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TRANSLATIONAL RESEARCH

Volatile anaesthetics reduce neutrophil inflammatory response by interfering with CXC receptor-2 signalling

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Editor's key points

- Accumulation of neutrophils contributes to ischaemia-reperfusion injury.
- The role of CXC signalling in the mechanism of sevoflurane- and desflurane-mediated protection was studied in human neutrophils.
- Sevoflurane and desflurane interfered with CXC receptor-2 signalling.

Background. Growing evidence suggests a protective effect of volatile anaesthetics in ischaemia-reperfusion (I/R)-injury, and the accumulation of neutrophils is a crucial event. Pro-inflammatory cytokines carrying the C-X-C-motif including interleukin-8 (IL-8) and CXC-ligand 1 (CXCL1) activate CXC receptor-1 (CXCR1; stimulated by IL-8), CXC receptor-2 (CXCR2; stimulated by IL-8 and CXCL1), or both to induce CD11b-dependent neutrophil transmigration. Inhibition of CXCR1, CXCR2, or both reduces I/R-injury by preventing neutrophil accumulation. We hypothesized that interference with CXCR1/CXCR2 signalling contributes to the well-established beneficial effect of volatile anaesthetics in I/R-injury.

Methods. Isolated human neutrophils were stimulated with IL-8 or CXCL1 and exposed to volatile anaesthetics (sevoflurane/desflurane). Neutrophil migration was assessed using an adapted Boyden chamber. Expression of CD11b, CXCR1, and CXCR2 was measured by flow cytometry. Blocking antibodies against CXCR1/CXCR2/CD11b and phorbol myristate acetate were used to investigate specific pathways.

Results. Volatile anaesthetics reduced CD11b-dependent neutrophil transmigration induced by IL-8 by >30% and CD11b expression by 18 and 27% with sevoflurane/desflurane, respectively. This effect was independent of CXCR1/CXCR2 expression and CXCR1/CXCR2 endocytosis. Inhibition of CXCR1 signalling did not affect downregulation of CD11b with volatile anaesthetics. Blocking of CXCR2-signalling neutralized effects by volatile anaesthetics on CD11b expression. Specific stimulation of CXCR2 with CXCL1 was sufficient to induce upregulation of CD11b, which was impaired with volatile anaesthetics. No effect of volatile anaesthetics was observed with direct stimulation of protein kinase C located downstream of CXCR1/CXCR2.

Conclusion. Volatile anaesthetics attenuate neutrophil inflammatory responses elicited by CXC cytokines through interference with CXCR2 signalling. This might contribute to the beneficial effect of volatile anaesthetics in I/R-injury.

Keywords: anaesthetics, inhalation, desflurane; anaesthetics, inhalation, sevoflurane; receptors, chemoreceptors

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Ischaemia-reperfusion injury (I/R-injury) during anaesthesia occurs as an unforeseen event, for example, transient myocardial ischaemia or as a planned step in surgery such as the Pringle manoeuvre during liver resection. Volatile anaesthetics protect different organs from I/R-injury such as liver, lung, and heart.[1–3] However, the mechanism of this protective effect remains unclear.

The restoration of blood flow after ischaemia activates innate and adaptive immune responses leading to an accumulation of neutrophils in the reperfused organ and subsequent tissue damage.[4] Consequently, inhibition of neutrophil invasion during reperfusion was demonstrated to reduce the extent of tissue damage.[5–7]

The accumulation of neutrophils in I/R-injury is the result of a three-step process: first, circulating neutrophils establish a low-affinity adhesive interaction with the endothelium called rolling.[8] This rolling is mediated by L-selectin (CD62L), which belongs to the family of glycoproteins on the neutrophil

surface that binds endothelial carbohydrate determinants. In a second step, the neutrophil adheres firmly to the endothelium through adhesion between integrins on the neutrophil surface and endothelial intercellular adhesion molecules. The most prominent integrin during this process is CD11b, which binds to endothelial intercellular adhesion molecule-1. The third and final step, which involves various adhesive glycoproteins including CD62L and CD11b, is transmigration of the neutrophil through the endothelium.

The family of CXC cytokines is defined by two N-terminal cystines separated by one amino acid (hence C-X-C). A subgroup of these CXC cytokines carries a Glu-Leu-Arg tripeptide (ELR) motif at the NH2-end and is termed ELR$^+$ CXC cytokines. This subgroup includes interleukin-8 (IL-8) and promotes the recruitment of neutrophils into inflamed tissues.[9] ELR$^+$ cytokines bind to two G protein-coupled receptors on the neutrophil surface, CXC receptor-1 (CXCR1) and CXC receptor-2 (CXCR2).[10 11] Signalling through these receptors is important during I/R-injury and pharmacological inhibition was demonstrated to reduce neutrophil infiltration and subsequent tissue damage.[12]

We hypothesized that the beneficial effects of volatile anaesthetics during I/R-injury are due to direct or indirect effects on CXCR1 and CXCR2 signalling that might alter the process of neutrophil accumulation. We chose IL-8 as a representative ELR$^+$ cytokine as it binds to both CXCR1 and CXCR2 with high affinity and is released by leucocytes and stromal cells such as fibroblasts in high concentrations during reperfusion.[13–16]

Methods

The study protocol was approved by the ethics committee for studies on humans of the University Hospital Zurich (KEK-ZH 2012-0274) and written informed consent was obtained. Twelve healthy volunteers (7 males/5 females, aged 19–52 yrs) were recruited from the Institute of Physiology in Zurich and blood samples (5 ml) were obtained from an antecubital vein into citrate tubes. Exclusion criteria were acute disease in the last 14 days or chronic disease with or without medical treatment of any type. Oral contraceptives were accepted for female donors. Red blood cells were lysed and neutrophils were isolated using Ficoll-Histopaque 1077 (Sigma-Aldrich, Buchs, Switzerland) as described previously.[17] The isolated neutrophils were resuspended at a concentration of 2×10^6 ml^{-1} in Ham's F-12 medium supplemented with 10% heat-inactivated fetal bovine serum, 5% penicillin/streptomycin (10 000 U litre^{-1}), and 5% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (all from Invitrogen/Life Technologies, Zug, Switzerland). All steps were performed at 4°C to prevent neutrophil activation.

Fifty microlitres of neutrophil cell suspension (10^5 neutrophils) were placed into sterile 96-well plates and exposed to 10 nM IL-8 (recombinant human IL-8, BD Pharmingen, Allschwil, Switzerland) or 200 ng ml^{-1} CXC-ligand 1 (CXCL1) (recombinant human CXCL1, R&D systems, Wiesbaden, Germany). Phorbol myristate acetate at 10 nM (Sigma-Aldrich) was used to activate protein kinase C in the corresponding experiments. These concentrations are commonly used and known to induce profound neutrophil activation and migration. To study the effect of I/R in our setting, human lung microvascular endothelial cells were exposed to 12 h of hypoxia (0.2% O_2), followed by 12 h of reoxygenation at 21% O_2. Neutrophils were then stimulated with the harvested supernatants at 1:1 dilution.

In some experiments, neutrophils were incubated for 15 min before stimulation with 10 μg ml^{-1} of anti-human CXCR1 and anti-human CXCR2 (Abcam, Cambridge, UK) or against the activation epitope of CD11b (anti-human CBRM1/5, Biolegend, Lucerne, Switzerland). Plates were then put in humified airtight chambers (Oxoid anaerobic jar; Oxoid AG, Basel, Switzerland).

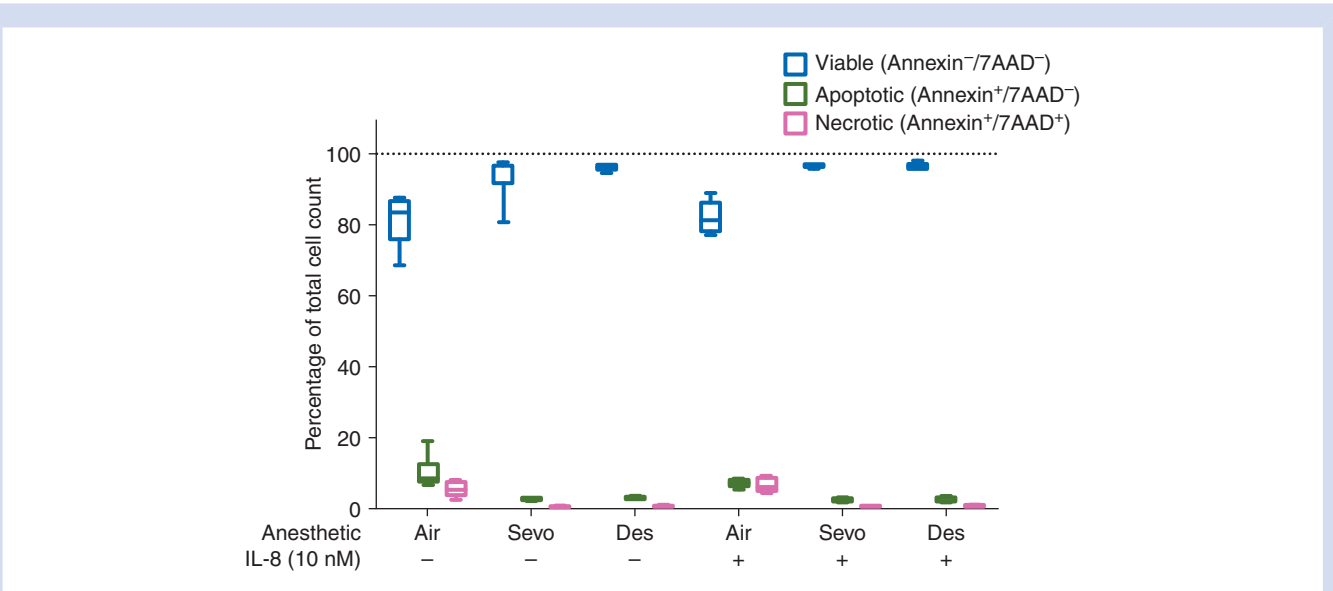

Fig 1 Viability of neutrophils exposed to IL-8 with and without sevoflurane or desflurane. Viable neutrophils are negative for annexin and 7-aminoactinomycin (7AAD, Annexin$^-$/7AAD$^-$, blue). Apoptotic neutrophils are positive for annexin and negative for 7AAD (Annexin$^+$/7AAD$^-$, green). Necrotic cells are positive for annexin and 7AAD (Annexin$^+$/7AAD$^+$, pink). Median, inter-quartile and full range shown (n=6).

Chambers were flushed with air/5% CO_2 containing 2.2 vol% sevoflurane (Sevorane®; Abbott AG, Baar, Switzerland, corresponding vaporizer: Sevotec5®; Abbott AG) or 6% vol% desflurane (Forene®, Baxter, Switzerland; corresponding vaporizer: Tec6, Carbamed, Switzerland). Control cells were exposed to air/5% CO_2 only. Neutrophils from each donor were investigated under all the three conditions. Concentrations of volatile anaesthetics were measured with the Ohmeda 5330 Agent Monitor (Abbott AG). After reaching the described concentrations, the chambers were sealed and kept in an incubator at 37°C (Bioblock, Ittingen, Switzerland). Volatile anaesthetic concentrations were checked again at the end of the incubation period to ensure that there was no loss of anaesthetic from insufficient sealing or evaporation.[17]

A 96-well migration plate with 3-μm pores (Millipore-Merck, Zug, Switzerland) was used to investigate neutrophil migration. Fifty microlitres of neutrophil suspension (10^5 cells) was added to the upper compartment of the plate. Medium supplemented with 10 nM IL-8 was added to the lower compartment. Plates were exposed to volatile anaesthetics as described above immediately after assembly of the plate and neutrophils were allowed to migrate for 1 h. Migrated neutrophils were quantified using an automated optical cell counter (TC-10, Biorad, Cressier, Switzerland).

Surface expression of CXCR1, CXCR2, CD11b, and CD11b activation epitope was quantified using flow cytometry. Neutrophils were kept on ice and fixed with 2% paraformaldehyde to prevent changes in receptor expression during staining. Cells were stained with specific antibodies for 30 min at 4°C, washed twice, and measured on a FACS Canto II (BD Pharmingen). Flow cytometry raw data were analysed using the FlowJo-Software for Macintosh (version 8.8.6, Tree Star Inc., Ashland, OR, USA). The following mouse anti-human antibodies were used (all antibodies from BD Pharmingen): APC-Cy7-conjugated CD11b (final staining concentration 2 μg ml^{-1}), APC-conjugated CXCR1 (1.25 μg ml^{-1}), and FITC-conjugated CXCR2 (0.25 μg ml^{-1}). FITC-conjugated CBRM1/5 (0.2 μg ml^{-1}) was obtained from Biolegend. Appropriate isotype-control antibodies were used to quantify nonspecific binding.

Neutrophil viability was quantified by staining the cells with annexin-V and 7-aminoactinomycin (PE Annexin V Apoptosis Detection Kit I, BD Pharmingen). IL-8 concentrations in supernatants were quantified using an enzyme-linked immunosorbent assay according to the manufacturer's protocol (R&D systems). This kit has a sensitivity range from 31.2 to 2000 pg ml^{-1} and all samples were diluted to achieve a concentration in the linear range.

Distribution of the data was assessed using the Kolmogorov–Smirnov test. Normally distributed data were analysed using a one-way ANOVA with Bonferroni post hoc test. Nonparametric data were analysed using Kruskal–Wallis and Dunn's multiple comparison tests. All experiments were performed with blood from three or more different donors with conditions repeated in duplicates. A P-value of <0.05 was considered statistically significant. Statistical analyses and graph creation were executed with Graphpad Prism 6 for Mac (Graphpad Software, La Jolla, CA, USA).

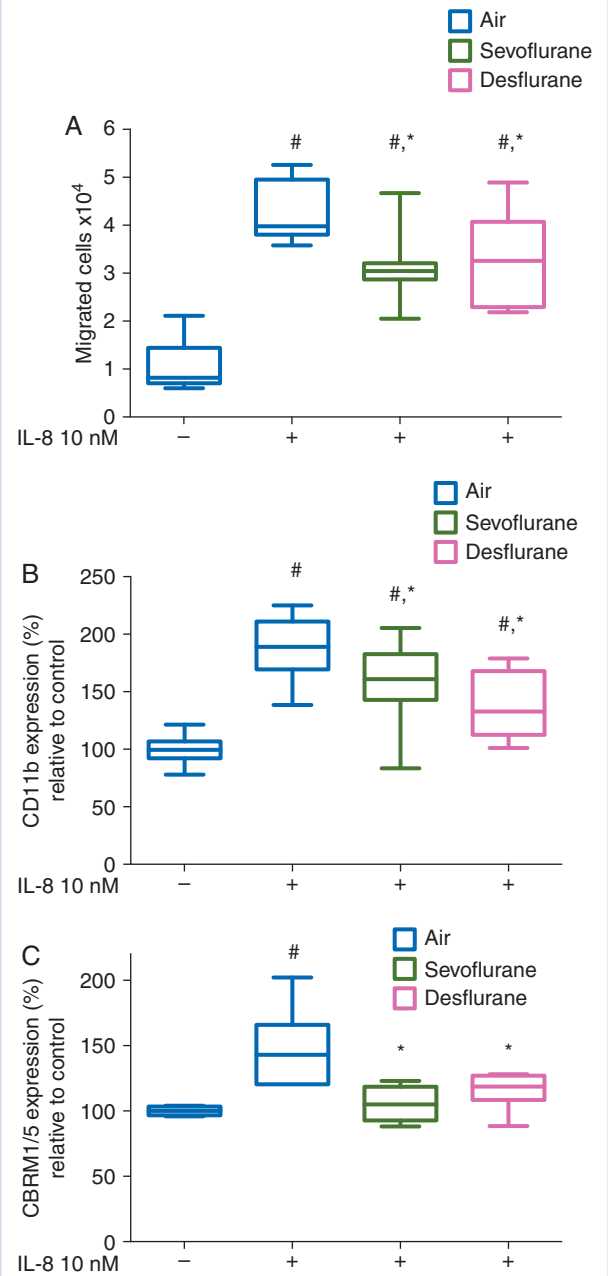

Fig 2 (A) Neutrophil transmigration in response to IL-8 in the presence of sevoflurane or desflurane for 1 h. $^{\#}P<0.0001$ *vs* control; $^{*}P<0.05$ *vs* IL-8 stimulated neutrophils exposed to air/5% CO_2 during transmigration. $n=9$. (B) Neutrophil CD11b expression after 1 h exposure to IL-8 with and without sevoflurane or desflurane. Mean fluorescence of CD11b relative to control neutrophils not exposed to IL-8 shown. $^{\#}P<0.0001$ *vs* control neutrophils without IL-8; $^{*}P<0.05$ *vs* IL-8 treated neutrophils without anaesthetic. ($n=12$). (C) Neutrophil activation-epitope of CD11b-specific CBRM1/5 expression. Mean fluorescence of the antibody clone relative to control neutrophils not exposed to IL-8 shown. $^{\#}P<0.001$ *vs* control neutrophils without IL-8; $^{*}P<0.05$ *vs* IL-8 treated neutrophils without anaesthetic. Median, inter-quartile, and full range shown ($n=6$).

Results

Our isolation procedure yielded neutrophils with a purity of >94% and a viability of >99% (data not shown). Exposure to volatile anaesthetics did not reduce neutrophil viability (Fig. 1).

Exposure to IL-8 induced transmigration of 43% of neutrophils within 1 h; exposure to sevoflurane or desflurane reduced migration by 36 and 32% (Fig. 2A; $P=0.012$ for sevoflurane, $P=0.032$ for desflurane). Neutrophil transmigration in this setting was dependent upon CD11b and abrogated by CD11b-blocking antibodies (Supplementary Fig. S1). Accordingly, upregulation of CD11b after stimulation with IL-8 was attenuated by both sevoflurane and desflurane (Fig. 2B; $P=0.013$ for sevoflurane, $P=0.001$ for desflurane). Staining for the activation-epitope of CD11b revealed a similar pattern with lower levels of activated CD11b in neutrophils exposed to sevoflurane and desflurane (Fig. 2C; $P=0.002$ for sevoflurane, $P=0.02$ for desflurane). Reduction of CD11b expression with volatile anaesthetics was also observed when neutrophils were stimulated with supernatants derived from hypoxia/reoxygenation-exposed endothelial cells (Supplementary Fig. S2).

Stimulation of neutrophils with IL-8 resulted in marked endocytosis and therefore reduction of surface expression of CXCR1 by 47% and CXCR2 by 81% (Fig. 3A; $P<0.0001$). Neither sevoflurane nor desflurane affected this process. Also, baseline CXC receptor expression in resting neutrophils was not affected by volatile anaesthetics (data not shown). The extent of endocytosis of the ligand–receptor complexes formed by IL-8 and CXCR1 or CXCR2 resulted in consumption and hence decrease of free IL-8 in the medium during incubation (Fig. 3B, $P=0.008$). Again, this was not influenced by sevoflurane or desflurane (Fig. 3B).

Direct stimulation of protein kinase C with phorbol myristate acetate resulted in upregulation of CD11b. No effect of sevoflurane and desflurane was observed in this setting (data not shown).

Blocking antibodies against CXCR1 and CXCR2 inhibited the upregulation of CD11b in response to IL-8 (Fig. 4A; $P<0.001$ and $P=0.01$, respectively). However, blocking of CXCR2 before stimulation neutralized the effect of volatile anaesthetics, while blocking of CXCR1 resulted in preserved attenuation of CD11b with sevoflurane and desflurane (Fig. 4B; $P<0.05$ for both sevoflurane and desflurane). In addition, stimulation with CXCL1, to activate CXCR2, reproduced the effect of volatile anaesthetics (Fig. 4C; $P<0.01$ for sevoflurane, $P<0.001$ for desflurane).

Conclusions

Our study demonstrates that volatile anaesthetics interfere with neutrophil inflammatory pathways, leading to decreased neutrophil migration and reduced expression of β_2-integrin CD11b. We locate the site of action of volatile anaesthetics to be downstream of CXCR2, a target receptor of ELR$^+$ CXC cytokines that are involved in I/R-injury.

We focused the current study on the specific impact of volatile anaesthetics on neutrophil signalling through CXCR1 and CXCR2. The importance of the ELR$^+$ family of cytokines that signals through these receptors is well established and their inhibition emerges as a new therapeutic option in I/R-injury.[9] IL-8 is a suitable cytokine to represent the ELR$^+$ family because of its high affinity to both of the ELR$^+$ target receptors, CXCR1 and CXCR2.[18] In addition, high levels of IL-8 are released during I/R-injury, and neutralization of IL-8 was found to attenuate tissue damage during I/R-injury.[16]

The accumulation of neutrophils in the reperfused organ after ischaemia is a multi-step process eventually leading to local tissue damage.[4] Inhibition of this event through various means such as depletion of neutrophils or antibodies against neutrophil adhesion molecules decreases tissue injury.[7 19] The ability of neutrophils to transmigrate through the endothelium is pivotal to their accumulation in the reperfused organ.

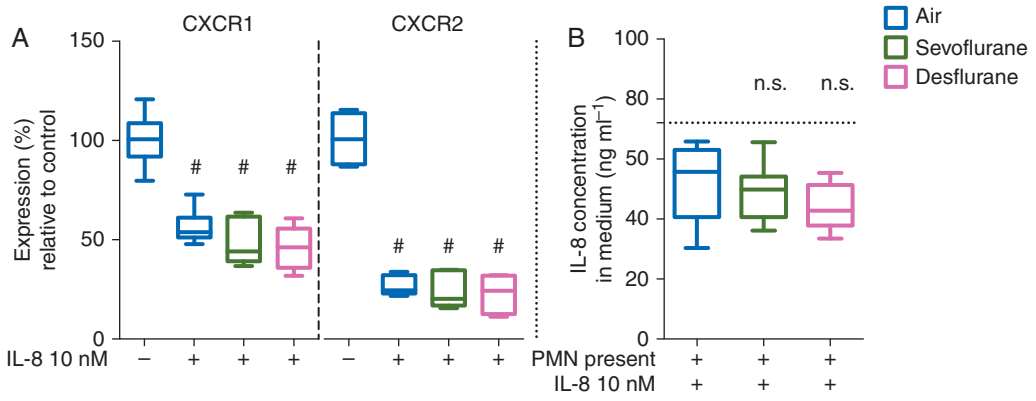

Fig 3 (A) CXCR1 (left panel) and CXCR2 (right panel) expression in neutrophils exposed to IL-8 for 1 h with and without sevoflurane or desflurane. Mean fluorescence relative to control neutrophils without IL-8 shown. $^{\#}P<0.001$ *vs* control neutrophils without IL-8 ($n=6$). (B) IL-8 concentrations in medium from neutrophils polymorphonuclear leucocyte (PMN) cultured with IL-8 for 1 h with and without sevoflurane or desflurane. Dashed line shows measured IL-8 concentration in the absence of neutrophils (expected concentration 80 ng ml^{-1}). No effect of sevoflurane or desflurane in IL-8 treated neutrophils. Median, inter-quartile, and full range shown ($n=8$).

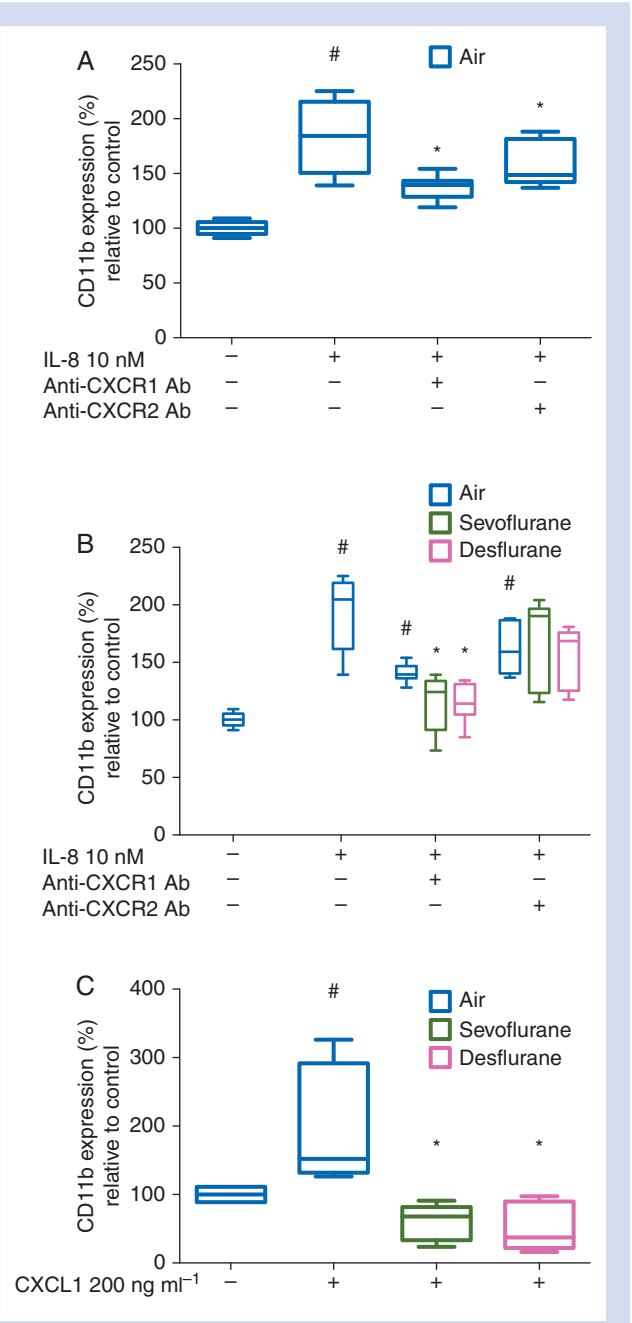

Fig 4 (A) Effect of anti-CXCR1- or anti-CXCR2-antibodies on CD11b expression in neutrophils with and without IL-8. Mean fluorescence of CD11b relative to control neutrophils without IL-8 shown. $^{\#}P<0.0001$ *vs* control, $^{*}P<0.05$ *vs* IL-8 alone ($n=8$). (B) Effect of anti-CXCR1- or anti-CXCR2-antibodies on CD11b expression in neutrophils with and without exposure to sevoflurane or of desflurane. Mean fluorescence of CD11b relative to control neutrophils without IL-8 shown. $^{\#}P<0.0001$ *vs* untreated control, $^{*}P<0.05$ *vs* IL-8 without anaesthetics ($n=6$). (C) Effect of CXCL1 on CD11b expression on neutrophils exposed to sevoflurane or desflurane. Mean fluorescence of CD11b relative to neutrophils without CXCL1 shown. $^{\#}P<0.05$ *vs* neutrophils without CXCL1, $^{*}P<0.01$ *vs* without anaesthetic. Median, inter-quartile, and full range shown ($n=6$).

We found that sevoflurane and desflurane reduced IL-8 induced transmigration of neutrophils.

The process of transmigration is mediated by several neutrophil and endothelial adhesive proteins. However, inhibition of the neutrophil–endothelial interaction between CD11b on the neutrophil surface and endothelial intercellular adhesion molecule-1 proved to be most important.[20] CD11b is critical to establish tight adherence of neutrophils to the endothelium, and inhibition of CD11b using antibodies blocks neutrophil recruitment and reduces tissue damage both *in vitro* and *in vivo*.[21 22] In our setting, neutrophil transmigration was dependent on CD11b expression and both sevoflurane and desflurane attenuated the upregulation of CD11b in response to IL-8. These findings concur with previous reports demonstrating the inhibition of neutrophil–endothelial interactions with isoflurane and sevoflurane.[23 24]

To elucidate the site of action of volatile anaesthetics, expression of the ELR^{+} receptors CXCR1 and CXCR2 which are activated by IL-8 was determined. These receptors undergo endocytosis upon ligand binding followed by a recycling step back to the membrane, which presumably serves to reduce neutrophil activity in response to high chemokine concentrations at inflammatory sites.[25] We found that IL-8 decreased the expression of CXCR1 and CXCR2. Exposure to volatile anaesthetics did not influence this effect, nor did it lead to a more pronounced downregulation in resting neutrophils. We further investigated the effect of endocytosis of the CXCR1 and CXCR2 ligand–receptor-complex on free IL-8 levels. The marked decrease in membrane CXCR1 and CXCR2, and hence high endocytosis of receptor-bound IL-8, resulted in diminished levels of IL-8 in the medium and volatile anaesthetics did not affect this. These observations suggest that volatile anaesthetics do not induce changes in CXCR1 or CXCR2 surface expression and endocytosis, which might have altered neutrophil activity.

Ligand binding to the G protein-coupled receptors CXCR1 and CXCR2 leads to dissemination of the G protein into the GTP-bound Gαi and the Gβγ subunit. Gαi then increases the activity of phosphatidyl-inositide-3 kinase while Gβγ activates phospholipase C. This leads to an increase of diacylglycerol that activates protein kinase C, which in turn induces CD11b expression.[26] We found that phorbol myristate acetate, an analogue of diacylglycerol that directly activates protein kinase C without involvement of surface receptors, upregulates CD11b. However, no effect of volatile anaesthetics was observed in this setting. This suggests that volatile anaesthetics alter the neutrophil ELR^{+} pathway at a site upstream of protein kinase C.

We next investigated whether the effects of volatile anaesthetics were attributable to downstream pathways specific to CXCR1 or CXCR2. Indeed, blocking of CXCR2 abrogated the impact of both sevoflurane and desflurane on CD11b expression. However, blocking of CXCR1 preserved the effects of volatile anaesthetics, although at a lower level of CD11b expression. We then used CXCL1, a cytokine of the same family as IL-8, but which binds only to CXCR2. In line with the above findings, we found that stimulation with CXCL1 reproduced neutrophil inhibition by volatile anaesthetics. Sevoflurane and desflurane

therefore seem to affect IL-8-induced neutrophil activation downstream of CXCR2. A particular role of CXCR2 during I/R-injury was also reported by Tarzami and colleagues who investigated the outcome of experimental myocardial infarction in CXCR2$^{-/-}$ (knockout) mice: not only were infarct sizes smaller, suggesting decreased tissue damage but also the number of infiltrating neutrophils was reduced in the infarcted area.[27] In line with our finding, this also suggests that CXCR2 signalling is important for leucocyte recruitment in I/R-injury.

Our experimental setup uses an *in vitro* system of isolated neutrophils and as such is limited as possible interactions of neutrophils with other cell types involved in I/R-injury *in vivo* cannot be quantified. We chose this approach as it allows us to investigate pathways and inflammatory responses specific to neutrophils while avoiding expected or unanticipated cell–cell signalling.

Our results provide insight into how volatile anaesthetics can influence the accumulation of neutrophils in I/R-injury through inhibition of neutrophil migration and β_2-integrin expression. Both sevoflurane and desflurane had comparable effects and seem to interfere within the CXCR2 signalling pathway at a site of action upstream of protein kinase C and downstream of CXCR2. Inhibition of CXCR2 signalling might therefore contribute to the well-established anti-inflammatory effect of volatile anaesthetics for the development of I/R-injury *in vivo*.

Authors' contributions

B.M.-E. designed and conducted the study, analysed the data, and drafted the manuscript; R.F. contributed to study design and conduct, data analysis, and manuscript preparation; T.P. contributed to data analysis and manuscript preparation; M.S. contributed to conducting the study and revision of the manuscript; B.R.-Z. and A.S. contributed to study design and conduct; B.B.-S. contributed to study design, data analysis, and manuscript preparation.

Supplementary material

Supplementary material is available at *British Journal of Anaesthesia* online.

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Declarations of interest

B.M.-E., R.F., T.P., M.S., B.R.-Z., and A.S. have no conflicts of interest to declare. B.B.-S. has received honoraria for advisory board meetings and research grants from Abbott, Switzerland, and Baxter Switzerland, but not for the current study.

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