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Ischemia-reperfusion injury is attenuated in VAP-1-deficient mice and by VAP-1 inhibitors

Jan Kiss[1,2], Sirpa Jalkanen[1,3], Ferenc Fülöp[4], Timo Savunen[2] and Marko Salmi[1,3]

[1] MediCity Research Laboratory, University of Turku, Turku, Finland
[2] Department of Surgery, Turku University Hospital, Turku, Finland
[3] Department of Bacterial and Inflammatory Diseases, National Public Health Institute, Turku, Finland
[4] Institute of Pharmaceutical Chemistry, University of Szeged, Szeged, Hungary

Neutrophils mediate the damage caused by ischemia-reperfusion both at the site of primary injury and in remote organs. Vascular adhesion protein-1 (VAP-1) is an ecto-enzyme expressed on endothelial cells and it has been shown to regulate leukocyte extravasation. Here we show for the first time using VAP-1-deficient mice that VAP-1 plays a significant role in the intestinal damage and acute lung injury after ischemia-reperfusion. Separate inhibition of VAP-1 by small molecule enzyme inhibitors and a function-blocking monoclonal antibody in WT mice revealed that the catalytic activity of VAP-1 is responsible for its pro-inflammatory action. The use of transgenic humanized VAP-1 mice also showed that the enzyme inhibitors alleviate both the ischemia-reperfusion injury in the gut and neutrophil accumulation in the lungs. These data thus indicate that VAP-1 regulates the inflammatory response in ischemia-reperfusion injury and suggest that blockade of VAP-1 may have therapeutic value.

Key words: Adhesion molecules · Inflammation · Ischemia-reperfusion injury

Introduction

Interaction between leukocytes and endothelium, which allows leukocyte extravasation to the tissues, forms an essential part of host defense. This interaction is mediated *via* adhesion molecules expressed on the surface of leukocytes and endothelium [1]. Although indispensable for normal immune response, increased leukocyte trafficking to the tissues has deleterious consequences in certain diseases [2–5].

Diseases caused by ischemia are the leading cause of death in the developed countries. Since ischemia causes tissue necrosis, its treatment consists of restoration of blood flow to the ischemic tissue. Although reperfusion is necessary to prevent further damage to the ischemic tissue, it triggers an inflammatory response that aggravates ischemia-induced tissue damage [6]. Therefore, ischemia-reperfusion (IR) injury (IRI) complicates treatment of many potentially fatal diseases. A hallmark of IRI is sequestration of neutrophils in the reperfused tissues. Excessive neutrophil extravasation is to a large extent a consequence of increased expression and/or avidity of adhesion molecules on the surface of endothelial cells and neutrophils [1, 7].

Inflammatory response to IR can cause damage not only in reperfused tissues but also in remote organs. Pro-inflammatory cytokines released at the site of reperfusion enter circulation and activate endothelium and circulating leukocytes in multiple vascular beds [2, 6]. Although systemic inflammatory response may lead to development of dysfunction anywhere in the body, lungs are the most frequently injured organ [8, 9]. Indirect acute lung injury (ALI) can appear alone or as a part of multiple organ dysfunction syndrome, in which it is a strong independent risk factor for death [10]. Since increased leukocyte trafficking plays a major role in both IR and remote organ injury,

Correspondence: Dr. Marko Salmi
e-mail: marko.salmi@utu.fi

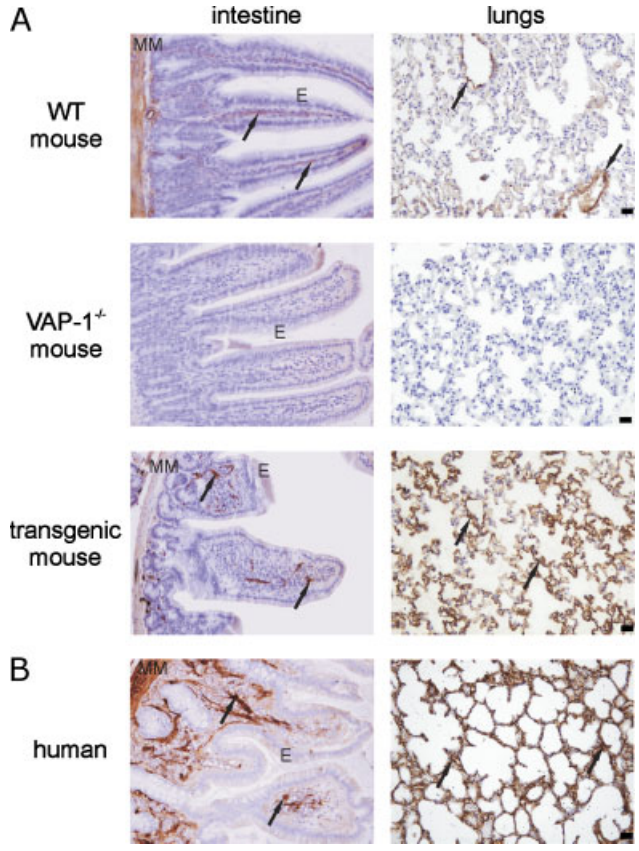

Figure 1. VAP-1 expression in the intestine and lungs of VAP-1$^{-/-}$, WT and transgenic mice and in humans. Intestine and lung sections from (A) VAP-1 deficient (VAP-1$^{-/-}$), WT, and humanized VAP-1 mice (transgenic) and (B) from humans were immunohistochemically stained for VAP-1. MM, muscularis mucosae, E, epithelial cells. Black arrows point to representative VAP-1^{+} (brown) blood vessels in the villi in the gut and in the lung interstitial tissue. Note that in WT lungs most vessels are faintly VAP-1^{+} (when compared with VAP-1$^{-/-}$ mice), but only a few express high level of VAP-1. Bars, 50 μm.

identification of the molecules involved in neutrophil infiltration is of paramount importance for the development of an effective treatment.

Vascular adhesion protein-1 (VAP-1) is a homodimeric sialoglycoprotein expressed in blood vessels [11, 12]. It is a cell-surface-expressed ectoenzyme belonging to semicarbazide-sensitive amine oxidases (SSAO) [13]. SSAO catalyze oxidative deamination of primary amines with NH_3, aldehydes and H_2O_2 as final products [14, 15]. Both hydrogen peroxide and aldehydes are biologically potent molecules capable of causing tissue damage due to their cytotoxicity at high concentrations [16]. However, at low physiological concentrations, H_2O_2 has signaling functions regulating leukocyte–endothelial interaction [17]. The adhesive function of VAP-1 mediates leukocyte rolling, firm adhesion and transmigration from the luminal surface of the endothelium to the tissue [17, 18]. This function can be blocked either by anti-VAP-1 antibodies or SSAO inhibitors. Notably, the antibodies do not inhibit the enzymatic activity of VAP-1 and the SSAO inhibitors do not affect the surface epitopes of the molecule seen by the neutralizing antibodies [19, 20]. Thus, VAP-1 is envisaged to have both enzyme-activity-dependent and enzyme-activity-independent functions in supporting leukocyte–endothelial interactions.

VAP-1-dependent leukocyte trafficking has been shown so far only in three inflammatory conditions (peritonitis, arthritis and mucosal vaccination) using VAP-1 deficient mice *in vivo* [21–23]. The role of VAP-1 in two mechanistically different and clinically relevant models, IRI and remote organ injury caused by systemic inflammation, remains unknown. Here we studied the involvement of VAP-1 in intestinal IRI and indirect ALI using VAP-1-deficient mice. Moreover, we separately examined the importance of adhesive and enzymatic activity of VAP-1 in both models using WT and transgenic humanized VAP-1 mice. Our results indicate that the catalytic activity of VAP-1 contributes to the pathogenesis of IRI.

Results

VAP-1 expression in WT, gene-modified mice and humans

To address the role of VAP-1 in IRI and ALI in different genetic models, we first analyzed the expression of VAP-1 in the intestine and lungs of WT, VAP-1 deficient and VAP-1 transgenic mice (VAP-1 deficient mice expressing human VAP-1 on endothelium) and in humans. In the gut of WT mice, the vessels in intestinal mucosa and smooth muscle layers are VAP-1^{+} (Fig. 1A). Intestines of VAP-1$^{-/-}$ mice are completely VAP-1^{-}, as expected. On the contrary, endothelial cells in the intestinal vasculature of transgenic mice strongly express VAP-1, while smooth muscle cells remain VAP-1^{-}. In humans, many of the vessels in lamina propria and smooth muscle cells are strongly VAP-1^{+} (Fig. 1B). In both species, the epithelial cells and leukocytes in lamina propria lack VAP-1.

In the lungs of WT mice VAP-1 has a patchy and weak expression pattern (Fig. 1A). The large vessels and interstitial capillaries are VAP-1^{+}, while alveolar epithelium and resident macrophages are negative. VAP-1 is completely absent in the lungs of VAP-1$^{-/-}$ mice. In contrast, VAP-1 is strongly expressed on numerous lung vessels including capillaries in transgenic, humanized VAP-1 mice (Fig. 1A). Also, in humans all the pulmonary vasculature is brightly positive (Fig. 1B). The same staining pattern was seen using another anti-VAP-1 antibody (data not shown). Moreover, the negative isotype-matched control antibodies did not show any specific staining (data not shown). Thus, VAP-1 appears to be more prominently expressed in the vessels of human lungs than in those of mouse, and the transgenic VAP-1 mice partially recapitulate the human type of VAP-1 expression.

VAP-1 is a critical molecule in intestinal IRI and subsequent ALI

We investigated the contribution of VAP-1 to intestinal damage caused by IR in WT and VAP-1$^{-/-}$ mice. Intestinal IR caused mucosal damage consisting of denudation and loss of intestinal villi (Fig. 2A).

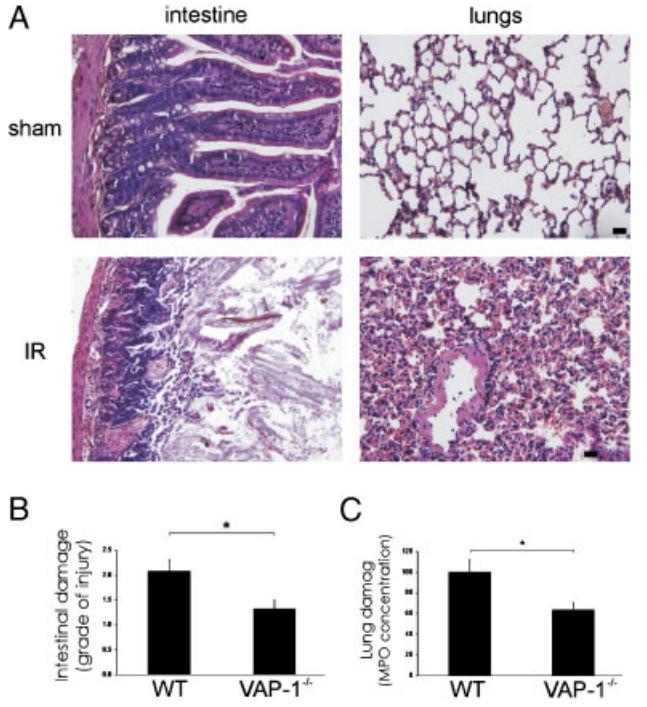

Figure 2. VAP-1 is crucial in the development of intestinal IRI and ALI. (A) VAP-$1^{-/-}$ mice and their WT controls underwent sham operation or 30 min of intestinal ischemia (IR). After 240 min of reperfusion, intestinal and lung samples were harvested, sectioned and stained with H&E. Micrographs show representative sections from a sham-operated and an ischemic WT animal. Bar, 50 μm. (B) Intestinal damage (mean ± SEM, $n = 10$ mice in both groups) was assessed from H&E-stained sections according to modified Park's scheme. (C) The lung damage (mean ± SEM, $n = 6$ mice in VAP-$1^{-/-}$ group and $n = 8$ in WT group) was assessed by measurement of lung MPO concentration, which was set to 100% in WT mice by definition. $^{*}p<0.05$, NS, non significant.

We found that VAP-$1^{-/-}$ mice had almost a 40% decrease in intestinal damage in comparison with their WT controls (Fig. 2B, $p = 0.02$).

Intestinal IR is an established model of indirect ALI [24, 25]. The most prominent features of the lung inflammation induced by intestinal IR were alveolar thickening due to edema, polymorphonuclear leukocyte infiltration into the interstitial space and intra-alveolar hemorrhage (Fig. 2A). Immunofluorescence staining for neutrophils showed a 48 ± 9% (mean ± SEM, $n = 7$–10 mice *per* group, $p = 0.001$) reduction in the number of infiltrating leukocytes in the ALI lungs in VAP-1-deficient mice. Quantification of the lung damage by myeloperoxidase (MPO) measurements also showed a similar significant inhibition in the mice lacking VAP-1 (Fig. 2C, $p = 0.02$). Together these data provide direct genetic evidence that VAP-1 contributes to the development of both IRI and ALI.

Enzymatic activity of VAP-1 is important in intestinal IRI

VAP-1 is an adhesion molecule with enzymatic activity [18]. Since these two functional modalities may play distinct roles in inflammation, we next investigated in WT mice, which of them mediates intestinal IRI. Intestinal IRI caused over 18-fold increase in intestinal damage when compared with sham-operated animals (Fig. 3A, $p = 0.007$). Enzymatic function of VAP-1 was inhibited in a group of animals by treatment with SSAO inhibitor (SZE 5302) prior to ischemia. Animals treated with saline served as a control. Strikingly, inhibition of enzymatic activity of VAP-1 caused almost 80% reduction of intestinal IRI when compared with vehicle-treated controls (Fig. 3A, $p = 0.03$). In contrast, inhibition of function of VAP-1 with anti-VAP-1 mAb had no effect on intestinal damage caused by IR when compared with controls (Fig. 3A). These data show that VAP-1 contributes to intestinal IRI *via* its enzymatic activity.

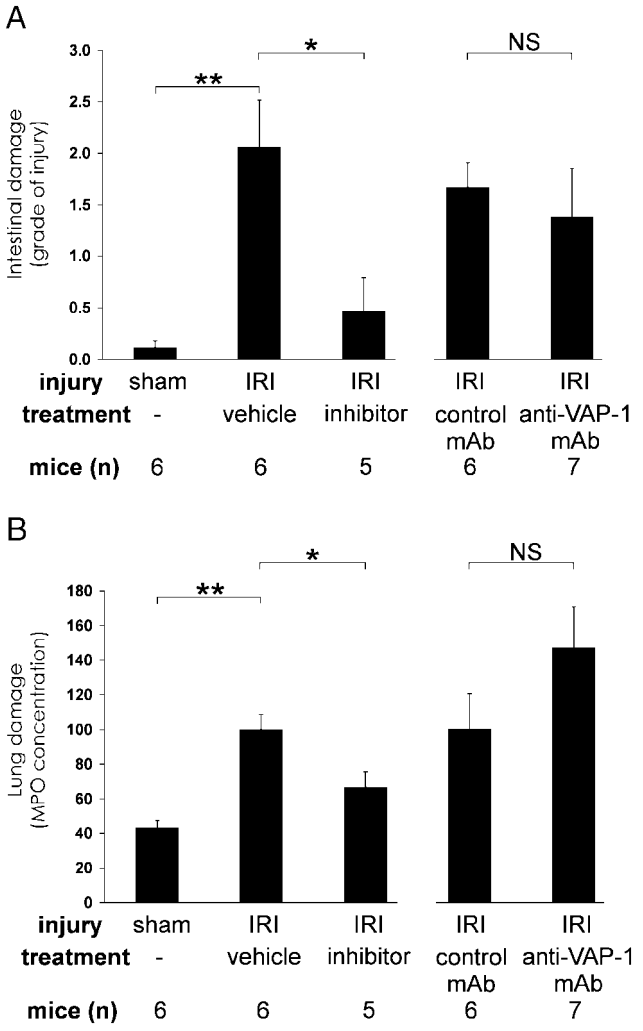

Figure 3. Enzymatic activity of VAP-1 is crucial for development of intestinal IRI and ALI. WT mice were subjected to sham operation or 30 min of intestinal ischemia and 240 min of reperfusion. The animals were treated prior to ischemia with SSAO inhibitor SZE5302, anti-VAP-1 mAb 7-106 or with controls. (A) Intestinal damage (mean ± SEM) was assessed from H&E-stained sections according to modified Park's scheme. (B) The lung damage (mean ± SEM) was assessed by measurement of lung MPO concentration, which was set to 100% in WT mice by definition; n = number of mice *per* group $^{*}p<0.05$, $^{**}p<0.01$.

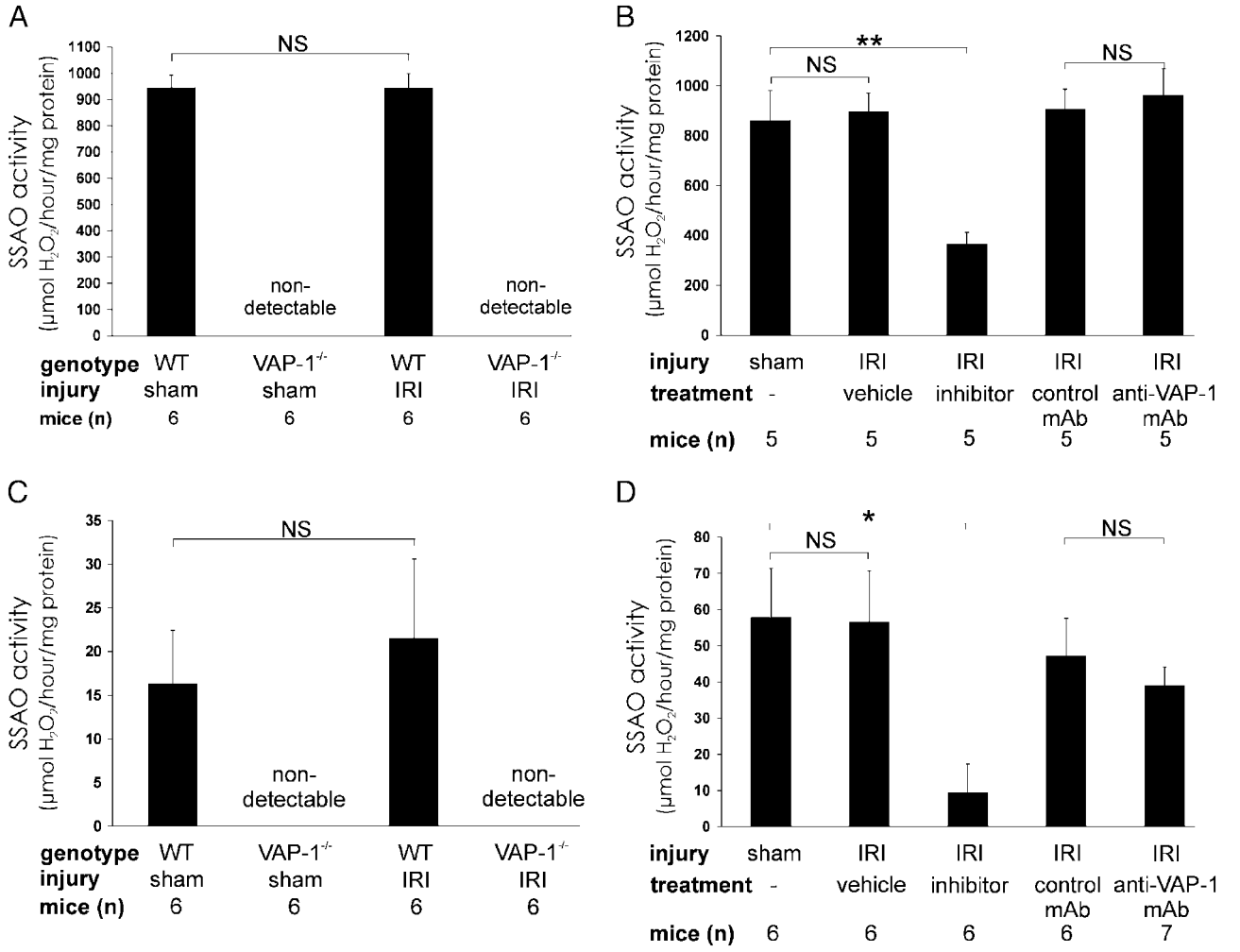

Figure 4. SZE 5302 but not the anti-VAP-1 antibodies block SSAO activity *in vivo*. Sham-operated animals underwent laparotomy, and the mice in the remaining groups underwent intestinal IR and were treated as indicated. SSAO activity (mean ± SEM) was measured from fat (A, B) and lungs (C, D). All mice in B and D were WT. $^{**}p<0.01$, NS, non significant.

Catalytic activity of VAP-1 contributes to the pathogenesis of indirect ALI

Intestinal IRI is an established model of systemic inflammation-induced indirect ALI [24, 25]. We confirmed this in our model by showing that ALI caused by intestinal IRI led to a 130% increase in lung MPO concentration when compared with sham-operated animals (Fig. 3B, $p = 0.002$). Inhibition of enzymatic activity of VAP-1 with the small molecule SSAO inhibitor SZE5302 caused over 30% attenuation of lung neutrophil infiltration when compared with saline-treated controls (Fig. 3B, $p = 0.03$). In contrast, blocking of VAP-1 adhesive function with anti-VAP-1 mAb did not alleviate lung damage (Fig. 3B). We conclude that the catalytic activity of VAP-1 is important also for the remote organ injury in the lungs after intestinal IRI.

SZE5302 but not the anti-VAP-1 mAb inhibits the catalytic activity of VAP-1 *in vivo*

VAP-1 has been shown to be inflammation inducible [12, 26]. However, it remains unknown whether IRI induces VAP-1 enzymatic activity at the site of primary insult and in remote organs. We therefore measured SSAO activity in the abdominal fat and in lungs. We found that VAP is the major source of SSAO activity in both anatomical locations since no SSAO activity was detectable in VAP-1-deficient mice (Fig. 4A and C). Interestingly, in WT mice the SSAO activity after intestinal IR remains unchanged both in the abdominal fat and in the lungs when compared with sham-operated animals (Fig. 4). Inhibitor treatment reduces SSAO activity in the abdominal fat of WT mice by 60% (Fig. 4B, $p = 0.0003$) and in the lungs by more than 80% (Fig. 4D, $p = 0.008$). Importantly, anti-VAP-1 mAb treatment had no effect on SSAO activity in the abdominal fat or in the lungs (Fig. 4B and D).

Targeting human VAP-1 alleviates IRI in transgenic mice

While VAP-1 expression on pulmonary endothelium in WT mice is spotty and weak, human pulmonary vessels are brightly VAP-1$^+$ (Fig. 1A and B). To study the importance of VAP-1 in IRI and indirect ALI in a setting similar to the human system, we used humanized VAP-1 mice. In these animals, inhibition of the enzymatic activity of human VAP-1 caused almost 50% reduction of intestinal IRI (Fig. 5A, $p = 0.01$). Inhibition of human VAP-1 with a function-blocking anti-human VAP-1 mAb had no significant effect on intestinal damage caused by IR (Fig. 5A).

Inhibition of catalytic activity of human VAP-1 with SZE5302 resulted in a 30% decrease of lung neutrophil infiltration when compared with vehicle-treated controls (Fig. 5B, $p = 0.05$) The anti-VAP-1 mAb also appeared to reduce lung neutrophil infiltration in this model, but this effect was not statistically significant (Fig. 5B). In conclusion, in the humanized VAP-1 mice, the catalytic function of this molecule appears to be more important than its mAb-defined epitopes especially in the development of IRI and possibly also in ALI.

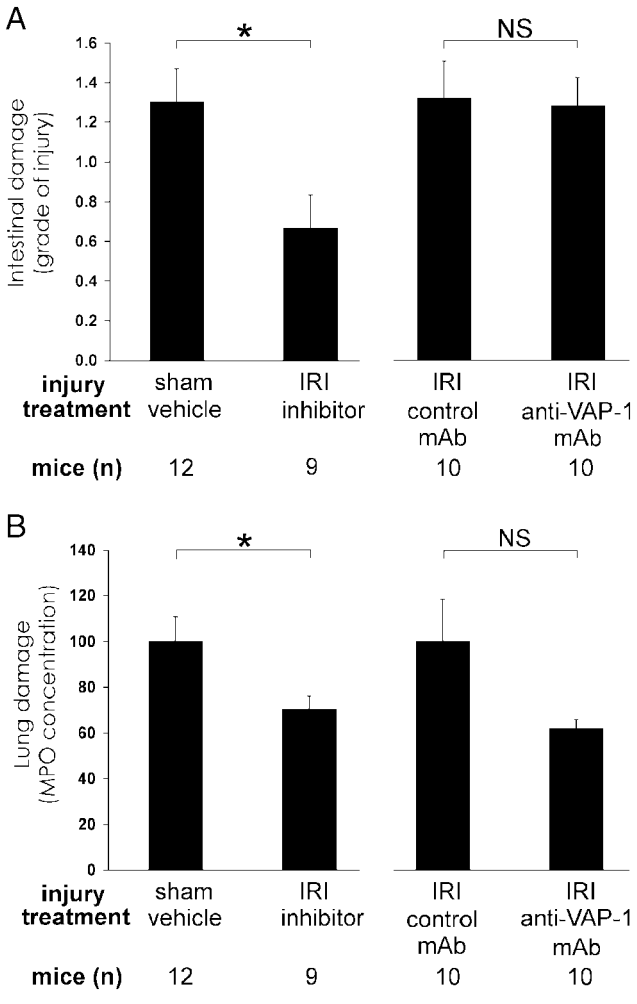

Figure 5. Inhibition of enzymatic activity in humanized VAP-1 mice prevents intestinal IRI and ALI. The effects of SSAO inhibition and anti-human VAP-1 mAb on the (A) intestinal and (B) lung injury were analyzed as in Fig. 3, n = number of mice *per* group.*$p<0.05$, NS, non significant.

Discussion

This is the first study to identify VAP-1 as a crucial molecule both in intestinal IRI and in indirect ALI. Genetic targeting of VAP-1 results in 40% reduction of IRI and ALI. Separate inhibition of the dual functions of murine and human VAP-1 *in vivo* revealed that SSAO activity rather than the mAb-defined epitopes is responsible for the tissue damage in both IRI and ALI. We have thus shown here using WT, VAP-1-deficient and transgenic humanized VAP-1 mice treated with anti-VAP-1 mAb and small molecule SSAO inhibitors that this ectoenzyme plays an important role in two clinically relevant models of IRI *in vivo*.

IRI represents a disease state in which the tissue damage is caused mostly by the extravasated leukocytes. Inhibition of leukocyte–endothelial interaction by blocking the classical adhesion molecules has shown to reduce tissue damage in IRI in numerous studies using gene-targeted animals [27]. Mice deficient in P-selectin and E-selectin, for instance, manifest with about 40% reduction of tissue damage in the model of myocardial IRI [28]. Similar reduction of myocardial IRI was observed after genetic targeting of endothelial Ig-family member ICAM-1 [28]. Indirect ALI is another example of a condition in which the tissue damage is caused by excessive leukocyte recruitment. Depletion of neutrophils has been shown to reduce ALI in experimental settings [29]. Moreover, interference with leukocyte–endothelial interaction reduces tissue damage in ALI in studies using P-selectin and ICAM-1 knockout mice [30]. Here we found that besides the traditional adhesion molecules also the cell-surface-expressed ectoenzyme VAP-1 contributes to IRI and ALI. Interestingly, other ectoenzymes, such as CD39 and CD73, have also recently been shown to be crucial for ALI development [25, 31]. Thus, this diverse class of ectoenzymes can contribute to the IR injury to the same extent as the classical adhesion molecules, and should therefore be considered as potential therapeutic target for these disorders.

VAP-1 has two functional modalities (enzyme-activity-dependent and enzyme-activity-independent) [17]. In all previous inflammatory models directly comparing the two functions, both functions have contributed to the adhesive role of VAP-1. Thus, leukocyte–endothelial interactions and development of inflammatory infiltrate *in vivo* have been inhibited to a similar extent either by anti-VAP-1 antibodies or small molecule SSAO inhibitors [20, 32, 33]. Moreover, combined use of both reagents has not led to additive or synergistic effects [20, 32]. This has led to a hypothesis, in which leukocytes first use a counter-receptor to interact with surface-exposed antibody-defined epitopes of VAP-1. Subsequently a leukocyte surface amine can then penetrate into the enzymatic channel of VAP-1 and serve as a substrate for the SSAO reaction [18]. Strikingly, in the IRI models reported here, only the catalytic activity of VAP-1

appears to be important. This observation may have several possible explanations. While the mAb only recognize VAP-1 expressed on endothelial surface, small molecular SSAO inhibitors diffuse to the tissues and interfere also with intracellularly stored VAP-1. The ability of the inhibitor to block VAP-1 expressed on non-endothelial cell types (pericytes, smooth muscle cells, adipocytes) better than that of anti-VAP-1 antibodies might also be important. However, we find this unlikely, since the inhibitor attenuated disease more effectively also in the transgenic mice, in which VAP-1 expression is confined to endothelial cells only. Finally, the enzymatic activity of VAP-1 has been shown to induce the expression or activity of other adhesion molecules, chemokines and transcription factors through the production of biologically active end-products [34–36]. This may be relevant in the current models, since the anti-VAP-1 mAb do not interfere with the catalytic activity of VAP-1, and thereby cannot prevent these secondary signaling effects. In any case, our data thus suggest for the first time that under certain conditions the two functions of VAP-1 can segregate.

Inhibition of enzymatic activity of VAP-1 caused an 80% reduction of tissue damage in IRI. This was superior to the reduction caused by genetic targeting of the molecule. SSAO inhibitors, including SZE5302, have been screened to be free from off-target effects with panels of kinases and phosphatases. However, the screenings cannot formally rule out all possible non-specific effects. Moreover, SZE5302 is known to inhibit other SSAO in addition to VAP-1. The SSAO family contains three genes, AOC1, AOC2 and AOC3 (VAP-1) [13, 37, 38], and no gene-specific inhibitors are available at the moment. Moreover, SZE5302 also inhibits a distantly related lysyl oxidase. Therefore, more effective inhibition of IRI by SZE5302 than by genetic deletion of VAP-1 might be partially attributed to its effects on other SSAO. Another factor potentially contributing to the more effective inhibition of development of IRI by the use of SSAO inhibitors is the acute onset of inhibition. While in the case of any knockout animal there is time to develop compensatory mechanisms to partially replace the inactivated molecule, acute inhibition does not allow for an extensive compensation.

The roles of VAP-1/SSAO in intestinal IRI and in indirect ALI have not been studied earlier. In a recent report, a different SSAO inhibitor LJP1207 was shown to decrease tissue damage in a stroke model in rats rendered exquisitely sensitive to IRI in the brain [39]. Moreover, administration of SSAO inhibitor has been shown to reduce leukocyte infiltration in LPS-induced pulmonary inflammation [40, 41]. Notably, while LPS inhalation in the lungs causes a direct lung injury and mimics pneumonia, intestinal IR triggers systemic inflammation and subsequent indirect ALI. Moreover, our current analyses are the first ones in which the involvement of VAP-1 and SSAO activity has been dissected in any type of IRI using genetic models. This is particularly important, since all SSAO inhibitors may have non-VAP-1-dependent effects.

In conclusion, we showed here for the first time with the use of genetically targeted animals that VAP-1 plays an important role both in IRI and in indirect ALI. Tissue damage in IRI and indirect ALI was caused by enzymatic activity of VAP-1. Therefore, these conditions can be partially prevented by inhibition of enzymatic activity of VAP-1. Third-generation SSAO inhibitors are highly selective and can be administered orally. These properties may make them suitable for treatment of several inflammation-mediated diseases, such as IR syndromes and ALI.

Materials and methods

Mice

VAP-1-deficient mice on 129S6 background were generated, validated and characterized as previously described [42]. Weight-, sex- and age-matched mice of the same background were used as controls. In certain experiments, WT mice of C57Bl background were used to exclude strain-specific phenotype. Transgenic humanized VAP-1 mice on FVB/129S6 background have been described previously [43]. Briefly, these animals were generated by crossing mice expressing human VAP-1 on endothelium under tie-1 promoter to VAP-1-deficient mice. Weight-, sex- and age-matched littermates were used as controls. All of the mice had access to standard mouse chow and water until the experiment. The protocol was approved by the Committee on Animal Ethics of Turku University and complies with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and the Statute 1076/85 § 3 and 1360/90 of The Animal Protection Law in Finland and the EU Directive 86/609.

Disease models

Intestinal IRI and indirect ALI were induced as described previously [24, 25]. Briefly, mice were anesthesized by intraperitoneal injection of ketamine and xylazine. Peroperative fluid loss was compensated by subcutaneous injection of saline. Superior mesenteric artery was occluded by microvascular clamp for 30 min. Sham animals underwent laparotomy only. Animals were sacrificed after 240 min of reperfusion and tissue samples were collected.

Inhibition of VAP-1 function by gene deletion, anti-VAP-1 antibodies and SSAO inhibitors

To investigate the importance of murine VAP-1 in intestinal IR and ALI, we studied VAP-1-deficient mice and WT controls. In these experiments, both genotypes underwent 30 min ischemia and 240 min reperfusion without any interventions.

To address separately the role of enzymatic and antibody-defined epitope function of VAP-1 in intestinal IR and ALI, we inhibited both functions of VAP-1 in WT mice. These animals were divided into five groups: (i) sham-operated animals without treatment, (ii) IRI animals with saline treatment (0.3 mL saline i.v.), (iii) IRI animals with SSAO inhibitor treatment (SZE 5302 also known as

BTT-2052 [23]) 50 mg/kg in 0.3 mL saline i.v.), (iv) IRI animals with control mAb treatment (9B5 (rat IgG), 100 μg/animal in 0.3 mL saline i.v.), (v) IRI animals with anti-mouse anti-VAP-1 mAb treatment (7-106 (rat IgG), 100 μg/animal in 0.3 mL saline i.v.). All treatments were given at the time of ischemia induction.

The potential role of human VAP-1 in IRI and ALI was investigated using transgenic VAP-1 humanized mice. These animals were divided into four groups: (i) IRI mice with PBS treatment (0.3 mL PBS i.v.), (ii) IRI mice with SSAO inhibitor treatment (SZE 5302, 50 mg/kg in 0.3 mL PBS i.v.), (iii) IRI mice with control mAb treatment (3G6 (mouse IgG), 100 μg/animal in 0.3 mL saline i.v.), (iv) ALI-induced animals with anti-human anti-VAP-1 mAb treatment (TK8-14 (mouse IgG), 100 μg/animal in 0.3 mL saline i.v.). All treatments were given at the time of ischemia induction.

Immunohistochemistry

VAP-1 was detected in paraffin sections using rat anti-mouse VAP-1 mAb (TK10-79), and polyclonal rabbit anti-human VAP-1 Ab [44]. Both antibodies recognize mouse and human VAP-1. As negative controls, rat mAb against human CD44 (9B5), not reactive with mouse antigens, and purified rabbit immunoglobulin (Dako) were used as appropriate. Staining of paraffin sections was performed as previously described [45]. Briefly, the sections received microwave treatment in a citrate buffer for antigen retrieval and were subsequently stained with the avidin-peroxidase method using Vectastain kits (Vector Laboratories, Burlingame, CA, USA). Intestine and lung samples from humans and mice of all the used genetic backgrounds (VAP-1-deficient, WT, transgenic humanized) were stained for VAP-1 and negative control. Images were obtained using an Olympus BX60 microscope (Japan) and a ColorView 12 camera (Olympus Soft Imaging Solutions, Mûnster, Germany).

In certain experiments, lung sections were stained using anti-mouse CD11a mAb (TIB217) and negative control followed by Alexa488 anti-rat IgG. The sections were embedded in Vectashield and examined using fluorescence microscopy.

Intestinal injury

Intestinal tissue was embedded in paraffin, cut into 4 μm slices, and stained with H&E. Intestinal injury was scored according to modified Park's grading [46] as follows: grade 0, normal mucosa; grade 1, subepithelial space and/or epithelial lifting in the villi; grade 2, villi denuded of epithelium and grade 3, loss of the villi. In each mouse, five randomly chosen fields of view under high magnification ($\times$ 400) were graded.

MPO assay

Pulmonary neutrophil sequestration was quantified using an MPO assay. Animals were euthanized and the lungs were harvested as described previously [25]. The lungs were mechanically homogenized in a buffer containing 200 mM NaCl, 5 mM EDTA, 10 mM Tris, 10% glycine, 1 mM PMSF and 28 μg/mL aprotinin (pH 7.4). The solution was centrifuged twice (1500 *g* at 4°C for 15 min) to separate tissue debris. The supernatant was then assayed for MPO activity using a commercially available ELISA kit according to manufacturer's instructions (HyCult, The Netherlands). To normalize the values from different sets of experiments, average lung MPO concentration in non-treated ALI-induced animals was arbitrarily assigned as 100%.

SSAO activity

Lung and abdominal fat were collected at the end of reperfusion (240 min). Tissue samples were cut into small pieces and lysed in a lysis buffer (PBS and 0.2% Triton X-100). The enzymatic activity of VAP-1 was determined by measurement of hydrogen peroxide production using fluoropolarimetric assay, as described previously [32]. Due to interference of intestinal flora with the assay, we were unable to measure SSAO activities directly in the intestinal villi.

Statistical analysis

The data were analyzed by non-parametric one-way ANOVA (Kruskall–Wallis and Mann–Whitney *U* tests) using SAS Enterprise Guide 3.0 (SAS Company, USA).

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Conflict of interest: Sirpa Jalkanen is a shareholder in Biotie. All other authors declare no financial or commercial conflict of interest.

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Abbreviations: **ALI:** acute lung injury · **IR:** ischemia-reperfusion · **IRI:** IR injury · **MPO:** myeloperoxide · **SSAO:** semicarbazide sensitive amine oxidase(s) · **VAP-1:** vascular adhesion protein-1

Full correspondence: Dr. Marko Salmi, MediCity Research Laboratory, University of Turku, Tykistökatu 6A, 20520 Turku, Finland
Fax: +358-2-3337000
e-mail: marko.salmi@utu.fi

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