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Clever-1/Stabilin-1 regulates lymphocyte migration within lymphatics and leukocyte entrance to sites of inflammation

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Clever-1/Stabilin-1 is a scavenger receptor present on lymphatic and sinusoidal endothelium as well as on a subset of type II macrophages. It is also induced on vasculature at sites of inflammation. However, its *in vivo* function has remained practically unknown and this work addresses those unknown aspects. We demonstrate using *in vivo* models that Clever-1/Stabilin-1 mediates migration of T and B lymphocytes to the draining lymph nodes *in vivo* and identify the adhesive epitope of the Clever-1/Stabilin-1 molecule responsible for the interaction between lymphocytes and lymphatic endothelium. Moreover, we demonstrate that Ab blocking of Clever-1/Stabilin-1 efficiently inhibits peritonitis in mice by decreasing the entrance of granulocytes by 50%, while migration of monocytes and lymphocytes into the inflamed peritoneum is prevented almost completely. Despite efficient anti-inflammatory activity the Ab therapy does not dramatically dampen immune responses against the bacterial and foreign protein Ag tested and bacterial clearance. These results indicate that anti-Clever-1/Stabilin-1 treatment can target two different arms of the vasculature – traffic *via* lymphatics and inflamed blood vessels.

Key words: Adhesion molecules · Cell trafficking · Endothelial cells · Inflammation

Supporting Information available online

Introduction

Immune cell trafficking between blood and lymphoid organs is an essential element in the proper functioning of the immune system. The mechanisms mediating lymphocyte entrance from the blood into the lymphoid organs and leukocyte trafficking to sites of inflammation are well known [1–3]. In contrast, mechanisms involved in lymphocyte migration from the periphery *via* the afferent lymphatics into the draining lymph nodes and their exit from the lymph nodes are poorly known. Besides lymphocytes, many tumors also use lymphatics for dissemination to distant sites of the body [4].

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Clever-1/Stabilin-1, also known as Feel-1, is a multifunctional molecule possessing scavenging ability on a subset of type II macrophages [5, 6]. It is also present both on afferent and efferent lymphatic endothelial cells and sinusoidal endothelial cells in the liver and spleen [7–10]. In man, it is brightly expressed on high endothelial venules (HEV), which are the specific vessels within organized lymphatic tissues mediating lymphocyte entrance into the tissues, and on sinusoidal macrophages. Moreover, at sites of inflammation its expression is induced in flat-walled vessels. On lymphatic vasculature it is able to bind lymphocytes and certain tumor cells and on inflamed blood vessel endothelium it can mediate adhesion of lymphocytes, granulocytes and monocytes [8, 11]. *In vitro* analyses have indicated it to be important in the transmigration step during the leukocyte extravasation process [12].

Clever-1/Stabilin-1 is involved in two intracellular trafficking pathways, namely in receptor mediated endocytosis and recycling as well as in shuttling between endosomal compartment and trans-Golgi network. These complex mechanisms require interactions with several molecules such as intracellular sorting adaptors [13]. For example, macrophage Clever-1/Stabilin-1 mediates uptake of acetylated low-density lipoprotein [5, 10] and secreted protein, acidic, rich in cysteine [14],] a regulator of tissue remodeling, cancer growth and dissemination. It is involved in intracellular sorting and lysosomal delivery of the Stabilin-1-interacting chitinase-like protein [15] and transcytosis of a GH-family hormone, placental lactogen [16].

It is currently unknown whether Clever-1/Stabilin-1 is involved in lymphocyte migration *via* afferent lymphatics into the draining lymph nodes and whether it can be used as a target for anti-inflammatory therapy. Therefore, this work was designed to elucidate the role of Clever-1/Stabilin-1 in lymphocyte trafficking and to study the therapeutic efficacy of anti-Clever-1/Stabilin-1 Ab in inflammation. As manipulating the leukocyte migration may cause deleterious effects to normal immune responses, we also studied the safety aspect connected to the therapeutic treatment.

Results

Anti-Clever-1/Stabilin-1 Ab prevents lymphocyte trafficking to draining lymph nodes

To study whether Clever-1/Stabilin-1 is involved in lymphocyte trafficking into the draining lymph nodes, 12–13 h time points were used because this time frame allows optimal recovery of migrated cells from the draining lymph node (*i.e.* the injected lymphocytes have had enough time to enter into the draining lymph nodes and most of them have not yet left the node *via* efferent lymphatics) [17, 18]. We first used our function-blocking anti-human Clever-1/Stabilin-1 Ab that cross-reacts with the rabbit homolog [8, 12]. Ab treatment efficiently blocked trafficking of normal lymphocytes from the footpad to the popliteal lymph nodes in rabbits (Fig. 1A and B). Like in the rabbit, the anti-mouse Clever-1/Stabilin-1 Ab treatment efficiently prevented migration of lymphocytes into the draining lymph nodes in mice (Fig. 1C). The subtype analyses revealed that trafficking of all major subtypes (CD4, CD8 and B cells) was inhibited (Fig. 1D). Thus, two Clever-1/Stabilin-1 targeting Ab in two different animal species inhibited lymphocyte migration to the draining lymph nodes *via* the afferent lymphatics.

The Ab 3-372 used in the rabbit experiments blocks lymphocyte binding to human lymphatic endothelium in *in vitro* adhesion assays and prevents the transmigration step in the capillary assays [11, 12]. To test whether the mechanism of action of the 1.26 Ab produced against N-terminal part of Stabilin-1 is comparable to 3-372, *in vitro* binding assays were performed on frozen sections of lymph nodes. In these assays 1.26 Ab effectively blocked the binding of lymphocytes to lymphatic endothelium (Fig. 1E), indicating that the Ab is a function-blocking mAb, which prevents a Clever-1/Stabilin-1 dependent component in the interactions between lymphocytes and lymphatic endothelial cells.

As 3-372 was raised against natural human Clever-1/Stabilin-1 and it contains several distinct protein domains that can potentially mediate cell adhesion, trafficking and scavenging functions, we wanted to clarify the mechanism behind the observed inhibitory effect of 3-372 Ab by determining the region of Clever-1/Stabilin-1 that the Ab recognizes. Several clones spanning the entire cDNA of Clever-1/Stabilin-1 were prepared (see Supporting Information Table 1) and used in defining the area as shown in Fig. 2. Two overlapping clones produced a polypeptide recognizable by the 3-372 Ab. The common region between the clones covers nucleotides 1945–3102 of the full-length molecule corresponding to aa 638–1024 and contains five EGF-like domains. Thus, these results suggest that the Clever-1/Stabilin-1 epitope recognized by 3-372 is also located within or in close proximity to the corresponding N-terminal fragment used as an Ag in production of the 1.26 Ab against mouse Clever-1/Stabilin-1.

Clever-1 mediates leukocyte extravasation to sites of inflammation

Next, we tested the *in vivo* role of Clever-1/Stabilin-1 on blood vasculature under normal and inflamed conditions. No inhibition was seen in short-term homing studies, in which the lymphocytes were intravenously injected and the lymphocytes were let to circulate for 18 h (Fig. 3A and B). This was consistent with the finding that unlike in rabbit (data not shown) and in man the murine HEV, which are the entry sites for lymphocytes from the blood, expressed very low levels of Clever-1/Stabilin-1 or lacked it completely when analyzed using conventional immunohistochemistry (Fig. 3C). When the *in vivo* injected Ab was detected *in vitro* with a second stage reagent, only occasional blood vessels showed faint positive staining 5 min after injection. After 20 h of injection no positive signal was detected on blood vessel endothelium. These data further suggest that Clever-1/Stabilin-1 is expressed at a very

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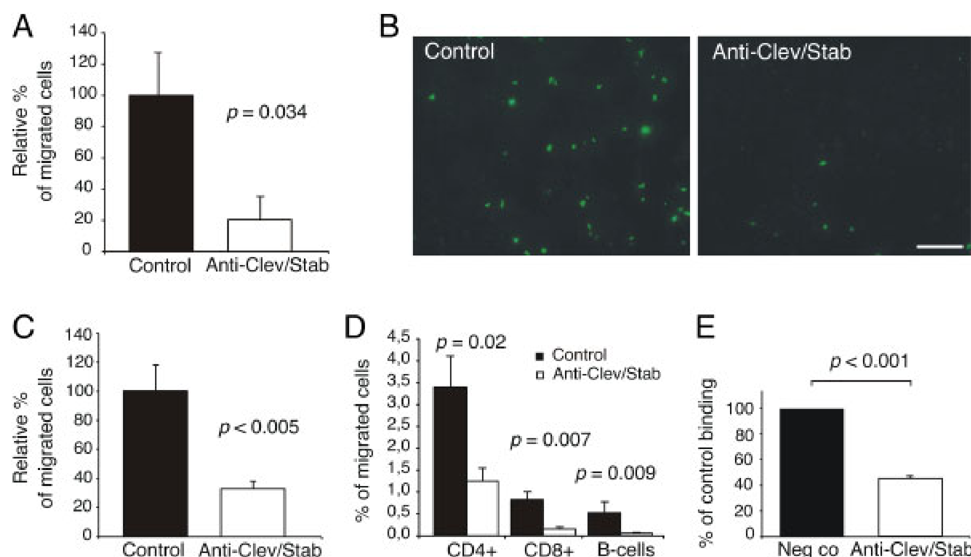

Figure 1. Clever-1/Stabilin-1 is important in lymphocyte trafficking *via* afferent lymphatics. (A) Lymphocyte migration in the rabbit. Fluorescently labeled lymphocytes were injected into the footpads of the rabbits treated either with anti-Clever-1/Stabilin-1 or control Ab ($n = 7$ in both groups). Popliteal lymph nodes were collected and the number of migrated lymphocytes was analyzed by flow cytometry. The results are expressed as percentage of migrated cells. Migration of the cells in control Ab treated rabbits was set as 100%. (Statistics: student's *t*-test). (B) Representative histological sections of popliteal lymph nodes of rabbits treated with the control Ab and anti-Clever-1/Stabilin-1 Ab. The green dots are injected cells, which have migrated into the nodes. (C) Lymphocyte migration in the mouse. Fluorescently labeled murine lymphocytes were injected into the footpads of mice treated either with anti-Clever-1/Stabilin-1 or control Ab ($n = 10$ in both groups) and the migrated cells were detected with flow cytometry. The results are expressed as percentage of migrated cells in comparison to the control treated mice (100% by definition). (Statistics: Student's *t*-test). (D) Percentages of migrated CD4, CD8 and B cells. The multicolor stainings were performed as explained in materials and methods. (Statistics: Mann–Whitney *U*-test). (E) Anti-Clever-1/Stabilin-1 Ab inhibits lymphocyte binding to lymphatic endothelium in *in vitro* binding assays. Frozen sections of lymph nodes were treated either with anti-Clever-1/Stabilin-1 (1.26) Ab or class matched negative control Ab (AK1) prior to lymphocyte incubations. Lymphocytes bound to lymphatic endothelium were counted and the combined results of three experiments are expressed as percentage+SD of control binding (the number of adherent cells/sinus in the presence of control mAb defines 100% binding). (Statistics: Student's *t*-test.)

low or undetectable level in the normal un-inflamed mouse blood vasculature. In contrast, after 20 h the injected Ab was abundantly present on lymphatic vasculature (Supporting Information Fig. 1). However, during the inflammatory reaction caused by s.c. footpad immunization with OVA also, HEV of the draining lymph nodes became Clever-1/Stabilin-1 positive in mouse (Fig. 3D).

Functionality of the inflammation-induced Clever-1/Stabilin-1 was tested in an 18-h peritonitis model. This time frame allows not only inflammation-induced entrance of granulocytes but also that of monocytes and lymphocytes into the peritoneum. Lymphocyte and monocyte trafficking was inhibited by the anti-Clever-1/Stabilin-1 Ab treatment nearly completely and 50% reduction was seen in the number of granulocytes (Fig. 3E and F). This inhibition is targeting only the endothelium as all leukocyte subpopulations in the blood and lymph nodes are Clever-1/Stabilin-1 negative (Supporting Information Fig. 2). Moreover, the Ab therapy does not affect the blood leukocyte counts demonstrating that the anti-Clever-1/Stabilin-1 treatment does not eliminate leukocytes (Fig. 4A). To further confirm that the Ab does not kill leukocytes, isolated mesenteric lymph node lymphocytes were incubated in normal mouse sera containing anti-Clever-1/Stabilin-1 (1.26) Ab or control Ab (both 50 μg/mL) for 1 h at 37°C. There was no difference in the viability of the cells under different Ab incubations when measured with Trypan blue (data not shown). Moreover, possible effects of the *in vivo* Ab treatment on the vascular integrity were tested using FITC-dextran injections. No vascular leakage of the 70 kDa FITC-dextran was found in any of the organs (lymph nodes, heart, liver, gut, lungs, spleen, kidneys, pancreas) analyzed of either group (Fig. 4B) indicating that anti-Clever-1/Stabilin-1 mAb treatment does not cause vascular damage.

Ab therapy does not significantly impair normal immune response

Since the Clever-1/Stabilin-1 blockade significantly prevents lymphocyte migration into the draining lymph nodes, it may also affect the normal immune response. We tested this possibility both in the rabbit and mouse models. Rabbits were treated either with anti-Clever-1/Stabilin-1 or a control Ab and immunized into the footpad with *Salmonella enteritidis* and *Escherichia coli* LPS (Fig. 5). No statistically significant differences were detected in specific Ab responses of IgM and IgG classes. The only exception was a slight decrease in the IgM response at day 11 in *S. enteritidis* in the rabbits treated with anti-Clever-1/Stabilin-1 Ab. Mice were immunized into the footpads with OVA. Absolute lymphocyte numbers and percentages of different subpopulations in lymph nodes and spleen of both treatment groups were comparable (Fig. 6A–C) and there were no

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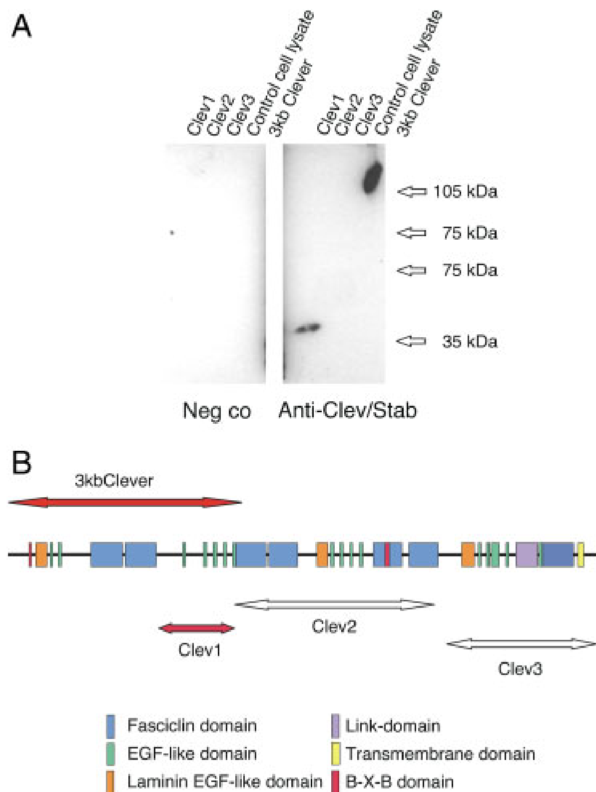

Figure 2. The anti-Clever-1 Ab 3-372 recognizes the polypeptide chains produced by the 3kbClever and Clev1 cDNA clones in Western blotting. (A) The cloned fragments were transiently transfected into HEK293 cells using the Ca-phosphate transfection method. Transfection efficiency was confirmed using a myc-specific Ab that recognizes all the expressed clones. Lysates of the transfected and control cells were run on a SDS-PAGE gel and blotted onto a Hybond ECL-membrane. ECL was performed using the anti-Clever-1 Ab 3-372 and the negative control Ab (Neg co). Above, a blot with four of the clones used is shown. (B) The epitope of the anti-Clever-1 Ab 3-372 resides in a region containing five EGF-like domains. A schematic representation of the protein structure of Clever-1/Stabilin-1 and the cDNA clones used in transfections are shown. The red arrows indicate the fragments recognized by the Ab 3-372.

statistically significant differences in OVA-specific T- and B-cell responses (Fig. 6D).

Anti-Clever-1/Stabilin-1 treatment does not affect bacterial clearance

We also tested whether the Ab therapy compromises the clearance of a bacterial infection. In these experiments we inoculated *Staphylococcus aureus* s.c. into the paws of mice treated with anti-Clever-1/Stabilin-1 or control Ab. These bacteria contain an operon that constitutively produces light allowing monitoring the extent of bacterial load using bioluminescent imaging (Fig. 7A). Kinetic analyses revealed comparable clearance rates of the bacteria in both Ab treated groups (Fig. 7B) indicating that anti-Clever-1/Stabilin-1 therapy does not impair the capacity of the host to control bacterial replication.

Discussion

This work demonstrates an important role for Clever-1/Stabilin-1 in migration of lymphocytes *via* afferent lymphatics into the draining lymph nodes. Moreover, it also shows that the Ab treatment is an effective way to prevent lymphocyte entrance into the lymphatics. Clever-1/Stabilin-1 on lymphatic endothelium can bind lymphocytes and cancer cells in *in vitro* adhesion assays and this interaction is prohibited by the 3-372 Ab [11]. In this work, we could directly show that the 1.26 Ab against mouse Clever-1/Stabilin-1 blocks lymphocyte binding lymphatic endothelium, thus acting in the similar fashion as 3-372. These results show that inhibition of the interaction between lymphocytes and lymphatic endothelium is a control point in cell trafficking into the lymphatics. This idea is also supported by our observation that no accumulation of injected cells can be seen in the subcapsular sinus of the draining lymph node. Instead, those injected cells that have been able to migrate into the nodes are dispersed throughout the draining lymph nodes as can be seen in Fig. 1.

In this work we also defined the epitope of human Clever-1/Stabilin-1 recognized by the migration-blocking Ab 3-372. This Ab has been shown to block both lymphocyte and cancer cell binding to the lymphatic vessels in *in vitro* adhesion assays [11]. The region carrying the 3-372 epitope corresponds to a region of the molecule containing five EGF-like domains. The exact function of these domains is still unclear, but they have been reported, *e.g.* to mediate homophilic and heterophilic protein–protein interactions [19, 20]. Recently, a similar domain in a homologous molecule Stabilin-2 has been shown to play a key role in the clearance of aged and apoptotic cells through recognition of the exposed phosphatidyl serine (PS) on the cell surface [21]. Since externalization of PS can also occur during many other cellular processes, like platelet activation and T-cell migration [22, 23] and the phenomenon has been observed on human tumor cells also[24], it can be hypothesized that the 3-372 Ab inhibits the interaction between these EGF-like domains of Clever-1/Stabilin-1 on the endothelium and externalized PS of the transmigrating cell. The recycling nature of Clever-1/Stabilin-1 also fits well to this idea, especially when the cells are transmigrating *via* the transcellular route. Moreover, our *in vivo* results together with earlier *in vitro* adhesion results indicate that adhesion is a critical step in lymphocyte migration into the afferent lymphatics and its inhibition leads to diminished lymphocyte traffic into the draining lymph nodes.

Only few molecules present on afferent lymphatics such as macrophage mannose receptor, sphingosine-1-phosphate receptor and CCL21 have been shown to mediate lymphocyte traffic *via* afferent lymphatic vessels [18]. Among those, Clever-1/Stabilin-1 is the first one, which is now shown to also be a good target at sites of inflammation *in vivo*.

The use of three different species, human, mouse and rabbit, in this work allowed us to study the role of Clever-1/Stabilin-1 molecule in different settings and also to compare different findings. Expression of Clever-1/Stabilin-1 on both afferent and efferent lymphatics is a shared feature in all these species. However, the studies revealed differences, which may need to be

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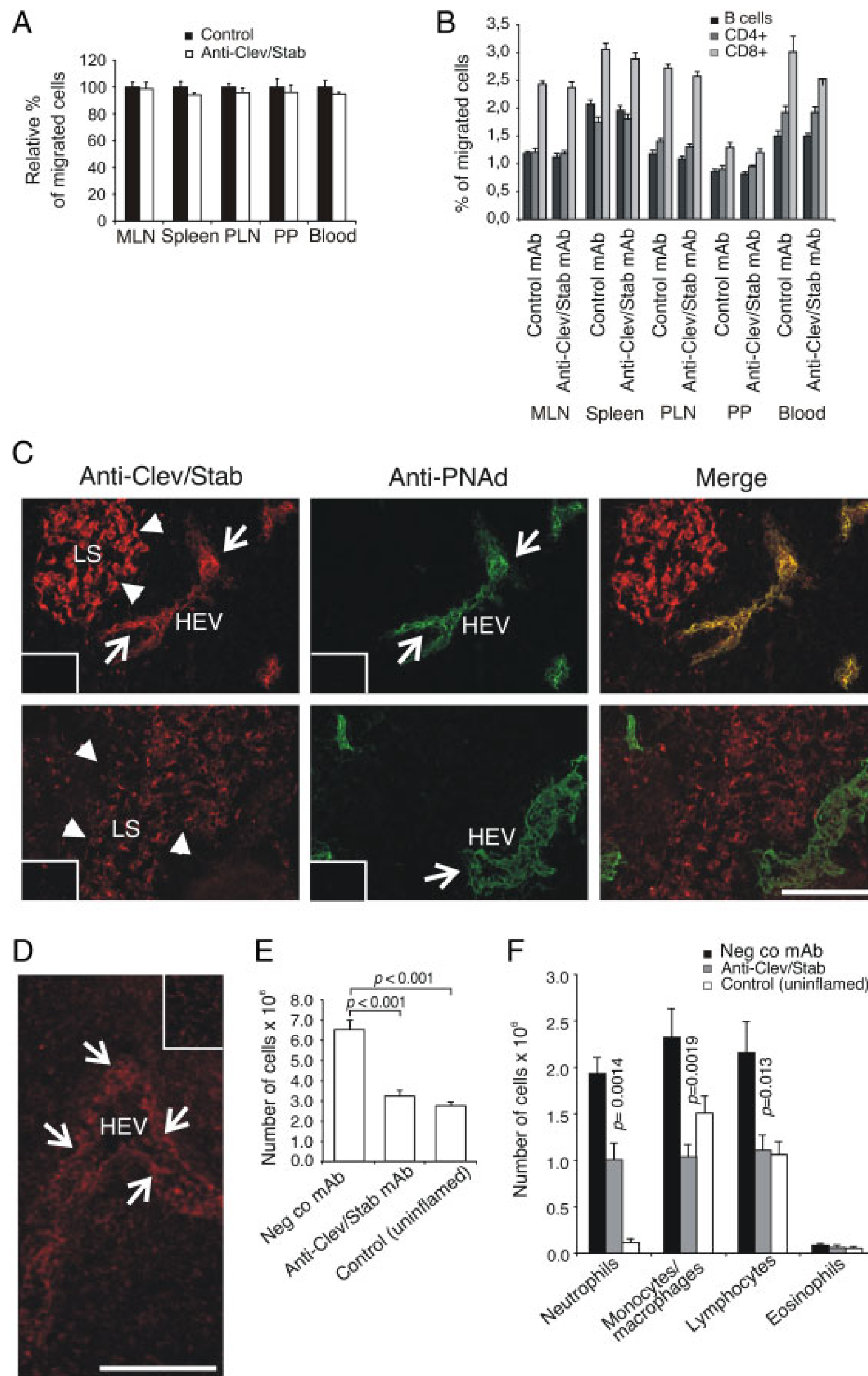

Figure 3. Clever-1/Stabilin-1 is not involved in lymphocyte homing but mediates leukocyte traffic to sites of inflammation in mouse. (A) Fluorescently labeled lymphocytes were intravenously injected into the tail vein of mice ($n=8$ in both groups) and the indicated lymphoid organs were collected after 18 h recirculation time. The number of homed cells was analyzed by flow cytometry. The results are expressed as percentage of migrated/homed cells in comparison to the control treated mice (100% by definition) (B) Percentages of migrated CD4, CD8 and B cells were analyzed as indicated in Fig. 1. (C) Immunohistochemical two-color stainings of normal human (upper panel) and mouse (lower panel) lymph nodes with anti-Clever-1/Stabilin-1 (red) and MECA-79 (anti-peripheral lymph node addressin (PNAd)) detecting HEV (green). Thick arrows point to the brightly Clever-1/Stabilin-1 positive lymphatic sinuses (LS) in both species and thin arrows to MECA-79 positive HEV bright in man and very faint or lacking Clever-1/Stabilin-1 in normal non-immunized mouse. Stainings with negative control Ab are shown in the insets. (D) Immunohistochemical staining of a popliteal lymph node after footpad immunization with OVA. Inflammation-induced Clever-1/Stabilin-1 positivity on HEV is pointed out by arrows. Staining with a negative control Ab is shown in the inset. (E) Peritonitis was induced with IL-1 and proteose peptone. Anti-Clever-1/Stabilin-1 ($n=10$) or control Ab ($n=11$) was intravenously given 1 h later. Eighteen hours after the induction of inflammation the cells present in peritoneum were collected and counted. (F) The leukocyte subsets were analyzed histochemically. Untreated mice ($n=6$) served as controls to analyze the number of resident cells normally present in peritoneum. Scale bars, 100 μm. (Statistics: Student's *t*-test).

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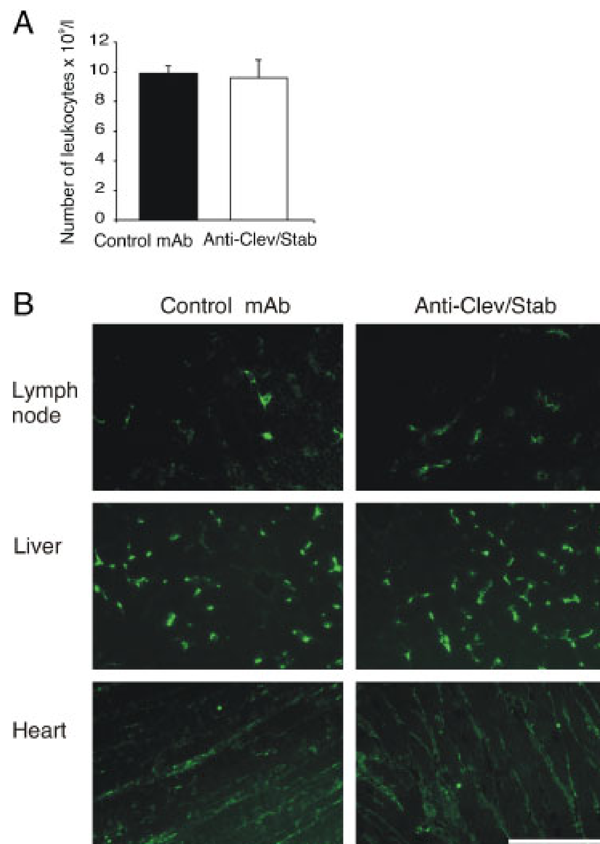

Figure 4. Anti-Clever-1/Stabilin-1 treatment with 1.26 Ab does not deplete leukocytes and does not impair vascular integrity. (A) Blood leukocyte counts of mice treated for peritonitis. Anti-Clever-1/Stabilin-1 ($n = 10$) or control Ab ($n = 11$) (100 μg/mouse) was given 17 h prior to analyzing the leukocyte counts. (B) Examples of FITC-dextran containing vessels (green) in frozen sections of lymph nodes, liver and heart. Anti-Clever-1/Stabilin-1 or control Ab ($n = 3$ in both groups) was given 20 h before FITC-dextran injections as explained in the *Materials and methods* section. Scale bar, 100 μm.

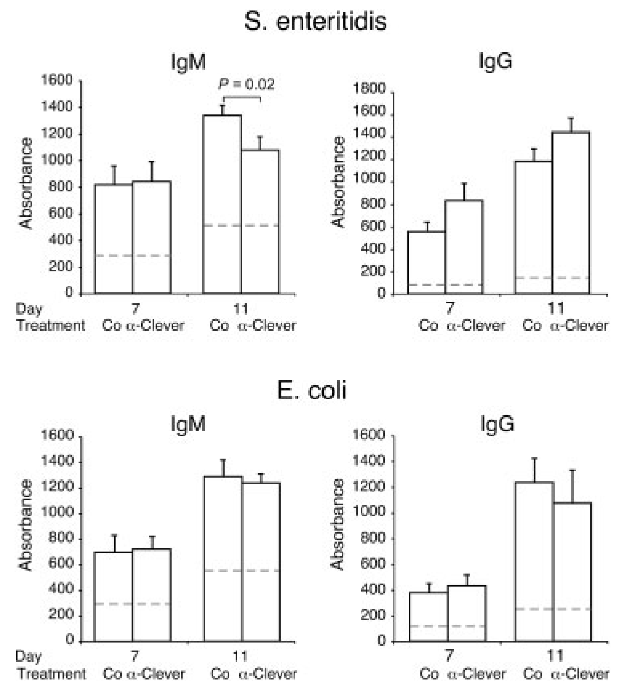

Figure 5. Anti-Clever-1/Stabilin-1 treatment does not significantly impair the Ab response. Rabbits were immunized with heat-killed *Salmonella enteritidis* and *E. coli* LPS and treated either with anti-Clever-1/Stabilin-1 or control Ab ($n = 5$ in both groups). The Ab titers were measured on days 7 and 11 after primary immunization using ELISA and presented as absorbance values. The dashed lines indicate the absorbance in non-immunized animals (receiving the Ab). (Statistics: Student's *t*-test.)

taken into account when designing pharmaceuticals to target Clever-1/Stabilin-1 and selecting the optimal disease(s) for clinical trials. The lack or low expression of Clever-1/Stabilin-1 on HEV in mouse is in a striking contrast to the situation in rabbit and man. This is in agreement to our earlier findings obtained using *in vitro* binding assays, in which we can efficiently block lymphocyte binding to human HEV with anti-Clever-1/Stabilin-1 Ab [8]. Based on our observations Clever-1/Stabilin-1 is practically absent from type II murine macrophages in normal conditions but according to the earlier findings are abundantly present in cancer and wound healing [25]. Moreover, we were not able to induce Clever-1/Stabilin-1 expression *in vitro* on the mouse macrophages, although human macrophages started to express Clever-1/Stabilin-1 after 4 days in culture in the presence of IL-4 and dexamethasone (data not shown).

Importantly, despite the Ab therapy was an effective anti-inflammatory treatment, it did not dramatically diminish the immune response against various types of Ag as slight decrease was only seen against *S. enteritidis*. Reasons behind this may be that the Ag get into the lymph nodes in sufficient quantities to create an immune response. Moreover, although the therapy presumably reduces lymphocyte trafficking into and out from the lymph node undergoing the immune response, it does not significantly alter the balance between the entrance of lymphocytes *via* HEV and their exit from the lymph nodes. Once created, Ab seem to circulate independently of Clever-1/Stabilin-1 in the body.

The Ab therapy did not significantly impair the clearance of *S. aureus* in our model, although it clearly affected migration of granulocytes in our other experiments. We believe that the redundancy of the leukocyte extravasation cascade can explain the lack of detrimental consequences on the overall anti-microbial immunity when one of the leukocyte adhesion molecules is inhibited. In severe microbial infection many inflammatory pathways become activated and several trafficking molecules are induced allowing sufficient leukocyte migration into the site of insult. In fact, comparable findings have also been reported in the absence (or therapeutic targeting) of other endothelial adhesion molecules such as P-selectin, ICAM-1 and VAP-1 in mice [26, 27]. These animal data are in line with clinical experience. Thousands of patients have been treated with mAbs against widely expressed adhesion molecules, alpha 4 integrin (Natalizumab) and CD11a (Efalizumab), and yet only surprisingly minor problems with bacterial infections have emerged. The most

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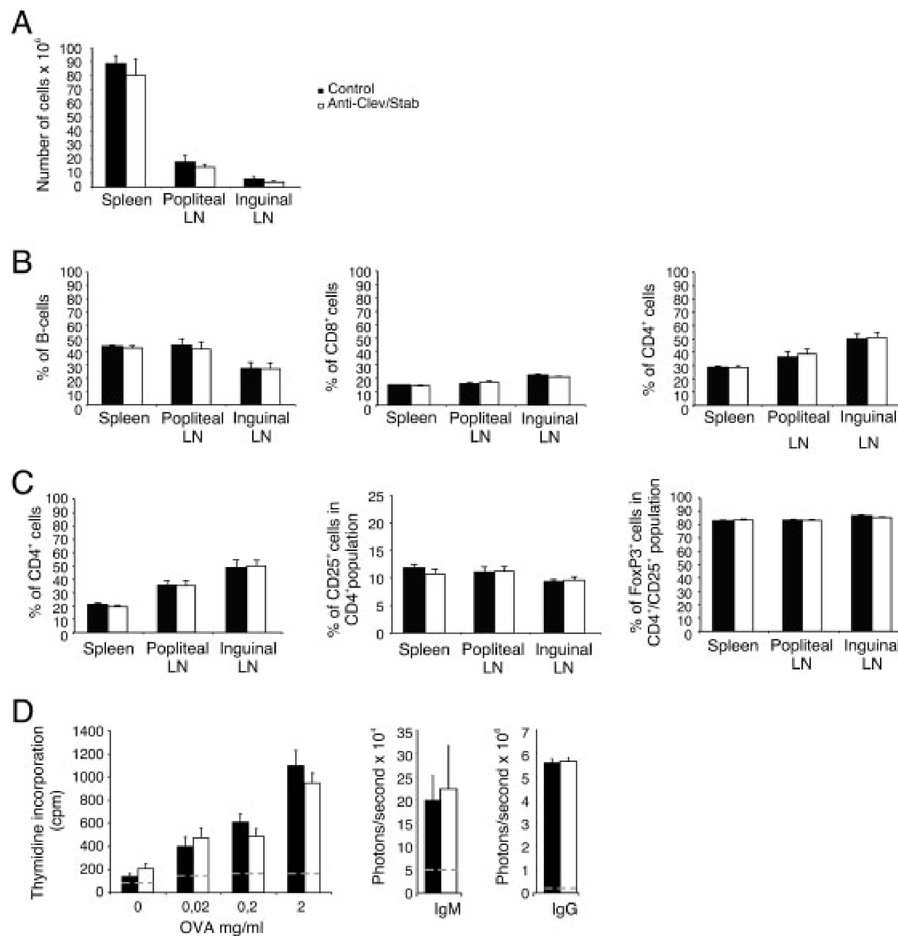

Figure 6. Anti-Clever-1/Stabilin-1 treated mice respond normally to OVA immunization. (A) Number of lymphocytes in the indicated organs. (B) Percentages of B cells, CD4 and CD8 positive T cells. (C) Percentages of regulatory T cells. (D) Proliferation responses of the splenic lymphocytes measured using thymidine incorporation and OVA-specific serum Ab of different immunoglobulin classes. $n = 6$ in Ab-treated groups. The dashed lines indicate the values in non-immunized animals (receiving the Ab).

severe cases have been few patients with reactivated JC virus infections [28, 29].

In summary, our results indicate that Clever-1/Stabilin-1 is involved in different control points determining immune cell trafficking in physiological and inflammatory conditions. However, it does not have marked effects on Ab production and T-cell response at least in our 17-day experimental setting. As a successful treatment of patients suffering from harmful inflammations requires a drug with sufficient potency but without deleterious side effects, anti-Clever-1/Stabilin-1 Ab may be a beneficial addition into the armamentarium used to fight against inflammations.

Materials and methods

Animals

Balb/C mice (6–9 weeks old) and New Zealand white (NZW) rabbits were used in the *in vivo* experiments. The Local Ethical Committee approved the experimental procedures that were used in this study.

Ab

The following anti-Clever-1/Stabilin-1 Ab were used: 3-372 Ab recognizing human and rabbit Clever-1/Stabilin-1 [8] and clone 1.26 [25] recognizing mouse Clever-1/Stabilin-1. Negative control Ab used were class-matched (AK1, from In Vivo BioTech Services and NS-1 from ATCC). Alexa Fluor 647-conjugated anti-mouse CD4, PerCP-Cy5.5-conjugated anti-mouse CD8a and Pacific Blue anti-mouse B220 were from BD Biosciences Pharmingen. MECA-79, detecting peripheral lymph node addressins and its negative control mAb (Hermes-1 against human CD44) were kind gifts from E. Butcher, Stanford University, CA, USA. The kit for detecting regulatory T cells was from eBioscience. FITC-conjugated anti-rat Ig was from Sigma, alkaline-phosphatase-conjugated anti-rabbit IgM was from Southern Biotechnology Associates and anti-rabbit IgG was from Dako Patts A/S.

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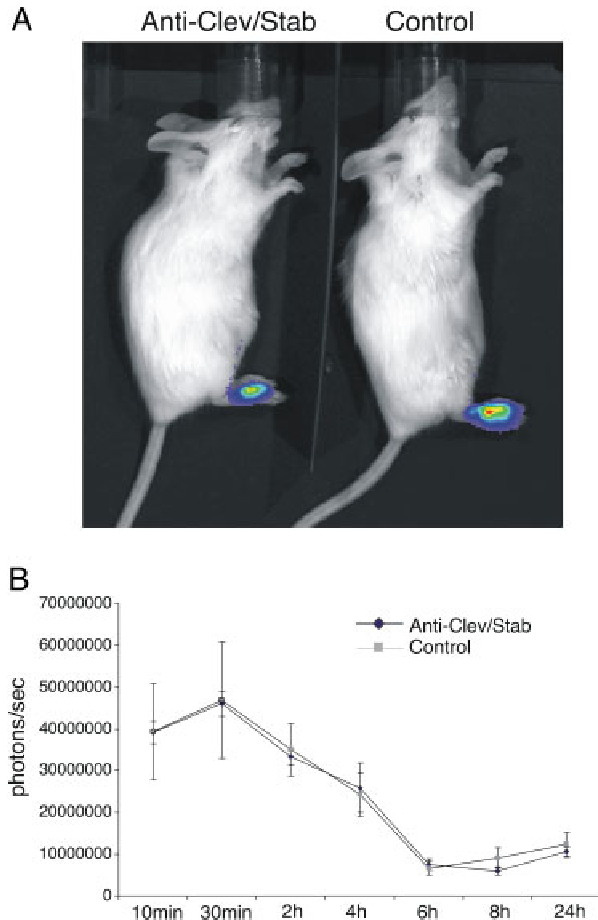

Figure 7. Anti-Clever-1/Stabilin-1 treatment does not impair clearance of *Staphylococcus aureus*. Real-time bioluminescent imaging was used to follow the replication of luciferase containing *Staphylococcus aureus* after footpad inoculation. (A) Representative images taken from anti-Clever-1/Stabilin-1 ($n = 6$) and control Ab ($n = 5$) treated mice 6 h after inoculation. (B) Bacterial load was kinetically followed in both Ab treated groups. Data are shown as photons/s (mean $\pm$ SEM).

In vivo homing experiments

The homing experiments were performed in rabbits and mice as described in the following sections. In each homing experiment the control Ab and anti-Clever-1/Stabilin-1 Ab-treated animals received identical aliquots from the same cell suspension. Migration of total lymphocytes in anti-Clever-/Stabilin-1 treated animals is expressed as percentage of migration in comparison to the control treated animals (set as 100% by definition). Actual percentages of migrated/homed cells are shown for different subpopulations of lymphocytes.

Lymphocyte migration *via* lymphatics in rabbits

Popliteal lymph nodes and spleens were collected and homogenized to obtain single cell suspensions. After hypotonic lysis of erythrocytes, the cells were labeled with 0.5 μM CFSE (Molecular Probes, Eugene, OR, USA). Then 40×10^6 cells were injected s.c. into the right hind footpads of recipient rabbits that were treated twice either with 3-372 Ab recognizing human and rabbit Clever-1/Stabilin-1 [8] or control Ab (30 and 6 h before cell transfer, 2 mg/kg i.v., $n = 7+7$). Thirteen hours after the cell transfer, popliteal lymph nodes were harvested from the recipients. Half of the lymph nodes were snap frozen for histology and the other half was passed through the wire mesh to obtain single-cell suspensions. Cell suspensions were analyzed by flow cytometry (FACSCalibur, BD Biosciences) to quantify the transferred cells.

Lymphocyte migration *via* lymphatics in mice

Spleens and lymph nodes were collected from Balb/C mice and homogenized to obtain single cell suspensions. After lysis of erythrocytes, cells were labeled with CFSE as described for rabbit lymphocytes. Thereafter, 20×10^6 cells were injected s.c. into the footpads. Two hours before lymphocyte injection recipient mice were treated either with anti-Clever-1/Stabilin-1 (clone 1.26) [25] or control Ab (50 μg/mouse, s.c.). Twelve hours after the lymphocyte injections, popliteal lymph nodes were collected and the cell suspensions were stained with Alexa Fluor 647-conjugated anti-mouse CD4, PerCP-Cy5.5-conjugated anti-mouse CD8a and Pacific Blue anti-mouse B220 (10 μg/mL). Stained cell suspensions were analyzed for percentage of $CFSE^+/CD4^+$, $CFSE^+/CD8^+$ and $CFSE^+/B220^+$ lymphocytes by LSRII flow cytometry (BD Biosciences).

Lymphocyte homing assay (i.v.) in mice

Normal healthy mice were treated either with anti-Clever-1/Stabilin-1 or control Ab (100 μg/mouse, i.v., $n = 8+8$). CFSE labeled lymphocytes were injected into the tail veins. After 18 h peripheral lymph nodes and MLN, blood, spleen and Peyer's patches were collected, homogenized and stained and analyzed as explained above.

In vitro binding assays

Binding assays were performed on frozen sections of murine lymph nodes as described [30]. Briefly, frozen sections were incubated with anti-Clever-1/Stabilin-1 Ab (1.26) or negative control Ab and thereafter, lymphocytes isolated from mesenteric lymph nodes were incubated on the sections for 15 min in static conditions, followed by 5 min of rotation at 60 rpm and then again for 15 min without rotation at 7°C. The adherent cells were fixed in 1% glutaraldehyde. Binding of lymphocytes to lymphatic sinuses was counted and the results are expressed as percentage $\pm$ SD of control binding (the number

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of adherent cells/sinus in the presence of control mAb defines 100% binding).

Epitope characterization

To characterize the epitope for the Ab 3-372 in human Clever-1 protein fragments of the full length cDNA of Clever-1 were prepared from the full-length cDNA [8] using PCR and primers containing the EcoR1-site in the 5′-end and XhoI-site in the 3′-end. In addition, a Kozak recognition sequence for efficient translation initiation was designed into the upstream primers (except for the 3kbClever fragment, which contains the endogenous translation start codon). The fragments obtained were cloned into EcoRI/XhoI-digested pcDNA3.1/*myc*-His A expression vector (Invitrogen). The primers used and the regions covered by the individual clones are shown in the Supporting Information Table 1.

Immunohistochemistry

Acetone-fixed frozen sections of human and mouse lymph nodes were stained with MECA-79 and with a negative control mAb (Hermes-1). FITC-conjugated anti-rat Ig diluted in PBS containing 5% normal mouse serum was used as the second stage Ab. In two color stainings, biotinylated anti-Clever-1/Stabilin-1 (3-372 for human and 1.26 for mouse) was followed by Streptavidin-Alexa Fluor 546. The sections were analyzed using Olympus BX60 microscope.

Peritonitis

Inflammation was induced in peritoneal cavities of Balb/C mice by 1 mL intraperitoneal injection of PBS containing 5% proteose peptone and 10 ng IL-1 (R&D Systems). To test the effect of the Ab after elicitation of inflammation (a treatment model), the mice were treated with either anti-Clever-1/Stabilin-1 or control Ab (NS-1, 100 μg/mouse, i.v., $n = 10+11$) 1 h after the induction of inflammation. Seventeen hours later the cells were collected from the peritoneal cavity by washing it with 10 mL RPMI 1640 containing 5 U/mL heparin (Løvens Kemiske Fabrik) and counted. Leukocyte subtypes were analyzed from lavage fluid smears after Reastain Quick-Diff (Reagena) stainings.

Vascular integrity

Mice were given either anti-Clever-1/Stabilin-1 (1.26) or control Ab intravenously (both Ab 100 μg/mouse = 3 mg/kg, $n = 3$ in both groups). Twenty hours later, 25 μg/kg of FITC-dextran (70 kDa) was injected intravenously and the mice were sacrificed 5 min thereafter. Frozen sections were prepared from lymph nodes, heart, liver, gut, lungs, spleen, kidneys and pancreas and analyzed using Olympus BX60 microscope.

Immunizations

Rabbits were immunized to the footpads with a cocktail (volume 200 μL) containing heat-killed *S. enteritidis* and *E. coli* LPS (10 mg). At the same time the rabbits received either anti-Clever-1/Stabilin-1 Ab (3-372, $n = 5$) or negative control Ab ($n = 5$) 2 mg/kg. Non-immunized rabbits were used as controls. The Ab treatments were repeated on days 2, 4, 7 and 9. Immunization was repeated on day 7. Serum samples were collected on days 7 and 11 and Ab titers were analyzed by ELISA. Briefly, polystyrene microtiter plates (Nunc) were coated with pre-tested concentrations of *E. coli* LPS (Difco Laboratories) and SDS-extract of *S. enteritidis*. After incubation with serum samples, IgM and IgG Ab in the wells were detected with alkaline phosphatase-conjugated anti-rabbit IgM and anti-rabbit IgG. The absorbances were detected with a Victor multilabel counter (Wallac) at a wavelength of 405 nm.

Mice were immunized with s.c. injection of 50 μg OVA (grade V; Sigma) in incomplete Freund's adjuvant into the footpads. Immunizations were repeated three times (on days 0, 7 and 14). Mice were treated 1 h before first immunization with s.c. injection of anti-Clever-1/Stabilin-1 or control Ab (50 μg/mouse, $n = 6+6$) and intraperitoneally three times a week (100 μg/mouse/injection). Mice were sacrificed on day 17 and popliteal lymph nodes, inguinal lymph nodes and spleens were collected and cells were isolated for flow cytometric analyses and for proliferation assay. Spleens were homogenized and red cells were lysed using hypotonic saline. T cells (0.2×10^6) were co-cultured with increasing concentration (0–2 mg/mL) of OVA in round-bottom 96-well plates. Co-cultures were incubated in culture medium for 3 days and pulsed with ^{3}H-thymidine (1 μCi [0.037 MBq] per well) for the final 6 h. Cells were harvested using semi-automated plate harvester (Tomtech MACH III) and counted with the 1450 Microbeta counter (Wallac). The Ab titers against OVA were determined by ELISA as described [31]. The phenotype analyses were carried out as explained above. In addition, FoxP3 positive regulatory T cells were detected using a kit from eBioscience according to the manufacturer's instructions.

S. aureus infection

Balb/C mice were treated either with anti-Clever-1/Stabilin-1 or control Ab (200 μg/mouse, i.v., $n = 6+5$). Luciferase containing *S. aureus* Xen36 (Xenogen/Caliper Life Sciences) were grown in Trypticase Soy Broth until absorbance of 0.5 at 600 nm was reached corresponding 1.0×10^8 cfu/mL of *S. aureus*. Five milliliters bacterial culture per mouse were pelleted and injected s.c. into the footpads under anesthesia. The viable bacterial counts in the footpads were followed using IVIS50

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bioluminescence imaging (Xenogen/Caliper Life Sciences) at time points 10 min, 30 min, 2 h, 4 h, 6 h, 8 h and 24 h.

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Abbreviations: **HEV:** high endothelial venules · **PS:** phosphatidyl serine

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