

Characterization of mac25/angiomodulin expression by high endothelial venule cells in lymphoid tissues and its identification as an inducible marker for activated endothelial cells

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Abstract

Previous results have indicated that mac25/angiomodulin (AGM) is expressed in lymph node (LN) high endothelial venules (HEV), the specialized venules that support efficient lymphocyte transendothelial migration. How mac25/AGM's endothelial expression pattern is regulated *in situ* remains unknown. Here, we demonstrate that in mouse LN blood vessels, including HEV, mac25/AGM is localized, unlike previous reports, not to the luminal or lateral regions bordering the endothelial cells (EC), but exclusively to the basal lamina that is in direct association with EC. In the spleen, mac25/AGM was expressed in the vascular basal lamina, in direct association with smooth muscle cells and pericytes, but not with EC. In addition, we report herein that mac25/AGM is an inducible marker for activated EC. In inflamed tissues, mac25/AGM expression was strongly induced in the abluminal region of blood vessels. *In vitro*, mac25/AGM was readily induced in EC upon activation with pro-inflammatory cytokines such as tumor necrosis factor- α , indicating that mac25/AGM is an activated EC marker. mac25/AGM binds vascular endothelial growth factor and, together with its strict abluminal localization, it is suggested that mac25/AGM has a specific function(s) in the subendothelium of activated blood vessels such as capturing humoral factors produced in the vicinity of HEV.

Introduction

The endothelium serves as a critical interface between the blood and the tissue parenchyma, regulating a number of processes, such as vascular permeability, blood flow, thrombogenesis, hematogenous tumor metastasis and leukocyte extravasation (1). Although various vascular endothelial cells (EC) share certain functions, there exists a considerable diversity of specialized functions that depend on the types of EC present and the properties of the underlying tissue. Nevertheless, the molecular mechanisms regulating the functional diversity of EC remain largely unknown (1,2).

One specialized type of microvasculature that has received much attention is the high endothelial venules (HEV) of the secondary lymphoid organs. The HEV, which are lined by morphologically and functionally distinctive EC, serve as the entrance for circulating lymphocytes into the parenchyma of the secondary lymphoid organs, such as the lymph nodes (LN), tonsils and Peyer's patches (PP), allowing lymphocytes to migrate and respond to antigens within the organ. The HEV mediate a highly selective lymphocyte recruitment process, termed lymphocyte homing, that involves a cascade of

specific steps: lymphocyte tethering, rolling and firm adhesion to the luminal surface, and, finally, transendothelial migration across the HEV (3,4). While there has been a great deal of progress in elucidating the adhesion molecules and chemokines involved in the cascade reactions, much remains unknown about the mechanisms by which the specific phenotype and functional properties of HEV are maintained. To investigate these issues, we have constructed a 3'-directed cDNA library from the mouse LN HEV cells expressing the peripheral lymph node addressins (PNA_d) or mucosal addressin cell adhesion molecule-1 (MAdCAM-1), and reported expression profiles of the active genes in HEV (5,6). Among the highly expressed genes in HEV, a mouse homologue of mac25/insulin-like growth factor binding protein-related protein 1 (IGFBP-rP1) (7)/angiomodulin (AGM) (8)/prostacyclin stimulating factor (PSF) (9) has been identified.

The cDNA encoding mac25/AGM was first cloned from human brain leptomenigeal cells (10). Essentially the same cDNA has been cloned from human fibroblasts as PSF, which stimulates prostacyclin (PGI₂) production in vascular EC (9,11). PGI₂ is a potent vasodilator and an inhibitor of platelet adhesion and aggregation, contributing to the vascular homeostasis (1,11). Human mac25/AGM was also isolated from the conditioned medium of a human bladder carcinoma cell line and found to be a secretory glycoprotein of ~30 kDa (12). This protein has the ability to regulate cell adhesion to and spreading on plastic plates and various extracellular matrix (ECM) components, and hence has also been designated as the tumor-derived adhesion factor [TAF (8); later renamed as AGM (13)]. Interestingly, mac25/AGM is also expressed in tumor vessels (8,14). These observations collectively indicate that mac25/AGM has diverse functions, while its precise role in individual tissues remains unknown.

In the present study, to gain further insight into the functional properties of mac25/AGM, we generated a biologically active recombinant mouse mac25/AGM using the baculovirus-mediated expression system, raised a specific polyclonal antibody (pAb) against it, and performed immunohistochemistry using both light and electron microscopy. We found that mac25/AGM localizes strictly to the basal side of small blood vessels including the HEV in mouse lymphoid tissues, in sharp contrast to the luminal and lateral expression of mac25/AGM in the HEV of human tonsils (15). We have also demonstrated that the endothelial expression of mac25/AGM is induced *in vivo* in inflamed tissues and *in vitro* by a pro-inflammatory cytokine tumor necrosis factor (TNF)- α . Hence, an apparently multifunctional protein mac25/AGM is an inducible marker for activated EC. In addition, mac25/AGM can bind vascular endothelial growth factor (VEGF) and, together with its strict abluminal localization, it is implied to function in the sub-endothelium of activated blood vessels by capturing humoral factors produced in the vicinity of HEV.

Methods

Animals

All animal experiments were performed under an experimental protocol approved by the Ethics Review Committee for Animal

Experimentation of Osaka University Graduate School of Medicine. C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan) and used at 10–12 weeks old. BALB/c mice were purchased from Clea Japan (Tokyo, Japan) and used at 9–12 weeks old. New Zealand White female 3-month-old rabbits were purchased from Kiwa Animal (Wakayama, Japan).

Reagents

Recombinant mouse VEGF₁₆₄, TNF- α , IFN- γ , IL-1 β and IL-6 were purchased from R & D Systems (Minneapolis, MN). Other biochemical reagents were purchased from Sigma-Aldrich (St Louis, MO), unless otherwise specified. The following antibodies were purchased: horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H + L) (American Qualex, San Clemente, CA), rhodamine-conjugated donkey anti-rabbit IgG (Chemicon, Temecula, CA), anti-mouse collagen IV pAb (Dako, Tokyo, Japan), FITC-conjugated and biotin-conjugated goat anti-rat IgG + M (Southern Biotechnology Associates, Birmingham, AL), FITC-conjugated mAb for mouse CD31 (PECAM-1, clone MEC13.3; BD PharMingen, San Diego, CA), mouse CD106 (VCAM-1, clone 429; BD PharMingen), mouse CD4 (clone RM4-5; BD PharMingen), anti-polyhistidine-, anti-myc-epitope (C-term) mAb (Invitrogen, Carlsbad, CA), and anti-mouse VEGF mAb (PeproTech, Rocky Hill, NJ). The MECA-79 mAb (16) was kindly provided by Dr E. C. Butcher (Stanford University, CA). Biotin or FITC conjugation of antibodies was performed using standard procedures.

Cell culture

The mouse brain EC line bEnd.3 (17) (obtained from ATCC, Rockville, MD; CRL-2299) was maintained in DMEM (Sigma-Aldrich) supplemented with 1 mM sodium pyruvate, 50 μ M 2-mercaptoethanol, 10 mM HEPES, 10 mM NaHCO₃, 2 mM L-glutamine, 1% (v/v) 100 \times non-essential amino acids (Gibco/BRL, Grand Island, NY), 100 U/ml penicillin, 100 μ g/ml streptomycin (Gibco/BRL) and 10% heat-inactivated FCS (Dainippon Pharmaceutical, Osaka, Japan). Primary calf pulmonary artery EC (CPAEC; Dainippon Pharmaceutical) were subcultured as described previously (18). The EC were passaged every 3–4 days and used at passages 3–8. Sf9 insect cells (Invitrogen) were maintained at 27°C in Grace's insect medium (Invitrogen). High Five cells (Invitrogen) were maintained at 27°C in EX-CELL 400 medium (JRH BioScience, Lenexa, KS). The culture medium for the insect cells was supplemented with 10% FCS, 100 U/ml penicillin and 100 μ g/ml streptomycin.

Preparation of recombinant mouse mac25/AGM and generation of pAb against mac25/AGM

The full-length mac25/AGM cDNA (GenBank AB012886) in the plasmid pBluescriptSKII-mac25 (19), kindly provided by Dr Y. Hayashizaki (RIKEN, Tsukuba, Japan), was excised out with *BsgI* and *XcmI*, and inserted into the corresponding multiple cloning site of the plasmid pcDNA3.1/Myc-His(+) (Invitrogen). Using the resulting plasmid as a template, the *SpeI*-*XhoI* fragment containing the mac25/AGM cDNA, polyhistidine and myc epitopes was generated by PCR with the primers, 5'-GGACTAGTTAAGCTTATGATGGAGCGGC-3' and 5'-CCGCTCGAGGCTGATCAGCGGGTTAACTCAATG-3', followed

by digestion with *SpeI* and *XhoI*. The resulting fragment was subcloned into the corresponding multiple cloning site of the pFastBac1 baculovirus transfer vector (Invitrogen). For large-scale production of the recombinant mac25/AGM protein, the Bac-to-Bac baculovirus expression system (Invitrogen) was used according to the manufacturer's instructions. The culture supernatant of the infected High Five cells was collected after 36 h of infection, and the secreted recombinant mac25/AGM with a molecular size of ~34 kDa was purified to homogeneity (Fig. 1A) by affinity chromatography with the Ni²⁺-based Probond resin (Invitrogen) and gel filtration chromatography (Sephadex-G200; Amersham, Little Chalfont, UK), and finally concentrated by ultrafiltration (CentriPlus YM-10; Millipore, Bedford, MA). The N-terminal sequence of the purified protein (Ser-Ser-Ser-Asp-Ala-Cys-Gly-Pro-Cys-Val) was found to be identical to that of mature mouse mac25/AGM (19), indicating that it was a mature full-length form of mac25/AGM. Addition of the recombinant mac25/AGM to cultured EC (CPAEC) resulted in the generation of a stable metabolite of PGI₂, 6-keto-PGF₁α, in a dose-dependent manner as has been reported previously (11) (Fig. 1B), indicating that the recombinant mac25/AGM thus obtained was biologically active.

pAb was raised against the recombinant mac25/AGM protein by subcutaneous immunization of New Zealand White female rabbits with the purified recombinant mac25/AGM (0.2 mg) emulsified in complete (for the initial injection) or incomplete (for the booster injection) Freund's adjuvant. The polyclonal IgG was affinity purified from the immunized serum using a Protein G-Sepharose CL-4B (Amersham BioSciences) column and a recombinant mac25/AGM-conjugated column. The affinity-purified pAb as well as a commercially available anti-polyhistidine mAb successfully detected the myc- and polyhistidine-tagged 34-kDa recombinant mac25/AGM protein on Western blots (Fig. 1C, lanes 1 and 2). It should be noted that the anti-mac25/AGM pAb specifically recognized the endogenous mac25/AGM produced by bEnd.3 cells (Fig. 1C, lane 3). Concerning the cross-reactivity of the pAb, we failed to detect its reactivity against human mac25/AGM that has high sequence homology (87%) to mouse mac25/AGM (data not shown). Furthermore, this antibody failed to react other myc-, His-tagged recombinant proteins such as L-selectin that we generated (data not shown), further verifying its specificity.

Immunohistochemistry

Immunostaining of frozen sections was performed as described previously (6). Briefly, cryosections (8 μm) were fixed in acetone and then in 4% paraformaldehyde (PFA)/PBS. The tissue sections were first incubated with the anti-mac25/AGM pAb (1 μg/ml), followed by rhodamine-conjugated goat anti-rabbit IgG (H + L) (1:200) and secondly incubated with FITC-conjugated MECA-79 mAb (2 μg/ml) or FITC-conjugated anti-mouse CD31 mAb (2 μg/ml). In some experiments, the tissue sections were first incubated with biotinylated anti-mac25/AGM pAb (1 μg/ml), followed by rhodamine-streptavidin (5 μg/ml; PharMingen), and then incubated with FITC-conjugated anti-mouse collagen IV pAb (1 μg/ml). For peroxidase detection, the tissue sections were first incubated with the anti-mac25/AGM pAb (1 μg/ml), followed by HRP-conjugated anti-rabbit IgG (H + L) (1 μg/ml) and developed

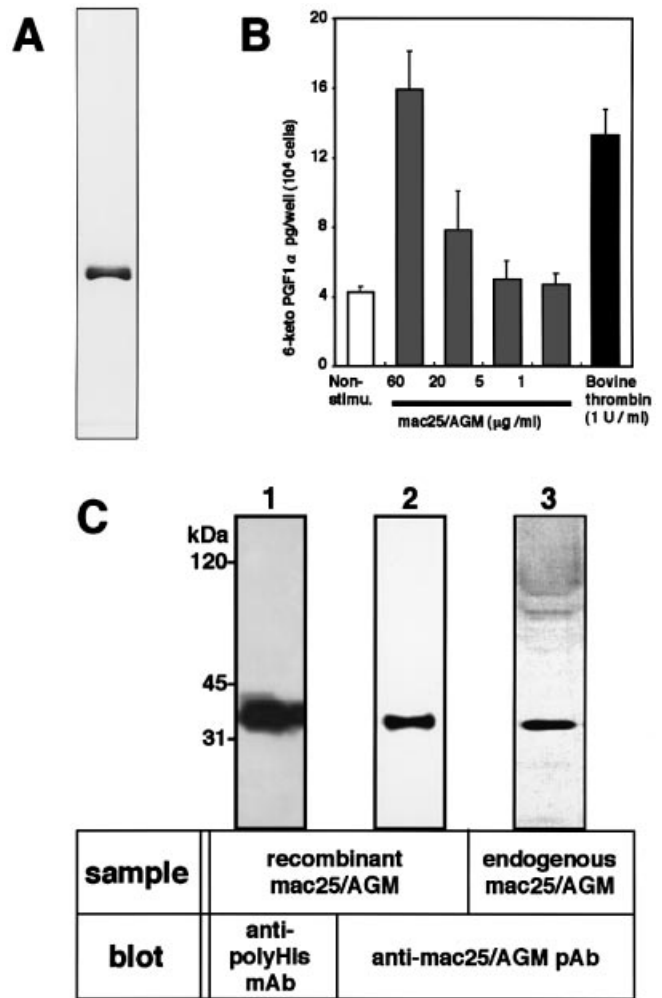


Fig. 1. Production of recombinant mouse mac25/AGM and generation of anti-mac25/AGM pAb. (A) Affinity purification of the recombinant mac25/AGM. A myc- and polyhistidine-tagged mac25/AGM was generated by the baculovirus-mediated protein expression system and purified using a Ni²⁺-chelated affinity column to homogeneity, which was confirmed by SDS-PAGE with Coomassie brilliant blue staining. (B) The bioactivity of the recombinant mac25/AGM as PSF. PGI₂ production in CPAEC upon mac25/AGM stimulation was measured by competitive ELISA to detect its stable metabolite, 6-keto-PGF₁α. mac25/AGM (1–60 μg/ml, for 1 h) increased PGI₂ synthesis in a dose-dependent manner (*n* = 6 in each group). Bovine thrombin was used as positive control for PGI₂ induction. Data obtained are the mean ± SD of three independent experiments. (C) Western blots of the mac25/AGM protein using various antibodies. The recombinant mac25/AGM (lanes 1 and 2) and the culture supernatant of bEnd.3 cells (lane 3) were subjected to SDS-PAGE, and electroblotted onto PVDF membranes. The blots were incubated with antibodies as indicated.

with an Immunopure Metal Enhanced DAB Substrate kit (Pierce, Rockford, IL). In each experiment, a negative control study was done by omitting the primary antibody or by using an irrelevant isotype- and concentration-matched primary antibody. To confirm the specificity of the anti-mac25/AGM pAb, a 20-fold molar excess of the recombinant mac25/AGM was added.

Immunoelectron microscopy

Mice were perfused with a 4% PFA and 0.1% glutaraldehyde mixture in 0.1 M cacodylate buffer (pH 7.4) containing 5% sucrose. LN, PP and spleens were removed and further fixed in the same fixative at 4°C for 4 h, and then immersed in 10, 15 and finally 20% sucrose solutions. The tissues were then cut into 50- μ m slices by a cryostat. Endogenous peroxidase in the slices was blocked by incubating them in a 0.5% sodium metaperiodate solution. The slices were then incubated with rabbit anti-mac25/AGM pAb (1 μ g/ml) for 48 h at 4°C, followed by incubation with HRP-goat anti-rabbit IgG (H + L) (1 μ g/ml). They were then fixed with 2% glutaraldehyde for 10 min and incubated in a diaminobenzidine solution containing H₂O₂ (DAB Reagent Set; KPL, Gaithersburg, MD). The DAB-reacted slices were post-fixed with 2% osmium tetroxide for 1 h and embedded in Epon 812 after dehydration in graded acetone solutions. Ultrathin sections were cut with an ultramicrotome (Ultracut, Reichert-Jung, Germany) at a thickness of ~100 nm. The sections were left unstained or stained with uranyl acetate and examined with a transmission electron microscope (200CX; Jeol, Tokyo, Japan).

Inflammation model

A chronic inflammation model with contact hypersensitivity was made as described previously (20). In brief, BALB/c mice were sensitized on the ear skin with 0.5% dinitrofluorobenzene (DNFB) and challenged repeatedly by applications of 0.2% DNFB on the same site every other day. Ear skin specimens were taken 48 h after the second challenge and examined by immunohistochemistry. Two independent experiments with three mice in each group were performed.

In vitro induction of mac25/AGM upon cytokine treatment

Quiescent bEnd.3 cells were treated with various cytokines for the indicated periods, then the conditioned medium was collected, concentrated by ultracentrifugation, subjected to SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). The blot was probed with anti-mac25/AGM pAb (0.5 μ g/ml), followed by HRP-goat anti-rabbit IgG (H + L) (0.5 μ g/ml). The blot was then developed with the ECL Western blotting detection reagents (Amersham). Densitometry was performed using NIH Image analysis software. For flow cytometry, cells were incubated with FITC-conjugated anti-VCAM-1 mAb (5 μ g/ml) and analyzed by an Epics-XL flow cytometer (Coulter, Hialeah, FL). Expression of VCAM-1 was assessed at 4 and 24 h after TNF- α stimulation. For RT-PCR, the total RNA from bEnd.3 cells was reverse transcribed with an oligo-dT₁₈ primer. A specific oligonucleotide sense primer 5'-ATCACTCTGGAGTTCAGC-3' and an antisense primer 5'-AAGAGAAGTGTGTCAGGC-3' were used for mac25/AGM, and 5'-ATGGATGACGATATCGCT-3' and 5'-ATGAGGTAGTCTGTGTCAGGT-3' were used for β -actin (5).

mac25/AGM binding to VEGF

For the detection of the interaction between mac25/AGM and VEGF, the recombinant mac25/AGM (1 μ g/ml) was immobilized onto 96-well microtiter plates (Sumilon H; Sumitomo Bakelite, Tokyo, Japan) at 4°C (50 μ l/well). The wells were blocked with 3% BSA and incubated with varying concentra-

tions of VEGF. VEGF bound to mac25/AGM was detected with anti-VEGF mAb and HRP-conjugated anti-rabbit IgG. To quantify the reaction, o-phenylenediamine was added and the optical density at 490 nm was read in a microtiter plate reader (InterMed, Tokyo, Japan). As control experiments, BSA (1 μ g/ml) or recombinant myc-, His-tagged human L-selectin (1 μ g/ml) (6) was immobilized instead of the recombinant mac25/AGM.

Results*mac25/AGM is constitutively expressed in the abluminal region of LN HEV*

We carried out immunohistochemical analyses of mouse LN, PP and spleen using the anti-mac25/AGM pAb. In accordance with previous reports (5,6,15), strong expression of mac25/AGM was observed in the HEV of all the peripheral LN tested, while moderate to weak expression was also observed in non-HEV-type blood vessels of mesenteric LN (Fig. 2A and B). Double immunofluorescence staining with a pan-EC marker anti-CD31 (PECAM-1) and the anti-mac25/AGM pAb confirmed that the majority of the blood vessels in LN were positive for mac25/AGM expression (Fig. 2C–E). Strong expression of mac25/AGM by HEV was also observed in the cervical and axillary LN as well as in the PP (data not shown).

While the above results are apparently concordant with those reported by Girard *et al.* (15), a closer investigation revealed that the pattern of expression of mac25/AGM in mouse HEV is quite different from that reported previously, i.e. mac25/AGM preferentially accumulated on the abluminal side of these blood vessels. Double immunofluorescence staining using the anti-mac25/AGM pAb and the MECA-79 mAb, which specifically reacts with PNAd (16), showed that the mac25/AGM-specific immunoreactivity was preferentially associated with the abluminal region of the HEV (Fig. 2G), whereas MECA-79's immunoreactive product was seen throughout the entire cell body of the HEV cells (Fig. 2F). A merged view of the mac25/AGM and MECA-79 staining clearly illustrates the abluminal accumulation of mac25/AGM in the HEV (Fig. 2H). In addition, two-color immunostaining with the anti-mac25/AGM pAb and an antibody against collagen type IV showed the co-localization of mac25/AGM and collagen IV, which is also present on the abluminal side of HEV (Fig. 2I–K). In all the staining experiments described above, an irrelevant primary antibody or secondary antibody alone gave no positive signal (data not shown), verifying the specificity of the observations obtained above.

To confirm the abluminal pattern of mac25/AGM expression in mouse HEV, we then performed immunoelectron microscopic analysis using the anti-mac25/AGM pAb. As shown in Fig. 3(A and B), the immunoreactive mac25/AGM densely accumulated in the basal lamina just beneath the HEV cells of peripheral LN and was uniformly distributed throughout the basal surface, but almost excluded from the luminal surface of the HEV. A similar staining pattern was observed in PP (Fig. 3C and D). In contrast, in the spleen, where no HEV are seen, mac25/AGM was specifically localized to the basal lamina just beneath the smooth muscle cells of the blood vessels and no positive signal was associated with the vascular EC (Fig. 3E

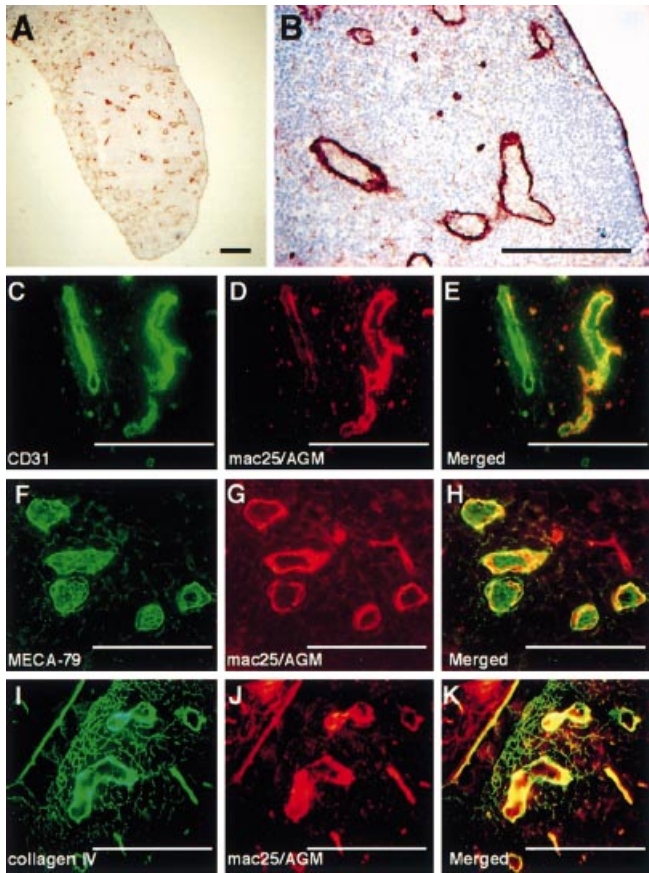


Fig. 2. Localization of mac25/AGM protein in LN. Fresh frozen sections of mouse LN were stained with anti-mac25/AGM pAb. (A) A low-power view: venular structures in the cortical area of the peripheral LN show intense staining with anti-mac25/AGM pAb. (B) A higher magnification shows strong signals around the HEV and the staining appears to localize mainly to the basal side of the HEV. (C–E) Two-color immunostaining with anti-mac25/AGM pAb (rhodamine) and anti-CD31 (FITC). The mac25/AGM expression is highly associated with blood vessels including HEV. (F–H) Two-color immunostaining with anti-mac25/AGM pAb (rhodamine) and MECA-79 mAb (FITC). The mac25/AGM protein is expressed by HEV. Note that the MECA-79 mAb gave strong staining of the whole cell body in HEV cells, whereas the mac25/AGM-specific immunoreactivity was confined to the basal side of the HEV. (I–K) Two-color immunostaining of mesenteric LN with anti-mac25/AGM pAb (rhodamine) and anti-collagen IV (FITC). The mac25/AGM protein co-localizes with collagen IV in the basal lamina of HEV. Collagen IV was also present in the reticular network structure, while mac25/AGM signals were exclusively associated with the endothelium. In all experiments, staining with irrelevant control antibodies produced no signal. Original magnification: A, $\times 40$; B–K, $\times 200$. Calibration bars: 100 μm .

and F). It could be that mac25/AGM is produced by smooth muscle cells and/or pericytes, but not by EC in the flat endothelial blood vessels of the spleen. These results confirm and extend our light microscopy observations that in HEV, mac25/AGM shows a restricted localization to the abluminal side of blood vessels.

mac25/AGM is induced in inflamed blood vessels

Because a few molecules, such as PNA^d (16), MAdCAM-1 (21,22) and vascular adhesion protein-1 (23,24), which are

expressed preferentially in HEV under physiological conditions, are often observed in blood vessels in chronically inflamed tissues, we examined whether mac25/AGM is also inducible in inflammatory lesions. In chronically inflamed skin lesions induced by repeated challenges with DNFB, mac25/AGM expression was clearly observed in the dermal blood vessels (Fig. 4A, C and H). In sharp contrast, mac25/AGM was only weakly, if at all, detected in the blood vessels of sham-sensitized (Fig. 4B, D and E) or unsensitized (data not shown) skin. Double staining with anti-CD31 mAb and anti-mac25/AGM pAb revealed that mac25/AGM was expressed in most blood vessels in the dermis of chronically inflamed DNFB-sensitized skin (Fig. 4H–J), particularly on the abluminal side of dermal blood vessels. These results suggest that mac25/AGM is inducible by inflammatory stimuli and that the pattern of expression in inflamed venules is quite similar to that observed in HEV.

mac25/AGM is induced upon stimulation with inflammatory mediators in vitro

While we found that mac25/AGM is induced in inflamed blood vessels, previous reports by others indicate that it is inducible in non-EC types (11,25–27). We therefore wished to determine whether mac25/AGM could be induced in EC *in vitro* using a mouse endothelioma cell line, bEnd.3. After treatment with TNF- α for 24 h, bEnd.3 cells strongly expressed VCAM-1 (Fig. 5A) and produced mRNA encoding mac25/AGM (Fig. 5B). Examination of the culture supernatant of the bEnd.3 cells revealed that the mac25/AGM protein was produced by the cells in a manner dependent on the time of incubation and dose of TNF- α used (Fig. 5C). While none of the stimulators we used, including TNF- α , IL-1 β , IL-6, VEGF, lipopolysaccharide, phorbol myristate acetate and IFN- γ , induced detectable levels of mac25/AGM protein expression at 4 h after stimulation (data not shown), at 24 h of stimulation, TNF- α induced a prominent production of mac25/AGM dose dependently and other stimulators, such as VEGF and IFN- γ , only slightly up-regulated the mac25/AGM production. After 48 h, TNF- α induced a greater production of mac25/AGM. These results indicate that TNF- α is particularly potent as an inducer of endothelial mac25/AGM.

mac25/AGM binds VEGF

Because mac25/AGM's expression is localized to the abluminal region of HEV that are highly metabolically active (28), we thought it likely that mac25/AGM captures angiogenic factors such as VEGF. When VEGF was added to immobilized mac25/AGM, it was found that VEGF binds to mac25/AGM dose dependently, and the binding was saturable at 0.3 $\mu\text{g}/\text{ml}$ of VEGF (Fig. 6). VEGF did not bind to a myc-, His-tagged control protein, the recombinant human L-selectin, indicating the specificity of the VEGF binding to mac25/AGM. These results indicate that mac25/AGM has the ability to interact with VEGF, if secreted locally, *in vivo*.

Discussion

Recent studies from our laboratory and others have demonstrated that mac25/AGM is abundantly expressed in HEV cells (5,6,15). The mouse homologue of mac25/AGM shares 87.5%

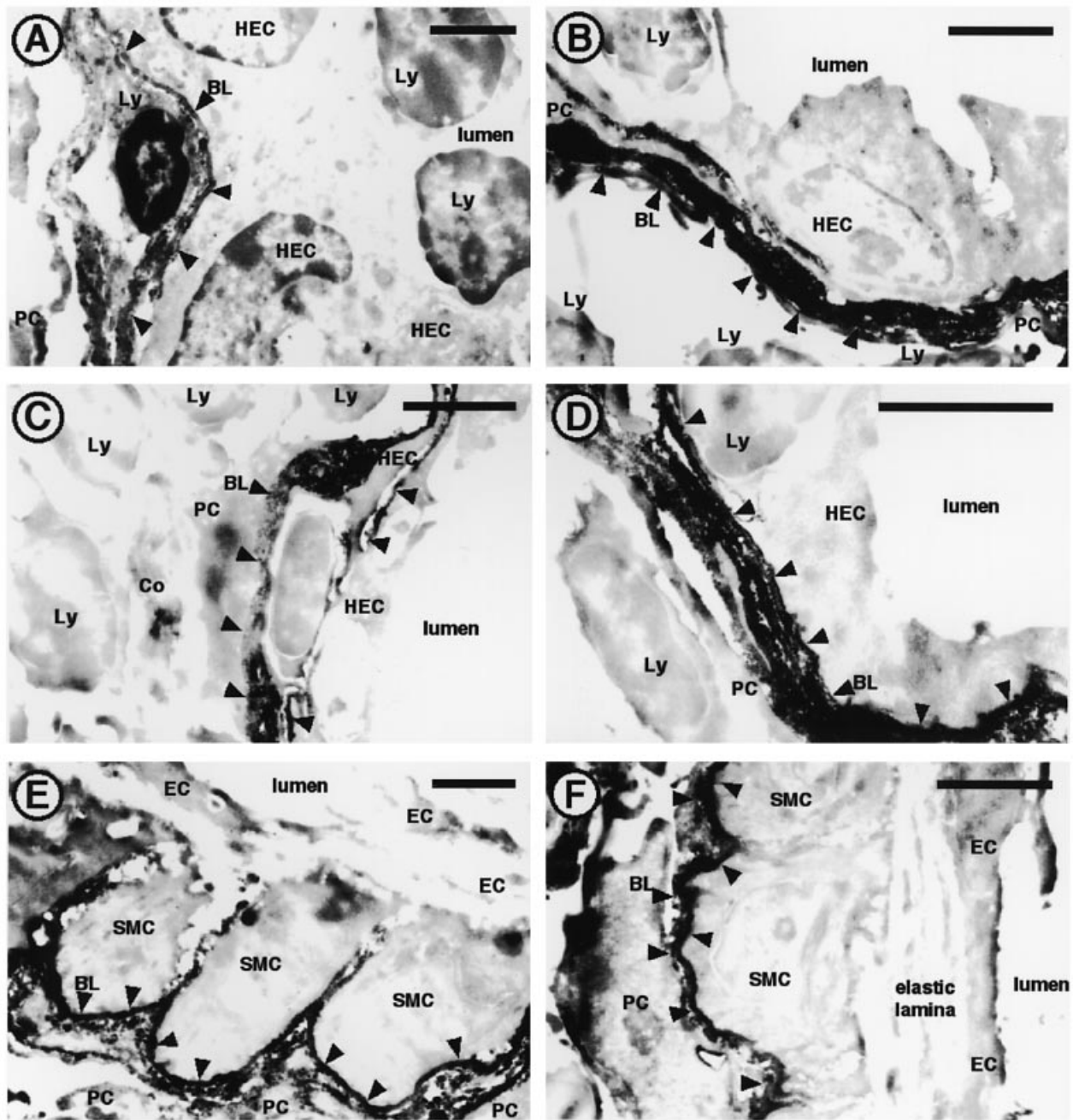


Fig. 3. Ultrastructural localization of *mac25/AGM*. Prefixed tissues were immunolabeled with anti-*mac25/AGM* pAb and the bound antibodies were detected by immunoelectron microscopy. (A and B) Mouse cervical LN HEV containing high EC (HEC), lymphocytes (Ly) and pericytes (PC). HRP staining for *mac25/AGM* showed a dense accumulation of peroxidase products exclusively in the basal lamina (BL, arrowheads) of the HEV. On the luminal side of the HEV, no positive staining for *mac25/AGM* was detectable. (C and D) In the HEV of PP, a similar abluminal localization of *mac25/AGM* was observed. Co, collagen fibers. (E and F) Splenic central artery. Positive staining for *mac25/AGM* was detected selectively in the basal lamina in direct association with smooth muscle cells (SMC), but not EC. In (A), an additional nuclear staining with uranyl acetate was performed. Calibration bars: 2 μ m.

nucleotide identity and 94.4% similarity with its human counterpart and is implicated in tumor cell growth (19,29). Although Northern analysis has indicated that *mac25/AGM* is distributed ubiquitously in almost all mouse and human tissues (5,7), several histochemical studies using anti-peptide antibodies in human tissues (30) have shown that its distribution in

each organ is restricted to certain components of the tissue, such as neurons, muscles, epithelial cilia and vascular endothelia. However, *mac25/AGM*'s definite function(s) *in situ* remains largely unknown.

In this study, we generated a recombinant *mac25/AGM* using the baculovirus expression system and raised a specific

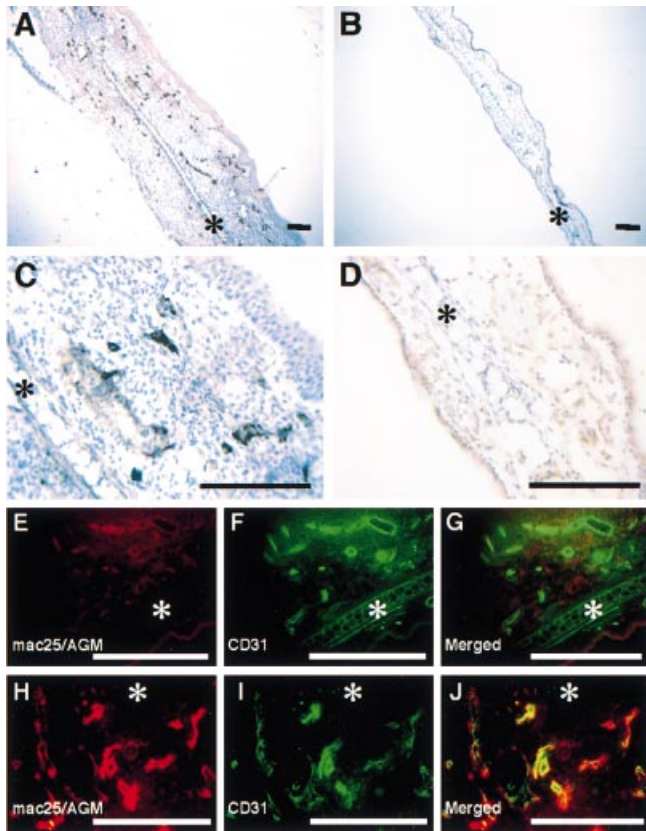


Fig. 4. Up-regulated expression of mac25/AGM in inflamed skin. (A–D) Immunoperoxidase staining of frozen sections of the ear skin. Mice were sensitized with DNFB on the ear and challenged repeatedly. Frozen sections of the ear skin from DNFB-sensitized mice (A and C) and control sham-operated mice (B and D) were stained with anti-mac25/AGM pAb, followed by counterstaining with Harris' hematoxylin. The sections from the inflamed mouse ear (A and C) showed an apparent up-regulation of mac25/AGM associated with a prominent infiltration of mononuclear cells and ear thickening. (E–J) Immunofluorescence staining of frozen sections of the ear skin from DNFB-sensitized mice with anti-mac25/AGM pAb (rhodamine) and anti-mouse CD31 mAb (FITC). (E–G) Control mouse dermis. (H–J) Inflamed mouse dermis. Note that the strong up-regulation of mac25/AGM appears to be localized to the abluminal side of blood vessels. *Cartilage. Original magnification: $\times 40$ (A and B), $\times 200$ (C–J). Calibration bars: 100 μm .

antibody (Fig. 1) to investigate the tissue localization of mac25/AGM in detail. One of the noteworthy findings of our study is the dense accumulation of mac25/AGM protein in the basal lamina of HEV (Figs 2 and 3). In LN and PP, mac25/AGM localized strictly to the HEV's basal lamina in direct association with EC, whereas in the spleen, mac25/AGM localized to the basal lamina in direct association with vascular smooth muscle cells, but not the EC. In all the lymphoid tissues tested, mac25/AGM was found on the abluminal side of blood vessels and was generally excluded from the luminal surface. These results are in sharp contrast to the study by Girard *et al.* (15), in which they reported a preferential localization of mac25/AGM to the luminal surface of HEV, particularly on the microvillous processes near the HEV junctions in human tonsils. This discrepancy may be explained in part by the fact that human tonsils are often exposed to microbes, whereas the

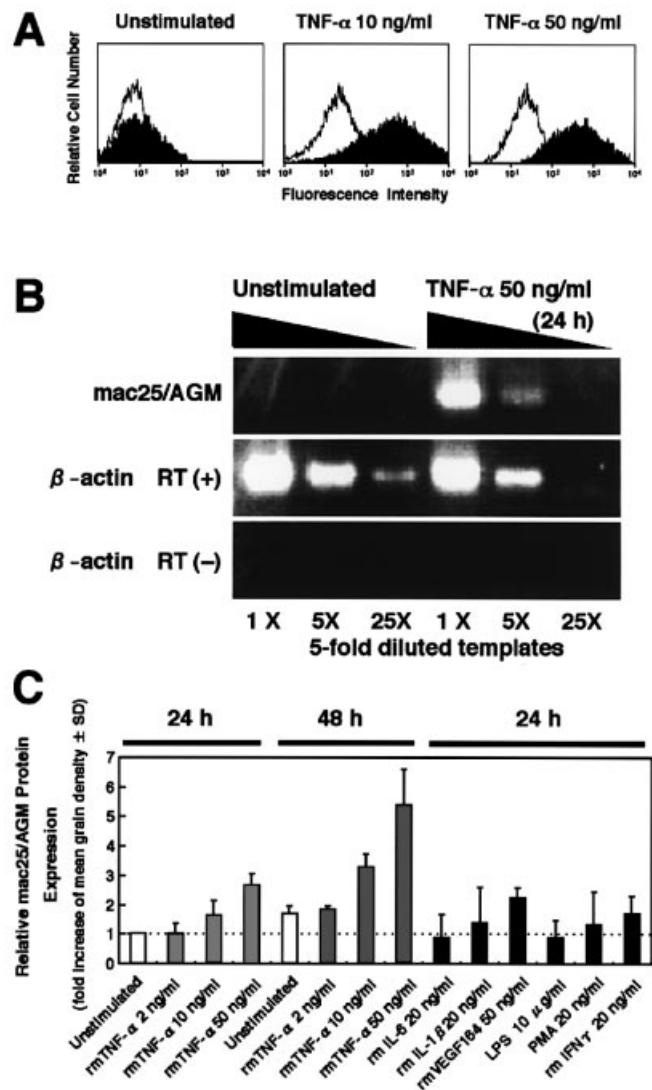


Fig. 5. Up-regulated expression of mac25/AGM in bEnd.3 cells by pro-inflammatory cytokines. The mouse brain EC line, bEnd.3 cells were treated with TNF- α , VEGF, lipopolysaccharide or phorbol myristate acetate for the indicated time period. (A) Flow cytometric analysis. VCAM-1 was strongly up-regulated on the bEnd.3 cells after 24 h of TNF- α stimulation. Filled profile: FITC-labeled anti-VCAM-1. Open profile: rat IgG1 (isotype-matched control). (B) RT-PCR analysis. The up-regulation of mac25/AGM in bEnd.3 cells upon TNF- α stimulation was observed at the mRNA level. (C) Effects of various cytokines on the mac25/AGM protein concentration in the culture supernatants of bEnd.3 cells. Western analysis using anti-mac25/AGM pAb and densitometry were performed. Each column represents the average \pm SD of the band intensities of four separate experiments. An increased secretion of mac25/AGM was detected upon stimulation with TNF- α . The maximum (equivalent to a 3-fold) increase was observed 48 h after stimulation with a dose of 50 ng/ml TNF- α .

LN of specific pathogen-free mice are not. However, deliberate antigenic challenges to the LN did not alter the expression pattern significantly (T. Usui, unpublished observation) and, hence, this possibility is not very likely. Another possibility is that the discrepancy is due to the difference in the animal species used, but our preliminary study with a polyclonal anti-

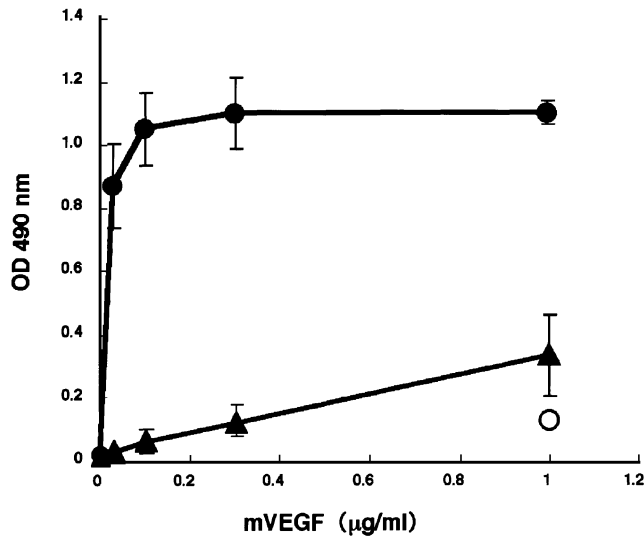


Fig. 6. Interaction of mac25/AGM with VEGF. Binding of VEGF to mac25/AGM *in vitro*. Microtiter plates (96-well) coated with 1 µg/ml of mac25/AGM (closed circles), BSA (closed triangles) or control recombinant myc-, His-tagged L-selectin (open circle) were incubated with indicated concentrations of VEGF. The amount of bound VEGF was determined by ELISA. The data represent the mean \pm SD of triplicate determinations.

human mac25/AGM antibody (kindly provided by Dr R. G. Rosenfeld) showed strong abluminal staining of HEV on the frozen sections of the human cervical LN (T. Usui, unpublished observation). Therefore, a simple difference in the animal species may not account for the discrepancy. It is also possible that a difference in the fine specificity of the antibodies used may partly account for the discrepancy in the results. For instance, certain epitopes on mac25/AGM may be immunologically cryptic and not readily detected under certain situations.

Concerning the biological significance of the abluminal localization of mac25/AGM, it is of note that mac25/AGM can interact with various ECM components (8,13) and VEGF (Fig. 6). Also, mac25/AGM has been reported to regulate cell adhesion (8) and to promote the production of the potent vasodilator PGI₂ in EC (9,11). Therefore, one may speculate that the abluminal localization helps mac25/AGM play multiple roles regulating the HEV's functions, e.g. by sequestering and presenting various humoral factors to the HEV, allowing lymphocytes to transit through and to detach from the substratum so that they can migrate further into the parenchymal compartment, and/or relaxing the vascular tonus to decrease the local blood flow and to alter the lymphocyte migration across the HEV, although further experimental verification will be required to resolve these issues.

We demonstrated for the first time in the present study the inducible expression of mac25/AGM in inflamed vessels *in vivo* (Fig. 4). Consistently, mac25/AGM production was induced in EC by a pro-inflammatory cytokine TNF- α *in vitro* (Fig. 5). TNF- α increased mac25/AGM secretion from the mouse EC line bEnd.3 in a time- and dose-dependent manner, and this induction was also observed at the transcriptional level (Fig. 5). TNF- α is the principal cytokine produced by activated T cells

to induce cell-mediated immune responses and activates nearby EC to enlist their participation in the immune response (31). The TNF- α -dependent mac25/AGM synthesis was observed at doses that modify other activities of bEnd.3 cells, such as the up-regulation of VCAM-1 (Fig. 5), indicating its physiological relevance. Taking into account the known mac25/AGM activities, its induction by a pro-inflammatory cytokine appears to be a plausible mechanism for regulating the vascular phenotype in immunological events.

TNF- α up-regulates various pro-inflammatory molecules, such as chemokines and cell adhesion molecules, in an NF- κ B-dependent fashion (32); however, a recent report on the characterization of the murine mac25/AGM gene revealed no NF- κ B site in its promoter region (33). In accordance with this report, none of the NF- κ B-specific inhibitors we tested suppressed the TNF- α -dependent mac25/AGM induction significantly (T. Usui, unpublished observation). Given that a number of factors have been reported to contribute to the mac25/AGM up-regulation (11,25–27), molecules other than pro-inflammatory cytokines may also contribute to the inflammation-induced up-regulation of mac25/AGM in EC. With respect to the high expression of mac25/AGM in HEV, although TNF- α signals can be detected in areas near the HEV, and mRNAs encoding TNF receptor (R)-I and TNFR-II respectively, are also expressed in purified HEV cells (T. Usui, unpublished observation), it is still unclear whether the TNF-TNFR pathway actually plays a role in the mac25/AGM's up-regulation in HEV *in situ*.

It should be noted that HEV cells, inflamed endothelium and tumor-associated endothelium (14), all of which are apparently activated and abundantly express mac25/AGM, share at least one property; specifically, an increased vascular permeability. However, our antibody did not inhibit any of the reported functions of mac25/AGM, including the ability to induce prostacyclin, and we were also unable to induce an increased endothelial permeability *in vitro* with the recombinant mouse mac25/AGM (data not shown); thus, we could not investigate this issue further.

Concerning the biological relevance of mac25/AGM-VEGF interaction, we have attempted to detect the mac25/AGM-VEGF interaction *in situ* without success, i.e. we could not detect the expression of VEGF in lymph nodes by immunohistochemistry. However, the observation that HEV show rapid and strong neoangiogenesis in inflammatory situations (34,35) indicates that HEV are highly sensitive to angiogenic factors such as VEGF in certain situations. Therefore, in the absence of *in vivo* data, we currently speculate that mac25/AGM can capture and present VEGF to its receptor *in situ*, if VEGF is produced locally.

In summary, we herein demonstrated the strict abluminal localization of mac25/AGM in activated endothelia, such as LN HEV cells and the EC in inflamed blood vessels using an anti-mac25/AGM pAb that we generated. While mac25/AGM is apparently absent in the blood vessels of normal tissues, it was found to be readily up-regulated in inflamed vessels *in vivo*, and also *in vitro* in response to pro-inflammatory cytokines, indicating that mac25/AGM is a novel marker of activated EC. Prominent binding of mac25/AGM with VEGF together with the restricted subendothelial localization of

mac25/AGM indicate that this unique protein may be involved in the regulation of angiogenesis in lymph nodes and PP.

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Abbreviations

6-keto-PGF ₁ α	6-keto-prostaglandin F ₁ α
AGM	angiomodulin
CPAEC	calf pulmonary artery endothelial cells
DNFB	dinitrofluorobenzene
ECM	extracellular matrix
EC	endothelial cells
HEV	high endothelial venule
HRP	horseradish peroxidase
IGFBP-rP1	insulin-like growth factor binding protein-related protein 1
LN	lymph node
MAdCAM-1	mucosal addressin cell adhesion molecule-1
PECAM-1	platelet-endothelial cell adhesion molecule-1
pAb	polyclonal antibody
PFA	paraformaldehyde
PGI ₂	prostaglandin I ₂ (prostacyclin)
PNAd	peripheral lymph node addressins
PP	Peyer's patches
PSF	prostacyclin stimulating factor
TAF	tumor-derived adhesion factor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor

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