte subsets

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ABSTRACT: Nitrogen dioxide (NO_2) is a free radical-producing oxidant gas. Inhalation of NO_2 could cause airway inflammation, and decrease immune function. This experiment tested the hypothesis that exposure to NO_2 would: 1) increase leukocytes in bronchoalveolar lavage (BAL); and 2) change the distribution of lymphocyte subsets and activation in BAL and peripheral blood (PB).

Using a counter-balanced, repeated-measures design, 15 healthy volunteers were exposed to filtered air (FA) or 2.0 parts per million NO_2 for 4 h·day^{-1} (4 × 30 min of exercise), for three consecutive days. Bronchoscopy was performed 18 h following each exposure set, and PB was drawn pre-exposure and pre-bronchoscopy. Flow cytometry was used to enumerate lymphocyte subsets and activation makers in BAL and PB.

In the bronchial fraction, there was an increase in the percentage of neutrophils following NO_2 exposure compared to FA (median (interquartile range): 10.6 (4.8–17.2)% *versus* 5.3 (2.5–8.3)%; p=0.005). In the BAL, there was a decrease in the percentage of T-helper cells following NO_2 exposure compared to FA (55.9 (40.8–62.7)% *versus* 61.6 (52.6–65.2)%; p=0.022). For PB, there were no between-condition differences in any leukocyte or lymphocyte subsets, or activation.

In conclusion exposure to nitrogen dioxide results in bronchial inflammation and a minimal change in bronchoalveolar lavage T-helper cells, and no changes in peripheral blood cells.

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Nitrogen dioxide (NO_2) is a free radical-producing oxidant gas. Atmospheric NO_2 is derived from the partial oxidation of nitric oxide, directly from combustion, and indirectly from reactions in ambient air [1]. The primary sources of NO_2 in indoor air are gas-burning equipment and tobacco smoke [2, 3], and in outdoor air are combustion engine and fossil-fuel burning emissions [4]. The concentration of NO_2 can range: up to 0.5–0.6 parts per million (ppm; 45 min period); 3.0–4.0 ppm (peak) in indoor air; 0.007–0.065 ppm (48 h mean); and 0.2–0.6 ppm (peak), in outdoor air [2, 5–7]. NO_2 has low water solubility (0.037 mL·mLH_2O^{-1} at 35°C) [8], therefore a large fraction of inhaled NO_2 could be deposited in the peripheral airways. Inhaled NO_2, or a component or reaction product of NO_2, could subsequently be delivered *via* tissue absorption and transfer across the blood-gas interface to the blood, therefore, systemic effects are possible.

Some epidemiological data suggest that exposure to ambient NO_2 is associated with an increased incidence of respiratory symptoms and illness [9–13]. However, other studies have indicated no association between NO_2 exposure and respiratory symptoms or illness [14–16]. A meta-analysis of the data from studies, involving children (age 5–12 yrs), indicated that a long-term increase in NO_2 exposure of 0.015 ppm was associated with a 20% increase in the odds of respiratory illness [17]. No such analysis is available for epidemiological data on adults.

Due to its cytotoxicity, inhalation of NO_2 can induce airway inflammation and change the leukocyte distribution in the airway lining fluid. Controlled single exposures of healthy individuals to 2.25, 3.5, 4.0, and 5.5 ppm NO_2 for 20 min, or 2.0 ppm NO_2 for 4 or 6 h have increased the number and percentage of neutrophils, decreased the percentage of macrophages in the bronchial fraction (BFx), increased the number of lymphocytes and mast cells, and decreased the number of macrophages in the bronchoalveolar lavage (BAL) at 4, 6, 8, 18, or 24 h post-exposure, compared to pre-exposure [18–22]. Controlled multiple-day exposures to 0.60 ppm NO_2 for 2 h on 4 out of 6 days, 1.5 ppm or 4.0 ppm NO_2 for 20 min every alternate day for 6 exposures did not change the percentage of macrophages, neutrophils, lymphocytes, or mast cells in either the BFx or BAL at 2 or 24 h post-exposure [23–25]. However, exposure to 4.0 ppm NO_2 for 20 min every alternate day for six exposures, or 2.0 ppm NO_2 for 4 h for 4 days, decreased the percentage of mast cells, and increased the percentage of neutrophils in the BFx 1.5 or 24 h post-exposure [25, 26].

Cellular immunity involves subsets of lymphocytes; T-cells, B-cells, and natural killer (NK) cells, which are

differentiated on the basis of surface-antigen cluster designations (CD). Lymphocytes become activated when stimulated by a specific antigen or nonspecific mitogens, resulting in cellular proliferation and differentiation. When activated, T-cells express specific surface proteins at higher levels; CD69 and CD25 are early and intermediate timeframe activation makers, respectively [27–29]. An NO_2-induced decrease in lymphocyte viability or activation could decrease immune function. Controlled single exposures to 3.5 ppm NO_2 for 20 min, or 2.0 ppm NO_2 for 4 h have increased the number of T-cells, B-cells, NK cells, and CD69+ T-cells in BAL at 6 or 24 h post-exposure [20, 21]. However, multiple-day exposures to 0.60 ppm NO_2 for 2 h on 4 of 6 days, or 1.5 or 4.0 ppm NO_2 on alternate days for 6 exposures, have decreased the percentage of T-suppressor cells and increased the T-suppressor/T-helper cell ratio, number of B-cells, and increased and decreased the number of NK cells [23–25]. Single and multiple day exposures to NO_2 have decreased the total lymphocyte number, as well as T-suppressor cells and CD4-CD8- T-cells in blood [21, 24, 25].

Due to free-radical activity, oxidation, and the formation of nitric and nitrous acid, NO_2 can affect cell function and viability by damaging lipids, proteins, and other biomolecules [30]. Exposure to NO_2 over a range of concentrations and durations has decreased total protein and albumin, the functional capacity of alpha-1-protease inhibitor, uric and ascorbic acids, and alveolar permeability, and has increased alpha-2-macroglobulin, in BFx and BAL [21, 26, 31–34].

Cumulatively, the reviewed data indicate that, for the exposure designs used, there are differences in the cellular response to multiple-day, compared to single-day NO_2 exposure, and that there is some degree of redistribution of lymphocyte subsets and activation following NO_2 exposure. There also appears to be a difference in the bronchial and bronchoalveolar regional cellular and protein responses to inhaled NO_2. This study was designed to test the hypothesis that serial-day exposure to 2.0 ppm NO_2 would change the distribution of leukocytes and lymphocyte subsets and activation of lymphocytes in BAL.

Methods

Design

This experiment used a repeated-measures, counterbalanced, single-blind design. Each subject was exposed, during separate exposure periods, to a control condition of filtered air (FA) and to 2.0 ppm NO_2 in FA. Subjects were unaware of the exposure condition. The exposure periods were for 4 h per day for three consecutive days. Total and differential cell counts, lymphocyte subset counts and activation, and total protein and lactate dehydrogenase (LDH) were measured in BAL post-exposure. Lymphocyte subset counts and activation were also measured in peripheral blood (PB) pre- and post-exposure. A minimum of 3 weeks separated the two exposure condition periods.

Subjects

The subject group consisted of 15 healthy volunteers who were characterized by physical characteristics, spirometric pulmonary function, specific airway resistance and nonspecific airway responsiveness (table 1). All subjects completed a medical history questionnaire, denied a history of pulmonary dysfunction, were nonsmokers, and had no respiratory tract illness in the 3 weeks preceding, or during, the experiment. Subjects abstained from strenuous exercise for 12 h and caffeine for 4 h prior to each session. Each subject was informed of the risks of the experiment and provided informed consent prior to participation. The procedures for this experiment were approved by the University of California, San Francisco Committee on Human Research (USA).

Equipment and measurements

Spirometric pulmonary function, specific airway resistance, and nonspecific airway responsiveness. Forced expired spirometry for the determination of forced vital capacity and forced expiratory volume in one second (FEV_1) was performed using a dry-rolling-seal spirometer (Model No. S400; Anderson Instruments, Spirotech Division, Atlanta, GA, USA) using standardized procedures [35]. Specific airway resistance was calculated as the product of total airway resistance and thoracic gas volume, which were both measured using a constant-volume body plethysmograph (Warren E. Collins, Braintree, MA, USA). Airway responsiveness was determined by the FEV_1 response to inhalation of nebulized (Model No. 646; Devilbiss, Somerset, PA, USA) phosphate-buffered saline (PBS) followed by increasing doses (0.26, 1.53, 4.09, 10.48 μmol) of methacholine in PBS delivered *via* a dosimeter (Rosenthal, Louisville, CO, USA) at the rate of 0.01 mL·breath^{-1} [36]. The airway responsiveness value was taken as either the cumulative dose of methacholine that produced a 20% decrease in FEV_1 from baseline (log-linear interpolation) or, when FEV_1 did not decrease by 20%, as the maximum dose of 10.48 μmol.

Exposure chamber and atmospheric monitoring. The exposure sessions were performed in a custom built steel and glass exposure chamber (Model No. W00327-3R; Nor-Lake Inc., Hudson, WI, USA), $2.5 \times 2.5 \times 2.4$ m in size, with an average airflow rate of 92 m^3·min^{-1}. The chamber air supply was sourced from ambient air that was filtered by passing through purifying (Model No. 6239; Purafil, Atlanta, GA, USA) and high efficiency particle (HEPA; Eco-Air, San Diego, CA, USA) filters. The filtered air was dehumidified by passing through a drier (Model No. HC-575; Cargocaire Engineering Corp., Amesbury, MA, USA). Subsequently, the air temperature was decreased with a chilled-water coil, and the humidity increased with steam (Model No. NHMC-050; Nortec, Ogdensbury, NY, USA), to obtain the preset temperature (20.0°C) and relative humidity (50%) conditions in the chamber. The temper-ature and relative humidity inside the chamber were monitored (3 min intervals) and controlled throughout the exposures (table 2: Model No. DSC 8500; Johnson Controls, Poteau, OK, USA). For the NO_2 exposures, NO_2 was supplied from gas cylinders containing 250–500 ppm NO_2 in air (Liquid Carbonic Corp., Oakbrook, IL, USA) which was piped through teflon tubing directly into the intake duct of the chamber. The NO_2 concentration in the chamber was monitored continuously (30 s intervals) throughout the exposures (table 2), chamber air being piped through teflon tubing directly to a chemiluminescent oxides of nitrogen (NO_x) analyser (Model No. 8840; Monitor Labs Inc., Englewood, CO, USA). The NO_x analyser was

Table 1. – Individual subjects physical, spirometric lung function and airway responsiveness characteristics

Subject	Sex	Age yrs	Height cm	Mass kg	FVC L	FEV1 L	FEV1/FVC %	S*R*aw L × $cmH_2O \cdot L^{-1} \cdot s^{-1}$	PD20 µmol
1	M	25	176	63.6	6.20	4.97	80	3.7	10.48
2	M	40	177	78.2	5.02	4.23	84	3.0	10.48
3	F	32	175	78.2	4.02	3.43	85	2.6	10.48
4	M	24	189	94.1	6.93	5.60	81	3.7	10.48
5	M	33	178	77.7	7.36	5.42	74	4.2	6.01
6	M	33	171	88.6	4.93	4.26	87	0.75	10.48
7	M	26	177	77.3	5.56	4.28	77	3.6	4.09
8	F	26	169	66.8	4.21	3.15	75	4.8	7.29
9	F	34	163	65.0	3.74	3.43	92	2.1	10.48
10	M	25	173	68.2	5.63	4.55	81	2.3	10.48
11	M	33	174	68.2	5.58	4.36	78	3.4	10.48
12	F	31	165	72.7	4.32	3.42	79	4.6	0.90
13	M	23	180	79.5	5.80	5.24	87	1.9	10.48
14	M	28	179	72.3	5.76	4.31	75	6.0	10.48
15	M	26	185	80.9	5.89	5.04	86	2.5	10.48
Mean±SD		29.3±4.8	175.4±6.8	75.4±8.6	5.40±1.04	4.38±0.78	81.4±5.3	3.3±1.3	8.90±2.99

Forced vital capacity (FVC), forced expiratory volume in one second (FEV1), FEV1/FVC %, specific airway resistance (S*R*aw) and provocative (cumulative) dose of methacholine causing a 20% fall in FEV1 (PD20) data are individual means of pre-exposure data for the filtered air and nitrogen dioxide conditions. M: male; F: female.

calibrated using a dynacalibrator (Model No. 340; Valco Instruments; Metronics, Huston, TX, USA) using ultrapure air (NO_x <0.001 ppm) and NO_2 of known concentration.

Exercise and pulmonary ventilation. During each exposure, exercise was utilized to induce mouth breathing and to increase minute ventilation ($\dot{V}_E$). The exercise consisted of either walking/running on a treadmill (Model No. M9.1; Precor, Bothel, WA, USA) or pedalling a cycle-ergometer (Model No. 90818e; Monark, Varberg, Sweden). The exercise intensity was adjusted for each subject to a target expired $\dot{V}_E$ of 25 $L \cdot min^{-1} \cdot m^{-2}$ body surface area. During exercise, $\dot{V}_E$ was calculated from tidal volume and breathing frequency measured using a pneumotachograph (Model No. 3; Fleisch; Rudolph Inst., Kansas City, MO, USA) at the 10 and 20 min interval of each 30 min exercise period. There was no significant difference in the mean $\dot{V}_E$ between the FA and NO_2 conditions (mean±SD; 47.8±5.6 $L \cdot min^{-1}$ *versus* 47.5±4.8 $L \cdot min^{-1}$; p=0.80).

Bronchoalveolar lavage and peripheral blood. The bronchoscopies were performed in a dedicated room at San Francisco General Hospital (CA, USA). Vital signs were measured pre- and post-bronchoscopy. Throughout the procedure, intravenous access was maintained, and arterial haemoglobin:oxygen (O_2) percentage saturation (Model No. Biox 3700; Ohmeda, Madison, WI, USA) and the electrocardiograph (Model No. SM-1; Physiocontrol, Redwood, IA, USA) were monitored. Atropine, to decrease airway secretions, and if required, midazolam, to maintain subject comfort, were administered intravenously. The posterior pharynx was anaesthetized using a 4% lignocaine gargle, a 1% lignocaine spray, and 4% lignocaine-soaked, cottontipped pledgets applied to the mucosa over the ninth cranial nerve. Supplemental O_2 was delivered *via* a nasal canula at 2 $L \cdot min^{-1}$. The bronchoscope (Model No. FB 18x; Pentax, Orangeburg, NY, USA), tipped with lignocaine jelly, was introduced through the mouth, and the larynx and airways were anaesthetized using 1% lignocaine solution as required. The bronchoscope was initially directed and wedged into the right middle lobe orifice (3 × 50 mL lavage), and subsequently the lingula (1 × 50 mL lavage). The purpose of the lingular lavage was to obtain alveolar macrophages for functional assays which will be the subject of another report. The lavages were performed using 0.9% saline heated to 37°C. The first 15 mL of lavage fluid returned was designated the BFx.

Table 2. – Exposure atmospheric characteristics

	Exposure condition	
	FA	NO_2
NO_2 ppm	–	1.95±0.14
Temperature °C	19.9±0.1	20.2±0.4
Relative humidity %	50.0±7.3	56.4±6.6

Values are mean±SD. FA: filtered air; NO_2: nitrogen dioxide.

Cell enumeration. All lavage samples were immediately placed on ice following collection. A 1 mL aliquot was removed for the total and differential cell counts. For the BAL and BFx fluids, total cell counts were performed using a haemocytometer (Hausser Scientific, Horsham, PA, USA), and differential cell counts were performed from cytospins subsequently stained (Diff-Quik; Baxter, San Juan, Puerto Rico). Total and differential (400 cells) cell counts were performed in duplicate by two counters. Total and differential cell counts in PB were performed by a certified commercial laboratory (PathLab, San Francisco, CA, USA).

In both the BAL and PB, three colour flow cytometry was used to enumerate lymphocyte subsets on the basis of CD as follows: B-cells (CD19+), T-cells (CD3+), T-helper cells (CD3+ CD4+), T-suppressor cells (CD3+ CD8+), and NK cells (CD3- CD16+ CD56+); and to examine the expression of the activation markers CD25 and CD69 on T- and NK cells. A 100 uL aliquot of either resuspended BAL cells (1×10^6 mL in PBS) or anticoagulated whole blood, were stained with saturating concentrations of anti-human leukocyte monoclonal antibodies (Caltag, Burlingame, CA, USA) for 30 min in the dark at 4°C. The cells were then washed using PBS, and erythrocytes were lysed (Coulter

lysis solution, San Francisco, CA, USA). Leukocytes were fixed using 500 mL of 1% paraformaldehyde for 5 min at 21°C, washed again with PBS, and stored in the dark at 4°C. Cells were analysed using a FACSort flow cytometer (Becton-Dickinson, San Jose, CA, USA) and CELLQuest software (San Jose, CA, USA). The proportion of lymphocytes that were T-cells was determined by measuring the ratio of T-cells to all lymphocytes (CD13- CD14- CD45 high). The ratio of T-cells to other subsets was determined by calculating the ratio of T-cells to B-cells, NK cells, T-helper cells and T-suppressor cells from samples stained in parallel with appropriate antibody combinations.

Biochemical assays. The BFx and BAL fluids were centrifuged to remove cells and debris; 1 mL aliquots of each fluid were removed for the LDH measurement; and the remaining supernatants were frozen at -80°C. The LDH assay was performed within 30 min of the lavage using a commercial kit (Sigma, St Louis, MO, USA) and a spectrophotometer (Model No. DU 65; Beckman, Fullerton, CA, USA). Total protein was measured in the previously frozen aliquots using the modified Lowry assay [37].

Procedure

Each subject completed nine laboratory sessions. Session 1 was for screening and characterization, subjects performing all pulmonary and airway function tests. Sessions 2–4 were the first exposure condition (FA or NO_2) period, and session 5 was for bronchoscopy, which was performed 18±1 h following the end of session 4. Following the minimum 3 week inter-condition period, sessions 6–8 were performed for the other exposure condition (FA or NO_2), and session 9 was for the bronchoscopy. Each exposure condition period consisted of 4-h exposures performed at the same time on three consecutive days. For each of the exposure sessions, pulmonary function tests were performed immediately pre- and post-exposure, and peak flow was monitored during the exposure at the end of each exercise period. During the exposure sessions, subjects alternated 30-min periods of rest and exercise. PB were collected from a forearm vein using standard procedures. Following the bronchoscopy sessions, subjects were transferred to the General Clinical Research Center (San Francisco General Hospital, CA, USA), where they were observed and released when recovered.

Statistical analyses

Most of the cell count and biochemical data were not normally distributed. Therefore, all BFx, BAL, and PB between-condition (FA *versus* NO_2) comparisons were performed using the Wilcoxon Signed Rank Test. For the PB data, the pre- to post-exposure change within each condition was used for comparisons between the two conditions. Statistical significance was set at $p<0.05$.

Results

Bronchoalveolar lavage

The volumes of lavage fluid returned in the FA and NO_2 conditions for the BFx were: median (interquartile range) 15.0 (14.4–15.0) mL and 15.0 (14.7–15.3) mL, respectively and for the BAL were 75.0 (63.8–83.0) mL and 63.6 (51.0–77.4) mL, respectively.

Total and differential leukocytes

All differential cell counts for the BFx, BAL, and PB were expressed as a percentage of total leukocytes. In both the BFx and BAL, there was no significant difference in the total leukocyte count between the FA and NO_2 conditions (table 3). In the BFx, there was a significant increase in the percentage of neutrophils after NO_2 exposure as compared to FA (table 3, fig. 1). In the BFx and BAL, there were no other significant differences in the total number or percentage of leukocytes, or the percentage of neutrophils, macrophages, eosinophils, or lymphocytes between the two conditions (table 3). In the PB, there was no significant difference in the percentage of monocytes, neutrophils, lymphocytes, or eosinophils between the FA and NO_2 conditions (all data within normal range; not shown).

Lymphocyte subsets and activation

In the BAL, there was a significant decrease in the percentage of T-helper cells after NO_2 exposure, as compared to FA (table 4). In the BAL, there were no significant differences in B-cells, T-suppressor cells, and NK cells, between the two conditions (table 4). There were also no

Table 3. – Leukocytes in bronchial fraction (BFx) and bronchoalveolar lavage (BAL), post-exposure to filtered air (FA) and nitrogen dioxide (NO_2)

	BFx		BAL	
Cell	FA	NO_2	FA	NO_2
Leukocytes				
$\times 10^4$ mL	16.3	17.0	13.8	15.8
IQR	8.0–19.3	10.3–22.5	8.0–17.5	12.3–20.3
95% CI	11.2–18.3	12.8–21.2	11.5–21.1	12.8–24.6
Leukocytes				
%	88.8	91.7	96.3	92.3
IQR	78.1–93.1	86.6–95.1	94.0–98.3	88.5–97.0
95% CI	80.0–91.4	90.3–95.2	92.3–97.6	85.6–95.9
Macrophages				
%	88.6	77.1	92.2	90.2
IQR	79.5–92.0	73.8–91.1	86.3–95.3	88.9–91.4
95% CI	81.4–91.1	72.0–87.1	85.7–94.6	80.3–92.9
Neutrophils				
%	5.3	10.6*	2.4	3
IQR	2.5–8.3	4.8–17.2	1.0–4.4	1.4–4.5
95% CI	3.7–8.3	6.7–20.1	1.6–3.8	1.7–7.0
Lymphocytes				
%	4.3	3.6	3.1	5.8
IQR	1.8–8.2	2.4–7.9	1.8–6.6	3.9–7.4
95% CI	2.8–8.3	3.3–6.8	2.2–8.2	3.5–10.5
Eosinophils				
%	0.3	1.1	0.5	0.6
IQR	0.0–1.8	0.0–1.8	0.0–1.0	0.0–1.9
95% CI	-1.0–5.2	0.0–4.0	-0.1–4.7	-0.3–4.4

Values are median and interquartile range (IQR). Leukocytes: percentage of total cells; all others: percentage of leukocytes. 95% CI: 95% confidence interval. *: significantly different from filtered air (p=0.005).

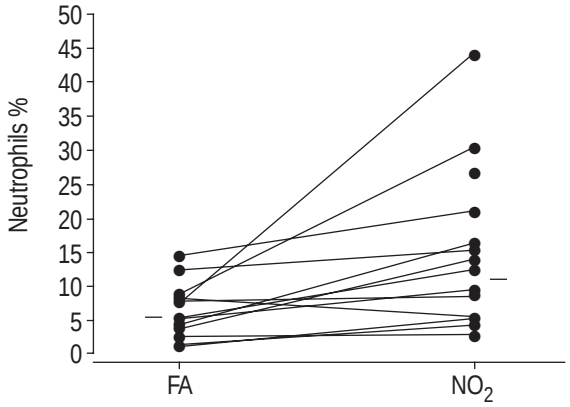

Fig. 1. – Individual (●) and median (horizontal bars) percentage differential neutrophil counts post-exposure to filtered air and nitrogen dioxide (NO_2).

significant differences for either of the two cell activation markers CD25 and CD69 on T-cells between the two conditions (table 4). In the PB, there were no significant differences in any of the lymphocyte subsets, or in activation of T- or NK cells, between the FA and NO_2 conditions (all data within normal range; not shown).

Total protein and lactate dehydrogenase

In the BFx, there was a significant decrease in total protein after NO_2 exposure as compared to FA (table 5). In the BAL, there was no difference in total protein between the exposure conditions. In both the BFx and BAL, there was no difference in LDH between the two conditions (table 5).

Discussion

The results of this experiment demonstrate that exposure to 2.0 ppm NO_2 for 4 h per day on three consecutive days produces an increase in neutrophils in the BFx and a decrease in T-helper cells in the BAL. These results indicate that this level of NO_2 exposure can induce mild bronchial airway inflammation and a minimal change in one lymphocyte subset involved in cellular immunity.

An increase in the number and percentage of neutrophils in the BFx has also been found following a single NO_2 exposure [20, 21]. Following other multiple-day exposures, the number or percentage of neutrophils was not changed, or the percentage of neutrophils was increased [23–26]. The total dose of NO_2 was lower in the experiments finding no change in neutrophils, compared to the experiment finding an increase in the percent of neutrophils and the current experiment. Therefore, the NO_2-induced increase in bronchial neutrophils could be a function of the total dose of NO_2, as opposed to the exposure format. The NO_2-induced increase in neutrophils in the BFx was not found in the BAL, suggesting a regional difference in NO_2 exposure (delivery), absorbance, clearance, or antioxidant defenses.

Single-day exposure to NO_2 increases lymphocyte and/or decreases macrophage counts [18–21]. There were no changes in the percentage of macrophages or lymphocytes in either the BFx or BAL in the current serial-day exposure experiment. Similarly, other multiple-day exposures did not change lymphocyte or macrophage numbers [23–26]. Hence, the leukocyte distribution response following single-day exposures could be decreased following multiple-day exposures. This difference may be due to an increase in the clearance of NO_2 from the airway epithelium or upregulation of antioxidant defenses.

The decrease in T-helper cells after NO_2 exposure in the BAL observed in the current experiment has not previously been reported, although other lymphocyte subsets have been changed following NO_2 exposure [20–25]. The B-cell, T-suppressor, and NK subsets were not-changed in the current experiment and have been variably changed in

Table 4. – Lymphocyte subsets and activation in bronchoalveolar lavage, post-exposure to filtered air (FA) and nitrogen dioxide (NO_2)

Cell	FA	NO_2
CD3+ T-cells		
%	93.3	93.8
IQR	90.1–95.7	90.9–96.2
95% CI	90.7–94.8	91.8–95.2
CD4+ T-cells		
%	61.6	55.9*
IQR	52.6–65.2	40.8–62.7
95% CI	54.7–68.1	46.0–60.4
CD8+ T-cells		
%	28.9	31.8
IQR	18.3–34.7	20.5–50.3
95% CI	21.4–32.6	23.1–40.7
CD4+/CD8+ T-cells		
ratio	2.3	1.7
IQR	1.5–3.2	0.8–3.2
95% CI	1.9–3.8	1.2–4.3
CD19+ B-cells		
%	1.3	1.3
IQR	0.8–1.9	0.4–2.0
95% CI	0.9–2.2	0.7–1.9
CD3- CD16+ CD56+ NK-cells		
%	0.3	0.2
IQR	0.2–0.9	0.1–0.4
95% CI	0.3–0.8	0.2–0.4
CD25+ CD4+ T-cells		
%	30.6	30.7
IQR	18.6–34.2	22.6–36.3
95% CI	22.1–32.6	23.7–35.4
CD25+ CD8+ T-cells		
%	7.5	8.9
IQR	5.8–9.6	6.0–13.9
95% CI	5.9–9.7	7.2–12.0
CD69+ CD4+ T-cells		
%	71.5	68.5
IQR	61.8–79.1	61.0–76.0
95% CI	65.0–76.2	63.1–75.0
CD69+ CD8+ T-cells		
%	78.9	81.5
IQR	73.4–87.5	70.6–89.9
95% CI	73.3–84.1	75.9–86.7

Values are median and interquartile range (IQR). Data for CD25+ and CD69+ subsets are percentages of CD4+ T-cell and CD8+ T-cell subsets. 95% CI: 95% confidence intervals; NK: natural killer; *: significantly different from filtered air (p=0.022).

Table 5. – Total protein (TP) and lactate dehydrogenase (LDH) in bronchial fraction (BFx) and bronchoalveolar lavage (BAL), post-exposure to filtered air (FA) and nitrogen dioxide (NO_2)

	BFx		BAL	
	FA	NO_2	FA	NO_2
TP mg·mL^{-1}	0.171	0.117*	0.114	0.105
IQR	0.124–0.232	0.099–0.189	0.086–0.134	0.066–0.144
95% CI	0.136–0.258	0.102–0.161	0.094–0.146	0.079–0.165
LDH mL	14.2	9.3	8.6	6.9
IQR	9.3–19.7	5.0–16.7	4.2–15.6	5.8–9.8
95% CI	10.5–16.9	6.3–14.4	5.6–16.9	6.0–11.4

Values are median and interquartile range (IQR). 95% CI: 95% confidence interval. *: significantly different from filtered air (p=0.004).

other experiments [20–25]. Taken together, the results of controlled human NO_2 exposure experiments show no consistent effect on a specific lymphocyte subset or group of subsets over a range of NO_2 exposure conditions. Although it is not known if different lymphocyte subsets have different specific sensitivities or responses to NO_2 exposure, it is probable that any oxidative or free-radical effects of NO_2, would be similar across all lymphocyte subsets, given the similarities in cell structure. The T-suppressor/T-helper cell ratio may be an important variable as an indication of cellular immune function. The decrease in T-helper cells did not result in any significant change in this ratio, suggesting that the current NO_2 exposure did not result in a functional change in cellular immunity. This is also apparent by the lack of change in activation of any of the lymphocyte subsets following NO_2 exposure. This finding could indicate a different response following multiple day exposure, compared to the increase in T-cell activation following a single exposure [21].

The finding in this experiment of only a small decrease in one cell population involved in cellular immune responses may help to explain why no NO_2-induced changes in response to experimental infection have been found in controlled human exposure experiments [38, 39]. The finding of no change in any of the leukocyte or lymphocyte subsets in the PB indicates that the inhaled NO_2 or a reaction product were either not transferred to the blood, or that the concentration of these molecules was too low to have an effect on the measured variables. This finding could be a function of a different response following multiple day exposure, compared to the increase in total lymphocytes, and the decrease in T-cells in blood following single exposures [21, 24, 25].

The findings of increased neutrophils and decreased total protein in the BFx, but not in the BAL, suggest regional differences in the response to inhaled NO_2. This differential response may be due to differences in NO_2 exposure (delivery), absorbance, clearance, or antioxidant defenses. Therefore it would be informative to include analysis of lymphocyte subsets in the BFx in future experiments. The decrease in total protein in the BFx may be due a decrease in epithelial permeability that has been demonstrated following NO_2 exposure [34]. It would appear that NO_2 decreases the transfer of proteins to the extracellular space, which could effect cellular function.

In conclusion, exposure to nitrogen dioxide under the conditions used in the current experiment resulted in an increase in neutrophils in the bronchial fraction and a decrease in T-helper cells in the bronchoalveolar lavage. The changes observed in the measured variables indicate that serial-day exposure to a high-ambient level of nitrogen dioxide is unlikely to produce any associated immunopathology. However, it cannot be excluded that higher concentrations (>2.0 parts per million), chronic exposure (>3 days), or exposure of specifically responsive individuals could result in larger nitrogen dioxide-induced changes in airway inflammation and cellular immunity.

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