Original Research

Effects of hypercapnia on T cells in lung ischemia/reperfusion injury after lung transplantation

Wei Gao[1], Dongdong Liu[1], Di Li[1], Xiangyu Che[2] and Guangxiao Cui[1]

[1]Department of Anesthesiology, The Second Affiliated Hospital of Harbin Medical University, 150000 Harbin, China; [2]Department of Anesthesiology, The Fifth Hospital of Harbin, 150000 Harbin, China
Corresponding author: Guangxiao Cui. Email: cuixiaoguang66@163.com

Abstract

T cells play a key role in lung ischemia/reperfusion injury (IRI). Hypercapnia has been indicated to decrease IRI and inhibit immunity. This study aimed to evaluate the effects of hypercapnia on T cells during lung IRI and to identify the underlying mechanism of these effects. In the *in vivo* study, rat recipients of lung transplants were randomized into a control group M and a hypercapnia group H. Peripheral blood T cells and cytokines were analyzed during reperfusion. In the *in vitro* study, we analyzed the T cells and cytokine levels in culture media from phytohemagglutinin-stimulated T cells from normal rats, stimulated under the normal (group C), hypercapnic (group H), or buffer hypercapnic (group BH) condition. In the *in vivo* study, the $CD3^+/CD4^+$ T-cell ratio and interleukin (IL)-2, IL-8, interferon (IFN)-γ, intracellular adhesion molecule (ICAM)-1, and P-selectin levels were decreased, but the IL-4 and IL-10 levels were increased, after reperfusion in group H compared to group M. In the *in vitro* study, groups H and BH exhibited a decreased $CD2^+/CD28^+$ ratio and IL-2 and IFN-γ levels, but elevated IL-4 and IL-10 levels, compared to group C. The $CD2^+/CD28^+$ ratio was not different between groups BH and H; however, group H evidenced a lower IL-2 level and higher IL-4 and IL-10 levels compared to group BH. Hypercapnia decreased the $CD3^+/CD4^+$ T-cell ratio and pro-inflammatory cytokine levels, but promoted anti-inflammatory factors in lung IRI. Hypercapnia inhibits CD2 and CD28 in T cells by CO_2 and modulates T-cell cytokines via acidosis.

Keywords: Hypercapnia, lung transplantation, reperfusion injury

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Introduction

Lung transplantation is an important therapy for advanced-stage lung disease. However, the mortality rate of lung transplantation after one year is high (20–30%),[1] largely due to failure of lung function. Ischemia/reperfusion injury (IRI) of the lung is a major risk factor for postoperative lung failure. Recently, T cells have been shown to be involved in IRI.[2–10] For example, de Perrot *et al.*[10] found that T cells were direct mediators of lung IRI after lung transplantation. Ysebaert *et al.*[7] reported that $CD4^+$ T lymphocytes infiltrated the kidney after renal ischemia. In an *ex vivo* model of liver reperfusion, Le Moine *et al.*[3] observed that livers harvested from nude mice had less severe injury 2 h after reperfusion than those from non-immunodeficient mice. In addition, Caldwell *et al.*[11] found that $CD4^+$ T cells were recruited rapidly during IRI and regulated inflammation positively by facilitating the recruitment of neutrophils by interleukin (IL)-17.

Several studies have indicated that hypercapnia can inhibit inflammation in IRI,[12] and that CO_2 pneumoperitoneum can inhibit immunity. However, no study has examined the effect of hypercapnia on T cells in lung IRI. In the present study, we performed hypercapnia in lung transplantation to investigate the effects of hypercapnia on T cells in IRI. We cultured T cells under the normal, hypercapnic, or buffer hypercapnic condition to evaluate the mechanism underlying the effect of hypercapnia on T cells.

Materials and methods

Animals

Ninety-six 10-week-old Wistar rats (250–300 g) were purchased from the Second Affiliated Hospital of Harbin Medical University, Harbin, China. All treatments were carried out in accordance with the Institutional Animal Care and Use Committee of Harbin Medical University and followed national guidelines for the treatment of animals.

In vivo study

Lung transplantation. All rats were fasted from food for 24 h before the study, but water was provided *ad libitum*. For each of three transplantation experiments, a group of 16 donor rats were anesthetized by intraperitoneal (i.p.) injection of 3% pentobarbital sodium (30 mg/kg) and ventilated with a tidal volume of 10 mL/kg. After heparinization and median laparosternotomy, the left lung was flushed through the main pulmonary artery with 20 mL of 0.9% sodium chloride at 4°C, injected at 20 cmH_2O pressure. The heart-lung block was recovered by inflating the lungs at end-tidal volume.

Meanwhile, for each of the three transplantation experiments, an additional 16 recipient rats (300–400 g) were anesthetized in the same manner as the donor rats, and each rat was intubated with a 14-gauge intravenous (i.v.) catheter. Recipients were ventilated with a mixed gas of 50% O_2 and 50% N_2, at a positive end-expiratory pressure of 2 cmH_2O (respiratory rate: 50/min, tidal volume: 10 mL/kg, inspiratory expiratory ratio: 1:1) for 15 min (T0). Under sterile conditions, the right femoral artery was cannulated, and 0.9% NaCl solution (10 mL/kg/h) was administered via the vena caudalis. The left pulmonary artery, left main bronchus, and pulmonary vein of recipients were anastomosed with donor lung by the Cuff technique.[13] The body temperature of the recipients was maintained at 37–39°C with a heated blanket and confirmed by a rectal thermometer.

The 16 recipient rats in each transplantation experiment were randomized into two groups ($n = 8$/group): a control/model group (M) and an experimental/hypercapnia group (H). Group M rats were ventilated with 50% O_2 and 50% N_2, while the partial pressure of carbon dioxide ($PaCO_2$) was maintained within 35–45 mmHg. Group H rats were ventilated with 50% O_2 and 50% mixed gas (45% N_2 and 5% CO_2) to keep the $PaCO_2$ within the range of 80–100 mmHg.[14] Blood and T-cell analyses were performed at the moment of reperfusion (T0) and 1 h (T1), 2 h (T2), and 4 h (T3) after reperfusion.

In the *in vivo* study, blood samples were collected of each of the three transplantation experiments for serum factor analysis and flow cytometry. These analyses required a large volume of blood. With the first transplantation procedure, $CD3^+/CD4^+$ T-cell ratios and arterial blood gas levels were analyzed in both groups. With the second transplantation procedure, peripheral blood levels of IL-8, intracellular adhesion molecule (ICAM)-1, and P-selectin were determined for both groups. Finally, with the third transplantation procedure, peripheral blood levels of interferon (IFN)-γ, IL-2, IL-4, and IL-10 were determined for both groups at T0, T1, T2, and T3.

Arterial blood gas analysis. Blood gas levels in the carotid artery were analyzed by a Bayer Rapidlab 248 analyzer (Diamond Diagnostics, Holliston, MA) at baseline and 30-min intervals after reperfusion.

Flow cytometry of peripheral blood. Peripheral blood was collected from recipients and analyzed by two-color flow cytometry at T0, T1, T2, and T3. To identify T cells, 100-μL aliquots of heparinized blood were incubated for 20 min with saturating concentrations of fluorescein isothiocyanate (FITC)-conjugated anti-CD3 and r-phycoerythrin (PE)-conjugated anti-CD4 mouse anti-rat monoclonal antibodies (mAbs) (Caltag Laboratories, CA, USA). Red blood cells were lysed with 1 mL of Flow Cytometric Analysis Cell Scan (FACS) lysing solution. Cells were washed with 2 mL of PBS and resuspended in 500 mL of PBS. Flow cytometric analysis was performed with a Becton Dickinson FACScan (Becton Dickinson, Belgium).

Cytokine levels in peripheral blood. At T0, T1, T2, and T3, peripheral blood levels of IL-8, ICAM-1, P-selectin, IFN-γ, IL-2, IL-4, and IL-10 were detected with specific enzyme-linked immunosorbent assay (ELISA) kits (Wuhan Boster Bio-Engineering Ltd. Co., Wuhan, China), according the manufacturer's instructions.

In vitro study

An *in vitro* study was performed to ascertain the mechanism by which hypercapnia induces its effects on T cells and to determine whether hypercapnia or CO_2 inhibits T cells. T cells isolated from eight normal rats were stimulated with phytohemagglutinin (PHA; BOC Sciences, Shirley, NY) under the normal, hypercapnic, or buffer hypercapnic condition. Peripheral blood mononuclear cells were isolated from the heparinized blood of normal Wistar rats by standard density centrifugation with Ficoll-Hypaque Solution (Hao Yang Biological Manufacturer, Tianjin, China). T cells were obtained by magnetic-activated cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) and resuspended in RPMI-1640 culture medium (GIBCO, Carlsbad, CA), containing 10% fetal bovine serum and 100 U/mL penicillin/streptomycin (at 1×10^6 cells/mL) in 16-well culture plates (Invitrogen, Carlsbad, CA).

The normal rat T cells were stimulated with 20 μg/mL PHA for 4 h at 37°C under three conditions. In the control group (group C), T cells were cultured in RPMI-1640 in a humidified atmosphere containing 2.5% CO_2, 21% O_2, and 76.5% N_2. In the hypercapnia group (group H), T cells were exposed to 21% O_2 and a mixed gas (5% CO_2 and 74% N_2), with the PCO_2 of RPMI-1640 kept between 80 and 100 mmHg. In the buffer hypercapnia group (group BH), T cells were cultured under the same gas condition as group H, but the RPMI-1640 was buffered with $NaHCO_3$ to maintain a pH of 7.3 ± 0.1. At baseline (T0) and 1 h (T1), 2 h (T2), and 4 h (T3) after stimulation with PHA, samples of T cells and supernatants of the culture medium were collected for further analysis.

Flow cytometry of incubated cells. PHA-stimulated T cells from normal rats in each group were sampled at T0, T1, T2, and T3. Samples were washed with 1 mL of PBS, incubated with FITC-conjugated anti-CD2 and PE-conjugated anti-CD28 mAbs (Caltag Laboratories) for 20 min, and analyzed by flow cytometry (FACScan, Becton Dickinson).

Cytokine levels in cell culture media. At T1, T2, and T3, samples of cell culture media were collected and centrifuged at 8000 × *g* and 4°C for 10 min. Levels of IFN-γ, IL-2, IL-4, and IL-10 were measured in the cell culture medium supernatants by ELISA kits, according to the manufacturer's instructions.

Statistical analyses

All data in this study were analyzed in SPSS13.0 software (SPSS Inc., Chicago, IL). Continuous variables were compared by repeated-measures analysis. Data were compared between groups (two groups in *in vivo* experiment and three groups in the *in vitro* experiment) at each experimental time point with Student's *t*-test if the data were normally distributed or the nonparametric Friedman test otherwise. Differences with a P value <0.05 were considered statistically significant.

Results

In vivo study

Forty-eight rats received lung transplants. There was no significant difference in the ischemic time of transplanted lungs between the hypercapnia group H and the control group M (23.8 ± 4.9 min and 22.4 ± 6.2 min, respectively).

Arterial blood gas analysis. There was no significant difference in pH or $PaCO_2$ at baseline between groups M and H. During reperfusion, the pH in experimental group H was significantly lower than that in control group M. Compared with group M, the PaO_2 in group H was significantly higher after reperfusion (Table 1).

Flow cytometry of peripheral blood. There was no significant difference in the $CD3^+/CD4^+$ T-cell ratio between groups H and M at T0. However, at T2 and T3, the $CD3^+/CD4^+$ T-cell ratio in experimental group H was significantly lower than that in control group M (Table 2, Figure 1).

Cytokine levels in peripheral blood. At T1, T2, and T3, the IL-2, IL-8, IFN-γ, and P-selectin levels in group H were significantly lower than those in group M. The IL-4 level at T2 and T3 and IL-10 level at T3 were significantly higher in group H compared to group M (Figure 2). At T1 and T2, the ICAM-1 level was significantly decreased in group H compared to group M (Figure 3).

In vitro study

Flow cytometry of cell culture media. The control group C, hypercapnia group H, and buffer hypercapnia group BH showed comparable $CD2^+$ and $CD28^+$ T-cell counts at T0 ($P > 0.05$). At T2 and T3, groups H and BH showed significantly fewer $CD28^+$ T cells compared to group C, with no significant difference between groups H and BH (Table 2,

Table 1 Comparison of pH, and $PaCO_2$ between two rat lung transplantation groups (mm Hg, $\bar{x} \pm SD$)

		Reperfusion time (min)							
	Baseline	30	60	90	120	150	180	210	240
$PaCO_2$									
Group M	31 ± 4	38 ± 5	40 ± 5	39 ± 5	42 ± 6	43 ± 5	45 ± 3	41 ± 5	41 ± 3
Group H	29 ± 2	83 ± 6*	80 ± 9*	98 ± 9*	85 ± 6*	88 ± 7*	86 ± 6*	86 ± 7*	83 ± 6*
pH									
Group M	7.42 ± 0.04	7.42 ± 0.04	7.39 ± 0.05	7.37 ± 0.05	7.37 ± 0.05	7.36 ± 0.03	7.33 ± 0.04	7.34 ± 0.05	7.32 ± 0.04
Group H	7.47 ± 0.05*	7.17 ± 0.03*	7.15 ± 0.08*	7.14 ± 0.06*	7.15 ± 0.06*	7.14 ± 0.07*	7.13 ± 0.05*	7.14 ± 0.08*	7.13 ± 0.07*

*$P < 0.05$, compared with group M.

Table 2 $CD3^+/CD4^+$ T-cell ratio in the *in vivo* groups, and the $CD28^+$ and $CD2^+$ T-cell numbers in the *in vitro* groups ($\bar{x} \pm SD$)

	Baseline (T0)	T1	T2	T3
$CD3^+/CD4^+$				
Group M	37.41 ± 3.09	43.37 ± 2.83	57.24 ± 5.27	65.53 ± 4.04
Group H	36.77 ± 2.47	42.42 ± 2.41	47.64 ± 2.11*	51.14 ± 2.76*
CD28				
Group C	73.60 ± 1.53	76.03 ± 2.68	78.95 ± 3.05	81.72 ± 2.02
Group B	72.78 ± 2.92	73.65 ± 2.49	73.39 ± 3.58†	74.66 ± 2.49†
Group H	74.65 ± 3.62	75.61 ± 2.61	73.4 ± 3.79†	71.63 ± 4.18†
CD2				
Group C	88.88 ± 1.11	90.62 ± 1.61	93.07 ± 1.33	94.54 ± 2.23
Group B	88.86 ± 1.11	89.34 ± 1.11	90.03 ± 0.92†	90.99 ± 0.99†
Group H	88.68 ± 0.96	89.45 ± 1.15	89.25 ± 1.41†	89.52 ± 0.97†

*$P < 0.05$ compared with group M, †$P < 0.05$ compared with group C.

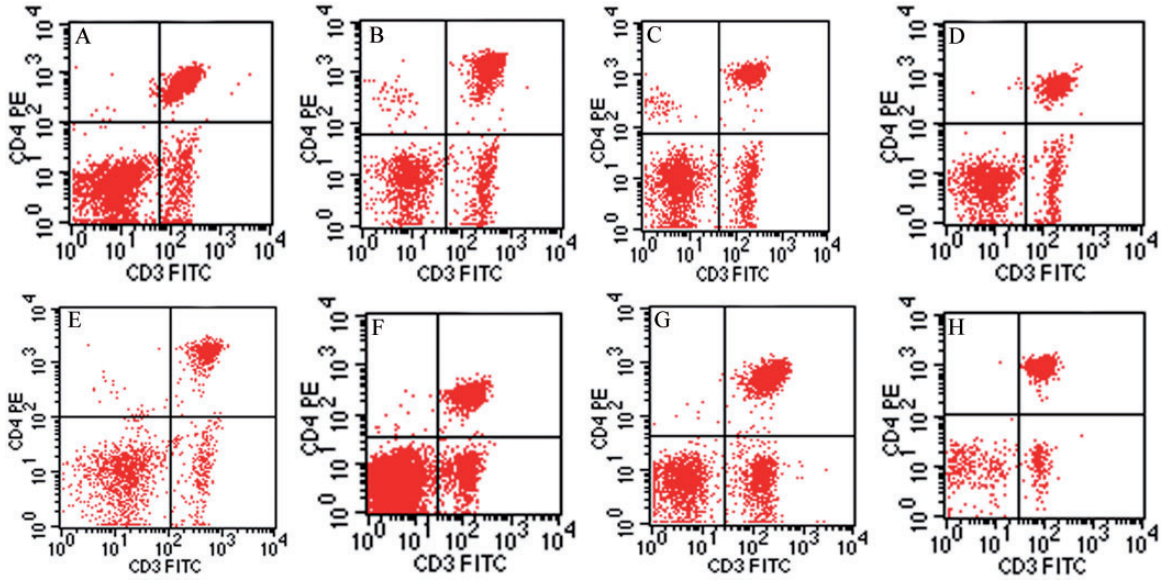

Figure 1 $CD3^+/CD4^+$ T cell ratio in peripheral blood from the rat lung transplantation groups. $CD3^+/CD4^+$ T cell ratio in group M(A–D) and group H (E–H) at T0, T1, T2, and T3, respectively

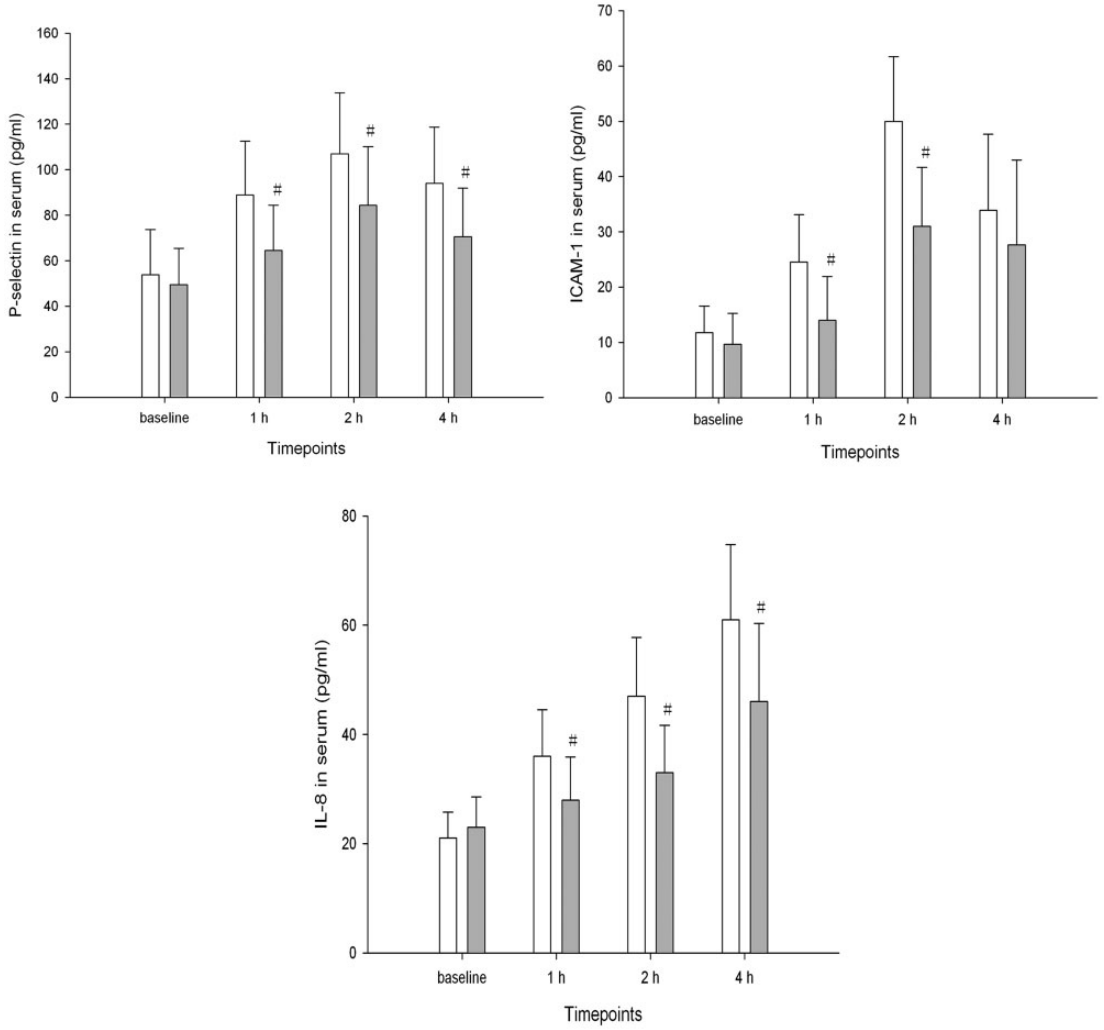

Figure 2 Comparison of P-selectin, ICAM-1, and IL-8 levels between in vivo groups M and H Group M (□), Group H (▒). $^{\#}P < 0.05$ compared with group M

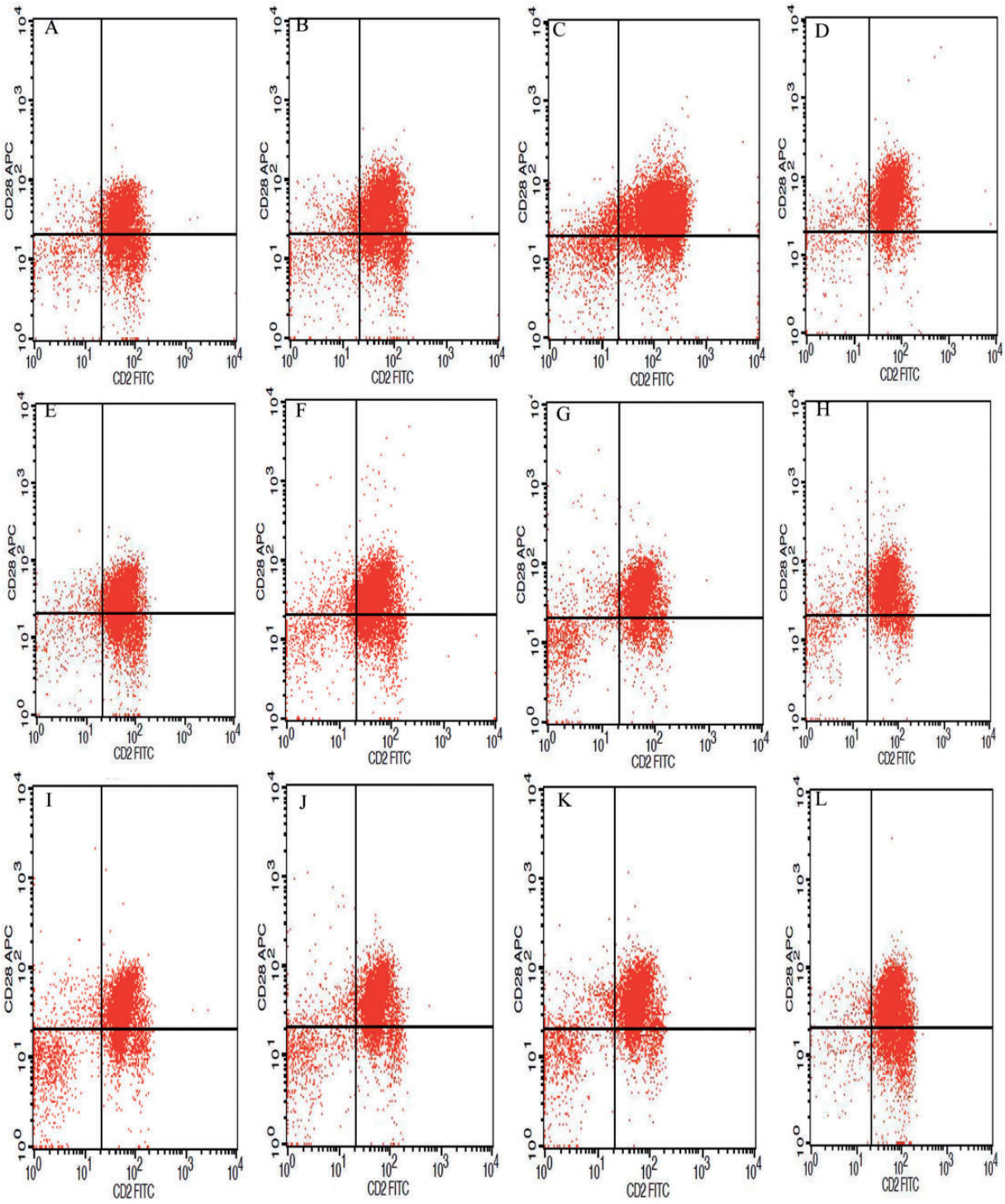

Figure 3 $CD28^+$ and $CD2^+$ T cell counts in the in vitro study. Control group (A–D), hypercapnia group (E–H), and buffer hypercapnia group (I–L) at T0 (A, E, I), T1 (B, F, J), T2 (C, G, K), and T3 (D, H, L)

Figure 4). Groups H and BH exhibited fewer $CD2^+$ T cells than group C at T1, T2, and T3, but the difference was only significant at T3. There was no significant difference in the $CD2^+$ T-cell count between groups H and BH at any analyzed time (Table 2, Figure 4).

Cytokine levels of cell culture media. The IFN-γ and IL-2 levels in the supernatants of the cell culture media were significantly lower in groups H and BH compared to group C at all evaluated times after PHA stimulation. There was no significant difference in IFN-γ level between groups H and BH, but the IL-2 level was significantly lower in group H compared to group BH at T1, T2, and T3. Compared to group C, the IL-4 level was significantly higher at T1, T2, and T3 in group H, and at T2 and T3 in group BH. At all evaluated times after PHA stimulation, the

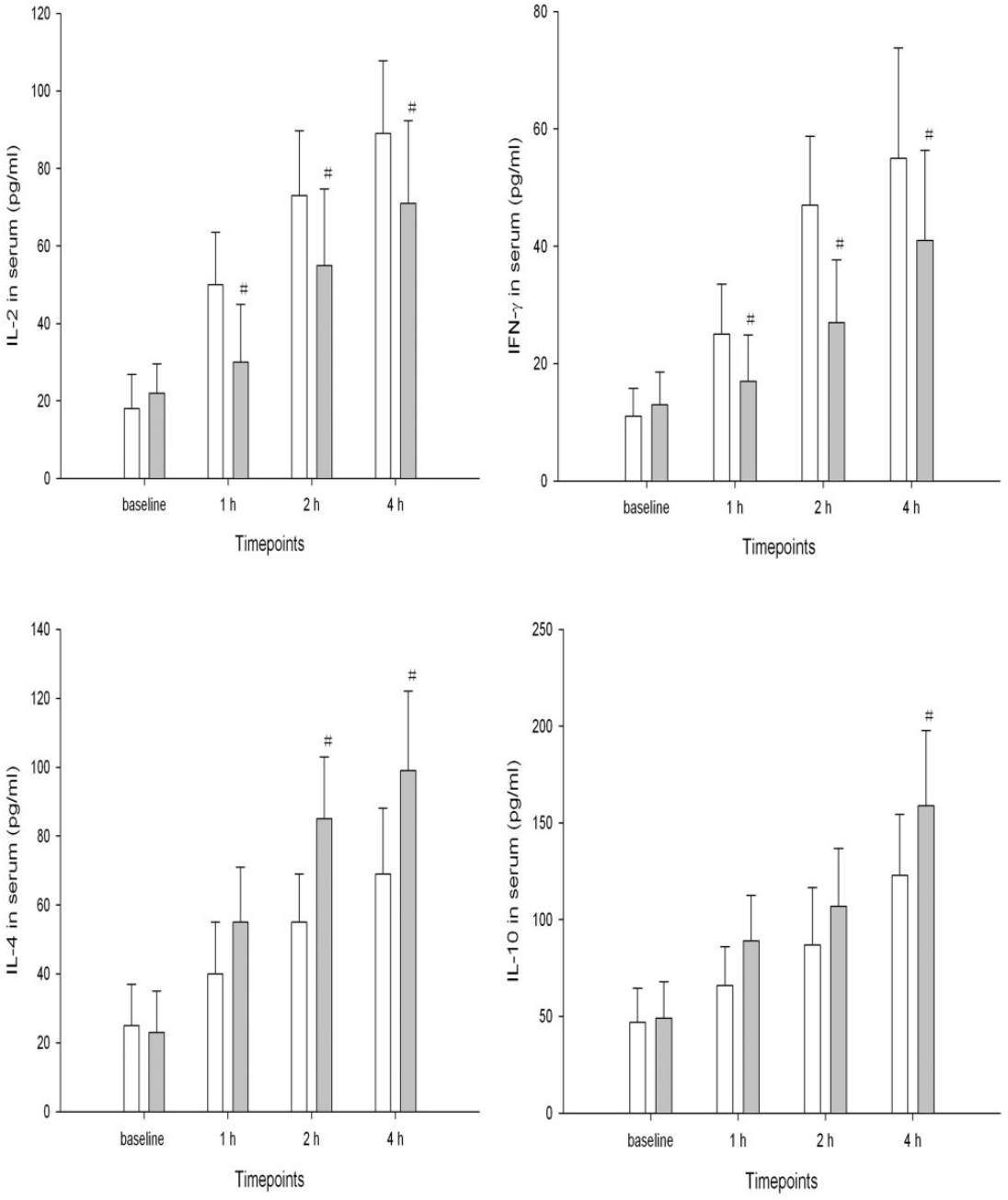

Figure 4 Comparison of IL-2, IFN-γ, IL-4, and IL-10 levels in peripheral blood between the two rat lung recipient groups (groups M and H). Group M (□), Group H (▨). $^{\#}P < 0.05$ compared with group M

IL-4 level was significantly higher in group H compared to group BH. Compared to group C, the IL-10 level was significantly higher at T1, T2, and T3 in group H, and at T3 in group B. The IL-10 level was significantly higher in group H compared to group BH at T1, T2, and T3 (Figure 5).

Discussion

In this study, we demonstrated that hypercapnia after lung IRI can decrease the level of pro-inflammatory factors of T cells, while increasing the level of anti-inflammatory factors. The effect of hypercapnia on T cells may be due to CD28, CD2, chemotactic factors, and/or adhesion molecules. In addition, CO_2 or acidosis can regulate T cells, but the combination of both is better than acidosis alone.

Many studies have indicated that $CD4^+$ T cells play a key role in IRI, not only by secreting factors but also via modulation of neutrophils.[2–6,8,9,11,15–17] For this reason, we chose to perform both *in vitro* and *in vivo* studies. $CD4^+$ T cells play dual, opposing roles in IRI,[11] which depend on the cytokines secreted by Th1 or Th2.[15,16] Boros and Bromberg[18] found that IRI is worse in $STAT6^{-/-}$ mice than in wild-type mice, whereas $STAT4^{-/-}$ mice have mildly improved function. T cells secrete cytokines in IRI, including both pro- and anti-inflammatory factors (e.g., IL-2

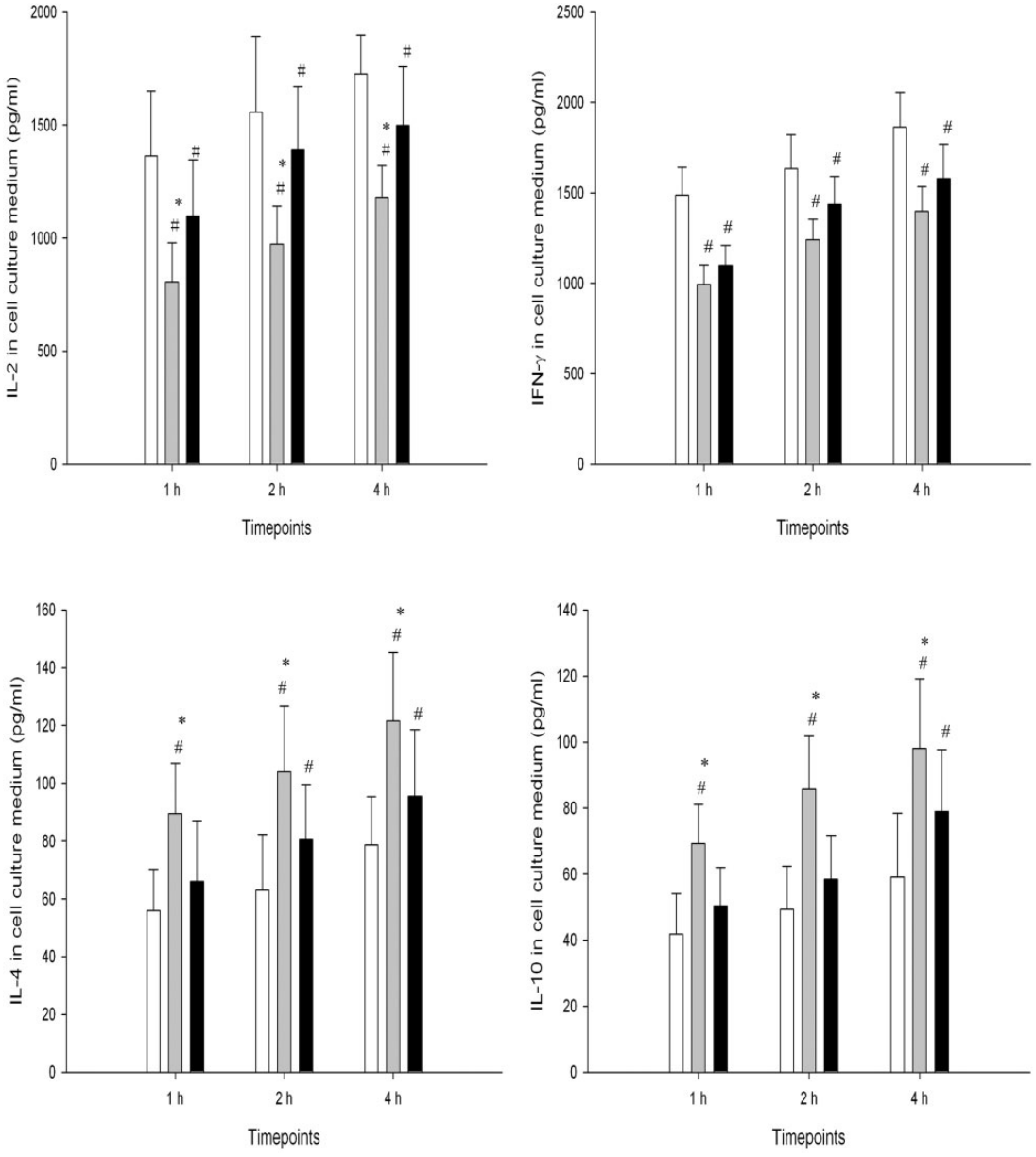

Figure 5 Comparison of IL-2, IFN-γ, IL-4, and IL-10 levels in the supernatants of cell culture media in the *in vitro* control group C, hypercapnia group H, and buffer hypercapnia group BH. Group C (□), Group H (▨), Group BH (■). $^{\#}P < 0.05$ compared with group M; $^{*}P < 0.05$ compared with group C

and IFN-γ vs. IL-4 and IL-10). IL-2 may induce the release of other cytokines, such as TNF-α, IL-1, and IFN-γ, which are key mediators in IRI and act directly on neutrophils to enhance their actions in IRI.[17,19,20] IL-4 and IL-10 had been proven to attenuate injury and preserve organ function after reperfusion.[15,21]

Some studies have demonstrated that CO_2 has an opposing effect on immunity,[22–27] causing an increase in IL-10 but a decrease in TNF-α levels.[27] The results of our animal analyses were in agreement with the latter study.[27] Hypercapnia downregulated the $CD3^+/CD4^+$ T-cell ratio and decreased the levels of IL-2 and IFN-γ, but increased IL-4 and IL-10. These effects may be associated with the inhibition of IL-8 and adhesion molecules (e.g., P-selectin and ICAM-1) that mediate the adhesion, chemotaxis, and activation of T cells. Hypercapnia can block these functions of T cells during IRI via inhibition of P-selectin, ICAM-1, and IL-8. However, no study to date has investigated whether hypercapnia can directly influence T cells by acidosis or CO_2.

CD28 and CD2 play key roles in T-cell activation.[2,6,28] In particular, several studies have indicated that CD28-B7 participates in T-cell activation in IRI, and that blockade of CD28-B7 can effectively attenuate IRI.[28] In another study, CD2 was found to be crucial for the adhesion and activation for T cells.[12] When we compared the number of $CD2^+$ and $CD28^+$ T cells under the normal and hypercapnic conditions, we found higher $CD2^+$ and $CD28^+$ cell counts

among the PHA-stimulated T cells in group C compared to group H. These results indicate that hypercapnia can directly inhibit T cells by CD2 and CD28.

Next, we compared the $CD2^+$ and $CD28^+$ T-cell counts and the IFN-γ, IL-2, IL-4, and IL-10 levels in cell culture media between the H and BH groups. The goal of this step was to investigate whether the effect of hypercapnia on T cells depends on respiratory acidosis or CO_2. We did not observe a significant difference in the number of $CD2^+$ and $CD28^+$ T cells between groups H and BH. This result suggests that the effect of hypercapnia on the CD molecules of T cells may depend on CO_2. However, the IL-2 level was significantly lower, and the IL-4 and IL-10 levels were significantly higher, in group H compared to group BH. These cytokine results indicate that both CO_2 and acidosis can influence the T cells, and that the combination has a stronger effect than CO_2 alone. Thus, we speculate that the mechanism by which hypercapnia exerts its effects on T cells is associated with both CO_2 and acidosis; CO_2 inhibits the CD molecules of T cells, and acidosis regulates the secretion of cytokines from T cells.

Unexpectedly, the levels of anti- and pro-inflammatory factors were increased and decreased, respectively, under both of the *in vitro* hypercapnic conditions (groups H and BH). The same results were also found by Jacobi *et al.*[27] Therefore, unknown factors may participate in the secretion of T cells and the hypercapnia-mediated immunoregulation of T cells. Our future studies will aim to elucidate the mechanism of this hypercapnia-mediated immunoregulation of T cells.

In conclusion, hypercapnia was involved in the immunoregulation of T cells, inhibiting pro-inflammatory factors and promoting anti-inflammatory factors in IRI. The effect of hypercapnia on T cells included indirect inhibition of chemotactic factors and adhesion molecules, as well as direct inhibition of CD28 and CD2. The effect of hypercapnia on the CD molecules of T cells appears to depend largely on CO_2, whereas the effect on cytokine release appears to depend on acidosis.

Author contributions: Wei Gao, Dongdong Liu, Di Li, Xiangyu Che and Guangxiao Cui have made a substantial contribution to the concept and design, acquisition of data, analysis and interpretation of data. Wei Gao, Dongdong Liu and Guangxiao Cui drafted the article or revised it critically for important intellectual content.

CONFLICT OF INTEREST

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion in this paper.

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