Published in final edited form as:

J Anesth. 2013 April ; 27(2): 261–268. doi:10.1007/s00540-012-1508-1.

Isoflurane inhibits neutrophil recruitment in the cutaneous Arthus reaction model

Carla Carbo, Ph.D.^{1,2,3,*}, Koichi Yuki, M.D.^{1,4,5,*}, Melanie Demers, Ph.D.^{1,2,3}, Denisa D. Wagner, Ph.D.^{1,2,3}, and Motomu Shimaoka, M.D., Ph.D.^{1,2,5,6}

¹Immune Disease Institute, Boston, Massachusetts, USA

²Program in Cellular and Molecular Medicine, Children's Hospital Boston, Boston, Massachusetts, USA

³Department of Pediatrics, Harvard Medical School, Boston, Massachusetts, USA

⁴Department of Anesthesiology, Perioperative and Pain Medicine, Children's Hospital Boston, Boston, Massachusetts, USA

⁵Department of Anaesthesia, Harvard Medical School, Boston, Massachusetts, USA

⁶Department of Molecular Pathobiology and Cell Adhesion Biology, Mie University, Graduate School of Medicine, Mie, Japan

Abstract

Purpose—Neutrophil recruitment to the inflammatory sites is regulated by a variety of adhesion molecules including $\beta 2$ integrins. The dependency of neutrophil recruitment on $\beta 2$ integrins is variable in different tissues, and not clarified in the cutaneous passive reverse Arthus reaction yet. We examined this question and also evaluated the impact of isoflurane on neutrophil recruitment to the skin because we previously showed *in vitro* that isoflurane binds and inhibits $\beta 2$ integrins.

Methods—The dependency on $\beta 2$ integrins in neutrophil recruitment to the skin in the Arthus reaction was examined using αL , αM and $\beta 2$ knockout mice. Then, we evaluated the effect of isoflurane on neutrophil recruitment to the skin. In addition, the effects of isoflurane on neutrophil binding to intercellular adhesion molecule-1 (ICAM-1), one of $\beta 2$ integrin ligands were studied *in vitro* using cell adhesion assays.

Results—Neutrophil recruitment to the skin in the Arthus reaction model was totally dependent on $\beta 2$ integrins, as $\beta 2$ knockout mice completely abolished it. However, the defect of only one of $\beta 2$ integrins was not sufficient to abolish neutrophil recruitment. Isoflurane reduced neutrophil recruitment to the skin by approximately 90%. Also isoflurane inhibited neutrophil adhesion to $\beta 2$ integrin ligand ICAM-1.

Conclusions—We demonstrated that 1) neutrophil recruitment to the skin was totally dependent on $\beta 2$ integrins, and 2) isoflurane significantly impaired neutrophil recruitment. Based on the previous studies on the contribution of other adhesion molecules in neutrophil recruitment, it is likely that isoflurane at least partially affects on $\beta 2$ integrins in this model.

Corresponding author: Koichi Yuki, M.D., Department of Anesthesiology, Perioperative and Pain Medicine, Children's, Hospital Boston, 300 Longwood Avenue, Boston, Massachusetts, 02115, USA, Tel: 617-355-6225, FAX: 617-730-0799, yuki.koichi@gmail.com And Motomu Shimaoka, M.D., Ph.D., Department of Molecular Pathobiology and Cell Adhesion Biology, Mie University, Graduate School of Medicine, 2-174 Edobashi, Tsu-City, Mie, 514-8507, Japan, Tel: +81-59-231-5031, FAX: +81-59-231-5209, shimaoka@doc.medic.mie-u.ac.jp.
*equally contributed

Keywords

$\beta 2$ integrin; reverse passive Arthus reaction; skin; isoflurane; neutrophil recruitment

INTRODUCTION

While general anesthesia has been used for more than 150 years, the mechanism of volatile anesthetics still has not been delineated yet (1, 2). Interestingly, these mysterious molecules have been reported to affect not only neuronal cells (3, 4), but also leukocytes (5). Acute inflammation due to tissue damage and/or pathogen invasion is a hallmark feature in surgery. Leukocytes are recruited to the inflammatory sites to remove dead cells, cellular debris and pathogens (6). This will not only enhance tissue repair and wound healing, but also reduce the perioperative infection. Therefore, it is important to know whether or not volatile anesthetics will alter the behavior of leukocytes, particularly that of the first defense cells neutrophils in the setting of tissue damage and/or pathogen invasion (7, 8). The off-target effect of anesthetics could potentially pose an impact on patients' outcome (9).

Neutrophils undergo several steps using various adhesion molecules to reach the inflammatory sites (7, 10). Initially, selectins help them to tether to and roll on the endothelium. Multiple chemoattractants are secreted from the wounded tissues to guide them to their destination. Chemokines, chemoattractants (such as C5a) and proinflammatory cytokines such as (TNF- α) activate adhesion molecules on neutrophils including $\beta 2$ integrins. These events cause neutrophil arrest on the endothelium and subsequent intravascular crawling to transmigration sites. Then, they transmigrate through the endothelium and reach the inflammatory sites.

$\beta 2$ integrins consist of noncovalently associated α - and β -subunits. Among four $\beta 2$ integrins ($\alpha L\beta 2$, $\alpha M\beta 2$, $\alpha X\beta 2$ and $\alpha D\beta 2$), $\alpha L\beta 2$ and $\alpha M\beta 2$ are predominantly expressed on neutrophils (11). $\alpha L\beta 2$ is involved in neutrophil arrest and transmigration, while $\alpha M\beta 2$ in intravascular crawling and transmigration (10). The importance of $\beta 2$ integrins is illustrated by the rare genetic disorder leukocyte adhesion deficiency (LAD) type-1, the loss of expression of the $\beta 2$ integrins (12–14). In LAD-1, which is characterized by recurrent severe soft tissue infections and chronic periodontitis, neutrophil mobilization into the extracellular, inflammatory sites is profoundly impaired (14, 15). Previously de Rossi et al. demonstrated *in vitro* that isoflurane attenuated the activation of $\beta 2$ integrins, suggesting that this may impact on neutrophil recruitment (16). We also demonstrated *in vitro* that isoflurane blocks the binding of $\alpha L\beta 2$ and $\alpha M\beta 2$ to the ligand intercellular adhesion molecule-1 (ICAM-1) (17–19). In addition, our nuclear magnetic resonance, X-ray crystallography, and photolabeling experiments found that isoflurane directly binds to $\alpha L\beta 2$ (17–19). However, the effect of volatile anesthetics on $\beta 2$ integrins *in vivo* remains to be determined.

Although the importance of $\beta 2$ integrins in neutrophil recruitment is well appreciated, the degree of dependency on them is variable in different tissues and organs (20, 21). The recruitment to the peritoneal cavity and the lung is not completely dependent on $\beta 2$ integrins. On the other hand, the recruitment to the skin is suggested to be predominantly dependent on $\beta 2$ integrins in some models (20, 21). In this study, we evaluate the degree of $\beta 2$ dependency in neutrophil recruitment to the skin in the reverse passive Arthus reaction, a well-established model for neutrophil study. We also evaluated the effect of isoflurane, a commonly used volatile anesthetic on neutrophil recruitment in this system.

MATERIALS AND METHODS

Mice

Six to eight-week-old C57BL/6J wild-type mice were purchased from the Jackson Laboratory (Bar Harbor, ME). α L, α M and β 2 knockout mice of C57BL/6J background were purchased from the Jackson Laboratory, bred and housed in our animal facility. Six to eight-week-old knockout mice were used for the experiments. The phenotypes of α L, α M, and β 2 knockout mice were shown in Table 1. Experimental procedures were approved by the Animal Care and Use Committees of the Immune Disease Institute and Harvard Medical School (Boston, MA).

Reverse passive Arthus reaction

First, mice were placed in the induction chamber and briefly anesthetized with isoflurane using isoflurane vaporizer (VetQuip; New South Wales, Australia). Then, mice were taken out from the induction chamber to induce the reverse passive Arthus reaction. The back of mice was shaved over a large area. The reverse passive Arthus reaction was elicited by intravenous injection of 1% BSA (75 μ g protein/g mouse in PBS) immediately followed by intradermal injection of anti-BSA antibody 20 μ l each (5 μ g/ μ L in PBS) (Sigma) at two sites of the center of the back as previously described (22). Nonimmunizing rabbit IgG was injected as a control. Once mice woke up from anesthesia, mice were divided into two groups. One group of mice were placed in the induction chamber again and exposed to isoflurane 2%. Soon mice were taken out from the induction chamber and placed on the nose cone to be continuously exposed to isoflurane for the duration of the experiment (2 or 4 hours). Mice were placed flat on their stomach on a warm pad (37°C) with ocular ointment. Another group of mice were not exposed to isoflurane. After 2 or 4 hours, mice were sacrificed. A 6 mm skin biopsy was taken from the site where anti-BSA antibody was injected, and weighed to assess edema formation. In addition, 100 μ l Evans Blue (1%) was injected in some mice with the BSA to visualize the inflammatory area.

Determination of myeloperoxidase (MPO) activity

MPO is a peroxidase enzyme that is most abundantly expressed in the granules of neutrophils (23, 24). It is one of the most abundant proteins in neutrophils, accounting for about 5% of the cell weight (24). It is also expressed in the granules of monocytes, but the MPO content of each monocyte is about 1/45 - 1/50 of that of neutrophil (25). The measurement of MPO of the skin is a well-established method to estimate neutrophil content in the cutaneous inflammation (25–27). Briefly, the dermis of 6 mm skin biopsies was blended in 50 mmol/L potassium phosphate buffer, centrifuged, resuspended, and sonicated in potassium phosphate buffer supplemented with 50 mmol/L hexadecyltrimethylammonium bromide (Sigma). After centrifugation of the cell lysates, MPO activity was assessed in the supernatant by adding tetramethylbenzidine and by absorbance reading at 450 nm after stopping the reaction with 0.3 N sulfuric acid.

Preparation of mouse neutrophils

Mouse neutrophils were isolated from bone marrow of wild-type (WT) mice using negative sorting with MACS separation columns (Miltenyl Biotec, Auburn, CA) as previously described (26, 28). Briefly, after red blood cell lysis, bone marrow cells were incubated for 10 minutes at 4°C with anti-CD45R antibody (10 μ g/ml), anti-CD5 antibody (10 μ g/ml), anti-CD8 antibody (5 μ g/ml), and anti-CD4 antibody (5 μ g/ml) (all from BD Biosciences, Franklin Lakes, NJ). After washing twice, cells were incubated with anti-rat IgG antibody coupled to magnetic beads (Miltenyl Biotec). The cells were run through a magnetic column. The eluted neutrophils were stained with anti Gr-1 antibody (BD Biosciences, San

Jose, CA), and the percentage of Gr-1 positive cells was determined by flow cytometry. Only samples with > 90% Gr-1 positive cells were used for adhesion studies.

Flow cytometry

Surface expressions of various integrins on neutrophils were evaluated using flow cytometry. Neutrophils were incubated for 30 min at room temperature with the following rat anti-mouse antibodies; anti- α L, anti- α M, anti- α X, anti- β 1, anti- β 2, anti-L-selectin antibodies (BD Biosciences, San Jose, CA). After washing, samples were subject to flow cytometry analysis using FACScan (BD Bioscience, San Jose, CA). Data are shown as mean $\pm$ S.E.M.

Neutrophil adhesion assay

Neutrophils adhesion assay using a U-bottom-well plate was performed as follows. U-bottom 96-well plates were coated with mouse ICAM-1-Fc (15 μ g/ml; R&D Systems, Minneapolis, MN) at 4°C overnight and then blocked with HEPES buffered saline (HBS) containing 2% bovine serum albumin (BSA) for 6 h at room temperature. The plate was washed with Tris-buffered saline containing Tween-20. 50 μ l each of HBS containing either 2 mM $\text{Ca}^{2+}/\text{Mg}^{2+}$ (final assay concentration 1 mM $\text{Ca}^{2+}/\text{Mg}^{2+}$) or 2 mM Mg^{2+} (final assay concentration 1 mM Mg^{2+}) was aliquoted to each well. Mouse neutrophils were incubated with anti-Fc blocking antibody, labeled with 2',7'-bis-(carboxyethyl)-5 (and -6)-carboxyfluorescein acetoxymethyl ester (Invitrogen, Carlsbad, CA) in L-15 medium (Cambrex Bioscience, East Rutherford, NJ) containing 2.5% fetal bovine serum, washed, and resuspended in HBS at the density of 1.0×10^5 cells/50 μ l. 50 μ l of cell suspensions were added to each well and incubated at room temperature for 15 min with mock, isoflurane (2 %) or α L β 2 and α M β 2 antagonist called compound #4 (10 μ M) (29). Isoflurane was administered to the cells in an airtight closed chamber using isoflurane vaporizer. The concentration of isoflurane was measured using Ultima infrared spectroscopy (Datex Instrument Corp., Helsinki, Finland). Following incubation, wells were washed, and fluorescence intensity of emission was measured using a fluorescence microplate reader at excitation of 485 nm and emission of 538 nm (Fluoroskan Ascent; Thermo Scientific, Waltham, MA). Binding percentage was calculated as [fluorescence intensity of emission following wash/input fluorescence intensity of emission] $\times$ 100 %.

Statistical analysis

Results were expressed as described in figures. One-way analysis of variance (ANOVA) with Tukey's *post hoc* was performed, and results were considered to be statistically significant when $p < 0.05$.

RESULTS

Neutrophil recruitment to the skin in the reverse passive Arthus reaction model is β 2-integrin dependent

The previous study of the rat reverse passive Arthus reaction model by Rote et al. demonstrated that neutrophil recruitment to the skin was not totally, but significantly inhibited by the administration of either β 2 antagonist or both α L β 2 and α M β 2 antagonists (26). This study certainly demonstrated that β 2 integrins play a central role in cutaneous neutrophil recruitment in this model, but did not rule out the possibility that β 2 integrin independent pathway may exist.

β 2 integrins on neutrophils were predominantly α L β 2 and α M β 2 (data not shown), as compatible with the previous report (11). To assess if neutrophil recruitment to the skin is purely β 2 integrins dependent, we evaluated neutrophil recruitment to the skin using α L,

α M and β 2 knockout mice. Mice were subjected to the reverse passive Arthus reaction for 4 h, and skin inflammation was evaluated by measuring both weight and MPO activity of the punch-biopsied skin. Neutrophils dominate the initial leukocyte influx to sites of acute inflammation (30, 31). This event is followed by the influx of monocytes. Monocytes infiltrate into inflamed tissue several hours after the onset of inflammation (31). In the reverse Arthus reaction model, neutrophils are the major cellular constituent during the first 12 hours and peaks at 4 hours, while monocytes predominate between 15 and 24 hours (32). In addition, the MPO is predominantly expressed in neutrophils as described in the Materials and Methods, and the MPO measurement is considered as a good surrogate of infiltrated neutrophils at our experimental time frame. β 2 knockout mice showed the complete inhibition of neutrophil recruitment to the skin and no edema formation (Figure 1A). However, α L and α M knockout mice did not show any reduction in neutrophil recruitment to the skin. These results demonstrated that neutrophil recruitment to the skin in this model was totally β 2 integrin-dependent, and inhibited only when both α L β 2 and α M β 2 integrins were blocked (Figure 1B and 1C).

Isoflurane inhibits neutrophil recruitment to the skin in the reverse passive Arthus reaction

Because the neutrophil recruitment to the skin in the Arthus reaction is completely β 2 integrin dependent, we examined neutrophil recruitment to the skin in the presence of isoflurane, which blocked α L β 2 and α M β 2 *in vitro* (17). Following the injections for the reverse passive Arthus reaction, mice were assigned to the group exposed or not exposed to isoflurane. Neutrophil recruitment and edema formation at the inflammatory site were evaluated at 2 h and 4 h. At 2 h, there was almost no neutrophil recruitment in the inflammatory site in mice exposed to isoflurane (89.2% reduction), while in non-anesthetized mice neutrophil recruitment was observed to a great extent (Figure 2A). At 4 h, neutrophil recruitment increased in both groups, but was still significantly lower in the isoflurane group (86.9% reduction) (Figure 2A). At 2 h, there was less edema formation in the anesthetized mice, which remained the same at 4 h (Figure 2B). Isoflurane significantly decreased vascular permeability compared to mice not exposed to anesthesia (Figure 3), supporting the less edema formation in the isoflurane group. This result is compatible with the previous *in vitro* data of isoflurane as an α L β 2 and α M β 2 antagonist.

Isoflurane inhibits β 2 integrin-mediated neutrophil adhesion to ICAM-1

Among various ligands for β 2 integrins, ICAM-1 plays a major role in neutrophil recruitment (33). Sequential binding of α L β 2 and α M β 2 to ICAM-1 helps neutrophils to capture and stably adhere to the endothelium (34), which is a requisite step for neutrophil recruitment. To see if isoflurane impairs the binding of β 2 integrins on neutrophils to ICAM-1, we tested the binding of neutrophils to ICAM-1 in both resting ($\text{Mg}^{2+}/\text{Ca}^{2+}$) and stimulatory (Mg^{2+}) conditions with or without isoflurane (12). The binding of β 2 integrins to ICAM-1 increases in a stimulatory condition (35). As expected, neutrophils bound to ICAM-1 more in Mg^{2+} than in $\text{Mg}^{2+}/\text{Ca}^{2+}$ in the absence of isoflurane (Figure 4). Isoflurane inhibited cell adhesion of neutrophils to ICAM-1 in the presence of Mg^{2+} to the comparable level with an α L β 2/ α M β 2 antagonist, called compound #4 (Figure 4).

DISCUSSION

In this study we demonstrated in the reverse passive Arthus reaction model that 1) neutrophil recruitment to the skin is completely dependent on β 2 integrins, 2) isoflurane significantly attenuates neutrophil recruitment, and 3) isoflurane attenuates the binding of neutrophils to ICAM-1.

The reverse passive Arthus reaction occurs in response to the immune complex mediated injury. Once the Arthus reaction occurs, neutrophils will be recruited to the skin with the help of cooperative actions of adhesion molecules. The identification of critical adhesion molecule is important to help to modulate the neutrophil recruitment process. The contribution of various adhesion molecules in neutrophil recruitment to the skin has been investigated using knockout mice or blocking antibodies. L-selectin knockout mice demonstrated approximately 33–34% reduction of neutrophil recruitment compared with the wild-type mice (36). E-selectin blocking antibody did not affect the degree of neutrophil recruitment (37). P-selectin blocking antibody reduced neutrophil recruitment by 32% (37). Also blocking P-selectin glycoprotein ligand-1, a ligand to all three selectins (38, 39) reduced only by 38% (37). The contribution of ICAM-1 was evaluated using ICAM-1 knockout mice (36). ICAM-1 knockout mice showed 43–54% reduction of neutrophil recruitment. Interestingly, the double knockout of L-selectin and ICAM-1 showed only 54% reduction, which is almost comparable to the result of ICAM-1 knockout mice. This suggests that ICAM-1 (i.e. binding to $\beta 2$ integrins) plays a significant role in neutrophil recruitment over selectin. Also it is easily speculated that there are other ligands to $\beta 2$ integrins other than ICAM-1 because $\beta 2$ integrins knockout mice abolished neutrophil recruitment completely, while ICAM-1 knockout mice inhibited only by about 50%. For example, fibrinogen is one of ligands to $\alpha M\beta 2$ and considered to be a important player in leukocyte migration (40). Our results suggest that $\beta 2$ integrins are critical adhesion molecules in neutrophil recruitment to the skin in this model. Isoflurane reduced neutrophil recruitment by approximately 90%. In the context of predominant role of $\beta 2$ integrins in neutrophil recruitment to the skin, the significant inhibition by isoflurane is considered to be at least partly due to the impairment of the function of $\beta 2$ integrins.

As shown in Figure 1, both αL and αM knockout mice did not show any reduction in neutrophil recruitment to the skin. This observation is in line with the previous report by Rote et al (26). Similarly, the dermal inflammation induced by interleukin-1 and lipopolysaccharide (LPS) was inhibited by combined administration of αL and αM antibodies, but each of these antibodies alone had minimal effect (41). These observations may indicate that $\alpha L\beta 2$ and $\alpha M\beta 2$ can independently mediate neutrophil recruitment in the cutaneous inflammation. Notably there was a statistically significant increase of neutrophil recruitment in αL knockout mice relative to wild-type mice. Although not statistically significant, there is also a trend of increased neutrophil recruitment in αM knockout mice. As previously described by Ding et al., there is no increased compensatory expression of $\alpha M\beta 2$ in αL knockout mice and $\alpha L\beta 2$ in αM knockout mice (15). The increased neutrophil recruitment in αL knockout mice over wild-type mice may reflect the underlying leukocytosis in αL knockout mice. αM knockout mice are reported to have reduction of apoptosis in neutrophils accumulating in the inflamed tissues (42). This may explain the increased neutrophil accumulation in αM knockout mice.

In the resting condition, $\beta 2$ integrin α - and β -cytoplasmic tails associate with each other and constrain itself in an inactive form, which has a low affinity to its ligands (35). Intracellular signaling pathways are activated by various mediators, impinge on the cytoplasmic domains of the integrin and induce it into an active conformation, which has a high affinity to its ligands. All biological integrin ligands are multivalent, and can also contribute to integrin clustering, which will further increase ligand binding capability. Therefore, isoflurane can impair the function of $\beta 2$ integrins in various ways; 1) by directly binding to and inhibiting $\alpha L\beta 2$ and $\alpha M\beta 2$, 2) inhibiting intracellular signaling pathways, 3) impairing clustering. Our result of the reverse passive Arthus reaction strongly suggests that isoflurane impairs the function of $\beta 2$ integrins, but whether or not isoflurane directly binds and inhibits $\beta 2$ integrins *in vivo* still remains to be clarified. We previously used photoactivatable isoflurane called azi-isoflurane to identify isoflurane binding sites on $\alpha L\beta 2$ protein (18). Because this

compound is very difficult to produce and is limited in amount, *in vivo* application has not been tested yet. In the future, using photoactivatable isoflurane *in vivo* may help to clarify the aforementioned questions in the future.

Surgical infections and postoperative sepsis are among the most serious complications after surgery (43). The loss or reduction of $\beta 2$ integrin expression is associated with recurrent soft tissue infections and periodontitis as illustrated in LAD-1, and functional alteration of $\beta 2$ integrin by isoflurane may pose clinical implication. There are several clinical studies that suggest a potential contribution of volatile anesthetics to immune disorders. For example, in long-term alcoholic patients, isoflurane anesthesia was associated with increased postoperative tracheobronchitis and pneumonia compared to propofol anesthesia (9). More cases of recurrent breast cancer were observed in patients who underwent resection of cancer with sevoflurane anesthesia vs. local anesthesia (44). The number of patients enrolled in these studies is small, and it is important to evaluate the significance of various anesthetics on immune functions and outcomes by large, randomized controlled studies in the future. However, it has to be kept in mind that clinical outcome studies themselves do not address the underlying mechanism of anesthetics induced immunomodulation. Therefore, it is extremely important to address the mechanism of isoflurane-induced immunomodulation. In the long run, the investigation into this question will help us to understand the still unresolved mechanism of volatile anesthetics as well as redesigning immunomodulation-free anesthetics.

In conclusion, we showed that isoflurane blocked neutrophil recruitment *in vivo*, and suggested that the impairment of $\beta 2$ integrin function is at least partly responsible. Further study will enhance our understanding the mechanism of this observation and clinical implication.

Acknowledgments

In part supported by National Institute of Health (NIH) grants HL041002 (D.W.), K08GM101345 (K.Y.) and CHMC Anesthesia Foundation (K.Y.). Acknowledge Dr. Roderic G. Eckenhoff (University of Pennsylvania) for valuable suggestions.

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Figure 1. Neutrophil recruitment was inhibited in $\beta 2^{-/-}$ mice, but not $\alpha L^{-/-}$ mice or $\alpha M^{-/-}$ mice

Neutrophil recruitment and skin weight of punch biopsied skin from mice with or without the reverse passive Arthus reaction were examined in WT, $\beta 2^{-/-}$ (A), $\alpha L^{-/-}$ (B) and $\alpha M^{-/-}$ mice (C). Neutrophil recruitment was assessed by myeloperoxidase activity level (MPO). Data represent mean $\pm$ standard deviation of MPO or skin weight of 4 – 6 mice. * represents $p < 0.05$.

Figure 2. Isoflurane inhibits neutrophil recruitment and reduces edema in the reverse passive Arthus reaction model

Neutrophil recruitment (A) and skin weight (B) of punch-biopsied skin were examined using mice exposed or not exposed to isoflurane. Neutrophil recruitment was assessed by myeloperoxidase activity level (MPO). Data represent mean $\pm$ standard deviation of MPO or skin weight of 7 – 9 mice. * $p < 0.05$.

Figure 3. Isoflurane inhibits vascular permeability in the reverse passive Arthus reaction model Evans Blue was injected to show areas of inflammation on mouse skin after the reverse passive Arthus reaction. Mice (A) not exposed to isoflurane and (B) exposed to isoflurane.

Figure 4. Isoflurane inhibits neutrophil adhesion to ICAM-1

Neutrophil binding to 96-well plates coated with ICAM-1 was examined in the presence of mock, isoflurane (2 %), or α L β 2 and α M β 2 antagonist compound #4 (1 μ M). Data are expressed as mean $\pm$ S.E. of 6 independent experiments. * $p < 0.01$ versus mock-treated samples in Mg condition. An α L β 2 and α M β 2 antagonist known as compound#4 (29) was used as a positive control for the inhibition.

Table 1

Reported phenotype of integrin knockout mice

Knockout mice	Phenotype
α L knockout mice	Leukocytosis, No gross abnormalities in growth and development, No increase rate of spontaneous infection ⁽¹⁵⁾ , Reduced T cell proliferation and natural killer cell cytotoxicity ⁽⁴⁵⁾
α M knockout mice	No gross abnormalities in growth and development, Spontaneous bacterial abscesses of salivary glands (1%) ⁽⁴⁶⁾
β 2 knockout mice	Leukocytosis, Lymphadenopathy, Splenomegaly, Increased immunoglobulin levels, Severe defect in T cell proliferation, Spontaneous skin ulceration (95%) ⁽²⁰⁾