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

Müller-Edenborn, B; Frick, R; Piegeler, T; Schläpfer, M; Roth-Z’graggen, B; Schlicker, A; Beck-Schimmer, B

Abstract: 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.

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

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Abstract

**Background:** Growing evidence suggests a protective effect of volatile anaesthetics in ischaemia-reperfusion (I/R)-injury. The accumulation of neutrophils is crucial for the development of I/R-injury. Pro-inflammatory cytokines carrying the C-X-C-motif including interleukin-8 (IL-8) and CXC-ligand 1 (CXCL1) activate CXC receptor-1 (CXCR1; IL-8) and/or CXC receptor-2 (CXCR2; IL-8, CXCL1) to induce CD11b-dependent neutrophil transmigration. Inhibition of CXCR1 and/or CXCR2 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 served 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 from 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 of 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.

**Number of words:** 248
Keywords:

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anaesthetics, inhalation, sevoflurane
Introduction

Ischaemia-reperfusion injury (I/R-injury) during anaesthesia occurs as an unforeseen event, e.g. 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.\textsuperscript{1-3} However, the mechanism of this protective effect has not been entirely elucidated.

The restoration of blood flow following ischaemia activates innate and adaptive immune responses leading to an accumulation of neutrophils in the reperfused organ and subsequent tissue damage.\textsuperscript{4} Consequently, inhibition of neutrophil invasion during reperfusion was demonstrated to reduce the extent of tissue damage.\textsuperscript{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.\textsuperscript{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 the transmigration of the neutrophil through the endothelium.

The family of CXC cytokines is defined by two N-terminal cystines seperated 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 pharmacologic 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 aforementioned 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 leukocytes and stromal cells such as fibroblasts in high concentrations during reperfusion.\(^{13-16}\)
Methods

The study protocol was approved by the ethic committee for studies on humans of the University Hospital Zurich (KEK-ZH 2012-0274) and written informed consent was obtained. Five ml of blood were taken from healthy volunteers by puncture of the cephalic vein. A total of 12 volunteers (7 male/5 female, aged 19 - 52 years) were recruited from the Institute of Physiology in Zurich. All institute members between 18 and 65 years old were considered eligible. Exclusion criteria were acute disease in the last 14 days or chronic disease with or without medical treatment of any kind. Oral contraceptives were accepted for female donors. All potential donors were interviewed by a medical doctor for these criteria and for informed consent prior to blood donation. Citrate was used as anticoagulant. Red blood cells were lysed and neutrophils were isolated using Ficoll-Histopaque 1077 (Sigma Aldrich, Buchs, Switzerland) as described before. The neutrophils were resuspended at a concentration of 2x10^6/ml in Ham´s F-12 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 5% penicillin/streptomycin (10000U/l) 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 µl of neutrophil cell suspension (10^5 neutrophils) were placed into sterile 96-well plates and exposed to 10nM IL-8 (recombinant human IL-8, BD Pharmingen, Allschwil, Switzerland) or 200ng ml^-1 CXCL1 (recombinant human CXCL1, R&D systems, Wiesbaden, Germany). Phorbol myristate acetate at 10nM (Sigma-Aldrich, Buchs, Switzerland) was used to activate protein kinase C in the corresponding
experiments. These concentration are commonly used and known to induce profound neutrophil activation and migration. To study the effect of ischaemia-reperfusion in our setting, human lung microvascular endothelial cells were exposed to 12h of hypoxia (0.2% O\textsubscript{2}), followed by 12h of reoxygenation at 21% O\textsubscript{2}. Neutrophils were then stimulated with the harvested supernatants at 1:1 dilution.

In some experiments, neutrophils were incubated for 15 min prior to stimulation with 10\(\mu\)g ml\(^{-1}\) of specific antibodies directed against CXCR1 or CXCR2 (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). Chambers were flushed with an air/5% CO\textsubscript{2} mixture for 5 min that was optionally augmented with sevoflurane (Sevorane\textregistered; Abbott AG, Baar, Switzerland, corresponding vaporizer: Sevotec5\textregistered; Abbott AG) or with desflurane (Forene\textregistered, Baxter, Switzerland; corresponding vaporizer: Tec6, Carbamed, Switzerland) to a concentration of 2.2 Vol% sevoflurane or 6 Vol% desflurane, respectively. Control cells were exposed to air/5% CO\textsubscript{2} only. Neutrophils from each donor were investigated under all three conditions (air/5% CO\textsubscript{2} only, air/5% CO\textsubscript{2} + sevoflurane, air/5% CO\textsubscript{2} + desflurane). Concentrations of volatile anaesthetics were measured with the Ohmeda 5330 Agent Monitor (Abbott AG, Baar, Switzerland). After reaching the described concentrations in the chambers, they were sealed and kept in an incubator at 37\textdegree C (Bioblock, Ittingen, Switzerland). Volatile anaesthetic concentrations were checked again at the end of the incubation
period to ensure there was no loss of anaesthetic concentration in the chamber from insufficient sealing and evaporation.\textsuperscript{17}

A 96-well migration plate with 3µm pores (Millipore-Merck, Zug, Switzerland) was used to investigate neutrophils migration. Fifty µl of neutrophil suspension (10\textsuperscript{5} cells) were added to the upper compartment of the plate. Medium supplemented with 10nM IL-8 was added to the lower compartment. Plates were exposed to volatile anaesthetics as described above immediately after assembly of the plate. Neutrophils were allowed to migrate for 1 hour. 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, Allschwil, Switzerland). Flow cytometry raw data was analyzed using the FlowJo-Software for Macintosh (version 8.8.6, Tree Star Inc., Ashland, USA). The following mouse-anti human antibodies were used (all antibodies from BD Pharmingen, Allschwil, Switzerland): APC-Cy7-conjugated CD11b (final staining concentration 2µg ml\textsuperscript{-1}); APC-conjugated CXCR1 (1.25µg ml\textsuperscript{-1}); FITC-conjugated CXCR2 (0.25µg ml\textsuperscript{-1}). FITC-conjugated CBRM1/5 (0.2µg ml\textsuperscript{-1}) was obtained from Biolegend, Lucerne, Switzerland. Appropriate isotype-control antibodies were used to quantify unspecific binding.
Neutrophil viability was quantified by staining the cells with annexin-V and 7-aminoactinomycin (PE Annexin V Apoptosis Detection Kit I, BD Pharmingen, Allschwil, Switzerland).

IL-8 concentration in supernatant was quantified using an enzyme-linked immunosorbent assay according the manufacturers protocol (R&D systems, Wiesbaden, Germany). This kit has a sensitivity range from 31.2pg ml\(^{-1}\) to 2000pg ml\(^{-1}\). All samples were diluted to achieve a sample concentration in the linear range of this assay.

Normal distribution of the data was assessed using the Kolmogorow-Smirnow test. Normally distributed data was analysed using one-way ANOVA with Bonferroni post-hoc test. Non-normally distributed data were analysed with a Kruskal-Wallis test with Dunn’s multiple comparison test. All data are expressed as box plots with whiskers representing the minimum and maximum of all the values. All experiments were performed with blood from 3 or more different donors with conditions repeated in duplicates. A \(p\)-value of \(<0.05\) was considered significant. Statistical analyses and graph creation were executed with Graphpad Prism 6 for Mac (Graphpad Software, La Jolla, USA).
Results

Our isolation procedure yielded neutrophils with a purity of >94% with <1% of dead cells (defined as cells staining positive for 7-aminoactinomycin, data not shown). Exposure to volatile anaesthetics did not reduce neutrophil viability (Figure 1).

We first investigated the functional effect of IL-8 on neutrophil transmigration. Interleukin-8 induced the transmigration of 43% of neutrophils within one hour. This was impaired to a comparable extent when neutrophils were exposed to sevoflurane or desflurane during migration (Figure 2A; 36% and 32% less transmigration with sevoflurane and desflurane, respectively; p=0.012 for sevoflurane, p=0.032 for desflurane). We found that neutrophil transmigration in this setting was dependent upon CD11b and abrogated by CD11b-blocking antibodies (supplemental Figure 1). Accordingly, upregulation of CD11b following stimulation with IL-8 was attenuated by both, sevoflurane and desflurane (Figure 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 (Figure 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 (supplemental Figure 2).

We next asked whether these effects of volatile anaesthetics might be attributable to alternations of the IL-8 target receptors CXCR1 and CXCR2 on the neutrophil surface or their downstream pathways. Stimulation with IL-8 lead to strong
endocytosis and therefore reduction of surface expression of CXCR1 by 47% and CXCR2 by 81% (Figure 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 (p=0.9 for sevoflurane vs air, p=0.6 for desflurane vs air; data not shown). The extent of endocytosis of the ligand-receptor complexes formed by IL-8 and CXCR1 or CXCR2 lead to a consumption and hence decrease of free IL-8 in the medium during incubation (Figure 3B, reduction of 31% after 1h of incubation compared to control medium without neutrophils, dashed line; p=0.008). Again, this was not influenced by sevoflurane or desflurane (Figure 3B, sevoflurane vs air p=0.999; desflurane vs air p=0.357).

Downstream effects of CXCR1 or CXCR2 activation include formation of diacylglycerol through phospholipase C, which activates protein kinase C. Direct stimulation of protein kinase C with phorbol myristate acetate, an analogue of diacylglycerol, lead to a strong upregulation of CD11b. No effect of sevoflurane and desflurane was observed in this setting (p>0.9 for sevoflurane vs air; p>0.9 for desflurane vs air, data not shown).

Using blocking antibodies against CXCR1 and CXCR2, we found that both receptors participate in the upregulation of CD11b in response to IL-8 (Figure 4A; 58% decrease following blocking of CXCR1, p<0.001; 36% decrease following blocking of CXCR2, p=0.01). However, the impaired upregulation of CD11b with sevoflurane and desflurane seems to be due to interference with pathways downstream of CXCR2 exclusively: blocking of CXCR2 prior to stimulation neutralized the effect of volatile anaesthetics, while blocking of CXCR1 resulted in a preserved attenuation of CD11b
with sevoflurane and desflurane (Figure 4B; p<0.05 for sevoflurane and desflurane). In addition, stimulation with CXCL1, a CXC cytokine that exclusively activates CXCR2, readily reproduced the aforementioned effect of volatile anaesthetics (Figure 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 β₂-integrin CD11b. We locate the site of action of volatiles 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 following 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 leads to decreased tissue injury.⁷ ¹⁹ The ability of neutrophils to transmigrate through the endothelium is pivotal for their accumulation in the reperfused organ. We found that sevoflurane and desflurane reduced the transmigration of neutrophils along an IL-8 gradient.
The process of transmigration is known to be 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.\textsuperscript{20} CD11b is critical to establish a tight adherence of the neutrophils to the endothelium, and failure to do so as by applying antibodies against CD11b significantly blocks neutrophil recruitment and reduces tissue damage both \textit{in vitro} and \textit{in vivo}.\textsuperscript{21,22} In our setting, neutrophil transmigration was dependent on CD11b expression. Accordingly, we found that both sevoflurane and desflurane attenuated the upregulation of CD11b in response to IL-8. These findings go in line with previous reports demonstrating an inhibition of the neutrophil-endothelial interaction with isoflurane and sevoflurane.\textsuperscript{23,24}

To elucidate the site of action of volatile anaesthetics, the expression of the ELR\textsuperscript{+} receptors CXCR1 and CXCR2 which are activated by IL-8 was quantified in this study. 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.\textsuperscript{25} We found that IL-8 decreases 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 strong decrease of membrane CXCR1 and CXCR2, and hence high endocytosis of receptor-bound IL-8, lead to a diminished concentration of remaining available IL-8 in the medium. This was evident to the same extent when neutrophils were exposed to volatile anaesthetics. 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 G\textsubscript{ai} and the G\textsubscript{ib\gamma} subunit. G\textsubscript{ai} then increases the activity of phosphatidyl-inositol-3 kinase while G\textsubscript{ib\gamma} activates phospholipase C. This leads to an increase of diacylglycerol that activates protein kinase C, which in turn induces CD11b expression.\textsuperscript{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\textsuperscript{+} pathway at a site upstream of protein kinase C.

We next aimed to investigate 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. On the contrary, blocking of CXCR1 preserved the effects of volatile anaesthetics, though at a lower level of CD11b expression. We then used CXCL1, a cytokine of the same family as IL-8, but which in contrast to IL-8 binds only to CXCR2. In line with the above findings, we found that stimulation with CXCL1 was sufficient to readily reproduce neutrophil inhibition with 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 et al. who investigated the outcome of experimental myocardial infarction in CXCR2\textsuperscript{-/-}(knockout) mice: not only did they find smaller infarct sizes as a correlate of decreased tissue
damage in CXCR2−/− mice, but they also observed a decreased number of infiltrating neutrophils in the infarcted area. In line with our finding, this suggest that CXCR2 signalling is important for leukocyte recruitment in I/R-injury.

The current experimental setup uses an in vitro system of isolated neutrophils. This is a limitation of the current study as possible interactions of neutrophils with other cell types involved in I/R-injury as observed 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 in how volatile anaesthetics can influence the accumulation of neutrophils in I/R-injury through inhibition of neutrophil migration and β2-integrin expression. Both, sevofoflurane 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.
Author contributions

BME designed the study, conducted the study, analysed the data and wrote the manuscript.

RF helped designing and conducting the study. He analyzed the data, and assisted in writing the manuscript.

TP helped analyzing the data and writing the manuscript.

MS helped conducting the study and revising the manuscript.

BRZ helped designing and conducting the study.

AS helped designing and conducting the study.

BBS helped designing the study, analyzing the data, and writing the manuscript.

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Declarations of interest

BME, RF, TP, MS, BRZ and AS have no conflicts of interest to declare. BBS has received honoraria for advisory board meetings as well as research grants from Abbott, Switzerland, and Baxter Switzerland, but not for the current study.

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Figure Legends

Figure 1

Volatile anaesthetics do not decrease neutrophil survival

Resting or IL-8 stimulated neutrophils were incubated with either an air/5% CO₂-mixture, 2.2 Vol% of sevoflurane or 6 Vol% of desflurane. Viable neutrophils are defined as staining both negative for annexin and 7AAD (7-aminoactinomycin) (Annexin⁻/7AAD⁻, blue box plots). The amount of apoptotic neutrophils (Annexin⁺/7AAD⁻) are represented by green box plots, pink box plots show the amount of necrotic cells (Annexin⁺/7AAD⁺). n=6/group.

Figure 2

Volatile anaesthetics attenuate neutrophil transmigration and CD11b-upregulation

A) 10⁵ neutrophils were seeded in the upper compartment of a modified Boyden chamber. Medium supplemented with 10nM IL-8 was added to the lower compartment. For control neutrophils, only medium was added to the lower compartment. Neutrophils were exposed to either an air/5% CO₂-mixture, 2.2 Vol% of sevoflurane or 6 Vol% of desflurane during the 1 hour incubation. The number of neutrophils in the lower compartment at the end of the incubation period is given. Data are shown as box plots with whiskers from minimal to maximal value. #p<0.0001 vs control; *p<0.05 vs IL-8 stimulated neutrophils exposed to air/5% CO₂ during transmigration; n=9/group.

B) Neutrophils were stimulated with 10nM IL-8 and incubated for 1 hour in the presence or absence of 2.2% sevoflurane or 6% desflurane, respectively. The mean fluorescence
as assessed by flow cytometry of CD11b relative to unstimulated control neutrophils is given. #p<0.0001 vs unstimulated control neutrophils; *p<0.05 vs stimulated neutrophils exposed to air/5% CO₂-mixture only; n=12/group.

C Neutrophils were stimulated with IL-8 and exposed to volatile anaesthetics as described for 2B. The mean fluorescence as assessed by flow cytometry of the activation-epitope of CD11b-specific CBRM1/5 antibody clone relative to unstimulated control neutrophils is given. #p<0.001 vs unstimulated control neutrophils; *p<0.05 vs stimulated neutrophils exposed to air/5% CO₂-mixture only; n=6/group.

Figure 3

A, B Volatile anaesthetics do not affect IL-8-induced CXCR1 and CXCR2 internalization or IL-8 uptake in neutrophils

A Neutrophils were stimulated with 10nM IL-8 and incubated for 1 hour in the presence of 2.2 Vol% of sevoflurane, 6 Vol% of desflurane or an air/5% CO₂-mixture. CXCR1 (left panel) and CXCR2 (right panel) mean fluorescence relative to unstimulated control neutrophils is given. Box plots with whiskers from minimal to maximal value are shown. #p<0.001 vs unstimulated control neutrophils; n=6/group.

B IL-8 concentration in medium was assessed by ELISA in the presence of neutrophils (PMN) after the addition of 10nM IL-8. Neutrophils were incubated at 37°C with either an air/5% CO₂-mixture, 2.2 Vol% of sevoflurane or 6 Vol% of desflurane for 1 hour. The dashed line shows the measured IL-8 concentration in the absence of neutrophils (expected concentration for 10nM IL-8 is 80ng ml⁻¹). p>0.3 for sevoflurane/desflurane vs stimulated neutrophils exposed to air/5% CO₂-mixture only. n=8/group.
Figure 4

Volatile anaesthetics affect CXCR2

A Neutrophils were either left untreated, stimulated with 10nM IL-8 alone or incubated with anti-CXCR1- or anti-CXCR2-antibodies prior to stimulation with 10nM IL-8. CD11b expression was assessed using flow cytometry. The mean fluorescence of CD11b relative to unstimulated control neutrophils is given. Box plots with whiskers from minimal to maximal value are shown. #p<0.0001 vs control, *p<0.05 vs IL-8 alone; n=8/group.

B Neutrophils were either left untreated or incubated with anti-CXCR1- or anti-CXCR2 antibodies prior to stimulation with 10nM IL-8 and exposure to either an air/5% CO₂-mixture, 2.2 Vol% of sevoflurane or 6 Vol% of desflurane. The mean fluorescence of CD11b relative to unstimulated and non-treated control neutrophils is given. #p<0.0001 vs untreated control, *p<0.05 vs air+IL-8; n=6/group.

C Neutrophils were stimulated with 200ng ml⁻¹ CXCL1 and incubated with either an air/5% CO₂-mixture, 2.2 Vol% of sevoflurane or 6 Vol% of desflurane. The mean fluorescence as assessed by flow cytometry of CD11b relative to unstimulated control neutrophils is given. #p<0.05 vs control, *p<0.01 vs air+IL-8; n=6/group.

Supplemental figure 1

Neutrophil transmigration is CD11b-dependent
Neutrophils were seeded in the upper compartment of a modified Boyden chamber. Medium supplemented with 10nM IL-8 was added to the lower compartment. Anti-CD11b blocking antibodies were added to the respective group of neutrophils. For control neutrophils, only medium was added to the lower compartment. **Box plots show migrated neutrophils after 1h migration with whiskers representing minimal and maximal values.** #p<0.001 vs control; *p<0.001 vs IL-8 stimulated neutrophils without Anti-CD11b antibodies; n=8/group.

**Supplemental figure 2**

**Volatile anaesthetics attenuate CD11b-upregulation following hypoxia/reoxygenation**

Human lung microvascular endothelial cells were exposed to 12h of hypoxia (0.2% O₂), followed by 12h of reoxygenation at 21% O₂. Neutrophils were then stimulated with the harvested supernatants at 1:1 dilution for 1h in the presence of 2.2 Vol% of sevoflurane, 6 Vol% of desflurane or an air/5% CO₂-mixture. The mean fluorescence as assessed by flow cytometry of CD11b relative to unstimulated control neutrophils is given. **Box plots with whiskers from minimal to maximal value are shown.** #p<0.001 vs unstimulated control neutrophils; *p<0.05 vs stimulated neutrophils exposed to air/5% CO₂-mixture only; n=6/group.
Figure 1

Figure 2A
Figure 2B

CD11b expression (%) relative to control

IL-8 10 nM

-  +  +  +

air sevoflurane desflurane

#, *

#
Figure 2C

![Graph showing CBRM1/5 expression (% relative to control) vs. IL-8 10 nM](image)

- **CBRM1/5 expression**: % relative to control
- **Y-axis**: CBRM1/5 expression (%)
- **X-axis**: IL-8 10 nM
- **Conditions**: air, sevoflurane, desflurane

Figure 3A

- **Graphs for CXCR1 and CXCR2**
- **Y-axis**: expression (%) relative to control
- **X-axis**: IL-8 10 nM
- **Conditions**: air, sevoflurane, desflurane

Figure 3B

- **Graph for IL-8 concentration in medium (ng/ml)**
- **Y-axis**: IL-8 concentration in medium (ng/ml)
- **X-axis**: PMN present
- **Conditions**: air, sevoflurane, desflurane

Legend:
- **n.s.**: not significant
- **#**: significant difference
Figure 4A

CD11b expression (%) relative to control

- IL-8 10 nM
- Anti-CXCR1 Ab
- Anti-CXCR2 Ab

- (-)
+ (+)

* * # air
Figure 4C

CD11b expression (%) relative to control

IL-8 10 [nM]
- + + +
Anti-CXCR1 Ab
- + - +
Anti-CXCR2 Ab
- - - +

CXCL1 200ng ml⁻¹
- + + +