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RESEARCH ARTICLE

Differential effects of volatile anesthetics on leukocyte integrin macrophage-1 antigen

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Abstract

Macrophage-1 antigen (Mac-1, $\alpha M\beta 2$) is a leukocyte adhesion molecule that plays a significant role in leukocyte crawling and phagocytosis, and is homologous to its sister protein leukocyte function-associated antigen-1 (LFA-1, $\alpha L\beta 2$). The authors have previously demonstrated that volatile anesthetics isoflurane and sevoflurane bound to and inhibited LFA-1. Here, the hypothesis tested was that isoflurane and sevoflurane would inhibit Mac-1. A binding assay of Mac-1 to its ligand inter-cellular adhesion molecule-1 (ICAM-1) using V-bottom plates was established. The effect of isoflurane and sevoflurane on Mac-1 was examined using this ICAM-1 binding assay and by probing exposure of activation-sensitive epitopes. The docking program Glide was used to predict anesthetic binding site(s) on Mac-1. The functional role of this predicted binding site was then assessed by introducing point mutations in this region. Lastly, the effect of anesthetic on activating mutants was evaluated. The results indicated that isoflurane inhibited binding of Mac-1 to ICAM-1, but sevoflurane did not. Isoflurane also attenuated the exposure of the activation-sensitive epitopes. The docking simulation predicted the isoflurane binding site to be at the cavity underneath the α 7 helix of the ligand binding domain (the αM I domain). Point mutants at this predicted binding site contained both activating and deactivating mutants, suggesting its functional significance. The binding of activating mutants α M-Y267A β 2-WT and α M-L312A β 2-WT to ICAM-1 was not affected by isoflurane, but binding of another activating mutant α M-WT β 2-L132A was inhibited supporting the binding of isoflurane to this cavity. The conclusion reached from these findings was that isoflurane inhibited Mac-1 binding to ICAM-1 by binding to the cavity underneath the α 7 helix of the α M I domain, but sevoflurane did not. Thus, because these common clinical volatile anesthetics demonstrated different effects on Mac-1, this implied their effects on the immune system might differ.

Introduction

Volatile anesthetics isoflurane and sevoflurane are used clinically to provide general anesthesia for surgical procedures. The exact mechanism of anesthetic action by these drugs remains to be determined, but it is considered that they directly bind to a subset of ligand-gated ion channel proteins in the central nervous system and alter their functions (Campagna et al., 2003; Dilger, 2002). Interestingly these anesthetics are suggested to generate an array of pleiotropic effects, even outside the central nervous system, including the effects on the immune system (Eckenhoff, 2001). Because adequate immune function is critical to prevent perioperative complications such as infection, understanding the impact and mechanism of volatile anesthetic-induced immunomodulation is important.

Adhesion to the endothelium is an essential process for leukocytes to transmigrate through endothelial cells and accumulate at the sites of infection and inflammation. Volatile anesthetics

Keywords

Integrin, isoflurane, sevoflurane

History

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isoflurane and sevoflurane at clinically relevant concentrations have been shown to reduce the ability of leukocytes to adhere to inflamed vascular endothelial cells, which is a requisite step for leukocyte recruitment (Kowalski et al., 1997; Mobert et al., 1999). As expected, isoflurane exposure attenuated the amount of accumulated neutrophils to the site of inflammation in the murine skin inflammation model (Carbo et al., 2012). The attenuation of neutrophil recruitment by anesthetics can be devastating in the setting of infection because they are the first defense innate immune cells to fight against infection.

Adhesion molecules leukocyte function-associated antigen-1 (LFA-1 *aka* $\alpha L\beta 2$, CD11aCD18) and macrophage-1 antigen (Mac-1 *aka* $\alpha M\beta 2$, CD11bCD18, complement receptor-3) are heterodimeric molecules consisting of α and β subunits and play a pivotal role in leukocyte arrest and intravascular crawling on the endothelium, both critical processes for leukocyte migration (Wagner & Frenette, 2008). In addition, Mac-1 facilitates bacterial phagocytosis. Both are expressed only on leukocytes and bind to the ligand called intercellular adhesion molecule-1 (ICAM-1) on the endothelium to facilitate these processes (Diamond et al., 1990). A functional defect in these molecules is significant as demonstrated in leukocyte adhesion deficiency characterized by severe infection, often leading to death

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Figure 1. Structural characteristics of I domain of the αL and αM subunits. (a) I domain of αL subunit (αL I domain) is shown. Structure is from protein data bank (PDB) 1ZOO. β -strands (yellow) are surrounded by α helices (red). Metal-ion dependent adhesion site (MIDAS) (= ICAM-1 binding site) is shown in cyan. (b) Surface of αL I domain is shown. Arrow represents the allosteric cavity underneath the α 7 helix to which isoflurane and sevoflurane bind. (c) I domain of αM subunit (αM I domain) is shown (PDB 1M1U). (d) αL I domain (light green) and aM I domain (cyan) are superimposed. (e) Surface of αM I domain is shown. The arrow represents the allosteric cavity underneath α 7 helix.



(Anderson & Springer, 1987). Their major ligand-binding domain (called I domain) is located at the top of the extracellular portion of the α subunit (called the α L I domain for LFA-1, the α M I domain for Mac-1). Our laboratory previously demonstrated both isoflurane and sevoflurane bound to and inhibited LFA-1 (Yuki et al., 2008, 2010, 2012). While ICAM-1 binds to LFA-1 at the metal ion-dependent adhesion site (MIDAS) located at the top of the α L I domain (Figure 1a), isoflurane and sevoflurane bind to the hydrophobic cavity underneath the α 7 helix of the α L I domain that acts as a functional regulator (Figure 1b). Unwinding of the C-terminal α 7 helix presumably leads to conformational rearrangements of the MIDAS from a low- to a high-affinity configuration, only the latter of which can tightly bind to ICAM-1 (Sen et al., 2013). By binding to this hydrophobic pocket, volatile anesthetics stabilize the helix of this region and impede the conformational conversion of MIDAS to bind to the ligand.

Mac-1 is highly homologous to LFA-1 including the αI domain (Figures 1c and d). The αM I domain contains a cavity underneath the $\alpha 7$ helix like the αL I domain does (Figure 1e). Therefore, it was hypothesized that isoflurane and sevoflurane would bind this cavity and inhibit Mac-1, as was the case for LFA-1. To test this, an assay system was established to test Mac-1/ICAM-1 binding and examined the impact of volatile anesthetics on Mac-1 function. Further, their potential anesthetic

binding sites were predicted using a docking stimulation called *Glide* and the function of corresponding site(s) was examined.

Materials and methods

Cells and transfection

Human embryonic kidney (HEK) cells (ATCC, Manassas, VA) were cultured in DMEM medium with HEPES modification and 10% fetal bovine serum (Atlanta Biological, Miami, FL) at 37 °C in an atmosphere containing 5% CO₂. Integrin α L, α M and β 2 plasmids, as well as ICAM-1 plasmid, were prepared are previously described (Jun et al., 2001; Shimaoka et al., 2002). Transfection with LFA-1 (combination of α L and β 2 plasmids), Mac-1 (combination of α M and β 2 plasmids) or ICAM-1 was performed using Fugene 6 (Promega; Madison, WI) following manufacturer protocols.

Antibodies

Mouse anti-human α L antibody TS1/22 and mouse anti-human β 2 antibody CBR LFA1/7 and m24 were obtained from the Immune Disease Institute (Boston, MA). Mouse anti-human α M antibody ICRF44 and CBR M1/5, and mouse anti-human β 2 antibody TS1/18 were purchased from Biolegend (San Diego, CA).

Flow cytometry-based ICAM-1 binding assay

Flow cytometric detection of ICAM-1 binding to LFA-1 or Mac-1 was assayed as described in Yuki et al. (2010). HEK cells transiently transfected with LFA-1 or Mac-1 were harvested and washed once with HEPES-buffered saline (HBS) containing 10 mM EDTA and then 3-times with HBS. Cells were then incubated for 30 min with ICAM-1-fragment crystallizable α (Fc α) in HBS containing 1 mM MgCl₂/CaCl₂ (inactivating condition) or 1 mM MnCl₂ (activating condition as Mn²⁺ is an activator for LFA-1 and Mac-1). The cells were then washed and incubated for 30 min with fluorescein isothiocyanate (FITC)-labeled anti-human IgA (Life Technologies, Grand Island, NY). After washing, bound ICAM-1 was detected by flow cytometry using an Accuri C6 system (BD Biosciences, San Jose, CA). A minimum of 10 000 events/sample was acquired.

V-bottom plate-based Mac-1 binding assay to ICAM-1

V-bottom plates were coated with 2µg ICAM-1/ml (R&D systems, Minneapolis, MN) using standard protocols. HEK cells transfected with Mac-1 were stained with 2',7'-bis-(2-carboxvethyl)-5- (and 6-)-carboxyfluorescein acetoxymethyl ester (BCECF-AM; Life Technologies) and incubated in HBS containing 10 mM EDTA, 1 mM MgCl₂/CaCl₂ or 1 mM MnCl₂ with or without isoflurane or sevoflurane at indicated concentrations at 37 °C for 30 min. Following incubation, the plates were centrifuged at $200 \times g$ for 5 min. Cells that did not bind to ICAM-1 were accumulated at the center of the well. Fluorescence was read at excitation = 485 nm and emission = 538 nm to assess HEK cell binding to ICAM-1. ICAM-1 binding percentage was defined as [(fluorescence intensity of EDTA sample) - (fluorescence intensity of samples)]/[fluorescence intensity of EDTA sample] \times 100 (%). In some experiments, 5 µg/ml of LFA-1 or Mac-1 protein (a generous gift from Dr Timothy Springer [Boston Children's Hospital]) was coated on the 96 well V-bottom plates and HEK cells transiently transfected with ICAM-1 were used.

Rigid docking simulation

Because the α M I domain is the major ligand binding domain (Diamond et al., 1993), it was postulated here that binding site(s) of volatile anesthetics localized within this domain. The structure of the α M I domain was obtained from the Protein Data Bank (PDB) ID number 1M1U (http://www.rcsb.org/pdb). The program *Glide* (Schrodinger, Cambridge, MA) was used to perform rigid docking of isoflurane with the α M I domain (Friesner et al., 2004). Isoflurane binding position was sought with the grid size of $20 \times 20 \times 20$ cubic angstrom, which is substantially larger than the size of isoflurane (144-cubic angstrom; Jenkins et al., 2001). No positional constraint was applied. *Glide* has a scoring system that ranks docked pairs based on predicted affinity. The docked pair with the most negative score was selected, as it is considered to have the highest affinity.

Alanine scanning mutagenesis

Site-directed mutagenesis was performed using Quikchange XL kit (Agilent Technologies, Lexington, MA) and DNA sequences were confirmed. Mutants were tested for surface expression and ICAM-1 binding.

Statistical analysis

Data were analyzed as indicated in the figure legends. Statistical significance was defined at p values < 0.05. All statistical calculations were done using PRISM 5 software (GraphPad Software, La Jolla, CA).

Results

ICAM-1 binding is greater to LFA-1 than to Mac-1

A flow cytometry-based LFA-1:ICAM-1 binding assay previously reported (Yuki et al., 2008, 2012) was used in which dimeric soluble ICAM-1 can bind to one or two LFA-1 on the cell surface (Figure 2a). Binding of Mac-1 to soluble ICAM-1 was not significant using this method (Figure 2b). When a leukocyte binds to the endothelium, multiple LFA-1 or Mac-1 on a single leukocyte can interact with multiple ICAM-1 on an endothelial cell. To simulate LFA-1 (or Mac-1) binding to ICAM-1 between cells, the binding of coated LFA-1 or Mac-1 protein to cells over-expressing ICAM-1 was evaluated (Figure 2a). In this plate-based assay, both LFA-1 and Mac-1 bound to ICAM-1 (Figure 2b). ICAM-1 binding was more robust onto LFA-1 than onto Mac-1, compatible with results of the flow-based assay.

Effects of anesthetics on LFA-1 and Mac-1 binding to ICAM-1

Previous studies reported that both isoflurane and sevoflurane reduced binding of LFA-1 to ICAM-1 in a flow cytometry-based assay (Yuki et al., 2008, 2010). Both anesthetics attenuated binding of ICAM-1 to LFA-1 in the plate-based binding assay using coated LFA-1 protein and ICAM-1-expressing cells (Figures 3a and b). Binding of ICAM-1-expressing cells to coated Mac-1 protein was also assessed; Mac-1 binding was impaired by isoflurane, but not sevoflurane (Figures 3a and b). A binding assay was also performed using HEK cells over-expressing Mac-1 and coated ICAM-1 protein. In tests of binding of Mac-1-over-expressing cells to coated ICAM-1 protein, Mac-1 binding was inhibited by isoflurane, but not by sevoflurane (Figures 3c and d), an outcome compatible with the results of binding of ICAM-1-expressing cells to coated Mac-1 protein

Effects of isoflurane and sevoflurane on Mac-1 activation

CBRM1/5 is an antibody with epitopes near the MIDAS and interacts with the α M I domain in an active conformation (Oxvig et al., 1999). The present study examined expression of CBRM1/5 epitopes with or without isoflurane or sevoflurane in the presence of Mn²⁺. The results indicated that isoflurane, not sevoflurane attenuated CBRM1/5 epitope expression, an outcome in line with results of the above-mentioned binding assays (Figure 3e).

Isoflurane docking to α M I domain cavity

LFA-1 and Mac-1 are quite homologous (Springer et al., 1985), with the amino acid homology between the αL I and αM I domains being \approx 34 %. As expected, the structures of the αL I and αM I domain are well-superimposed (Figure 1d). ICAM-1 binds to LFA-1 at the MIDAS of the αL I domain (Shimaoka et al., 2003). The exact ICAM-1 binding site on Mac-1 has not been completely determined, but the αM I domain is the major recognition site if not the solo binding site (Diamond et al., 1993). It was previously reported that both isoflurane and sevoflurane inhibited LFA-1 by binding to the allosteric cavity of the α L I domain (lovastatin site). As Mac-1 has a similar cavity in the α M I domain called 'SILEN (socket for isoleucine)' (Xiong et al., 2000), it was predicted that isoflurane would bind to the cavity. Docking simulation indicated isoflurane bound to the pocket (Figure 4), with the trifluoromethyl head of isoflurane interacting with α M-Asp-294 and α M-Gln-311. The diffuormethyl fluorine showed hydrophobic interaction with α M-Lys-315. Interestingly, sevoflurane did not show docking in this condition, which is in line with our binding data.



Figure 2. Flow cytometry- and plate-based ICAM-1 binding assays. (A) Schemes of flow cytometry- and plate-based ICAM-1 binding assays. In plate-based assay using ICAM-1 over-expressing HEK cells and coated LFA-1 (or Mac-1) protein is illustrated. Binding assay using LFA-1 (or Mac-1) over-expressing HEK cells and coated ICAM-1 protein are not shown. (B, left) Representative result of flow cytometry-based ICAM-1 binding to LFA-1 (or Mac-1) over-expressing HEK cells. Mock indicates HEK cells transiently transfected with empty vector. Mg/Ca = 1 mM MgCl₂/CaCl₂, Mn = 1 mM MnCl₂. Three independent experiments were performed. (B, right) Representative result of V-bottom plate-based ICAM-1 binding assays using ICAM-1 over-expressing HEK cells and coated LFA-1 (or Mac-1) protein. 5 μ g/ml of LFA-1 (or Mac-1) protein was coated on wells. Data shown are mean \pm SD of triplicates. Three independent experiments were performed.

Presumed isoflurane binding site on Mac-1 is functionally important

In the docking simulation, this study identified the amino acids within 4 angstrom from isoflurane and mutated them to alanine. Surface expression and activation status of all mutants were then examined (Figures 5a and b) and the degree of activation probed with CBRM1/5 antibody. α M-Y267A β 2-WT and α M-L312A β 2-WT mutants were constitutively active as they demonstrated significant activation-sensitive epitope exposure, even in an inactivating condition (Mg²⁺/Ca²⁺), while the α M-K315A β 2-WT mutant remained inactive even in an activating condition (Mn²⁺), demonstrated as limited activating epitope exposure. Binding of these mutants to ICAM-1 was also examined using a



Figure 3. Effect of isoflurane and sevoflurane on ICAM-1 binding to LFA-1 or Mac-1. Impact of (a) isoflurane and (b) sevoflurane on ICAM-1 binding to LFA-1 or Mac-1 with Mn^{2+} stimulation was examined using ICAM-1 over-expressing HEK cells and coated LFA-1 (or Mac-1) protein [5 µg/ml LFA-1 (or Mac-1) coated on V-bottom wells]. Statistical significance between groups with and without anesthetics was assessed using two-tailed student *t*-test. Impact of (c) isoflurane and (d) sevoflurane on binding of ICAM-1 to Mac-1 with Mn^{2+} stimulation was examined by using Mac-1 over-expressing HEK cells and coated ICAM-1 protein. 2 µg ICAM-1/ml was coated on wells. For (c), significance was tested using 1-way analysis of variance with Bonferroni *post-hoc* analysis. For (d), significance was tested using 2-tailed Student's *t*-test. (e) Effect of isoflurane and sevoflurane on epitope exposure of activation-sensitive antibody CBRM1/5. MFI = mean fluorescence intensity. Significance was tested using 2-tailed Student's *t*-test. **p* < 0.05; n.s. = not significant. Data shown are mean ± SD of triplicates. In (a–e), three independent experiments were performed.

Figure 4. Isoflurane docking site on the α M I domain. Docking of isoflurane on the α M I domain (PDB 1M1U) using *Glide*. Right panel shows blowup of the docking site. In isoflurane: red, oxygen; green, chloride; light blue, fluoride.



plate-based assay (Figure 5b). This result was compatible with the one of activation-sensitive epitope exposure (Figure 5c). These results reveal the presumed isoflurane-binding site was extremely important in Mac-1 activation and binding.

Effect of isoflurane on Mac-1-activating mutants

The effect of isoflurane on activating mutants α M-Y267A β 2-WT and α M-L312A β 2-WT, both of which have alternation at the allosteric cavity of the α M I domain, was evaluated. Any attenuation of ICAM-1 binding by isoflurane might indicate the presence of any other potential isoflurane binding site outside the allosteric pocket in the α M I domain. Isoflurane did not attenuate binding of α M-Y267A β 2-WT and α M-L312A β 2-WT to ICAM-1 (Figure 6a). The study then searched for a Mac-1-activating mutant without modification of the allosteric cavity in the α M I domain. Previously, it was demonstrated that α L-WT β 2-L132A and α X-WT β 2-L132A mutants were constitutively active (Sen et al., 2013; Yuki et al., 2012). β 2-Lys-132 is located at the cavity in the top domain of the β 2 subunit called the β 2 I domain. Here, tests were done to ascertain if α M-WT β 2-L132A behaved similarly. As seen in Figure 6b, this mutant was constitutively active, as demonstrated by activation-sensitive epitope exposure in the β I domain probed by m24 (Sen et al., 2013) and ICAM-1 binding even under Mg²⁺/Ca²⁺ conditions. β 2-Lys-135 is located at the same pocket where β 2-Lys-132 is located.



Figure 5. Functional role of predicted isoflurane binding site in Mac-1. (a) Surface expression of alanine scanning mutants at the predicted isoflurane binding site (the α M I domain) was probed by ICRF44 antibody. β 2-WT was paired with all α M-mutants. MFI = mean fluorescence intensity. (b) Activation level of mutants probed by CBRM1/5 antibody. CBRM1/5 expression was normalized by expression of activating-insensitive antibody ICRF44 as follows: Normalized CBRM1/5 expression = [(MFI probed by CBRM1/5 antibody in WT or mutants) – (MFI probed by CBRM1/5 antibody in mock)]/[(MFI probed by ICRF44 antibody in WT or mutants) – (MFI probed by ICRF44 antibody in mock)]. Thereafter, relative value to normalized CBRM1/5 expression in WT, 1 mM Mg²⁺/Ca²⁺ was calculated. One-way analysis of variance with Bonferroni *post-hoc* analysis was performed to compare data within the 1 mM Mg²⁺/Ca²⁺ or 1 mM Mn²⁺ group. *p<0.05 versus WT Mg²⁺/Ca²⁺ and **p<0.05 versus WT Mn²⁺. Data shown are mean ± SD of triplicates. In (a–c), representative data from three independent experiments are shown.

Our previous data showed both α L-WT β 2-L135A and α X-WT β 2-L135A were deactivating mutants. Likewise, α M-WT β 2-L135A was deactivating (Figure 6b). The β I domain is considered functionally important as it relays the activation signal to the α I domain and induces its conformational change; results here suggested that LFA-1, Mac-1, and $\alpha X\beta 2$ use a common mechanistic approach to activate the α subunit from the $\beta 2$ subunit. Isoflurane exposure reduced the degree of ICAM-1 binding to α M-WT β 2-L132A, which does not have any mutation in the allosteric cavity of the α M I domain (Figure 6c). Taken together, it appears isoflurane would bind to this cavity (Figure 6c). These results are in contrast to our previous LFA-1 study. With LFA-1, binding of the activating mutant α L-I306A β 2-WT (α L-IIe-306 is amino acid residue in allosteric cavity of α I domain) was attenuated by isoflurane; isoflurane bound to the β 2 I domain in addition to the α L I domain (Yuki et al., 2012). The isoflurane binding pocket in the $\beta 2$ I domain of LFA-1, where β 2-Lys-132 and β 2-Lys-135 are located, can be probed by TS1/18 antibody as its epitopes (β 2-Arg-133 and β 2-Gln-322 (Huang et al., 2000)) are present in this pocket (Yuki et al., 2008). Because isoflurane was unlikely to bind to the $\beta 2$ subunit of Mac-1 based on binding studies, it was hypothesized the β 2 I domain conformation might be not exactly the same between LFA-1 and Mac-1. We examined TS1/18 antibody epitope exposure in

relative to the expression of another $\beta 2$ antibody (CBR LFA1/7) that probes more C-terminal residues of the $\beta 2$ subunit (residue 432–487) (Lu et al., 2001). The epitopes of TS1/18 were less exposed in Mac-1 than in LFA-1 as predicted (Figure 6d), which likely hindered isoflurane binding to the $\beta 2$ I domain of Mac-1.

Discussion and conclusion

In the study reported here, it was shown that isoflurane inhibited binding of both LFA-1 and Mac-1 to ICAM-1, but that sevoflurane inhibited only LFA-1. Docking stimulation suggested to us that isoflurane bound to the allosteric cavity located underneath the α 7 helix of the α M I domain. A mutagenesis experiment confirmed the functionality of this cavity and also supported the binding of isoflurane to this cavity. In all the binding assays, plate-based binding assays were used, as the functional role of soluble ICAM-1 is yet to be determined (Witkowska & Borawska, 2004) and the assay system using soluble ICAM-1 may not reflect the binding of Mac-1 to ICAM-1 *in vivo*.

Both sevoflurane and isoflurane are volatile anesthetics with an ether group that are widely used in clinical practice. However, their anesthetic potencies differ, as demonstrated in their minimum alveolar concentration (MAC) values (sevoflurane



Figure 6. Effect of isoflurane on activating mutants. (a) Binding of WT and activating mutants α M-Y267A β 2-WT and α M-L312A β 2-WT to ICAM-1 with or without 2% isoflurane in presence of Mn²⁺. A 2-tailed Student's *t*-test was performed between groups with or without isoflurane in either Mac-1 WT, α M-Y267A β 2-WT or α M-L312A β 2-WT. *p<0.05; n.s. = not significant. (b) Mac-1 WT, α M-WT β 2-L132A and α M-WT β 2-L135A were subject to ICAM-1 binding assay and probing activation-sensitive epitopes in β 2 I domain using m24. Expression of m24 epitopes was normalized by CBR LFA1/7 expression as [(MFI of m24 of sample) – (MFI of m24 of mock)]/[(MFI of CBR LFA1/7 of sample) – (MFI of CBR LFA1/7 of mock)]. Statistical significance was tested using 1-way analysis of variance with Bonferroni *post-hoc* analysis to compare data within 1 mM Mg²⁺/Ca²⁺ or 1 mM Mn²⁺. *p<0.05 vs WT Mg²⁺/Ca²⁺ and **p<0.05 vs WT Mn²⁺. (c) Effect of isoflurane on ICAM-1 binding in Mac-1 WT and α M-WT β 2-L132A. Statistical significance tested using 2-tailed Student's *t*-test. *p<0.05. (d) TS1/18 antibody epitope exposure in LFA-1 and Mac-1. TS1/18 expression normalized by CBR LFA1/7 of mock)]. Statistical significance was analyzed using 1-way analysis of variance was analyzed using 1-way analysis of variance was analyzed using 1-way analysis. *p<0.05; n.s. = not significance tested using 2-tailed Student's *t*-test. *p<0.05. (d) TS1/18 of sample) – (MFI of TS1/18 of mock)]/[(MFI of CBR LFA1/7 of mock)]. Statistical significance was analyzed using 1-way analysis of variance with a Bonferroni *post-hoc* analysis of variance with a Bonferroni *post-hoc* analysis. *p<0.05; n.s. = not significant. Data shown are mean \pm SD of triplicates. In (*a*-*d*), representative data from three independent experiments are shown.

≈ 1.8% versus isoflurane 1.2 % in adults), suggesting that the underlying mechanism of their anesthetic actions may not be exactly the same or there exists a difference in their affinity with the same molecules. For example, sevoflurane and isoflurane demonstrated different effects on 5-HT_{3AB} receptors (Stevens et al., 2005). While sevoflurane inhibited 5-HT_{3AB}, isoflurane minimally affected it. Volatile anesthetics presumably interact with multiple targets including targets outside the central nervous system (Eckenhoff, 2001). Therefore, it is natural to think that volatile anesthetics may not have the same functional profiles outside the central nervous system, possibly having different target molecules. Mac-1 is a good example to suggest that isoflurane and sevoflurane may have different immunological effects.

The present study did not demonstrate the direct interaction of isoflurane with Mac-1 using X-ray crystallography or nuclear magnetic resonance (NMR). While α M-Y267A β 2-WT and α M-L312A β 2-WT mutants have altered allosteric cavity in the α M I domain, this cavity is presumably intact in the α M-WT β 2-L132A mutant. The result showing that isoflurane inhibited α M-WT β 2-L312A, not α M-Y267A β 2-WT and α M-L312A β 2-WT, in conjunction with the docking simulation, strongly supports the proposition that isoflurane inhibits Mac-1 directly by binding to the cavity in the α M I domain. In the future, detailed analyses using X-ray crystallography will be important, particularly studies performing co-crystallization of full-length Mac-1 with isoflurane. Unfortunately, there is no reported X-ray crystallographic

structure of full-length Mac-1 available, but co-crystallization with isoflurane will likely provide significantly valuable information because the α 7 helix of α M I domain is considered to be extremely flexible in associated with the β 2 subunit, as suggested in $\alpha X\beta$ 2, another sister protein of Mac-1, and the conformation of and around the allosteric cavity in the α M I domain can change significantly. An alternative approach will be to use dynamic NMR using the α M I domain to assess the interaction with isoflurane or sevoflurane, especially at around the α 7 helix. The use of full-length Mac-1 in dynamic NMR is impractical due to the size of the molecule. Sevoflurane is larger in size than isoflurane. Detailed structural analysis including the size of the pocket using full-length Mac-1 in the future will allow us to understand the underlying mechanism of differential effects of volatile anesthetics on Mac-1 from a structural perspective.

Similar to volatile anesthetics, Mac-1 interacts with several ligands such as ICAM-1, iC3b, fibrinogen and Factor X. As a result, it is involved in a wide range of immunological events such as leukocyte intravascular crawling (Dunne et al., 2002), phagocytosis (Harris et al., 2000) and neutrophil apoptosis (Coxon et al., 1996; Mayadas & Cullere, 2005). In this study, binding of Mac-1 to ICAM-1 was evaluated because this interaction is critical for the recruitment of leukocytes (particularly neutrophils) in patients who undergo surgery. Although both LFA-1 and Mac-1 bind to ICAM-1, they reveal different binding kinetics; the binding affinity for LFA-1 is much stronger than that for Mac-1 (Li et al., 2013), which is in line with our binding results. It is

generally noted that the binding of LFA-1 to ICAM-1 allows neutrophils to arrest on the endothelium, while the binding of Mac-1 to ICAM-1 will allow neutrophils to crawl on the endothelium (Phillipson et al., 2006). However, differences in neutrophil recruitment among different tissues have been recognized (Kolaczkowska & Kubes, 2013), and it is still yet to be determined how each tissue uses these adhesion molecules for neutrophil migration.

We recently found that, in a polymicrobial sepsis model, Mac-1 knockout mice had a significant reduction in neutrophil migration to their liver, but LFA-1 knockout mice did not show any reduction (unpublished data, paper in submission). This suggests to us that Mac-1 is a dominant factor in neutrophil migration to the liver in the model. Neutrophils are responsible for various, acute inflammatory liver injuries such as ischemicreperfusion mediated liver injury, alcoholic hepatitis and acetaminophen liver toxicity (Ramaiah & Jaeschke, 2007). Ischemic reperfusion of liver can be observed during liver transplantation, liver surgery using Pringle maneuver and hemorrhagic shock. In those cases, isoflurane or sevoflurane may be given to patients for surgical anesthesia. Attenuation of neutrophil extravasation is important to alleviate the severity of liver injury. Based on our in vitro results, isoflurane anesthesia may be preferable over sevoflurane anesthesia in these clinical scenarios to attenuate the degree of inflammatory-related liver injury. In vivo examination is warranted in the future. In addition, whether or not there is any difference in bacterial phagocytosis as a result of exposure to the different anesthetics still needs to be determined.

Additional interesting findings in the present study were related to integrin biology. First, the $\beta 2$ I domain structure of Mac-1 was not exactly the same with the one of LFA-1. Isoflurane binding pocket in the $\beta 2$ I domain of LFA-1 was less exposed in Mac-1 when probed by TS 1/18 antibody. This may hinder access of isoflurane to the $\beta 2$ I domain in Mac-1. Second, there exists a common activation mechanism from the $\beta 2$ I domain to the α I domain among LFA-1, Mac-1 and $\alpha X\beta 2$. When mutations at the cavity of the $\beta 2$ I domain were studied, α L-WT $\beta 2$ -L132A, α M-WT $\beta 2$ -L132A and αX -WT $\beta 2$ -L132A were all activating, while α L-WT $\beta 2$ -L135A, α M-WT $\beta 2$ -L135A and αX -WT $\beta 2$ -L135A were all deactivating, mutants. The role of this cavity needs to be explored more in detail in the future. Information about the full-length LFA-1 and Mac-1 structures would be critical for that purpose.

In conclusion, the present study showed that two commonly used volatile anesthetics, isoflurane and sevoflurane, affected an important immunological molecule, i.e. Mac-1, differently. This suggested to us that widely used volatile anesthetics potentially have different immunotoxic effects.

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Declaration of interest

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References

Anderson, D. C., and Springer, T. A. 1987. Leukocyte adhesion deficiency: An inherited defect in the Mac-1, LFA-1, and p150,95 glycoproteins. *Annu. Rev. Med.* 38:175–194.

- Campagna, J. A., Miller, K. W., and Forman, S. A. 2003. Mechanisms of actions of inhaled anesthetics. *New Engl. J. Med.* 348:2110–2124.
- Carbo, C., Yuki K., Demers, M., et al. 2012. Isoflurane inhibits neutrophil recruitment in the cutaneous Arthus reaction model. *J. Anesth.* 27: 261–268.
- Coxon, A., Rieu, P., Barkalow, F. J., et al. 1996. A novel role for the β2 integrin CD11b/CD18 in neutrophil apoptosis: A homeostatic mechanism in inflammation. *Immunity* 5:653–766.
- Diamond, M. S., Garcia-Aguilar, J., Bickford, J. K., et al. 1993. The I domain is a major recognition site on the leukocyte integrin Mac-1 (CD11b/CD18) for four distinct adhesion ligands. J. Cell Biol. 120: 1031–1043.
- Diamond, M. S., Staunton, D. E., de Fougerolles, A. R., et al. 1990. ICAM-1 (CD54): A counter-receptor for Mac-1 (CD11b/CD18). J. Cell Biol. 111:3129–3139.
- Dilger, J. P. 2002. The effects of general anaesthetics on ligand-gated ion channels. *Br. J. Anaesth.* 89:41–51.
- Dunne, J. L., Ballantyne, C. M., Beaudet, A. L., and Ley, K. 2002. Control of leukocyte rolling velocity in $TNF\alpha$ -induced inflammation by LFA-1 and Mac-1. *Blood* 99:336–341.
- Eckenhoff, R. G. 2001. Promiscuous ligands and attractive cavities: How do the inhaled anesthetics work? *Mol. Interv.* 1:258–268.
- Friesner, R. A., Banks, J. L., Murphy, R. B., et al. 2004. Glide: A new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. J. Med. Chem. 47: 1739–1749.
- Harris, E. S., McIntyre, T. M., Prescott, S. M., and Zimmerman, G. A. 2000. The leukocyte integrins. *J. Biol. Chem.* 275:23409–23412.
- Huang, C., Zang, Q., Takagi, J., and Springer, T. A. 2000. Structural and functional studies with antibodies to the integrin β 2 subunit. A model for the I-like domain. *J. Biol. Chem.* 275:21514–21524.
- Jenkins, A., Greenblatt, E. P., Faulkner, H. J., et al. 2001. Evidence for a common binding cavity for three general anesthetics within the GABAA receptor. J. Neurosci. 21:RC136.
- Jun, C. D., Shimaoka, M., Carman, C. V., et al. 2001. Dimerization and the effectiveness of ICAM-1 in mediating LFA-1-dependent adhesion. *Proc. Natl. Acad. Sci. USA* 98:6830–6835.
- Kolaczkowska, E., and Kubes, P. 2013. Neutrophil recruitment and function in health and inflammation. *Nat. Rev. Immunol.* 13: 159–175.
- Kowalski, C., Zahler, S., Becker, B. F., et al. 1997. Halothane, isoflurane, and sevoflurane reduce post-ischemic adhesion of neutrophils in the coronary system. *Anesthesiology* 86:188–195.
- Li, N., Mao, D., Lu, S., et al. 2013. Distinct binding affinities of Mac-1 and LFA-1 in neutrophil activation. J. Immunol. 190:4371–4381.
- Lu, C., Ferzly, M., Takagi, J., and Springer, T. A. 2001. Epitope mapping of antibodies to the C-terminal region of the integrin β2 subunit reveals regions that become exposed upon receptor activation. *J. Immunol.* 166:5629–4637.
- Mayadas, T. N., and Cullere, X. 2005. Neutrophil β2 integrins: Moderators of life or death decisions. *Trends Immunol.* 26: 388–395.
- Mobert, J., Zahler, S., Becker, B. F., and Conzen, P. F. 1999. Inhibition of neutrophil activation by volatile anesthetics decreases adhesion to cultured human endothelial cells. *Anesthesiology* 90:1372–1381.
- Oxvig, C., Lu, C., and Springer, T. A. 1999. Conformational changes in tertiary structure near the ligand binding site of an integrin I domain. *Proc. Natl. Acad. Sci. USA* 96:2215–2220.
- Phillipson, M., Heit, B., Colarusso, P., et al. 2006. Intraluminal crawling of neutrophils to emigration sites: A molecularly distinct process from adhesion in the recruitment cascade. *J. Exp. Med.* 203: 2569–2575.
- Ramaiah, S. K., and Jaeschke, H. 2007. Role of neutrophils in the pathogenesis of acute inflammatory liver injury. *Toxicol. Pathol.* 35: 757–766.
- Sen, M., Yuki, K., and Springer, T. A. 2013. An internal ligand-bound, metastable state of a leukocyte integrin, $\alpha X\beta 2$. *J. Cell Biol.* 203: 629–642.
- Shimaoka, M., Lu, C., Salas, A., et al. 2002. Stabilizing the integrin α M inserted domain in alternative conformations with a range of engineered disulfide bonds. *Proc. Natl. Acad. Sci. USA* 99: 16737–16741.
- Shimaoka, M., Xiao, T., Liu, J. H., et al. 2003. Structures of the α L I domain and its complex with ICAM-1 reveal a shape-shifting pathway for integrin regulation. *Cell* 112:99–111.

- Springer, T. A., Teplow, D. B., and Dreyer, W. J. 1985. Sequence homology of the LFA-1 and Mac-1 leukocyte adhesion glycoproteins and unexpected relation to leukocyte interferon. *Nature* 314:540–542.
- Stevens, R., Rusch, D., Solt, K., et al. 2005. Modulation of human 5-hydroxytryptamine type 3AB receptors by volatile anesthetics and n-alcohols. *J. Pharmacol. Exp. Ther.* 314:338–345.
- Wagner, D. D., and Frenette, P. S. 2008. The vessel wall and its interactions. *Blood* 111:5271–5281.
- Witkowska, A. M., and Borawska, M. H. 2004. Soluble intercellular adhesion molecule-1 (sICAM-1): An overview. *Eur. Cytokine Network* 15:91–98.
- Xiong, J. P., Li, R., Essafi, M., et al. 2000. An isoleucine-based allosteric switch controls affinity and shape shifting in integrin CD11b Adomain. J. Biol. Chem. 275:38762–38767.
- Yuki, K., Astrof, N. S., Bracken, C., et al. 2008. The volatile anesthetic isoflurane perturbs conformational activation of integrin LFA-1 by binding to the allosteric regulatory cavity. *FASEB J.* 22:4109–4116.
- Yuki, K., Astrof, N. S., Bracken, C., et al. 2010. Sevoflurane binds and allosterically blocks integrin lymphocyte function-associated antigen-1. Anesthesiology 113:600–609.
- Yuki, K., Bu, W., Xi, J., et al. 2012. Isoflurane binds and stabilizes a closed conformation of the leukocyte function-associated antigen-1. *FASEB J.* 26:4408–4417.