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ORIGINAL ARTICLE

Cardiopulmonary Bypass

Rolipram plus Sivelestat inhibits bone marrow-derived leukocytic lung recruitment after cardiopulmonary bypass in a primate model

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Abstract

Cardiopulmonary bypass (CPB) recovery is complicated by lung inflammation from bone marrow (BM)-derived polymorphonuclear leukocytes (PMNs) and monocytes (MO). Although Sivelestat reduces inflammatory mediators and Rolipram inhibits PMN and MO activation, any kinetic effects to improve CPB recovery in vivo are unknown. We hypothesized that intraoperative co-administration of these compounds would reduce CPB-induced lung inflammation through downregulation of PMN and MO recruitment. A 2-h CPB was surgically established in cynomolgus monkeys ($n=13$), and BM leukocyte release and lung recruitment were monitored postoperatively by flow cytometry with 5′-bromo-2′-deoxyuridine (BrdU) and cytokine ELISA. Either Sivelestat, Sivelestat plus Rolipram, or saline (control) was administered intraoperatively and both peripheral and perfusion sampling courses revealed BrdU-labeled cells representative of activated leukocyte infiltration. Levels of cytokines CD11b and CD18 were leukocytic activation markers. Sivelestat plus Rolipram attenuated increases in CPB-associated circulating band cells, prolonged BM-transit time (PMN: 121.0 ± 3.7 to 96.2 ± 4.3 h [control], $p=0.012$; MO: 84.4 ± 4.1 to 61.4 ± 3.0 h [control], $p=0.003$), and reduced their alveolar appearance. CD11b-mediated PMN and MO changes during CPB and the post-surgical increases of Interleukin (IL)-6 and IL-8 in the bronchoalveolar lavage fluid were suppressed. Sivelestat alone increased PMN transit time to 115.8 ± 6.6 h, but monocytes were unaffected. Therefore, Rolipram has additive inhibitory effects with Sivelestat on the CPB-induced activation and release of BM-derived PMNs and MO and their recruitment to the lungs. Co-administration of these compounds could, therefore, hold value for preventing CPB-induced lung injury.

Keywords Acute respiratory distress syndrome · Animal model · Cardiopulmonary bypass, CPB · Lung · Stem cells

Introduction

Bone marrow (BM)-derived leukocytes, such as polymorphonuclear leukocytes (PMNs), are a major contributor to post-surgical pulmonary injury and we previously reported that cardiopulmonary bypass (CPB) accelerates the release of both PMNs and monocytes which are recruited to the lungs from BM in a monkey model [1]. These CPB-induced, BM-derived leukocytes, rather than circulating leukocytes, are critical for the pathogenesis of post-CPB lung injury resulting from a whole-body inflammatory reaction [1, 2]. Therefore, prophylactic suppression of BM-derived leukocyte recruitment to the alveolar spaces could attenuate CPB-related lung injury. However, it is well documented that circulating leukocytes are activated by both direct contact with the CPB apparatus and the serum cytokines/

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proteases produced during CPB [3, 4]. Thus, the potent milieu of resultant inflammatory mediators from alveolar macrophages and lung epithelial cells due to CPB-activated circulating leukocytes could further recruit BM-derived leukocytes to the lungs.

These activated PMNs sequester in the lung and secrete toxic oxygen species and proteolytic enzymes, including cytotoxic neutrophil elastase, that are implicated in severe tissue injury and subsequent multiple organ dysfunction by promoting the release of cytokines such as tumor necrosis factor-alpha, interleukin (IL)-6, or IL-8 from lung epithelium [3, 5]. Using a simulated, extracorporeal circulation to model such inflammatory responses to CPB, we recently reported that neutrophil elastase inhibitors such as Sivelestat (a synthetic, specific, low-molecular weight neutrophil elastase inhibitor) reduce inflammatory mediators and Rolipram, a selective phosphodiesterase (PDE) type 4 inhibitor, inhibit activation of circulating PMNs or monocytes [6–9]. Sivelestat reduces both neutrophil elastase levels and IL-8 production while preserving neutrophil deformability [6] and Rolipram acts through intracellular messenger cyclic adenosine monophosphate (cAMP) to suppress leukocyte activation [10]. As PDE type 4 is the predominant PDE isozyme in leukocytes [10], Rolipram-mediated cAMP increases in leukocytes, therefore, control a pivotal event in leukocyte activation [11]. We previously reported that, in an in vitro model of PMN-mediated lung injuries, Rolipram inhibited PMN functional changes and the crucial step of initial PMN sequestration in microvessels during CPB [7, 8].

Sivelestat is clinically approved in Japan for acute lung injury cases although there is controversy regarding its clinical benefits [12]. Rolipram acts via an alternative pathway and can also inhibit elastase release from PMNs [7, 8]. Hence, combining these drugs could additively enhance suppression of CPB-induced leukocyte activation. In this study, we hypothesized that, during CPB, combining Sivelestat and Rolipram (versus Sivelestat alone) would inhibit the activation of and inflammatory response from circulating PMNs and monocytes while suppressing BM-derived leukocyte recruitment to the lungs in our established primate CPB model.

Materials and methods

Animal care

Male cynomolgus monkeys (*Macaca fascicularis*) ($n = 13$; 4–6 years old, 4.6 ± 0.9 kg) born in the Tsukuba Primate Center were used in this study. The study strictly adhered to the Rules for Animal Care and Management of the Tsukuba Primate Center [13] and to the Guiding Principles for Animal Experiments Using Nonhuman Primates formulated by the Primate Society of Japan [14]. The protocol including the ethical principles of laboratory animal care was approved by the Animal Welfare and Animal Care Committee of the National Institute of Infectious Diseases (Tokyo, Japan) and by the Animal Experimentation Committee of the University of Tsukuba.

Experimental design

CPB effect on leukocyte kinetics, including BM-derived leukocytes, was evaluated by using our established nonhuman primate model as previously described [1]. Briefly, CPB was established with an ascending aortic cannulation for arterial inflow and superior and inferior vena cava cannulations for venous outflow in 13 monkeys for 2 h through a median sternotomy. To label dividing cells in the BM, 5′-bromo-2′-deoxyuridine, BrdU (100 mg/kg; Sigma-Aldrich, St. Louis, MO) was intravenously infused 24 h before surgery [1]. After anesthetization, continuous intravenous *infusion* of normal saline (control, $n = 5$) or Sivelestat (2 mg/kg/h, Elaspol, ONO-5046-Na; Ono Pharmaceutical Co., Osaka, Japan; S group, $n = 4$), or Sivelestat plus Rolipram (20 µmol/L, BIOMOL Research Laboratories, Inc, Plymouth Meeting, PA; SR group, $n = 4$) was administered by infusion pump during the operation and removed before recovery. Peripheral blood samples were obtained just before (time − 24) and at intervals from 24 to 192 h after BrdU injection. An arterial-blood gas test was performed from the right femoral artery at the same time point. Perfusion blood samples were obtained from the circuit just before and at 30, 60, and 120 min after CPB induction. Total white blood cell (WBC) counts were determined with a Sysmex KX-21 (Sysmex, Kobe, Japan) and the number of BrdU-labeled PMNs (PMN^{BrdU}) and monocytes (MO^{BrdU}) was determined by flow cytometry as described below. Differential WBC counts were done by counting 200 leukocytes in randomly selected fields of view on Wright-Giemsa-stained blood smears. Bronchoalveolar lavage fluid (BALF) samples were simultaneously collected for PMN^{BrdU}/MO^{BrdU} infiltration monitoring as previously described [1] by using a flexible soft catheter (10 Fr, SF-ND1013OS; Terumo, Tokyo, Japan) connected to a specimen collection container (2586-M, Argyle; Covidien, Shizuoka, Japan). Total nucleated cell counts were performed for each sample. BALF was filtered through a 42-µm nylon mesh, and then centrifuged. Supernatants were aspirated and cells resuspended in PBS for flow cytometry as described below. Supernatant levels of IL-6 (Invitrogen/Life Technologies, Grand Island, NY) and IL-8 (Abnova, Taipei City, Taiwan) were measured just before, at 24, and 48 h after surgery by enzyme-linked immunosorbent assay according to the manufacturers' instructions.

Flow-cytometric analysis

BrdU-positive PMNs and monocytes in the peripheral blood and BALF samples were determined by two-color immunofluorescent flow cytometric analysis as previously described [1] using a fluorescein isothiocyanate (FITC)-conjugated, monoclonal mouse, anti-BrdU antibody (BrdU Flow Kit; BD, Franklin Lakes, NJ) with peridinin-chlorophyll protein (PerCP)–conjugated CD14 antibody (mouse anti-human CD14 mAb Tuk4; Miltenyi Biotec, Tokyo, Japan) to distinguish monocytes. A total of 3,000 cells per specimen were evaluated and results were expressed as the percentage of BrdU-labeled PMNs or monocytes with a high FITC fluorescence intensity. The transit times of leukocytes through BM were calculated as described elsewhere [1, 15]. In addition, surface expression changes in CD11b and CD18 of PMNs and monocytes were measured as biomarkers of leukocyte activation in the perfusion blood samples obtained from the CPB circuit [16]. Four-color analysis via BrdU Flow Kit on FITC/PerCP-stained cells was conducted by simultaneous staining of leukocyte CD11b and CD18 with allophycocyanin-conjugated, rat monoclonal anti-human CD11b antibody (Miltenyi Biotec) and phycoerythrin (PE)-conjugated, mouse monoclonal, anti-human CD18 antibody (BD Biosciences Pharmingen; San Diego, Calif) according to the manufacturers' instructions. CD11b and CD18 expression was measured via mean fluorescent intensity and CD11b and CD18 changes were normalized against a baseline value of 100 (just before the CPB).

Statistical analysis

Results were expressed as means ± SE and analyzed with repeated analyses of variance (ANOVA) over time while the effect of multiple comparisons was corrected using the Bonferroni method. Changes in CD11b or CD18 expression and the transit times of PMNs or monocytes were compared by one-way ANOVA followed by a post-hoc Tukey's test among the groups. $P < 0.05$ was considered significant. Statistical analyses were performed using IBM Statistics SPSS 25 (IBM Corporation; NY, USA).

Results

Dual administration reduces band cell counts

Leukocyte counts were normalized to hematocrit values because significant dilution occurred after CPB initiation in all groups. Circulation hematocrit levels equilibrated within 7 days post-surgery. The administration of Sivelestat or Rolipram during surgery did not change white blood cell (WBC), red blood cell, and platelet counts (data not shown) or PMN and monocyte counts compared to controls (Fig. 1a). However, the percentage of circulating, non-segmented PMNs (band cells, a BM stimulation indicator) was significantly lower from 24 to 72 h post-surgery in the SR group and from 72 h post-surgery in the S group compared to controls (Fig. 1b). Figure 1C shows the temporal changes of arterial blood gas amounts (under room air) following CPB. The blood oxygen levels after surgery tended to be higher in the SR group ($P = 0.075$), but not significantly among the groups.

BrdU-labeled leukocytes are released from the BM after CPB

The circulatory release of PMN^{BrdU} and MO^{BrdU} from the BM following CPB surgery is shown in Fig. 2a, b. The circulatory fraction of PMN^{BrdU} changed similarly in the S and SR groups (where appearance/disappearance was found slower than controls) and peaked at 96 h post-surgery (versus 72 h for control) (Fig. 2a). In contrast, although the appearance of MO^{BrdU} in the S and SR groups shared the temporal dynamics of PMN^{BrdU}, their washout from S group's circulation was faster than in the SR group which was similar to controls (Fig. 2b).

Calculated transit times of PMN^{BrdU} and MO^{BrdU} through BM were significantly prolonged in the SR group compared to controls (PMN^{BrdU}: 121.0 ± 3.7 h versus 96.2 ± 4.3 h, $p = 0.012$. MO^{BrdU}: 84.4 ± 4.1 h versus 61.4 ± 3.0 h, $p = 0.003$, respectively) (Table 1). Administration of Sivelestat also prolonged PMN^{BrdU} transit time through the BM ($p = 0.041$) while not significantly changing monocyte BM transit time.

Dual Administration Reduces BM-derived Leukocytes in BALF

The absolute number of BrdU-labeled cells in BALF following CPB surgery significantly increased within 24 h, reaching a peak at 96 h post-surgery in controls (Fig. 2c). Values were significantly lower at 48 h post-surgery in the S group and from 48 to 96 h postoperatively in the SR group when compared with controls. A spike in values on the 4th postoperative day was also significantly suppressed in the SR group.

Both IL-6 (Fig. 3a) and IL-8 (Fig. 3b) levels in the BALF significantly increased from the surgical baseline (Time 0) in controls. This increase of IL-8 was significantly reduced at 24 h in both S and SR groups (E: 740.6 ± 220.4 U/mL versus control: 1444.2 ± 250.7 U/mL; $p = 0.044$. SR: 612.6 ± 178.8 U/mL versus control: 1444.2 ± 250.7 U/ mL; $p = 0.032$) and at 48 h in the SR group (SR: 518.3 ± 190.6 U/mL versus control: 1199.1 ± 200.3 U/mL; $p = 0.028$) compared with

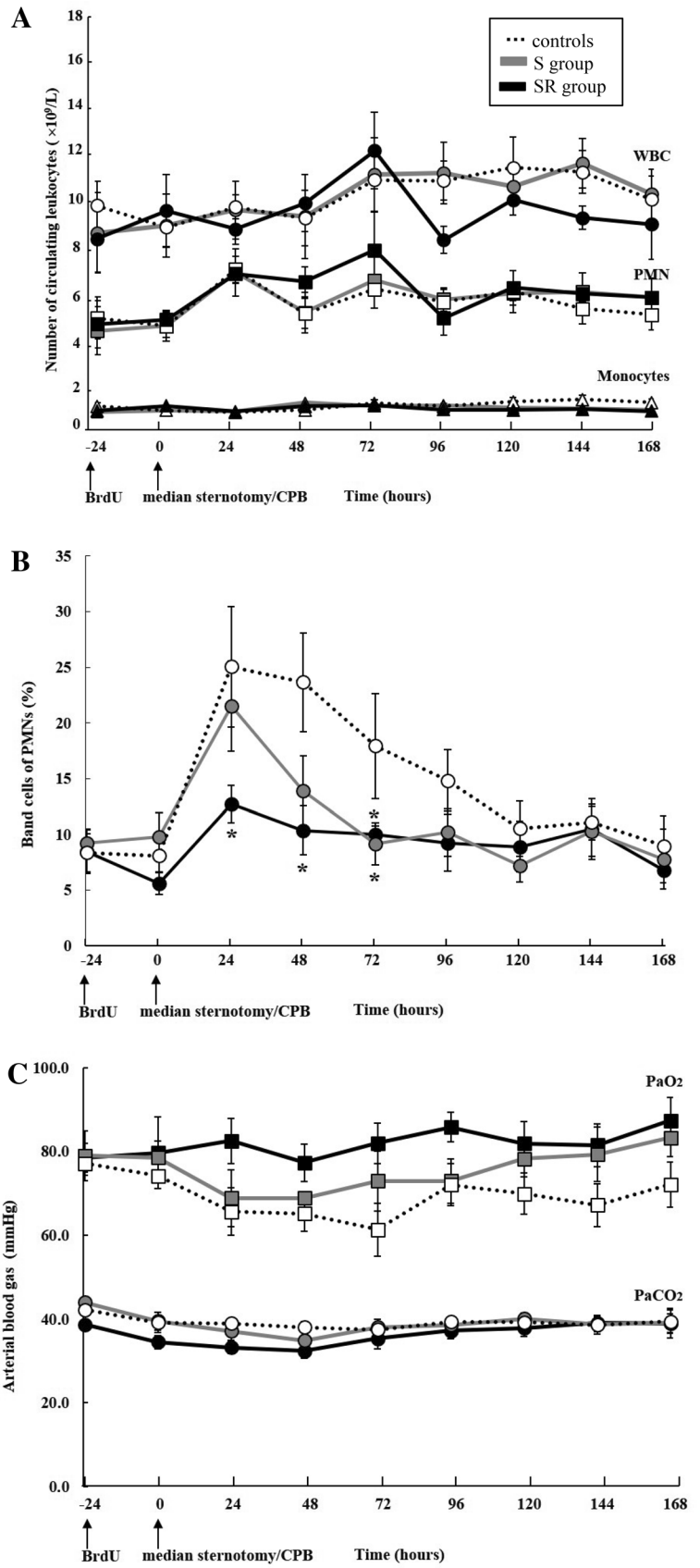

Fig. 1 Changes in **a** circulating white blood cell (WBC) counts, polymorphonuclear leukocytes (PMNs), and monocytes, **b** the percentage of band cells (non-segmented PMNs), and **c** the amounts of arterial gases (room air) following CPB (controls, dashed line, $n=5$; S group, grey line, $n=4$; SR group, solid line, $n=4$). Each value represents the mean ± standard error (SE). $^*P<0.05$ versus the control group. BrdU = 5-bromo-2′-deoxyuridine, S = Sivelestat, SR = Sivelestat + Rolipram

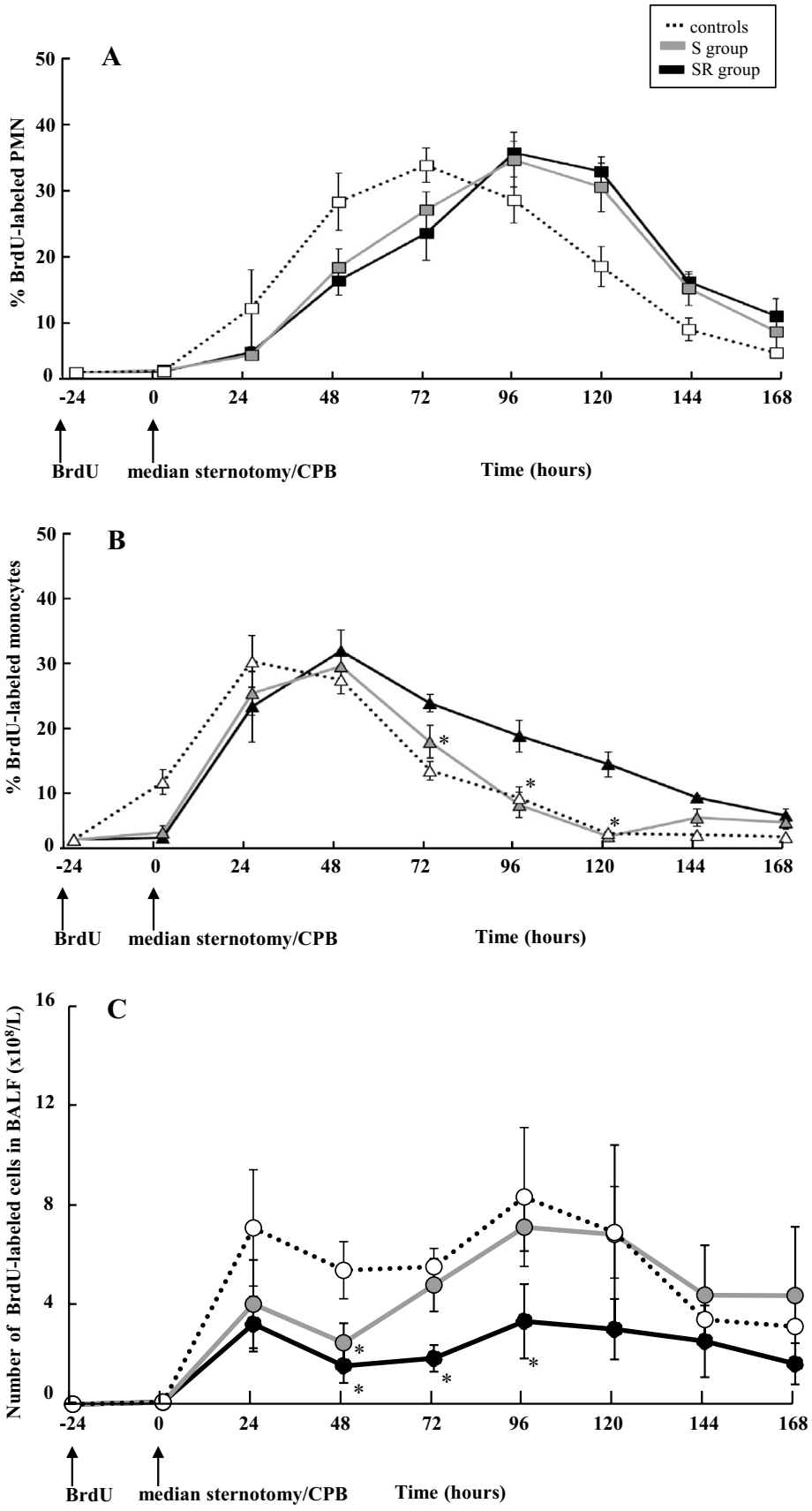

Fig. 2 Percentages of circulating **a** 5-bromo-2′-deoxyuridine (BrdU)-labeled PMNs and **b** monocytes post-surgery with cardiopulmonary bypass (CPB), and **c** number of 5-bromo-2′-deoxyuridine (BrdU)-labeled cells in the bronchoalveolar lavage fluid (BALF) after CPB surgery (controls, dashed line; S group, gray line; SR group, solid line). Each value represents mean ± standard error (SE) obtained for 4–5 monkeys. $^{*}P < 0.05$ versus control group. S = Sivelestat, SR = Sivelestat + Rolipram

Table 1 Leukocyte transit times through bone marrow

Group (*n*)	PMNs (h)	Monocytes (h)
CPB (controls: 5)	96.2 ± 4.3	61.4 ± 3.0
Sivelestat (S: 4)	115.8 ± 6.6*	69.1 ± 4.0
Sivelestat + Rolipram (SR: 4)	121.0 ± 3.7*	84.4 ± 4.1*

All values represent the means ± SE

CPB cardiopulmonary bypass, *n* number of experimental monkeys, *h* hours

*$P < 0.05$ versus control group

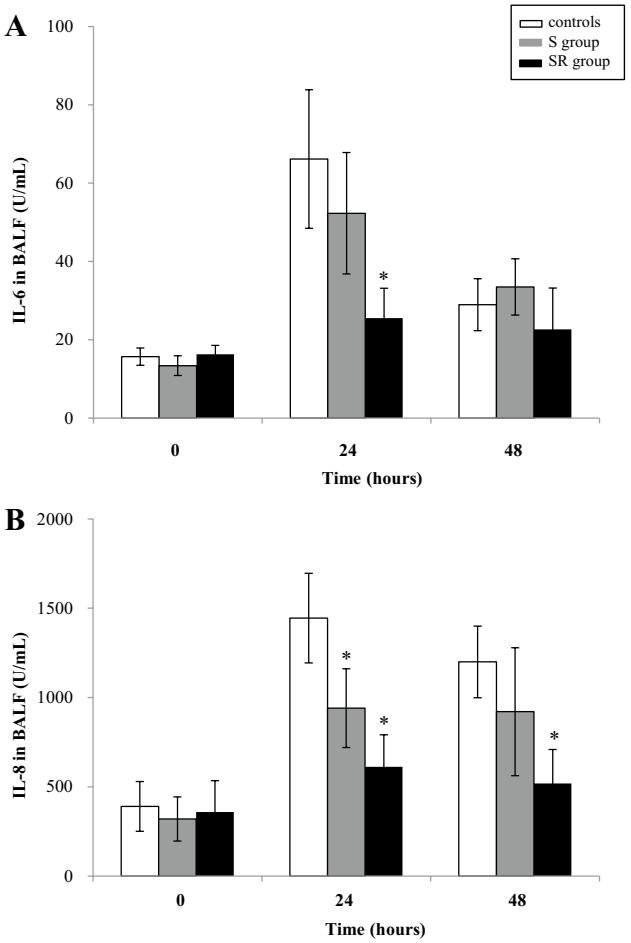

Fig. 3 **a** Interleukin (IL)-6 and **b** IL-8 levels in bronchoalveolar lavage fluid (BALF) after CPB (controls, unfilled bars; S group, gray bars; SR group, filled bars). Surgery was performed at the 0-h time point. Each value represents mean standard error (SE). *$P < 0.05$ versus control group. S = Sivelestat, SR = Sivelestat + Rolipram

controls. The IL-6 increase was also suppressed at 24 h in the SR but not S group.

Dual Administration Suppresses Leukocytic CD11b and CD18 Expression during CPB

In controls, CD11b and CD18 expression on PMNs and monocytes consistently increased from baseline values over the 2-h CPB duration (Fig. 4, unfilled bars) while being significantly inhibited in the SR group ($p < 0.05$, at 2 h), except for CD18 expression on PMNs (Fig. 4, filled bars). There were, however, no significant differences in expression between the S group (Fig. 4, gray bars) and controls.

Discussion

BM-derived leukocyte recruitment following CPB is a systemic recovery response and contributes to the pathogenesis of post-CPB lung injury. We recently developed a precise in vivo model of CPB that exploits thymidine analogue BrdU to label dividing leukocyte precursors in and quantify this BM response [1]. This method evaluates both leukocyte transit time through the marrow as well as circulatory behavior and recruitment into lung tissues. Using this model, we reported that CPB causes rapid release of BM-resident PMNs and monocytes, shortens their transit through BM, and further induces migration into alveolar spaces after surgery [1]. These recruited cells were mostly BM-derived and highly proteolytic PMNs [1] that are thought to damage tissue more than their mature counterparts. Previous reports confirmed the specificity of Sivelestat for inhibition of these damaging proteolytic enzymes such as neutrophil elastase [6, 9] and we reported that Sivelestat controls immune cascade activation via suppression of IL-6 and IL-8 [3, 5]. In our model, Sivelestat was shown to control both neutrophil elastase levels and IL-8 production during a 2-h circulation while preserving neutrophil deformability [6]. Reduction of IL-8, a potent BM leukocyte release activator, downregulates the inflammatory cascade [17]. Therefore, in this study, the next step was to evaluate the in vivo effect of combined Sivelestat and Rolipram on BM-derived leukocyte kinetics induced by CPB.

Sivelestat alone reduces IL-8 levels in the alveolar spaces at 24 h after CPB (Fig. 3B) while decelerating PMN release from and increasing transit time through the BM (Table 1). Although the in vitro efficacy of Sivelestat on post-perfusion lung, ischemia–reperfusion, and endothelial cell injuries has been reported [18–20], there are conflicting conclusions regarding its single-use benefit in human acute lung injury. This discrepancy might be supported by our findings that continuous administration of Sivelestat alone during surgery could not effectively inhibit BM-derived leukocyte lung recruitment and attenuate the exaggerated inflammatory response associated with CPB.

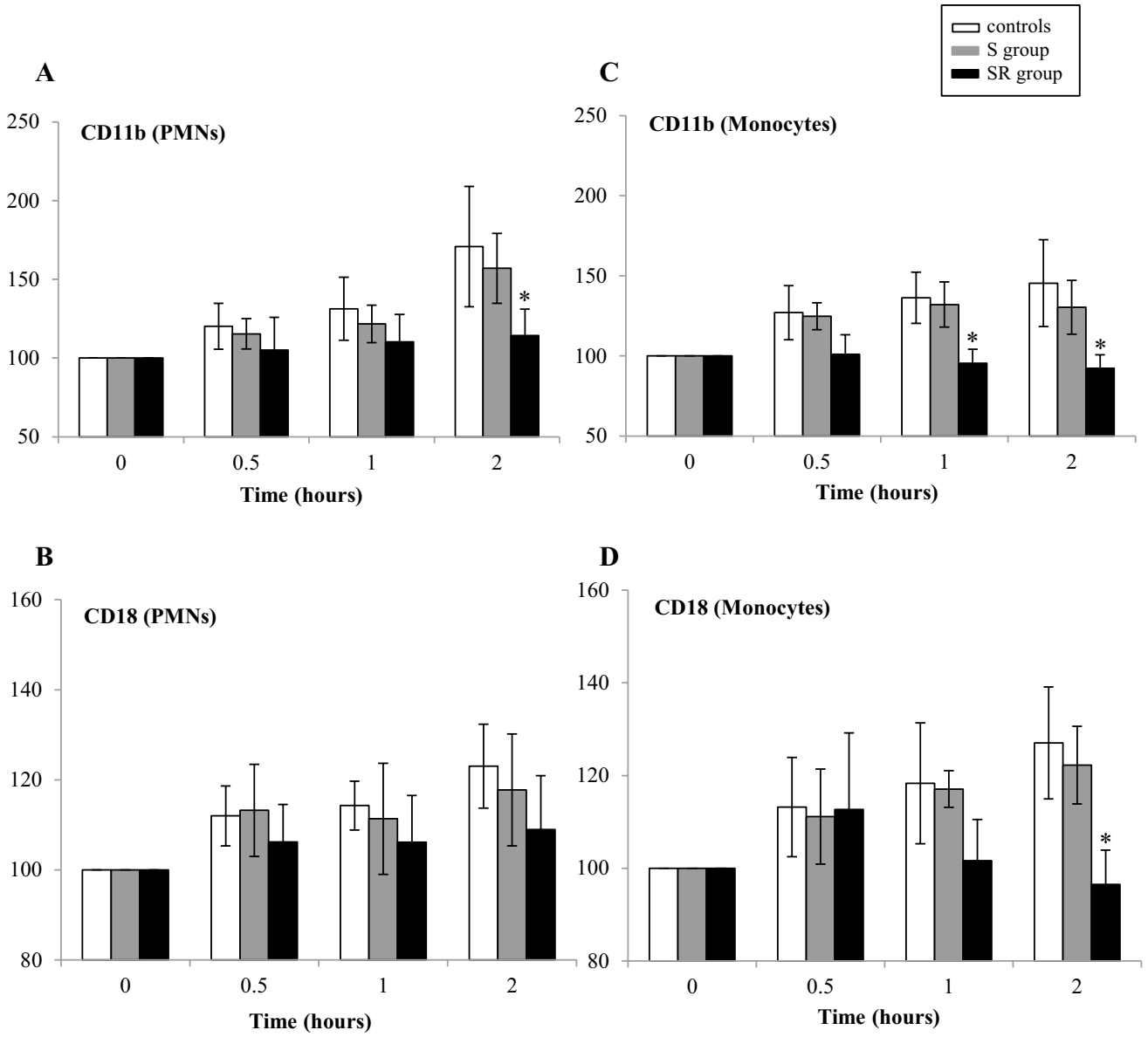

Fig. 4 CD11b and CD18 expression in PMNs (**a, b**, respectively) and monocytes (**c, d**, respectively) during CPB (controls, unfilled bars; S group, gray bars; SR group, filled bars). CD11b and CD18 expression were measured via mean fluorescent intensity and CD11b and CD18 changes were normalized against a baseline value of 100 (Time 0, just before the CPB). Results are expressed as mean ± SE. *$P < 0.05$ versus control group

As for Rolipram, PDE type 4 is the predominant PDE isozyme in leukocytes and Rolipram-mediated increase of cAMP has been reported to play a critical role in suppression of such PDEs [10, 11]. Additionally, we have reported in vitro inhibition of PMN-mediated lung injuries due to Rolipram's effect on initial PMN sequestration in microvessels during CPB [7, 8]. Furthermore, by inhibiting PMN elastase release, Rolipram may prevent endothelial injury [8] and additively reduce neutrophil elastase levels in the alveolar space when paired with Sivelestat. As expected, we found that co-administration during CPB additively decelerated PMN and monocyte release from BM while reducing alveolar space recruitment via suppression of both IL-6 and IL-8 levels (Fig. 3). This was associated with a decrease in bone marrow turnover and an increase in marrow transit time (Table 1), supported by our finding that band cell percentages (indicative of BM stimulation) were significantly decreased. This falls in line with previous reports that Rolipram inhibits monocytic IL-6 [8] which is produced by lung cells to mediate leukocytic production and mobilization from the BM [21]. These multiple angles of biochemical attack from Sivelestat and Rolipram, therefore, regulate

both BM-derived monocytes and PMNs, as IL-6 and IL-8 (which are induced by the initial accumulation of activated, circulating leukocytes during CPB) can activate production and migration of both cell types from the BM into lung tissues. Together, our results indicate that simultaneous, intraoperative administration of these drugs inhibits leukocyte activation during CPB and subsequent lung inflammatory responses at least partially by cytokine downregulation.

Additionally, we investigated CD11b as its increase during in vitro CPB is an accepted biomarker of PMN or monocyte activation [8, 9, 16]. CD11b causes changes in integrins that control firm cellular adhesion [22] resulting in subsequent extravasation of PMNs through endothelial cells, prolonged sequestration in microvessels, and eventual injury [23]. Our in vivo results show that CPB increases CD11b expression in PMNs and monocytes (Fig. 4a, c, respectively) which is in line with former in vitro studies [8, 16] and most clinical studies [24, 25]. In this study, Sivelestat plus Rolipram significantly inhibited the increase of PMN and monocytic CD11b during CPB (Fig. 4, filled bars). However, Sivelestat alone did not suppress CD11b significantly, in line with previous studies showing that only Rolipram attenuates expression of CD11b in PMNs and monocytes [6–8]. These data, therefore, support the idea of co- administration of Sivelestat and Rolipram during CPB to inhibit CD11b-mediated sequestration of PMNs in microvessels.

Limitations

This report is based on our previous in vitro studies [6–9] and verifies those results in our established monkey model [1]. As our dose regimen of Sivelestat and Rolipram was determined based on these previous studies and extrapolated to the in vivo model with an almost doubled circulation volume, we relied on hematocrit values during CPB (data not shown) and this may not directly translate to humans. Moreover, in this experiment, due to the lack of in vivo kinetic assessments within the Rolipram single administration group, our results may demonstrate a more additive than synergistic effect with regard to simultaneous administration of Rolipram and Sivelestat. Sivelestat administration is clinically a part of standard therapy for lung injury and it would be valuable to focus the additional effect of Rolipram with sole administration of Sivelestat as a clinical control, but further exploration is required to elucidate their pharmacological interaction and mechanism in vitro or in vivo.

Conclusions

We investigated the effect of Sivelestat with or without Rolipram on the BM response associated with CPB in an in vivo monkey model and showed that Rolipram has additive inhibitory effects to Sivelestat. Accelerated release of new BM PMNs and monocytes as well as their infiltration into lung tissues were significantly suppressed. In light of this, we propose that pharmacological modulation of BM-derived leukocytes in the alveolar spaces is a feasible therapeutic strategy to prevent CPB-induced lung injury.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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