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*J Immunol* 2007; 179:6160-6168; doi: 10.4049/jimmunol.179.9.6160

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VAP-1-Deficient Mice Display Defects in Mucosal Immunity and Antimicrobial Responses: Implications for Antiadhesive Applications

Kaisa Koskinen, Suvi Nevalainen, Marika Karikoski, Arno Hänninen, Sirpa Jalkanen, and Marko Salmi

VAP-1, an ecto-enzyme expressed on the surface of endothelial cells, is involved in leukocyte trafficking between the blood and tissues under physiological and pathological conditions. In this study, we used VAP-1-deficient mice to elucidate whether absence of VAP-1 alters the immune system under normal conditions and upon immunization and microbial challenge. We found that VAP-1-deficient mice display age-dependent paucity of lymphocytes, in the Peyer’s patches of the gut. IgA concentration in serum was also found to be lower in VAP-1−/− animals than in wild-type mice. Although there were slightly less CD11a on B and T cells isolated from VAP-1-deficient mice than on those from wild-type mice, there were no differences in the expression of gut-homing-associated adhesion molecules or chemokine receptors. Because anti-VAP-1 therapies are being developed for clinical use to treat inflammation, we determined the effect of VAP-1 deletion on useful immune responses. Oral immunization with OVA showed defective T and B cell responses in VAP-1-deficient mice. Antimicrobial immune responses against Staphylococcus aureus and coxsackie B4 virus were also affected by the absence of VAP-1. Importantly, when the function of VAP-1 was acutely neutralized using small molecule enzyme inhibitors and anti-VAP-1 Abs rather than by gene deletion, no significant impairment in antimicrobial control was detected. In conclusion, VAP-1-deficient mice have mild deviations in the mucosal immune system and therapeutic targeting of VAP-1 does not appear to cause a generalized increase in the risk of infection. The Journal of Immunology, 2007, 179: 6160–6168.

Lympocyte trafficking between the blood and tissues is crucial for mounting proper immune responses. This continuous process of lymphocyte recirculation is needed for productive contacts between APCs and responding lymphocytes in secondary lymphoid organs and for dispersal of effector lymphocytes into peripheral organs. The most important function of this system is to generate protective immune responses against microbes.

Lymphocyte traffic is governed by multiple activation and adhesion molecules expressed both on lymphocytes and vascular endothelial cells (1, 2). One of the endothelial adhesion molecules involved in the multistep extravasation cascade is VAP-1 (3). It belongs to a unique group of cell surface-expressed amine oxidases known as semicarbazide-sensitive amine oxidases (SSAO) (4). These enzymes mediate oxidative deamination of primary amines in a reaction that results in the production of aldehyde, ammonium and hydrogen peroxide (5). All end-products of the SSAO-catalyzed reaction are biologically active compounds that may alter the function of the endothelial cells or affect other nearby cells in a paracrine manner. The importance of low levels of hydrogen peroxide, in particular, as a local signaling molecule has become increasingly evident during the past few years (6, 7).

Previous in vitro and in vivo studies by several groups have shown that VAP-1 mediates rolling, firm adhesion and transmigration of leukocytes under normal conditions and in inflammation (8–15). Mechanistic analyses have revealed that VAP-1 displays both enzyme-activity-dependent and enzyme-activity-independent adhesive functions. Thus, anti-VAP-1 Abs, which do not interfere with the SSAO activity of VAP-1, block leukocyte extravasation. On the other hand, SSAO inhibitors also suppress VAP-1-dependent extravasation without affecting the Ab-defined epitopes. Moreover, a point mutation that renders VAP-1 catalytically inactive, abolishes VAP-1-dependent leukocyte-endothelial contacts (12).

We have recently generated VAP-1-deficient mice (16). These animals show impaired lymphocyte traffic into mesenteric lymph nodes and spleen in short term homing experiments. The influx of granulocytes into inflamed peritoneum and joints is also diminished in the absence of VAP-1. Here our aim was to assess whether VAP-1 deficiency causes other aberrations in the immune system. Moreover, we subjected VAP-1-deficient mice to microbial (bacterial and viral) infections to analyze the role of this adhesion molecule in the generation of proper antimicrobial responses. Our results revealed mild but significant changes in the mucosal immune system and in the control of infections in the absence of VAP-1. Since efforts aiming at blocking VAP-1 for therapeutic purposes are in progress (17, 18) it was interesting to observe that
neutralization of VAP-1 by Abs and enzyme inhibitors did not alter the course of microbial infections.

Materials and Methods

Mice

VAP-1-deficient mice in 129S6 background have been described (16). VAP-1 mice in C57BL/6 background were produced by crossing VAP-1−/− mice (in 129S6 background) with C57BL/6 Bom wild-type (wt) animals and then backcrossing the animals for >10 generations. In certain experiments, WT BALB/c mice were used. In all experiments, age- and sex-matched animals were used. All studies were approved by the Committee for Animal Experimentation in University of Turku.

The immune phenotyping (lymphocyte subsets, adhesion molecules, Ig measurements) were performed in WT and VAP-1−/− 129S6 mice of different ages. Oral immunizations were performed with 6-wk-old WT and VAP-1−/− 129S6 mice. For Staphylococcus aureus infections three different mouse strains were used (129S, C57BL/6, and BALB/c). Cox sackie virus infections were performed with C57BL/6 background due to its sensitivity for this virus.

Flow cytometry

Single cell suspensions were prepared from minced pieces of thymus, spleen, Peyer’s patches (PP), mesenteric lymph nodes (MLN) and peripheral lymph nodes (PLN; pooled axial, cervical, and inguinal nodes) by mechanical teasing through a metal meshwork. Erythrocytes were removed from spleen cells using hypotonic lysis. Cell numbers were determined by microscopic counting using trypan blue stainings.

Leukocyte phenotyping was done with mAbs against CD4 (CT-CD4-PE), CD8 (CT-CD8a-PE) and F4/80 (CL: A3-1-FITC) from Caltag Laboratories, anti-B220 (TIB-146, American Type Culture Collection, biotinylated), and anti-syndecan-1 (281.2). Streptavidin-PE (BD Pharmingen) was used as a second-stage reagent for the biotinylated mAbs, and 281.2 was detected using anti-rat IgG-Pe (Southern Biotechnology). Regulatory T cells were identified using mouse regulatory T cell staining kit (eBioscience; CD4-FTTC, CD25-allophocycin and FoxP3-PE). Adhesion molecules were stained with mAbs against CD62L (MEL-14-FITC), CD162 (2PH1-PE), CD44 (IM7-FITC), CD49d (R1-2-PE), αβ (DATK32-PE), CD11a (2D7-FTTC; all obtained from BD Pharmingen). In certain experiments, cells were triple-stained with CD11a-FTTC, CD3-PE and B220-Pacific Blue (all obtained from BD Pharmingen). Chemokine receptors were analyzed with mAbs against CCR9 (clone 242503-allophycocyanin), CCR10 (clone 248918 + anti-rat IgG-FITC), and CXCXR4 (clone 247506-allophycocyanin; all obtained from R&D Systems) in three-color stainings with CD3-PE and B220-Pacific Blue. In all stainings, appropriate negative control mAbs were included.

Cells were analyzed using FACSCalibur and CellQuestPro software or with LSRII instrument (at 405, 488, and 635 nm lasers) using BDFACTS- Diva software. The percentage of positive cells and mean fluorescence intensities (MFIs) were calculated by subtracting the values obtained with negative control mAbs from the values obtained with the specific mAbs.

Immunohistochemistry

Expression of VAP-1 in frozen sections was analyzed using indirect immunoperoxidase staining with anti-VAP-1 (7-106) and negative control (9B5) mAbs, as described (13).

Determination of soluble Ig (sIg) levels

Concentrations of different Ig isotypes in the serum and intestine were determined using a sandwich ELISA. Blood was collected through cardiac puncture, and serum was separated after clotting and stored at −70°C. The sIgs were extracted as described (19). In certain experiments, serum was collected and stored at −70°C. sIg concentrations were analyzed with IgA, IgG, and IgM ELISA kits (Bethyl Laboratories) according to the manufacturer’s instructions. In brief, the 96-well plates were coated with the capturing anti-mouse IgA, IgG, or IgM Abs. After blocking, diluted specimens were incubated in the wells for 1 h. HRP-conjugated anti-mouse IgA, IgG, or IgM detection Ab was then added. The detection reactions were started by adding TMB and stopped using H2SO4. The absorbances at 450 nm were then measured using Multiscan microtiter plate reader. The Ig concentrations were determined on the basis of standard curves using four-parameter logistic curve-fit program. For the intestinal samples, the results were expressed as microgram of Ig per gram of intestinal content.

ELISPOT assay

Individual cells secreting IgA (so called spot-forming cells) were visualized using a modification of a previously described ELISPOT assay (20). In brief, flat-bottom 96-well microtiter plate wells were coated with rat anti-mouse IgA, IgM, or goat anti-mouse IgG (Southern Biotech) diluted 1/100, 1/200, or 1/1000, respectively. After a 2 h incubation at +37°C, the wells were blocked using PBS containing 1% BSA. Mouse splenocytes, bone marrow cells, and PP lymphocytes were then added at four serial dilutions (10^5 to 10^6 cells) and further incubated for 30 min at +37°C. Cells were then lysed by washing with PBS containing 0.05% Tween 20. Ig secreted during the incubation time were detected with alkaline-phosphase-conjugated goat anti-mouse IgA, IgG, or IgM (Southern Biotech) diluted 1/2000 in PBS using an overnight incubation at room temperature. Thereafter the substrate, 5-bromo-4-chloro-3-indolyl phosphate diluted in 2-amin-2-methyl-1-propanol buffer, was mixed with hot agar solution and overlaid to wells. The plates were incubated at room temperature for 1 day. The blue spots were microscopically read under a low magnification. Each of the four dilutions was analyzed in four replicate wells and the number of spot-forming cells was the average from all 16 wells.

In vivo homing assay

The homing assay was performed as previously described (21). In brief, spleens and lymph nodes were collected from WT and VAP-1−/− mice and homogenized to obtain single cell suspensions. After lysis of erythrocytes from splenic cell suspensions, WT cells were labeled for 20 min with 0.5 μM CFSE (Molecular Probes) and VAP-1−/− cells with 5 μM TRITC (tetramethylrhodamine-5-isothiocyanate; Molecular Probes) at 37°C. After washings, WT and VAP-1−/− cells were pooled at 1:1 ratio and 20 × 10^6 cells in RPMI 1640 were injected i.v. into age-matched VAP-1−/− mice (n = 9) and WT (n = 6) recipients. Cells were allowed to recirculate for 18 h. Thereafter, PP were harvested from recipients, lymphocyte suspensions were stained with Pacific Blue anti-mouse B220 and analyzed for the percentage of immigrated B cells (CFSE/TRITC−, B220+) using LSRII flow cytometry.

Oral OVA immunizations

Mice were immunized intragastrically three times at 1 wk intervals with 6 μg (1 mg/dose) and cholera toxin (10 μg/dose) as an adjuvant (22). One week after the last immunization, the mice were killed and cells were isolated from spleen and mesenteric lymph node and subjected to thymidine incorporation assay, as described (23). In brief, different concentrations of OVA or medium only were used to restimulate cells in vitro for 3 days in the presence of [3H]thymidine for the final 6 h. The incorporated radioactivity in the cells (three replicate wells from each animal) was measured with a beta-counter. A proliferation index was determined for each mouse by dividing the mean of OVA-induced thymidine incorporation count by that measured in the wells containing medium only.

The appearance of OVA-specific Abs was determined from serum samples as described (24). In brief, baseline serum samples were drawn before starting the immunizations, and samples from immunized mice were obtained at a 4 wk time point. Diluted serum samples were added into wells precoated with OVA, and the binding of OVA-specific Abs was detected using isotype-specific second-stage Abs and chemiluminescence. The specific relative light units were obtained by subtracting the counts from baseline samples from the postimmunization samples.

S. aureus infections

S. aureus (parental strain American Type Culture Collection 49525 isolated from a bacteremic patient) containing a stable copy of modified Photobacterium luminescens luxABCDDE operon was purchased from Xenogen. These S. aureus Xen 36 were grown in trypticase soy broth medium under kanamycin (200 μg/ml) selection until absorbance of 0.5 at 600 nm was reached. The bacteria were pelleted and diluted in PBS. Wild-type and VAP-1−/− 129S6 female mice, BALB/c, and C57BL/6 were injected with 1 × 10^6 CFU of the bacteria s.c. in a volume of 50 μl under isoflurane anesthesia. Immediately after the injection, the mice were imaged using IVIS50 bioluminescence workstation (Xenogen) and 1 min exposure time. Bioluminescence values were collected from the region of interest. The in vivo imaging procedure was then repeated at the indicated time points. The bioluminescence values obtained from bacteria correlate closely both to the OD (absorbance at 600 nm) and to viable counts (CFU/ml), and therefore represent a reliable measure of the bacterial numbers (25).
Coxsackie B4 infections

Coxsackie virus CBV4-E2 infection model was performed as described (26–28). Wild-type or VAP-1-deficient C57BL/6 female mice were injected i.p. with 1000 PFU in 100 μl of PBS. After 6 days, the mice were sacrificed and pancreata were collected, formalin-fixed and paraffin-embedded for sectioning and staining with H&E. The numbers of inflammatory leukocytes in pancreata was blindly scored using the following semiquantitative scale: 0 = no or very occasional (<5 cells/microscopic field) leukocytes; 1 = low to moderate numbers of infiltrating leukocytes; and 2 = high numbers of infiltrating cells. Generally, the inflammatory damage in the pancreatic cells correlated well to the extent of leukocytic infiltration.

Infection models in anti-VAP-1 mAb and SSAO inhibitor-treated mice

In the bacterial infection model, WT BALB/c mice (and WT C57BL/6) were treated with a combination of function blocking anti-VAP-1 mAbs and an SSAO inhibitor at the time point –2 h. Anti-mouse VAP-1 mAbs 7–106 and 7–88 were used as a pool at a saturating concentration determined in previous studies (100 μg of each mAb/animal; i.v.) (13). As a chemical SSAO inhibitor BTT-2052, a selective, hydrazine-based VAP-1 inhibitor was administered (1.5 mg/mouse, in PBS; i.p.) (12, 29). The negative control group was treated with mAb HB-151 against human HLA-ABC (200 μg/mouse, i.v.) and vehicle (PBS, i.p.). At time point 0 h, S. aureus bacteria were injected s.c. At time point 3 h the Ab and SSAO inhibitor treatments were repeated. The mice were used for bioluminescence imaging as described above. In the end of the experiment, serum and fat samples were collected for enzyme assays.

For Coxsackie B4 infection model, the animals were first infected with the Coxsackie virus exactly as described above. The group for neutralization of VAP-1 was then treated with daily i.p. injections with BTT-2052 (0.3 mg/mouse, in PBS), and anti-VAP-1 mAb 7–106 (180 μg/mouse) was given i.p. every second day. The control group received the control Ab HB-151 and vehicle at the same time points. On day 5, the pancreata were collected for histological analyses and fat and serum samples for SSAO activity measurements.

Enzymatic SSAO analyses

Adipose tissue was lysed in 0.1% Nonidet P-40 containing buffer, and the clarified supernatants were used for determination of SSAO activity using fluorescent assay as described (16, 29). In brief, samples were incubated with methyamine (an SSAO substrate) in the presence or absence of semicarbazide (an SSAO inhibitor), and the formation of hydrogen peroxide was determined using Amplex Red reagent. Specific SSAO activity was determined as picomoles of H2O2 formed per milligram of protein in 1 h. Protein concentrations were determined using DC-Protein assay (Bio-Rad).

Enzymatic SSAO activity measurements.

Comparisons between WT and VAP-1−/− mice were done using Mann-Whitney U test (FACS, Ig, oral immunizations, Coxsackie infections) and the kinetic curves (Staphylococcus infections) were analyzed using variance analysis of repeated measurements. Pancreatic inflammation (score 0 vs scores 1–2) were done using χ2 or Fisher’s exact test. Values of p < 0.05 were considered significant. Analyses were done using SAS Enterprise Guide 3.0 program. Unless otherwise mentioned, all data are given as mean ± SEM.

Results

VAP-1−/− mice have diminished numbers of lymphocytes in PP

To study the role of VAP-1 in homeostatic control of lymphoid organs, we analyzed the numbers of major lymphoid subclasses in lymphoid organs in VAP-1-deficient mice. Because the composition of lymphoid organs is heavily influenced by the age of the animals (30–32), we performed these analyses in three different age groups (Table I). These data revealed that there were no significant differences in the numbers of lymphocytes between WT and VAP-1−/− littermates in thymus, spleen, MLN, or PLN at any age. In contrast, in 6-wk-old animals the lymphocyte numbers in PP of the gut were reduced by >50% in VAP-1-deficient mice when compared with the WT littermates (Table I). There was also a significant difference in the numbers of lymphocytes per PP (0.46 ± 0.05 × 106 cells/PP in VAP-1-deficient mice and 0.96 ± 0.12 × 106 cells/PP in WT animals; p < 0.01). The numbers of PP in 6-wk-old mice were not significantly different between the genotypes (9.2 ± 0.4 in WT and 8.8 ± 0.2 in VAP-1−/−, n = 5). In 20- to 22-wk-old mice there was still a trend toward decreased cell numbers in PP in VAP-1-deficient mice, but the difference disappeared in old (1.5 year) mice. The different phenotypes seen at 6 wk vs the older age groups (20 wk, 1.5 year) were not caused by possible age-dependent differences in VAP-1 expression in WT mice, because semiquantitative immunohistochemistry showed identical levels of VAP-1 in vessels in all three age groups (n = 3 mice/group, data not shown).

The phenotype of PP lymphocytes was then analyzed in more detail. At 6 wk ~67% of the cells were B220-positive B cells in WT mice, whereas 62% of PP cells expressed this marker in VAP-1-deficient mice (p < 0.01; Table I). In line with this, the proportions of CD4 and CD8 T cells were somewhat higher among PP cells isolated from VAP-1−/− mice than in those from WT. A trend toward decreased numbers of B cells in PP was still seen in 20- to 22-wk-old VAP-1-deficient mice but not in the 1.5-year-old animals. The numbers of CD4+CD25+ (effector) T cells and CD4+CD25+FoxP3+ regulatory T cells were similar in both genotypes (Table II). Together these data show that the absence of VAP-1 leads to a reduction in the numbers of lymphocytes in the PP of young animals, and that the B cells appear to be affected more than T cells.

Homing receptor expression does not explain diminished cell numbers in VAP-1−/− PP

Altered lymphocyte composition in PP of VAP-1-deficient mice could be due to defective homing. Therefore we first studied the expression of certain homing receptors on lymphocytes of the VAP-1−/− mice (Table III). These analyses showed no significant changes in the levels of CD62L/leectin, CD162/P-selectin glycoprotein ligand 1, CD49dαL integrin, αβ integrin or CD44 in any lymphoid organ in either young or old VAP-1-deficient mice when compared with WT animals. However, a small, but statistically significant, decrease in the percentage of cells positive for CD11a/LFA-1 integrin was found in PP, MLN, and PLN of 6-wk-old VAP-1-deficient mice (Table III). The same reduction in LFA-1 expression was evident when analyzing the MFI values. Specific MFIs for CD11a in 6-wk-old WT and VAP-1−/− animals were 38.8 ± 0.2 and 33.7 ± 0.4 in PP, 50.4 ± 0.3 and 42.8 ± 0.3 in PLN, 51.9 ± 0.3 and 45.0 ± 0.7 in MLN and 49.9 ± 0.5 and 44.8 ± 0.5 in spleen, respectively (p < 0.05 for each comparison). Also when lymphocyte subpopulations were analyzed separately, 6-wk-old VAP-1-deficient mice had fewer CD11a positive B and T cells than WT mice. The decrease was 20.9 ± 2.7 and 23.6 ± 5.0% in PP CD3+ and B220+ cells, respectively (p < 0.03 for both), whereas no significant change was seen in PLN CD3+ or B220+ cells. Moreover, the expression level of CD11a (MFI) in B cells and T cells both in PP and PLN was diminished by 10–25% in the absence of VAP-1 (n = 4/group). Nevertheless, the slightly decreased expression of the nontissue selective adhesion molecule LFA-1 cannot explain the selective decrease in lymphocyte numbers in PP of VAP-1-deficient mice.

Altered chemokine receptor expression (especially that of CCR9, CCR10, and CXCR4) (33, 34) could lead to less effective homing and/or retention of lymphocytes in PP of VAP-1-deficient mice. However, we found no significant differences between 6-wk-old WT and VAP-1-deficient mice in the expression of these three
chemokine receptors on PP or PLN lymphocytes (Table IV). Finally, we performed short-term in vivo homing experiments to directly study the B cell immigration into PP. The results showed that B lymphocytes isolated from WT and VAP-1−/− mice homed equally well both into WT PP (0.22 ± 0.02 and 0.20 ± 0.03% immigrated B cells, respectively) and into VAP-1−/− PP (0.21 ± 0.03% and 0.18 ± 0.03% immigrated B cells, respectively; n = 6–9/group). These data strongly suggest that entry of B cells into PP is not altered in the absence of VAP-1, and therefore either B cell proliferation or retention is likely to account for diminished B cell numbers in VAP-1-deficient mice.

Serum IgA level is decreased in VAP-1-deficient mice

To analyze whether altered B cell numbers in VAP-1−/− mice have functional consequences for Ig synthesis, we studied the levels of various Ig subclasses in the serum and intestine of these animals. The concentration of serum IgA in VAP-1-deficient mice (pooling all age groups) was, on average, only 75.6 ± 7.9% of that in WT mice (p = 0.02). In all age groups, the serum IgA levels in VAP-1-deficient mice showed a decreasing trend when compared with those in WT mice (slgA was 15, 10, 46, and 27% lower in 6, 8, 16- to 20-wk-old, and 1.5-year-old VAP-1-deficient mice, respectively) (Fig. 1A). However, the difference reached statistical significance only in 16- to 20-wk-old mice. In the intestine, no significant difference in slgA levels was found between VAP-1-deficient and control mice (Fig. 1A). There were also no consistent differences in the levels of other Ig subclasses either in the serum or intestine (Fig. 1, B and C).

ELISPOT assays were then performed to study the capacity of B cells to produce Igs at a single cell level. We found that the number of IgA-secreting cells in spleen (8-wk-old), bone marrow or PP (6-wk-old) was not statistically significantly different between the genotypes (Fig. 2). The numbers of cells positive for cell surface immunoglobulins (IgA, IgM, and IgG) in spleen, PP, PLN, and MLN were also similar in both genotypes (data not shown). Also the expression of syndecan, a marker for plasma cells, in 8-wk-old mice, was comparable (19.9 ± 0.6% and 18.9 ± 1.0% in spleen of 8-wk-old WT and VAP-1−/− mice, respectively). Together these analyses thus show that the slightly lower concentrations of serum IgA levels in VAP-1-deficient mice are likely not due to differences in Ig synthesis and, therefore, suggest that IgA is possibly cleared from the circulation faster in the absence of VAP-1.

VAP-1-deficient mice show impaired immune responses after oral vaccination

To analyze the functional consequences of diminished lymphocyte numbers in PP of VAP-1-deficient mice further, we immunized 6-wk-old mice perorally with OVA and cholera toxin. After 4 wk, OVA-specific T cell proliferation was decreased up to 50% in MLN (p < 0.05) and up to 35% in spleen (p < 0.05) in the VAP-1-deficient mice when compared with WT mice (Fig. 3, A and B). The OVA-specific B cell responses also appeared attenuated in the absence of VAP-1 (Fig. 3C), although oral immunization induced variable responses also in WT mice (each of which showed good T cell responses) and generated measurable OVA-specific Abs of IgG class only. Nevertheless, these data show that
VAP-1 deficiency clearly impairs mucosal immune responses after oral immunization.

Deletion of VAP-1 impairs control of Staphylococcus aureus infection

To study the role of VAP-1 in control of microbial infections, we inoculated S. aureus bacteria subcutaneously into the paws of VAP-1-deficient and WT mice. These bacteria express an operon that constitutively produces light at 37°C allowing real-time monitoring of the numbers of bacteria using bioluminescent imaging (Fig. 4A). Kinetic analyses showed that S. aureus bacteria proliferate more vigorously during the early phases of infection in VAP-1-deficient mice than in WT animals (Fig. 4B). The enhanced growth of bacteria in VAP-1−/− mice reached statistical significance at 5.5 and 11 h time points. In the subacute phase of the infection (from 20 h onward up to 8 days) the numbers of bacteria were comparable in both genotypes. These data thus show that VAP-1-deficient mice have a transient defect in controlling the replication of S. aureus in skin.

Aggravated inflammation in the Coxsackie B4 virus infection in the absence of VAP-1

We next determined whether absence of VAP-1 also alters the course of viral infections. We chose a model of Coxsackie B4 infection.
infection that causes pancreatitis upon i.p. infection (Fig. 5, A–C).

Histological analyses revealed that Coxsackie B4 inoculation caused more severe inflammation in the pancreata of VAP-1-deficient animals than in their WT controls (Fig. 5D). Notably, when the pancreata were divided into healthy (score 0) and inflamed (scores 1 and 2), there were more inflammatory cells in the VAP-1-deficient mice than in WT controls (\( \chi^2 \) test, \( p < 0.05 \)). Hence VAP-1 mice show mild defects in controlling Coxsackie B4 infection.

Therapeutic blocking of VAP-1 does not impair antimicrobial control

VAP-1 is being targeted in preclinical and clinical trials for developing new anti-inflammatory drugs (17, 18). Therefore, the impaired control of bacterial and viral infections in the VAP-1-deficient mice raises concerns about the safety of this approach. To mimic a scenario where a patient under anti-VAP-1 therapy contracts an infection, we applied our two infection models to mice that were concurrently treated with a saturating dose of function blocking anti-VAP-1 mAb and a small molecule inhibitor of VAP-1 enzyme activity.

When WT BALB/c mice expressing endogenous VAP-1 were treated with the combination of anti-VAP-1 mAb and BTT-2052 (an SSAO inhibitor) or with a negative control mAb and vehicle, and then infected with \( S. aureus \), there was no significant difference in the multiplication of the bacteria (Fig. 6A). The efficacy of VAP-1 blocking was shown by demonstrating a nearly complete inhibition of SSAO activity in the serum and in fat lysates in the groups that got BTT-2052 (Fig. 6B). To control for potential host strain differences, the same experiment was repeated in C57BL/6 mice with similar results (data not shown).

Finally, we applied Coxsackie B4 virus model to mice treated with anti-VAP-1 blocking reagents. In this model, there was only minimally more non-inflamed specimens in the control group than
in the VAP-1 targeted group (Fig. 6C), which almost completely lacked VAP-1/SSAO activity (Fig. 6D). Moreover, when the samples were divided into healthy (score 0) and inflamed (scores 1 and 2) there was no difference between the two genotypes (Fisher’s exact test, $p = 0.6$). Collectively, these infection models suggest that acute blockade of VAP-1 through combined anti-VAP-1 and SSAO inhibitor treatment does not aggravate the course of concomitant microbial infection, at least with these model microbes.

**Discussion**

We have shown here that VAP-1-deficient animals have fewer lymphocytes, and B cells in particular, than WT controls in PP at a young age. VAP-1-deficient mice show diminished levels of serum IgA. Moreover, T and B cell dependent immune responses upon oral vaccination are diminished in the absence of VAP-1. The ability of VAP-1-deficient mice to control bacterial and viral infections is also compromised. However, when the function of VAP-1 is acutely neutralized by function blocking anti-VAP-1 mAbs and SSAO enzyme inhibitors, the course of *S. aureus* and Coxsackie B4 infections are comparable to those in control animals. These data suggest that therapeutic blocking of VAP-1 for antiadhesive purposes should not cause a generalized impairment in control of microbial infections.

VAP-1-deficient animals lack completely VAP-1 protein and all detectable SSAO activity (16). So far only two reports using these gene-modified animals have been published. These studies showed that deletion of VAP-1 results in defective rolling, firm adhesion, and transmigration of granulocytes and lymphocytes at sites of inflammation. The homing of lymphocytes to spleen and MLN was also reduced, and there was a trend of impaired homing to PP and PLN in the absence of VAP-1 (16). Lack of VAP-1 also alleviates inflammation in chemically induced peritonitis and in anti-collagen Ab-induced arthritis (16, 29).

Although not evident in a previous study (16), our data now imply that lack of VAP-1 has mild but significant consequences on the numbers and phenotypes of lymphocytes in PP under normal conditions. However, acute blockade of VAP-1 through combined anti-VAP-1 and SSAO inhibitor treatment did not potentiate S. aureus or Coxsackie B4 infections, suggesting that therapeutic blocking of VAP-1 for antiadhesive purposes should not cause a generalized impairment in control of microbial infections.

**FIGURE 5.** Coxsackie B4 virus causes aggravated pancreatitis in VAP-1-deficient mice. Mice were infected i.p. with Coxsackie B4 virus and the extent of inflammation in pancreas was analyzed 6 days later. A–C. Representative sections from pancreata showing no inflammation (score 0), moderate inflammation (score 1), and strong inflammation (score 2). Original magnification, $\times 400$. D. The percentage of infected mice falling into each category of inflammation is shown. $n = 17$ for WT mice and $n = 16$ for VAP-1$^{-/-}$ mice.

**FIGURE 6.** Acute inhibition of VAP-1 by anti-VAP-1 mAbs and enzyme inhibitors does not potentiate microbial infections. A. Kinetic analyses of *S. aureus* growth were performed as detailed in Fig. 4 in WT animals treated with a function blocking anti-VAP-1 mAb and an SSAO inhibitor BTT2052 (VAP-1 inh) or with a negative control Ab and vehicle (control). $n = 10$ mice/group. B. The SSAO activity (mean $\pm$ SEM) in the serum and fat of all *S. aureus* infected animals from both groups was determined using enzymatic assays. C. The extent of tissue inflammation after Coxsackie B4 infection was determined in WT animals treated with the function blocking anti-VAP-1 mAb and an SSAO inhibitor BBT2052 or with a negative control Ab and vehicle (as detailed in Fig. 4). $n = 8$ mice/group. D. The SSAO activity (mean $\pm$ SEM) in the serum and fat of all Coxsackie B4-infected mice from both groups were determined using enzymatic assays.
conditions. The apparent discrepancy between the results is explained by the age of mice used for the experiments. In the previous report age-matched WT and VAP-1-deficient mice were used, but all animals were >16 wk old. Now we have analyzed the cell numbers and phenotypic differences in a more detailed manner in different age groups and observe significant differences only in the young mice.

The diminished lymphocyte numbers and alterations in the proportion of different subsets in young VAP-1-deficient mice is likely not due to differences in the homing efficiency. This conclusion is based on similar expression of gut-homing receptors and gut-selective chemokine receptors and similar homing efficiency in vivo assays in both genotypes. These data thus indirectly suggest that the differences are due to lymphocyte proliferation or retention in the tissue. Although VAP-1 is absent from leukocytes (3), the reaction products generated from trace amines during VAP-1/SSAO catalytic activity (aldehydes, ammonium, and hydrogen peroxide) might affect other cell types in a paracrine fashion. Hydrogen peroxide, in particular, is known to be a powerful signaling molecule at low concentrations (6, 7). For instance, it directly modulates B cell receptor signaling by inhibiting phosphatases, which then are less able to negatively regulate BCR signaling (35). Because hydrogen peroxide has a short half-life of ~1 ms in the cells and it is freely diffusible across cell membranes, it would only act locally in the vicinity of VAP-1 catalytic activity. Interestingly, catalytic activity of VAP-1 has very recently been shown to induce the expression of endothelial adhesion molecules, such as P- and E-selectin and ICAM, and a chemokine CXCL8, by stimulating PK3, MAPK, and NF-kB pathways (36, 37). These or other molecules regulated by VAP-1 activity in a paracrine fashion might therefore tune mechanisms in VAP-1 negative cell types that are involved in proliferation and/or retention of cells in PP.

Reduced T cell proliferation in response to oral immunization was observed in VAP-1-deficient mice. This could be caused by impaired homing of naive lymphocytes to PP (leading to aberrant ratio of incoming and resident cells) during ongoing immune response. Alternatively, migration of effector lymphocytes into MLN and spleen or their retention and/or proliferation may be defective in the absence of adhesive and/or signaling functions of VAP-1 (see above).

VAP-1-deficient mice do not suffer from any spontaneous infections. Nevertheless, upon infection with either S. aureus or Coxsackie B4 we saw a slight impairment in controlling the infection in absence of VAP-1. The redundancy of the vital leukocyte extravasation cascade apparently explains the lack of more severe outcome in the absence of one adhesion molecule. In an overt microbial infection it is likely that many inflammatory pathways become maximally activated and thus induce the expression and function of all molecules involved in leukocyte influx into the site of insult. The lack of other adhesion molecules, like any of the three selectins or ICAMs has not led to the increased susceptibility to opportunistic infections either. Only when multiple molecules are simultaneously targeted, e.g., both endothelial selectins, opportunistic infections are seen (38). Moreover, mice lacking other endothelial adhesion molecules often show stimulus-dependent changes in responses upon infectious challenge. P-selectin-deficient mice, for instance, show no impairment in handling of Listeria monocytogenes, Mycobacterium bovis, Leishmania major, Borrelia burgdorferi or lymphocytic choriomeningitis virus, whereas they suffer from more severe Streptococcus pneumoniae, Porphyromonas gingivalis, andecal flora infections, but from less severe S. aureus-induced arthritis and Plasmodium berghei infections than WT animals (39–48). Notably, in microbial diseases, which are not dependent on either P- or E-selectin or ICAM-1 (46), VAP-1 might contribute to the formation of normal leukocyte infiltrates.

The function of VAP-1 can also be blocked by anti-VAP-1 mAbs or SSAO inhibitors. These reagents have also proved useful in controlling multiple inflammatory reactions (such as peritonitis, arthritis, ischemia-reperfusion injury, lung inflammation, septic shock, and organ transplantation) in in vivo mice models (8–15). Therefore, anti-VAP-1 therapy can be envisioned as a potential new form of anti-inflammatory medication. In this context, the more severe infections seen in the VAP-1-deficient mice raises concerns about the safety of VAP-1 targeting. This issue has become even more critical after the findings of serious JC-virus infections in patients treated with another adhesion molecule antagonist against α4 integrin either alone or in combination with other immunomodulatory agents (49). Therefore, it was of crucial importance to compare the effect of acute VAP-1 blockade to that of genetic deletion on the control of infectious diseases. We found that administration of function blocking anti-VAP-1 mAbs and small molecule SSAO inhibitors simultaneously did not lead to any exacerbation of infections when compared with WT animals. The difference in control of infections after genetic and therapeutic blockade of VAP-1 can have many explanations. For example, in VAP-1-deficient animals, life-long absence of VAP-1 may have led, through compensatory mechanisms, to slow alterations in other cellular or humoral components of the immune system that impair antimicrobial defense. Moreover, there is still residual VAP-1/SSAO activity left (5–10%) in pharmacologically treated animals (there is virtually no SSAO activity in VAP-1-deficient mice) that might be sufficient to trigger some critical VAP-1 dependent antimicrobial responses. Finally, because no existing SSAO inhibitor is absolutely specific for VAP-1, they may theoretically also cause non-VAP-1 specific changes (in the host or even in microbes) that alter the course of infections. In any case, these data suggest that short-term neutralization of VAP-1 using protocols that show clear beneficial anti-inflammatory responses in experimental inflammations do not compromise the ability of the host to defend against microbial infections. However, the role of any adhesion molecule in antimicrobial control is likely to be stimulus- and site-dependent, and therefore it should be emphasized that the current results can only be used to infer that neutralization of VAP-1 does not lead to a greater increase in the susceptibility to infections.

Acknowledgments

We thank biostatistician Tero Wahlberg for help with the statistical analyses. Raisa Turja, Mari Mäkinen, Etti-Liisa Viinänen, Pirjo Heiniälä, and Minna Santanen are acknowledged for technical assistance and Anne Sovikoski-Georgieva for secretarial help.

Disclosures

S.J. has stock ownership exceeding 5000 USD in Biotic Therapies Limited.

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