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ORIGINAL ARTICLE

Suppressive effects of sivelestat on interleukin 8 and TNF-α production from LPS-stimulated granulocytes in whole blood culture

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Abstract
Purpose The goal of the study was to examine the effects of sivelestat sodium hydrate (sivelestat), a neutrophil elastase inhibitor, on production of cytokines in granulocytes and monocytes, using flow cytometry after cytokine staining in whole blood culture.
Methods Blood samples were collected from healthy volunteers. Vehicle (control group), lipopolysaccharide (LPS) (LPS group), or LPS + sivelestat (sivelestat group) were added to the whole blood, followed by addition of a protein transport inhibitor in each group. After incubation, staining for cytokines retained in the cells was performed by addition of an anti-interleukin 8 (IL-8) or anti-tumor necrosis factor-α (TNF-α) antibody. The cells were then analyzed using flow cytometry.
Results Granulocytic production of IL-8 induced by 1 ng/ml LPS was significantly ($P < 0.05$) inhibited by treatment with 1 μg/ml sivelestat, and upregulation of IL-8 by 10 ng/ml LPS was also significantly ($P < 0.05$) suppressed by 1 and 10 μg/ml sivelestat. Addition of 10 or 100 μg/ml sivelestat significantly ($P < 0.05$) inhibited the production of TNF-α from granulocytes induced by 10 ng/ml LPS. Sivelestat did not significantly inhibit LPS-induced monocytic production of TNF-α and IL-8.
Conclusion Suppression of granulocytic production of IL-8 and TNF-α by sivelestat suggests that this drug may be useful for treatment of morbid conditions involving IL-8 and TNF-α at onset.

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Introduction

Systemic inflammatory response syndrome (SIRS) refers to a condition in which excessive biological reactions occur systemically in response to infectious disease, trauma, burns, or surgical stress [1]. Inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), interleukin 6 (IL-6), and interleukin 8 (IL-8) form complex networks in SIRS that activate neutrophils and induce organ failure [2–6]. Neutrophil elastase (polymorphonuclear leukocyte elastase) released from activated neutrophils has many actions, including decomposition of pulmonary connective tissue proteins such as elastin, collagen, fibronectin, and proteoglycans, induction of vascular hyperpermeability, and induction of leukocyte migration factors (C5a, IL-8).

Sivelestat sodium hydrate (sivelestat; Ono Pharmaceutical, Osaka, Japan) is a selective neutrophil elastase inhibitor that has been found to attenuate pulmonary disorders and improve pulmonary function in animal experiments. Clinically, the drug is effective for pulmonary disorders associated with SIRS [7–9]. Blood levels of inflammatory cytokines were decreased in patients treated with sivelestat [10], indicating an inhibitory effect of sivelestat on neutrophil elastase and possible suppression of cytokine production. In clinical studies, however, it is unclear whether monocytes or granulocytes release cytokines in these patients and which cells are influenced by sivelestat, and there are several factors that have effects on the cytokine inhibition (for example, patients with SIRS have already received not only sivelestat but also other

drugs such as antibiotics, and/or patients' backgrounds such as diseases or severities were not exactly the same). Therefore, it remains uncertain if suppression of cytokine levels was caused in vivo by sivelestat. There are several reports on the cytokine inhibitory effects of sivelestat in vitro following treatment with lipopolysaccharide (LPS) [11, 12]. Sivelestat may suppress cytokine production in monocytes isolated from human blood [11], but one report has suggested that a clinical dose of sivelestat has no suppressive effect in cultured type II pulmonary epithelial cells (A549 cells) [12]. Therefore, the cytokine inhibitory effects of sivelestat in vitro are not necessarily revealed in detail, and especially, the effects in granulocytes are unknown.

Neutrophilic cytokines play important roles in induction of pulmonary disorders such as acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). ALI is inhibited by suppression of phosphoinositide 3-kinase (PI3-K) and p38 mitogen-activated protein kinase (p38 MAPK) activity in neutrophils, with an accompanying decrease in downstream TNF-α and IL-8 levels [13–15]. Therefore, reduction of IL-8 and TNF-α production in neutrophils by administration of sivelestat is likely to be significant for suppression of progression of ALI. In the present study, we investigated the cytokine inhibitory effects of sivelestat in granulocytes and monocytes in whole blood, using flow cytometry with intracellular cytokine staining.

Methods

This study was approved by our institutional committee, and written informed consent was obtained from each volunteer. The ability of cells of whole blood to produce cytokines was assessed by flow cytometry with intracellular cytokine staining, as described previously [16]. Briefly, blood samples were collected in heparinized tubes from ten healthy volunteers. Aliquots of the whole blood specimens (1 ml) were mixed with vehicle (phosphate-buffered saline) (control group), 1 or 10 ng of LPS (*Escherichia coli* 0111: B4; Sigma-Aldrich, St. Louis, MO, USA) (LPS group), or 1 or 10 ng of LPS + 1, 10, or 100 μg/ml sivelestat (sivelestat group). A protein transport inhibitor, brefeldin A (10 μg/ml, Sigma-Aldrich), which inactivates the Golgi apparatus and prevents cytokine secretion, was added to each mixture to facilitate intracellular cytokine accumulation [17], followed by incubation at 37°C for 4 h. After incubation, the mixture was subjected to staining with fluorescein isothiocyanate (FITC)-labeled anti-CD33 monoclonal antibody (BD Bioscience, San Jose, CA, USA). Then, after erythrocytic lysis with a fluorescence-activated cell sorting (FACS) lysing solution (BD Bioscience) and permeabilization of the cell membrane with a FACS permeabilizing solution (BD Bioscience), the mixture was treated with phycoerythrin (PE)-labeled anti-IL-8 or anti-TNF-α monoclonal antibody (BD Bioscience). The procedure for measurement of the concentrations of cytokines required 100 min after the 4-h incubation.

Data were acquired by FACSCalibur (Becton-Dickinson, San Jose, CA, USA) and analyzed with CellQuest software (Becton-Dickinson). Cells with the highest FITC fluorescence intensity were regarded as monocytes ($CD33_{bright}$), those with intermediate intensity as granulocytes ($CD33_{dim}$), and those with negative staining as lymphocytes ($CD33_{negative}$). By gating each cell, a histogram of the fluorescence intensities of PE, which reflects cytokine production, was prepared. Differences in the histograms between two groups were compared using the index of similarity [D/S(n)] [18] calculated by the Kolmogorov–Smirnov test in the CellQuest software. The Kolmogorov–Smirnov (K-S) two-sample test is used for overlaid histograms: it tests whether two selected histograms are different. The calculation commutes the summation of the curves and finds the greatest difference between the summation curves [19, 20]. An increase in cytokine production compared to the reference value in the control group (control = 0) was expressed with a plus sign, and a decrease in cytokine production compared to the control value was expressed with a minus sign. Comparison of the three groups was conducted by Friedman test, and comparison of two groups was conducted by Wilcoxon signed-rank test. Differences at $P < 0.05$ were considered to denote significance. Statistical analysis was performed using Dr. SPSS II software (SPSS, Chicago, IL, USA).

Results

Histograms illustrating increased monocytic and granulocytic production of IL-8 and TNF-α induced by LPS (1, 10, and 100 ng/ml) are shown in Fig. 1a–d. The indices of similarity [D/S(n)] calculated by comparing the histograms in the control and LPS groups for monocytic and granulocytic production of IL-8 ($n = 5$) and TNF-α ($n = 5$) are shown in Fig. 2a–d. Intracellular IL-8 and TNF-α were upregulated in granulocytes and monocytes after stimulation with LPS. The upregulation of IL-8 and TNF-α in granulocytes reached a maximum at 10 ng/ml LPS ($P < 0.05$, 1 ng/ml LPS vs. 10 ng/ml LPS, for both IL-8 and TNF-α). In contrast, no obvious dose-dependency was detected in monocytes (there were no significant differences in the levels of upregulation induced by 1, 10, and 100 ng/ml LPS).

We then examined effects of sivelestat on LPS-stimulated cytokine production (Figs. 3, 4, 5). Histograms illustrating

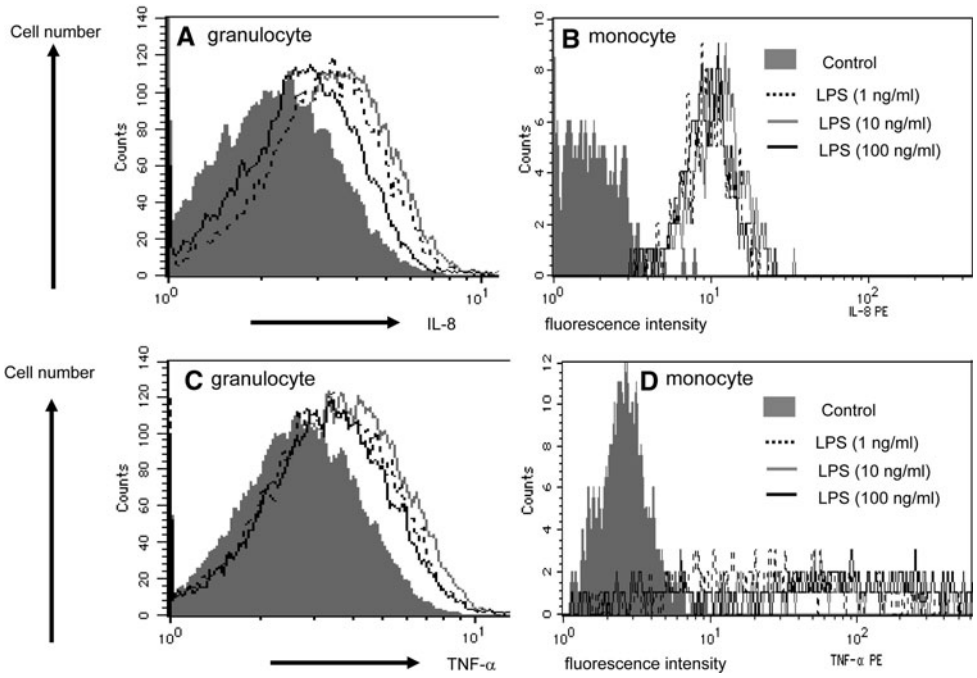

Fig. 1 Histograms illustrating increased monocytic and granulocytic production of interleukin 8 (IL-8) and tumor necrosis factor (TNF)-α induced by lipopolysaccharide (*LPS*) (1, 10, and 100 ng/ml). **a** Granulocytic IL-8; **b** monocytic IL-8; **c** granulocytic TNF-α; **d** monocytic TNF-α

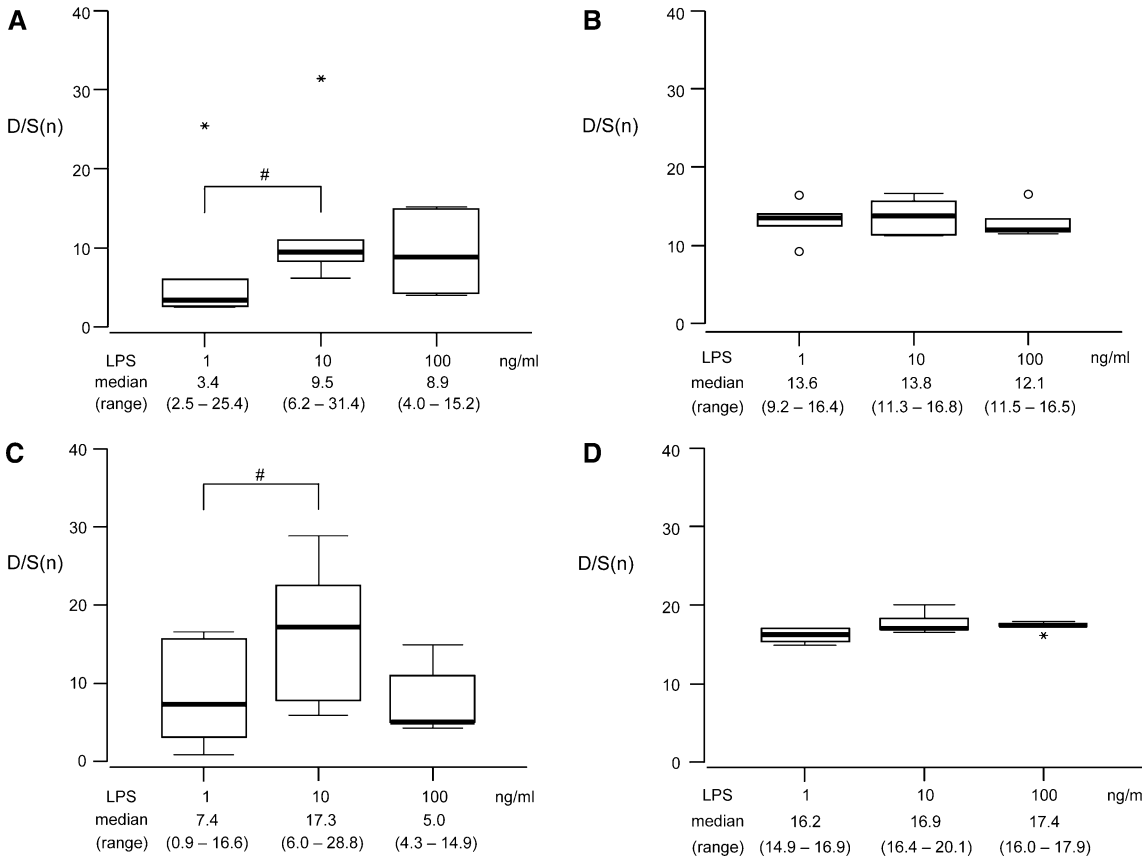

Fig. 2 The indices of similarity [D/S(*n*)] calculated by comparing the histograms in the control and LPS groups for monocytic and granulocytic production of IL-8 ($n = 5$) and TNF-α ($n = 5$). **a** Granulocytic IL-8; **b** monocytic IL-8; **c** granulocytic TNF-α; **d** monocytic TNF-α. Ds(n): index of similarity for intracellular cytokines before any stimulation (control) was designated as zero. *Boxes* show interquartile range (IQR), *middle thick lines* show medians, and *whiskers above* and *below* the *box* show the range between highest and lowest values, excluding outliers. Values plotted with *circles* are greater than 1.5 IQR outside the box, and values plotted with *asterisks* are greater than 3 IQR outside the box. $^{\#}P < 0.05$

the effects of sivelestat at clinical concentrations (1 and 10 μg/ml) on suppression of granulocytic IL-8 production induced by 10 ng/ml LPS are shown in Fig. 3a. Indices of similarity for the effects of sivelestat on IL-8 production in granulocytes induced by 1 and 10 ng/ml LPS ($n = 5$) are shown in Fig. 3b, c. Upregulation of IL-8 by 1 ng/ml LPS was inhibited with 1 μg/ml sivelestat ($P < 0.05$, 1 ng/ml LPS vs. 1 ng/ml LPS + 1 μg/ml sivelestat). Moreover, upregulation of IL-8 by 10 ng/ml LPS was also suppressed with 1 and 10 μg/ml sivelestat ($P < 0.05$, 10 ng/ml LPS vs. 10 ng/ml LPS + 1 and 10 μg/ml sivelestat).

Histograms illustrating the effects of sivelestat at 1 and 10 μg/ml on granulocytic production of TNF-α induced by stimulation with 10 ng/ml LPS are shown in Fig. 4a. Indices of similarity reflecting differences in histograms for TNF-α production in granulocytes induced by stimulation with 1 and 10 ng/ml LPS ($n = 5$) in the absence and presence of sivelestat are shown in Fig. 4b, c. Upregulation of TNF-α by 1 ng/ml LPS was little attenuated with sivelestat. However, upregulation of TNF-α by 10 ng/ml LPS was inhibited by 10 and 100 μg/ml sivelestat ($P < 0.05$, 10 ng/ml LPS vs. 10 ng/ml LPS + 10 and 100 μg/ml sivelestat). Sivelestat exerted no significant effect on suppression of monocytic IL-8 or TNF-α production induced by LPS (Fig. 5a–d).

Discussion

In the present study, we showed that LPS-induced granulocytic IL-8 and TNF-α production was suppressed by sivelestat, but that the drug had only a slight suppressive effect on monocytic cytokine production. In previous studies of the effects of sivelestat on cytokine production, the cytokine levels were determined in isolated monocytes and cultured type II pulmonary epithelial cells (A549 cell) by enzyme-linked immunosorbent assay (ELISA) [11, 12]. Haga and Ogawa [11] reported that sivelestat suppressed production of IL-1β, IL-6, and TNF-α from monocytes isolated from human peripheral blood and stimulated with LPS. However, Misumi et al. [12] found that the production of IL-8 and monocyte chemoattractant protein-1 (MCP-1) from cultured A549 cells with endotoxin or TNF-α was not suppressed by sivelestat at clinical concentrations (1 and 10 μg/ml) but was

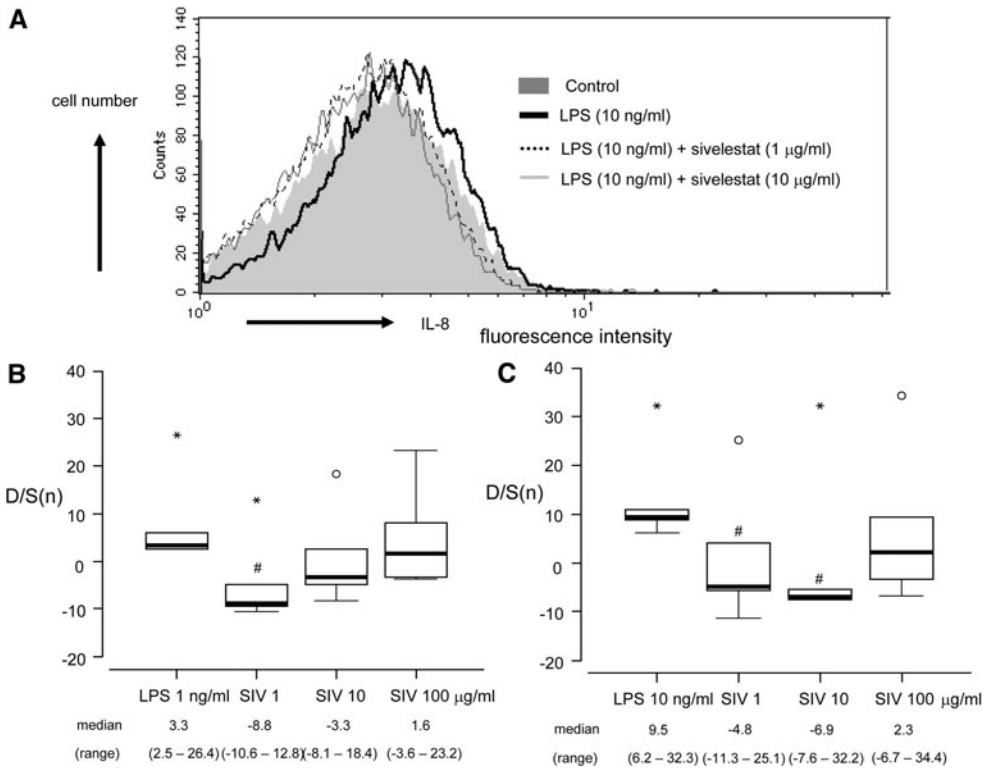

Fig. 3 **a** Histograms illustrating the effects of sivelestat at clinical concentrations (1 and 10 μg/ml) on suppression of granulocytic IL-8 production induced by 10 ng/ml LPS. **b**, **c** Indices of similarity for the effects of sivelestat on IL-8 production in granulocytes induced by 1 (**b**, *left panel*) and 10 (**c**, *right panel*) ng/ml LPS ($n = 5$). D/S(n): index of similarity for intracellular cytokines before any stimulation (control) was designated as zero. *Boxes* show interquartile range (IQR), *middle thick lines* show medians, and *whiskers above* and *below* the *box* show the range between highest and lowest values, excluding outliers. Values plotted with *circles* are greater than 1.5 IQR outside the box, and values plotted with *asterisks* are greater than 3 IQR outside the box. ($n = 5$). $^{\#}P < 0.05$ versus LPS. *LPS*, lipopolysaccharide; *SIV*, sivelestat

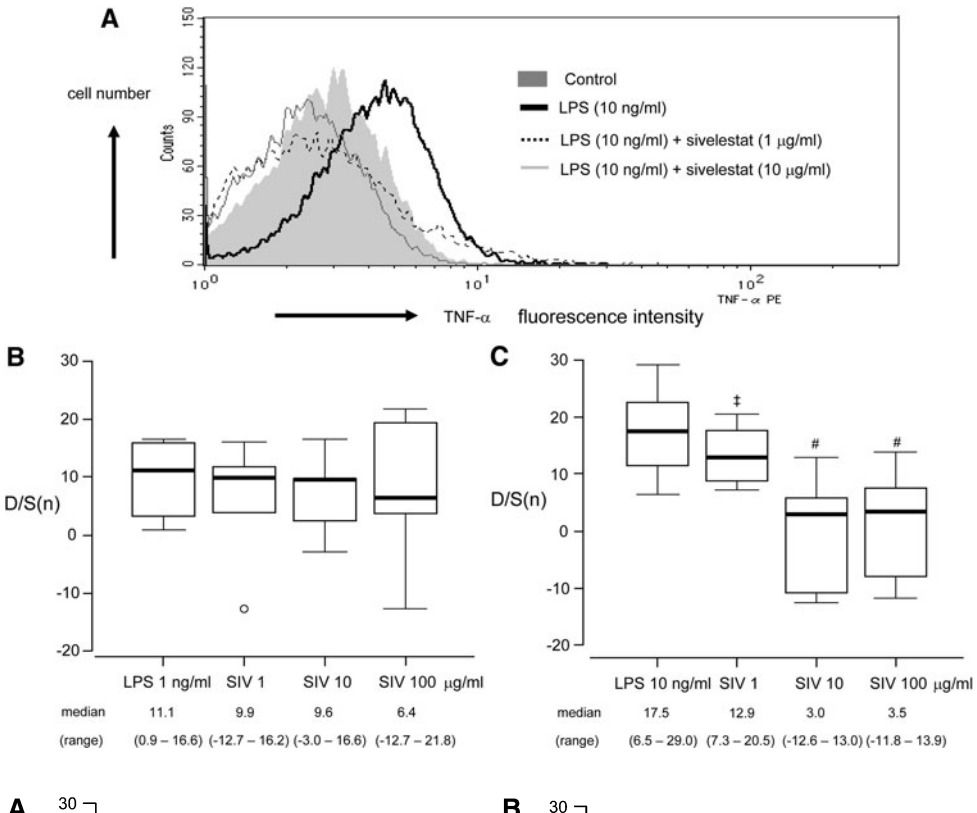

Fig. 4 **a** Histograms illustrating the effects of sivelestat at 1 and 10 μg/ml on granulocytic production of TNF-α induced by stimulation with 10 ng/ml LPS. **b**, **c** Indices of similarity for the effects of sivelestat on TNF-α production in granulocytes induced by 1 (**b**, *left panel*) and 10 (**c**, *right panel*) ng/ml LPS ($n = 5$). Ds(n): index of similarity for intracellular cytokines before any stimulation (control) was designated as zero. *Boxes* show interquartile range (IQR), *middle thick lines* show medians, and *whiskers above* and *below* the *box* show the range between highest and lowest values, excluding outliers. Values plotted with *circles* are greater than 1.5 IQR outside the box. $^{\#}P < 0.05$ versus LPS. $^{\ddagger}P < 0.05$ versus control. *LPS*, lipopolysaccharide; *SIV*, sivelestat

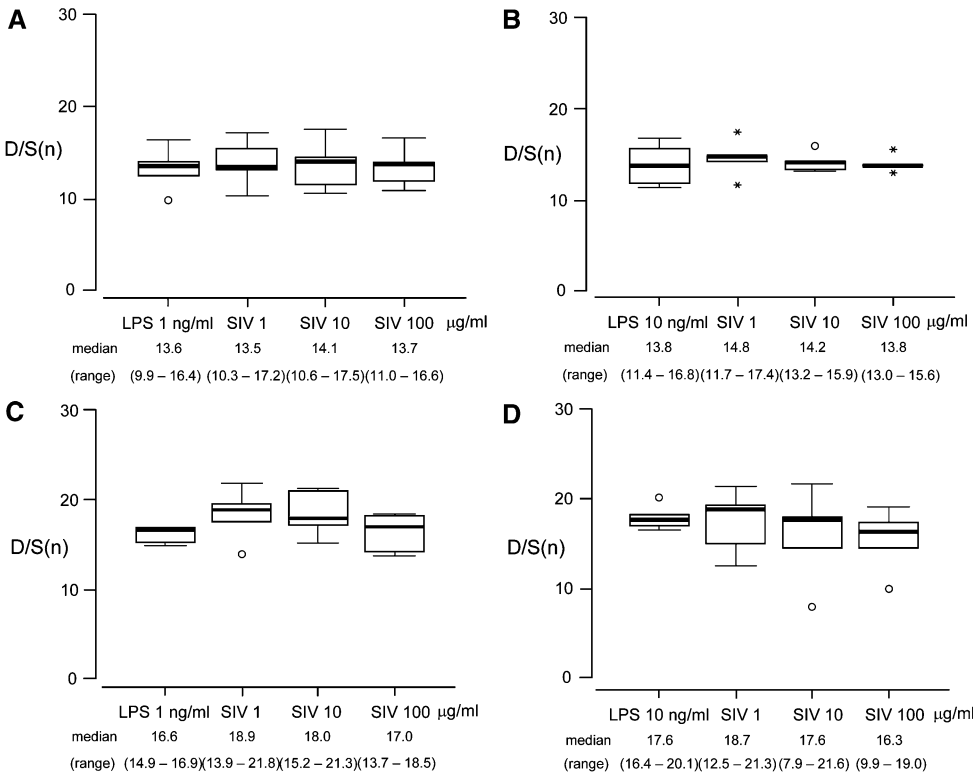

Fig. 5 Few suppressive effects of sivelestat were observed on LPS-induced TNF-α and IL-8 production in monocytes. **a** IL-8; 1 ng/ml LPS. **b** IL-8; 10 ng/ml LPS. **c** TNF-α; 1 ng/ml LPS. **d** TNF-α; 10 ng/ml LPS. Ds(n): index of similarity for intracellular cytokines before any stimulation (control) was designated as zero. *Boxes* show interquartile range (IQR), *middle thick lines* show medians, and *whiskers above* and *below* the *box* show the range between highest and lowest values, excluding outliers. Values plotted with *circles* are greater than 1.5 IQR outside the box, and values plotted with *asterisks* are greater than 3 IQR outside the box. *LPS*, lipopolysaccharide; *SIV*, sivelestat

suppressed at a high concentration (100 μg/ml). Similar results were obtained by quantification of the mRNA for IL-8 and MCP-1. It has been reported that the whole blood concentration of sivelestat after 2-h continuous intravenous administration (C_{2h}) at the dose of 0.5 mg/kg/h is 11.7 μg/ml [21].

The discrepancies between previous studies and our study may result from differences in the study design.

In experiments using cells isolated from blood, cell-to-cell interaction and cytokine networks might have been blocked. For example, whole blood contains endogenous protease inhibitors such as α-1 protease inhibitor (α1-PI). Because α1-PI functions as a scavenger for neutrophil elastase in plasma [22] and inhibits LPS-stimulated TNF-α released from monocytes [23], the effects of sivelestat on monocytes might decrease. Furthermore, it has recently been shown that neutrophil elastase induces IL-8 production via toll-like receptor 4 (TLR4) [24]. Because α1-PI may rapidly inactivate neutrophil elastase after LPS stimulation, neutrophil elastase might fail to induce IL-8 production via TLR4 on monocytes. At inflammatory sites, protease inhibitors, typically α1-PI, are inactivated by neutrophil-derived reactive oxygen species [25]. Therefore, sivelestat may be able to reduce not only neutrophilic but also monocytic cytokine production activated by neutrophil elastase at inflammatory sites such as ARDS or sepsis. We cannot dismiss the possibility that sivelestat had directly suppressed signals such as phosphorylation after intracellular transition. However, it has been reported, from a preliminary experiment for evaluation of the characteristics of intracellular transition of sivelestat, that sivelestat shows little intracellular transition and does not exert any inhibitory effect on intracellular elastase [26].

The suppressive effect of sivelestat on the neutrophilic production of IL-8 or TNF-α was qualitatively similar. In a quantitative aspect, however, the suppression of IL-8 production was observed at 1 μg/ml sivelestat, whereas more than 10 μg/ml sivelestat was required for TNF-α production (see Figs. 3, 4). The different potency of sivelestat might be derived from different sensitivity for inhibiting the production of IL-8 or TNF-α. Alternatively, different mechanisms might be involved in the suppressive effect of sivelestat on different cytokines. Further studies are clearly necessary to elucidate this point.

Neutrophil elastase has recently been recognized as an etiological factor in pulmonary disorders associated with SIRS [27]. We have reported high levels of neutrophil elastase and IL-8 in ARDS associated with sepsis [6] and high levels of neutrophil elastase in the bronchoalveolar lavage fluid of ARDS patients [28]. It has also been reported that administration of sivelestat decreases the blood levels of neutrophil elastase, IL-8, and TNF-α and improves the respiratory condition in patients with acute respiratory disorder [10]. These observations lend support to the results of the present study, which showed that sivelestat does not merely inhibit neutrophil elastase, but also suppresses production of IL-8 and TNF-α. Suppression of IL-8 and TNF-α production results in reduced activation of neutrophils, which probably leads to a decrease in the production of neutrophil elastase and reduction in the severity of respiratory disorders.

In conclusion, the present study has suggested that sivelestat suppressed LPS-induced granulocytic IL-8 and TNF-α production, and may be useful not only as a neutrophil elastase inhibitor, but also for treatment of various morbid conditions that may be induced by IL-8 and TNF-α.

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