Analysis of CD97 Expression and Manipulation: Antibody Treatment but Not Gene Targeting Curtails Granulocyte Migration

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Analysis of CD97 Expression and Manipulation: Antibody Treatment but Not Gene Targeting Curtails Granulocyte Migration

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The heptahelical receptor CD97 is a defining member of the EGF-TM7 family of adhesion class receptors. In both humans and mice, CD97 isoforms are expressed with variable numbers of tandemly arranged N-terminal epidermal growth factor-like domains that facilitate interactions with distinct cellular ligands. Results from treatment of mice with mAbs in various disease models have suggested a role for CD97 in leukocyte trafficking. Here, we aimed to thoroughly characterize the expression profile of CD97, and delineate its biological function. To this end, we applied a novel polyclonal Ab, which is the first antiserum suitable for immunohistochemistry, and combined this analysis with the study of Cd97-lacZ knock-in mice. We show that similar to the situation in humans, hematopoietic, epithelial, endothelial, muscle, and fat cells expressed CD97. Despite this broad expression pattern, the Cd97−/− mouse that we created had no overt phenotype, except for a mild granulocytosis. Furthermore, granulocyte accumulation at sites of inflammation was normal in the absence of CD97. Interestingly, application of CD97 mAbs blocked granulocyte trafficking after thioglycollate-induced peritonitis in wild-type but not in knock-out mice. Hence, we conclude that CD97 mAbs actively induce an inhibitory effect that disturbs normal granulocyte trafficking, which is not perturbed by the absence of the molecule. The Journal of Immunology, 2008, 181: 6574–6583.

The adhesion long N-terminal extracellular region-containing clade B seven-transmembrane (LNB-TM7) class of heptahelical receptors comprises 33 members in humans (1, 2). These molecules are characterized by a long N-terminal part that is linked by a stalk to a TM7 region. A structural hallmark is the presence of a proteolytic G protein-coupled receptor-processing site that gives rise to autocatalytic processing of the polypeptide into an extracellular α subunit and a TM7 region or β subunit (3). Non-covalent association of these subunits leads to expression at the cell surface as heterodimeric glycoprotein. The extracellular N-terminal part of various receptors contains many different functional protein domains, which are thought to provide cell–cell or cell–matrix contacts. A subfamily of the adhesion class of receptors is the EGF-TM7 family, in humans consisting of CD97 and epidermal growth factor (EGF) module-containing mucin-like hormone receptor (EMR) 1 through 4 (4).

Human CD97 (hCD97) was first identified and cloned as a lymphocyte activation-induced protein (5, 6). Later analyses revealed its presence on many leukocytes with highest expression levels found on myeloid cells, like macrophages, monocytes, and granulocytes (7). In addition, smooth muscle cells and various epithelial tumors express hCD97 (8–11). Through the process of alternative splicing, three isoforms of the β subunit of CD97 can be formed, which differ in the number of EGF-like repeats. In humans, they are designated as hCD97(EGF1,2,5), hCD97(EGF1,2,3,5), and hCD97(EGF1,2,3,4,5) (12). In mice, the longest isoform contains four EGF domains interrupted by a domain of unknown origin, called mouse CD97 (mCD97) (EGF1,2,X,3,4). The two other isoforms are mCD97(EGF1,2,3,4) and mCD97(EGF1,2,4) (13, 14). The EGF and transmembrane domains of CD97 are highly conserved between humans and mice.

To date, three ligands have been identified for CD97. The first one is CD55 (also known as decay-accelerating factor), which binds to the small CD97 isoform(s) (13–18). CD55 is a GPI-linked membrane protein, which plays a role in protection from complement-mediated attack and lysis (19). The second known ligand is chondroitin sulfate, a glycosaminoglycan, which binds specifically to the large isoform(s) of CD97 and affects cell attachment...
(20–22). The third ligand for CD97 is the αβ1 integrin. This ligand binds via the Arg-Gly-Asp motif in the stalk region of hCD97 (23), which is not found in mCD97 (14).

Due to its unusual structure and different cellular ligands, a role for CD97 in migration and adhesion has been proposed. By making use of mAbs specific for different EGF domains, interaction between CD97 and its ligands, CD55 and chondroitin sulfate, and their role in migration of immune cells was studied. In a mouse model of pneumococcal pneumonia, as well as experimental colitis, migration of neutrophils was inhibited (24). In addition, mCD97 mAbs ameliorated collagen-induced arthritis (see Ref. 25 and J. Dulos and B. Owens, unpublished observation) and impaired IL-8-induced stem cell mobilization (26). Genetic models for CD97 became available only recently. A CD97-deficient mouse developed by Kelly and coworkers displayed an increased resistance to systemic infection by Listeria monocytogenes, possibly caused by a mild granulocytosis (27). Unexpectedly, however, granulocyte recruitment to the peritoneum in response to inflammatory stimuli was normal in this mouse. The different consequences of gene targeting and Ab treatment raised questions about the biological function of CD97 and its specific role in leukocyte trafficking.

Thus, we set out to perform a rigorous analysis of the CD97 expression pattern and to delineate its biological function. To this end, we applied immunohistochemistry using a novel polyclonal Ab against mCD97, in combination with a Cd97-LacZ knock-in mouse, showing CD97 to be widely expressed. Surprisingly, despite this broad expression pattern, analysis of the Cd97<sup>−/−</sup> mice showed very limited phenotypic changes compared with wild-type mice. Importantly, direct comparison of mAb treatment in Cd97<sup>−/−</sup> mice was initially initiated at the Leiden University Medical Center (Leiden, The Netherlands) and the colony is currently maintained at the Academic Medical Center (Amsterdam, The Netherlands). Cd97<sup>−/−</sup> lacZ (Cd97<sup>tm1Dgen</sup> on http://www.informatics.jax.org/external/kol/deltagen/867) mice in which a bacterial lacZ gene was inserted together with a nuclear localization signal into Cd97, such that the endogenous gene promoter drives expression of β-galactosidase (generated by Deltagen) were obtained from The Jackson Laboratory. This strain is housed at the Medical Experimental Center, University of Leipzig. All animal work was approved by the Animal Ethics Committees of our institutions.

**Immunological reagents**

Biotinylated hamster anti-mCD97 mAbs from the hybridomas 1A2 and 1B2 (14, 24) were produced in house. For Western blot and immunohistochemistry, a polyclonal Ab, produced in goats immunized with purified recombinant mCD97(EGF1,2,X,4,5) (amino acids 24 to 477), was obtained from R&D Systems. This Ab detects the α-chain of mCD97. Furthermore, for Western blot, a rabbit-anti-mCD97 polyclonal Ab was raised against the synthetic peptide TSQTRALRSSESG corresponding to the C-terminal intracellular part of the β-chain. Both polyclonal Abs were affinity-purified using their respective Ag.

**Flow cytometry**

Peripheral blood was collected in 5 mM EDTA or heparin by heart puncture. Single cell suspensions of spleen and thymus were made by smashing the organs through a 70 μm cell strainer. Bone marrow cells were harvested from dissected femurs by flushing the bone marrow plug with PBS/0.5% BSA. Erythrocytes were lysed with a buffer containing 155 mM ammonium chloride, 10 mM potassium bicarbonate, and 1 mM EDTA in all these cell preparations, and 5 × 10⁶ cells were used per staining. Unspecific binding of mAbs was blocked by adding 10% normal mouse serum and 1.25 μg/ml anti-CD16/32, together with the appropriately diluted mAbs. Flow cytometric analysis was performed using a FACS Calibur (BD Biosciences) and the FlowJo software package (Tree Star).

**Western blotting**

Total lysates of spleen, lung, heart, and liver were prepared in tissue protein extraction reagent (T-PER; Perbio) and size fractionation was performed using equal amounts of protein on 12% SDS-PAGE gel. Proteins were transferred by semidry blotting to nitrocellulose membranes, and CD97 was detected by incubation with polyclonal Abs overnight, followed by HRP–conjugated secondary Ab and ECL reaction (SuperSignal West Pico detection kit; Perbio). Specific recognition of the β-chain-directed mCD97 Ab was confirmed by blocking with the peptide used for immunization.

**Immunohistology**

Dissected tissues were embedded in Tissue Tek (Sakura Finetek) and snap frozen in the vapor phase of liquid nitrogen. Six-μm cryostat sections were dried overnight on silicon gel and fixed in acetone in the presence of 0.03% H₂O₂ to quench endogenous peroxidase activity.

For CD97 staining, cryostat sections were treated with a biotin blocking system (DakoCytomation) to reduce background staining by endogenous biotin and avidin. Sections were incubated with 5% normal rabbit serum, followed by the α-chain-specific polyclonal mCD97 Ab overnight. Then sections were incubated with a biotinylated rabbit-anti-goat Ab and streptavidin-conjugated HRP (DakoCytomation), and staining was visualized with diaminobenzidin substrate and counterstained with hematoxalin. For immunofluorescence staining, the α-chain-specific polyclonal mCD97 Ab was detected with a rabbit-anti-goat-Alexa Fluor 564 F(ab′)₂ (Invitrogen), followed by an incubation with 10% normal goat serum. The second primary Ab was visualized with goat-anti-rat-Alexa 488 F(ab′)₂, or chicken-anti-mouse-Alexa-488 F(ab′)₂ (Invitrogen).

**Materials and Methods**

**Generation of Cd97-gene targeted mice**

To construct a targeting vector, an 8.5-kb HindIII fragment of the murine Cd97 gene spanning intron 1 to exon 12 was isolated and cloned into pBluescript. This vector and the vectors pBS-loxP-PGKneo-loxP and pV-loxP were provided by Dr. J. Jonkers (Netherlands Cancer Institute, Amsterdam, The Netherlands). The targeting vector was generated by a two-step procedure (Fig. 1A), in which the loxP-PGKneo-loxP cassette was inserted into an EcoRI site in exon 1, followed by the insertion of a third loxP site, derived from pV-loxP, into an XbaI site in exon 5. Accuracy of the construct was verified by restriction analysis and sequencing.

The targeting construct was linearized by XhoI digestion and electroporated into 129/Ola-derived embryonic stem (ES) cells following standard procedures. These cells were selected for 8 days with G418, and PCR analysis was used to screen G418-resistant ES cell clones for the occurrence of homologous recombination. First, 5′-homologous recombination was confirmed by PCR, using a forward primer located 18 bp upstream of the targeting construct (5′-GGCAGAGAGGGCTTGTCTCTGC-3′) and a reverse primer located within the neomycin cassette (5′-GCTGAGCTA AACTCCCTTTCA-3′); PCR1 in Fig. 1, A and B). Second, presence of the third loxP site was verified with two independent primer combinations (forward: 5′-CTGGGATATTGATAGCTGCCGCTG-3′ or 5′-CTTCCCCT ATATCTACCTTGATACG-3′; the underlined sequence in the latter primer is the inserted loxP site; reverse: 5′-GTCGGAGGCTCCA CGGAGTTCTC-3′; PCR2 and PCR3 in Fig. 1, A and B). This led to the isolation of targeted clone IIH10. ES cells of clone IIH10 were microinjected into C57BL/6 blastocysts and chimeric offspring was mated with C57BL/6J mice (Charles River, Maastricht, The Netherlands). Germline transmission resulted in agouti offspring generated at the Leiden University Medical Center (Leiden, The Netherlands) and the colony is currently maintained at the Academic Medical Center (Amsterdam, The Netherlands). Cd97<sup>−/−</sup> lacZ (Cd97<sup>tm1Dgen</sup> on http://www.informatics.jax.org/external/kol/deltagen/867) mice in which a bacterial lacZ gene was inserted together with a nuclear localization signal into Cd97, such that the endogenous gene promoter drives expression of β-galactosidase (generated by Deltagen) were obtained from The Jackson Laboratory. This strain is housed at the Medical Experimental Center, University of Leipzig. All animal work was approved by the Animal Ethics Committees of our institutions.

**Generation of Cd97-gene targeted mice**

**Generation of Cd97-gene targeted mice**
For Ly6G staining, lungs were incubated for 24 h in 4% formaldehyde and embedded in paraffin. Four-μm sections were deparaffinized, endogenous peroxidase activity was blocked with 0.3% H2O2 in methanol, and slides pretreated with 0.025% pepsin in 0.1 M HCl for 15 min at 37°C. After incubation in 10 nM Tris, 5 mM EDTA, 0.15 M NaCl, 0.25% gelatin, and 0.05% Tween 20 for blocking, slides were incubated with anti-Ly-6G-FITC for 1 h. Subsequently, slides were incubated with rabbit-anti-FITC (DakoCytomation) in 5% normal mouse serum in PBS for 30 min. Staining was visualized with diamino-benzidin and counterstained with methyl green.

**Histological detection of β-galactosidase activity**

Tissues from heterozygous Cd97-lacZ mice were fixed in 2% paraformaldehyde (PFA), 2 mM MgCl2, 1.25 mM EDTA in PBS in 5 h, cryoprotected in 15% and 30% sucrose in PBS with 2 mM MgCl2 for 4 and 12 h respectively, and shock-frozen in 2-methylbutane at −20°C. Before staining, 4-μm cryostat sections were postfixed in 2% PFA for 5 min, rinsed in PBS containing 2 mM MgCl2 for 10 min and PBS containing 2 mM MgCl2, 0.01% sodium deoxideycholate, and 0.02% Nonidet P-40 for 10 min. Expression of β-galactosidase was visualized by staining at 37°C for 16 h in 1 mg/ml 5-bromo-4-chloro-3-indoly β-galactopyranoside (X-Gal; Sigma-Aldrich), 5 mM K4Fe(CN)6, 5 mM K3Fe(CN)6, 2 mM MgCl2, 0.01% sodium deoxideycholate, and 0.02% Nonidet P-40 in PBS. Subsequently, sections were counterstained with nuclear fast red.

**Histopathology**

Four-month-old Cd97−/− mice (3 females and 3 males) were sacrificed by CO2 asphyxiation and perfused with 10 ml 4% formalin, or a fixative containing anti-rabbit-poly-HRP (Immunologics) in PBS for 30 min. Stained sections were postfixed in 2% PFA for 5 min, rinsed in PBS containing 2 mM MgCl2 for 10 min and PBS containing 2 mM MgCl2, 0.01% sodium deoxideycholate, and 0.02% Nonidet P-40 for 10 min. Expression of β-galactosidase was visualized by staining at 37°C for 16 h in 1 mg/ml 5-bromo-4-chloro-3-indoly β-galactopyranoside (X-Gal; Sigma-Aldrich), 5 mM K4Fe(CN)6, 5 mM K3Fe(CN)6, 2 mM MgCl2, 0.01% sodium deoxideycholate, and 0.02% Nonidet P-40 in PBS. Subsequently, sections were counterstained with nuclear fast red.

**Results**

**Generation of the Cd97 knock-out mouse**

The murine Cd97 gene consists of 20 exons spanning ~17.8 kb of DNA (http://www.ensembl.org). To generate mice with a dysfunctional Cd97 gene, we made use of a Cre/loxP-mediated DNA recombination technique. This approach was chosen to allow for conditional inactivation of Cd97 in a later phase. A targeting construct was generated in which a loxP-PGKneo-loxP cassette was inserted upstream of exon 2 and a third loxP site was placed downstream of exon 5 (Fig. 1A). Recombination between the outer loxP sites eliminates exon 2 to 5, resulting in removal of the exons that encode the signal peptide and the first two, CD55-binding EGF domains (24). Furthermore, this recombination leads to the introduction of a frame shift.

129/Ola-ES cells were electroporated with the linearized targeting construct and selected with G418. Of the 192 G418-resistant clones picked, 7 were found to contain a 5′-homologously recombined targeting construct. One of these clones (IIH10) also contained the third loxP site (Fig. 1B). This clone was injected into C57BL/6J-derived blastocysts to generate chimeric offspring, which were mated with C57BL/6J mice. In 14 of 33 agouti progeny, germline transmission of the targeted allele was found. To induce inactivation of Cd97, the mice were bred with the “deleter” strain, in which the promoter of the EIIa gene drives ubiquitous expression of Cre-recombinase during early embryogenesis (28). Cre-mediated recombination was found in 35 of 56 progeny with a targeted Cd97 allele. In 7 of these 35 mice, recombination had occurred between the outer loxP sites (Δ1–3), resulting in deletion of the gene. In addition, we obtained 3 Δ1–2 mice, no Δ2–3 mice, and 25 mice with a “mosaic” genotype. Cd97-deficient mice were crossed back on a C57BL/6J background and after four (F4), eight (F8), and twelve (F12) generations, homozygous colonies were established for experimental usage. All experimental data presented here are verified at least once in F8 mice.

To verify the successful deletion of the Cd97 gene, we tested the expression of Cd97 on leukocytes by flow cytometry (Fig. 1C). Spleenocytes from Cd97−/− and wild-type littermate control mice were stained for Cd97 and lineage markers CD11b (myeloid cells), Gr-1 (monocytes and granulocytes), F4/80 (macrophages), B220 (B cells), and CD3 (T cells). No expression of Cd97 was found on immune cells of Cd97−/− mice, whereas myeloid cells, B cells, and T cells of wild-type mice showed various intensities of Cd97 staining.

To further confirm that expression of both Cd97 subunits was abrogated in the Cd97−/− mouse, we analyzed lysates from various organs on Western blot with chain-specific polyclonal Abs (Fig. 1D). A specific signal for CD97 β-chain with a molecular mass of ~28 kDa was obtained from lysates of lung, spleen, and heart. Using the Ab directed against the α-chain of CD97, we obtained specific signals in lung and spleen lysates, where two α-chains with a molecular mass of 75 and 80 kDa were detectable. In contrast, tissue lysates from Cd97-deficient mice showed no specific signals confirming the absence of the protein in these animals.

**Mouse Cd97 is expressed on a wide variety of hematopoietic and non-hematopoietic cells**

To enable a rational analysis of the biological function of Cd97, we first unraveled the tissue distribution of mCD97. In accordance with the results obtained by Western blotting (Fig. 1D), quantification of transcript levels in mouse tissues by real-time PCR revealed highest amounts of Cd97 mRNA in spleen and lung.
whereas lower levels were found in heart, liver, kidney, and brain.

To study the expression pattern of CD97, we performed immuno-histochemistry in wild-type mice and compared the staining with β-galactosidase expression in heterozygous Cd97-lacZ mice. Cd97+/− mice were used as negative controls. All data described below are shown in Figs. 2 and 3 and summarized in Table I. As reported before (14), we could show CD97 expression in several hematopoietic cell lineages. In the spleen, CD97 expression was widespread, found predominantly on leukocytes present in both the white and red pulp (Fig. 2, a and b). In the thymus, expression of CD97 was observed in nearly all T cells; however, expression was lower in cells in the medulla as compared with those present in the cortex (Fig. 3a). Tissue-localized F4/80+ macrophages also expressed CD97, shown here for Kupffer cells in the liver (Fig. 3b). Overall, CD97 was expressed in subsets of tissue-localized hematopoietic cells throughout the organism, including granulocytes, monocytes/macrophages, dendritic cells, B cells, T cells, and NK cells.

Like in humans, expression of CD97 in mice was not restricted to hematopoietic cells. CD97 was also detected in endothelial and epithelial cells. In liver, CD97 expression was found to colocalize with a subpopulation of CD31+ endothelial cells (data not shown).

In the lung, most pneumocytes and epithelial cells of the respiratory tracheal and bronchial epithelium labeled strongly. Endothelial cells within a portion of lung vessels also expressed CD97 (Fig. 2, c and d, and Fig. 3, c–e). In the kidney, very strong expression of CD97 was seen in part of the cells forming the glomeruli (Fig. 2, e and f). Colocalization with desmin and lack of CD31 expression suggested these cells are most likely podocytes or renal mesangial cells (Fig. 3f and data not shown). In addition, CD97 expression was evident in arterial, arteriolar, and capillary intertubular endothelium, as shown by co-staining with CD31 in the medulla of the kidney (data not shown) and in a portion of duct cells and intralobular small vessels in the pancreas (Fig. 2r). Epithelial cells of the ampulla of the oviduct (data not shown) and a small subset of seminiferous tubule epithelium and interstitial cells, probably Leydig cells, in testis stained very strongly for CD97 (Fig. 2r). Furthermore, epithelial cells of the epididymis labeled strongly for CD97 in their apical region, as shown by co-staining with EpCAM (Fig. 2r and Fig. 3g). Finally, expression of CD97 was seen in CD31+...
small vessels within the myocard (Fig. 2g (arrow) and Fig. 3h), where cardiomyocytes also expressed moderate levels of CD97 (Fig. 2, g and h (arrow)).

Different types of muscle cells were shown to express CD97 apart from cardiomyocytes. In lung, there was a little but significant expression of CD97 on smooth muscle cells associated with bronchi as well as larger vessels (data not shown). In addition, smooth muscle cells of the tunica muscularis of the small and large intestines were slightly positive for CD97. CD97 expression was also detected in smooth muscle cells of the myometrium (Fig. 2, k and l) and the muscular coat of the urinary bladder (Fig. 2q). In the ovary, theca cells surrounding the follicles expressed CD97 (Fig. 2, m and n). These cells did not co-stain for CD31 (data not shown). Furthermore, CD97 is very likely expressed in skeletal muscle cells of the gastrointestinal tract expressed CD97, here shown in the large intestine (i). Additionally, cells of the lamina propria mucosae in the large intestine (i and j) were labeled in both mice. The same was true for smooth muscle cells of the myometrium in the uterus (k and l). Within the ovary (m and n), theca cells surrounding the follicles were CD97\(^-\). The urothel within the bladder was CD97\(^-\), but the Ag was detectable in the lamina propria mucosae and the muscular coat. Strongly stained cells (arrow) were present within the lamina propria mucosae and the tela submucosa (q). Subsets of pancreatic duct and endothelial cells (arrow) stained for CD97 (r). \(\beta\)-galactosidase activity was detectable in the male reproductive system in basal sitting cells of the tubuli seminiferi and few interstitial cells (s) and in epididymidal cells (t). Finally, fat cells were CD97\(^-\) (o and p). Data represent typical results of six mice analyzed (3 males and 3 females). Scale bar a–f and i–t, 50 \(\mu\)m; g and h, 25 \(\mu\)m.

**FIGURE 2.** Expression analysis of murine CD97. Representative staining patterns of CD97 (a, c, e, g, i, k, m, o, q, r; brown staining with blue hematoxalin counterstaining) and nuclear \(\beta\)-galactosidase (b, d, f, h, j, l, n, p, s, t; blue staining with pink nuclear fast red counterstaining) in tissues from wild-type and Cd97-lacZ mice, respectively. In spleen (a and b), stained subsets of several leukocyte subtypes were found throughout the organ in both red and white pulp. Centrocytes and centroblasts were mainly CD97\(^-\). In the lung (c and d), most pneumocytes and bronchial epithelial cells (arrow) were positive. In the kidney (e and f), cells located in the glomerula strongly expressed CD97. Heart muscle cells and the endothelium of smaller vessels in the heart (arrow, g and h) slightly expressed the molecule. The muscular coat of the gastrointestinal tract expressed CD97, here shown in the large intestine (i). Additionally, cells of the lamina propria mucosae in the large intestine (i and j) were labeled in both mice. The same was true for smooth muscle cells of the myometrium in the uterus (k and l). Within the ovary (m and n), theca cells surrounding the follicles were CD97\(^-\). The urothel within the bladder was CD97\(^-\), but the Ag was detectable in the lamina propria mucosae and the muscular coat. Strongly stained cells (arrow) were present within the lamina propria mucosae and the tela submucosa (q). Subsets of pancreatic duct and endothelial cells (arrow) stained for CD97 (r). \(\beta\)-galactosidase activity was detectable in the male reproductive system in basal sitting cells of the tubuli seminiferi and few interstitial cells (s) and in epididymidal cells (t). Finally, fat cells were CD97\(^-\) (o and p). Data represent typical results of six mice analyzed (3 males and 3 females). Scale bar a–f and i–t, 50 \(\mu\)m; g and h, 25 \(\mu\)m.
muscle, as β-galactosidase is weakly present in the nuclei. However, staining for CD97 failed in immunohistology (data not shown).

In contrast to humans, mCD97 expression was found in the lamina propria mucosae of several organs. In most cases, the expression was weak or moderate; like in the urinary bladder (Fig. 2q) and the large intestine (Fig. 2, i and j). These cells usually did not express F4/80 or CD31 and most likely made up the connective tissue. In the urinary bladder, strong CD97 expression was also found in a small number of single cells or cell groups located within the lamina propria of the urinary bladder (Fig. 2q, arrow). These cells may represent endothelial cells and macrophages because co-staining with F4/80 and CD31 was seen (data not shown).

Unexpectedly, we found that fat cells strongly expressed CD97 (Fig. 2, o and p). Both, s.c. and visceral fat was found to be CD97+. A careful examination of various human tissues revealed that CD97 also resides in human fat cells (G. Aust and S. Becker unpublished observation). Together, CD97 showed a broad tissue distribution in mice that largely resembled the situation in humans.

To further study the hematopoietic compartment of Cd97−/− mice, we isolated peripheral blood leukocytes, splenocytes, thymocytes, and bone marrow cells from 10- to 16-wk-old F4 Cd97−/− and wild-type littermate control mice. Cells were stained for CD11b, Gr-1, F4/80, B220, CD3, CD4 (CD4+ T cells), CD8 (CD8+ T cells), and CD97 and analyzed by flow cytometry. Absolute numbers were calculated from percentage of lineage marker-positive cells and live cell count harvested from specific organs. For the most part, no differences were found in percentages or absolute numbers of immune cells in Cd97−/− mice, although we did observe a higher frequency of granulocytes in circulation in Cd97−/− animals (Fig. 4A). These findings lead us to perform an extensive analysis of the myeloid compartment in F8 and F12 Cd97−/− mice. Indeed, an increased percentage of circulating granulocytes was found in ~40% of Cd97−/− animals (p = 0.002). Cd97−/− mice also had increased numbers of granulocytes in spleen (p = 0.001), but not in bone marrow (Fig. 4B). Similar results were found in 15- to 16-mo-old mice (data not shown). We concluded that CD97 is not involved in the development of the immune cell compartments tested, but may be involved in the homeostasis or distribution of granulocytes.

Leukocyte trafficking is affected by CD97 mAbs but not by CD97 deficiency

Because the Cd97−/− mice showed no overt phenotype at the steady state except for a mild granulocytosis, we decided to analyze the behavior of these animals in models of inflammation. First, we subjected Cd97−/− mice to S. pneumoniae-induced pneumonia, a model system of lung inflammation. Previously, we showed that pneumonia was aggravated by prophylactic treatment of wild-type mice with CD97-specific mAbs, which was attributed to blocking migration of granulocytes into the inflamed lungs (24).
However, in lungs, blood, or spleen of wild-type and \( Cd97^{-/-} \) mice, no differences in bacterial outgrowth were seen at either time-point tested (24 and 44 h) (Fig. 5A and data not shown). Furthermore, there was no effect on the accumulation of phagocytic cells as indicated by myeloperoxidase levels in lung homogenates (Fig. 5B) and numbers of Ly6G\(^+\) granulocytes in lung tissue (data not shown). Thus, absence of CD97 did not affect the

### Table I. \( CD97 \) expression in wild-type and \( Cd97\text{-LacZ} \) mice

<table>
<thead>
<tr>
<th>Organ System/Cell Type</th>
<th>( CD97 ) Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td>Macrophages, granulocytes subsets ++/+ + +</td>
</tr>
<tr>
<td>B, T, and NK cells subsets +/+ +</td>
<td></td>
</tr>
<tr>
<td>Thymus</td>
<td>Cortical CD4(^+)/CD8(^-) thymocytes +</td>
</tr>
<tr>
<td>Medullary CD4(^+) or CD8(^-) T cells subset +</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>Hepatocytes –</td>
</tr>
<tr>
<td>Kupffer cells subset ++</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>Bronchial epithelium large subset +++</td>
</tr>
<tr>
<td>Bronchial smooth muscle ++/+ +</td>
<td></td>
</tr>
<tr>
<td>Pneumocytes large subset +++</td>
<td></td>
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<tr>
<td>Pulmonary vessels, EC some ++</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>Podocytes/or mesangial cells +++</td>
</tr>
<tr>
<td>Glomerular EC –</td>
<td></td>
</tr>
<tr>
<td>Proximal and distal tubules –</td>
<td></td>
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<tr>
<td>Collecting ducts –</td>
<td></td>
</tr>
<tr>
<td>Vessels, EC subset ++</td>
<td></td>
</tr>
<tr>
<td>Pancreas</td>
<td>Exocrine epithelial cells –</td>
</tr>
<tr>
<td>Islets of Langerhans –</td>
<td></td>
</tr>
<tr>
<td>Vessels, capillaries, EC subset ++</td>
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<td>Ductal epithelial cells subset ++</td>
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<td>Stomach</td>
<td>Lamina propria mucosae ++</td>
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<tr>
<td>Tunica muscularis +</td>
<td></td>
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<tr>
<td>Small and large intestine</td>
<td>Epithelium –</td>
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<tr>
<td>Paneth cells +</td>
<td></td>
</tr>
<tr>
<td>Lamina propria mucosae +++</td>
<td></td>
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<tr>
<td>Tunica muscularis ++/+ +</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>Cardiomyocytes +</td>
</tr>
<tr>
<td>Myocard capillaries, EC some ++</td>
<td></td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>IHC –, ( \beta)-galactosidase (( \beta)-gal) activity was analyzed in 3-mo-old ( Cd97\text{-lacZ} ) mice (3 males and 3 females). Comparable results were obtained with both methods if not indicated otherwise. Scores are estimates. –, –, –, +, +, and ++ indicate no, slight, moderate, and strong staining, respectively. EC, endothelial cells.</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>Epithelial cells (urothel) –</td>
</tr>
<tr>
<td>Lamina propria mucosae ++/+ +</td>
<td></td>
</tr>
<tr>
<td>Tunica muscularis +</td>
<td></td>
</tr>
<tr>
<td>Uterus</td>
<td>Endometrium –</td>
</tr>
<tr>
<td>Myometrium +/+ +</td>
<td></td>
</tr>
<tr>
<td>Ovary</td>
<td>Follicles, corpus luteum –</td>
</tr>
<tr>
<td>Theca cells subset ++</td>
<td></td>
</tr>
<tr>
<td>Oviduct</td>
<td>Ampulla, epithelial cells large subset +++</td>
</tr>
<tr>
<td>Testis</td>
<td>Seminiferous tubules few cells ++</td>
</tr>
<tr>
<td>Interstitial cells few cells ++</td>
<td></td>
</tr>
<tr>
<td>Capillaries some ++</td>
<td></td>
</tr>
<tr>
<td>Epididymis</td>
<td>Epithelial cells +++</td>
</tr>
<tr>
<td>Fat</td>
<td>Adipocytes ++/+ + +</td>
</tr>
</tbody>
</table>

\( ^a \) Immunohistochemistry (IHC) was performed in 3-mo-old wild-type mice (3 males and 3 females). \( \beta\)-galactosidase (\( \beta\)-gal) activity was analyzed in 3-mo-old \( Cd97\text{-lacZ} \) mice (3 males and 3 females). Comparable results were obtained with both methods if not indicated otherwise. Scores are estimates. –, –, –, +, +, and ++ indicate no, slight, moderate, and strong staining, respectively. EC, endothelial cells.
FIGURE 5. Models of inflammation show that leukocyte trafficking is affected by CD97 mAbs but not by CD97 deficiency. A, Outgrowth of pneumococcal bacteria was not different in CD97−/− compared with wild-type mice at either 24 or 44 h after intranasally inoculation with 8 × 10⁴ CFU S. pneumoniae. Bars indicate median bacterial counts (n = 8 in all groups). B, Concentration of myeloperoxidase in lung homogenates of wild-type and CD97−/− mice at 24 or 44 h after pneumococcal bacteria infection was not different. Indicated is the mean ± SEM of the concentration of myeloperoxidase in lung homogenates (n = 8 in all groups). C, Recruitment of granulocytes into the peritoneum 4 h after i.p. injection of 1 ml 4% thioglycollate was not different in CD97−/− compared with wild-type mice. Indicated is the mean ± SD of the percentage granulocytes in peritoneal exudates. Graphs represent pooled data from four independent experiments (n = 11–13). D, CD97-specific mAb treatment had no effect on the granulocyte compartment in the circulation of either wild-type or CD97−/− animals. Indicated is the mean ± SD of the percentage granulocytes in peripheral blood one day after i.v. injection of 250 µg mAb per animal (n = 3–4). E, Treatment with CD97-specific mAbs of wild-type animals, but not CD97−/− mice, resulted in significant inhibition of granulocyte recruitment to the peritoneum, 4 h after i.p. injection of thioglycollate. The difference between control (3D7; anti-human EMR3) and the mAb to the first EGF domain (1B2) showed the strongest inhibition, whereas the inhibition with the mAb raised against the third EGF domain (1C5) was somewhat less. Indicated is the mean ± SD of the percentage granulocytes in peritoneal exudates (n = 3–4). One of two independent experiments is shown. * p < 0.05; *** p < 0.0005.

early immune response to pneumococcal pneumonia as compared with our previous data using Ab treatment, showing a robust and unambiguous result (24).

To test whether lack of CD97 can alter leukocyte trafficking in an independent model, we chose to compare CD97−/− and wild-type mice in thioglycollate-induced sterile peritonitis, an established model for leukocyte recruitment upon an inflammatory stimulus. At steady state, the number of granulocytes is normally very low and was unaffected in CD97−/− animals (7.6 vs 9.1 × 10³ for wild-type and CD97−/−, respectively; p = 0.53). Four hours after thioglycollate injection, there were no differences between wild-type and CD97−/− mice in granulocyte migration to the peritoneum (Fig. 5C). Furthermore, monocytes/macrophage accumulation, assessed 48 h after thioglycollate injection, was also not affected by the loss of CD97 (83.6% vs 87.5% for wild-type and CD97−/−, respectively; p = 0.52; n = 2–3). Prophylactic Ab treatment was never studied in this model, so we performed thioglycollate-induced peritonitis in either wild-type or CD97−/− mice, injected with control mAb (3D7, raised against human EMR3) or two different mAbs that recognize either EGF domain 1 (1B2) or EGF domain 3 (1C5) of mCD97, 1 day before peritonitis induction. In this experiment, mAbs had no effect on the percentages of granulocytes present in blood (Fig. 5D). Furthermore, no evidence of increased activation or apoptosis could be found after application of 1B2 in vivo (data not shown). However, treatment of wild-type mice with CD97-specific mAbs 1B2 or 1C5 (but not the control mAb 3D7) reduced granulocyte recruitment to the peritoneum at least 2-fold, whereas CD97−/− mice again showed no significant differences whatsoever (Fig. 5E). Interestingly, the most potent inhibition in wild-type animals was seen with 1B2, raised against the first EGF domain, which is present in all CD97 isoforms. The less effective 1C5 recognizes the third EGF domain, which is only present in the two largest isoforms and thus only in part of the total amount of mCD97 protein (13, 14). Because CD97 is widely expressed in the body, we are currently investigating whether Ab treatment against CD97 on immune cells affects granulocyte migration into the peritoneum. Preliminary data (not shown), obtained from thioglycollate-induced peritonitis in bone marrow chimeras of wild-type and CD97−/− mice, revealed that CD97 indeed needs to be present on immune cells for 1B2 to perform its action. Together, we conclude that Ab treatment to CD97 results in a potent inhibition of granulocyte migration, whereas lack of CD97−/− has no discernible effect on granulocyte recruitment.

Discussion

CD97 is the evolutionary oldest and most conserved member of the EGF-TM7 family (J. Hamann, unpublished observation) and it can be anticipated that it possesses a function that is archetypical for this subfamily of adhesion class heptahelical receptors. As yet, the application of mouse models for the investigation of CD97 was hampered by uncertainties about the tissue distribution of mCD97. Existing hamster mAbs confirmed the presence of mCD97 on various subsets of leukocytes by flow cytometry (14) but unanimously failed to visualize expression by immunohistology. Applying a new polyclonal Ab, we show here that endothelial, epithelial, smooth and heart muscle, and fat cells also express mCD97. At most sites, results obtained by immunohistochemistry correlated
well with β-galactosidase activity in the Cd97-lacZ mouse. A possible explanation for the failure of the polyclonal Ab to detect mCD97 on skeletal and (partially) smooth muscle might be a different posttranslational modification of CD97 in these cells. Likewise, we previously showed that hCD97 on normal smooth muscle cells is not N-glycosylated and consequently is not recognized by certain mAbs (30). Together, the overall comparability between CD97 expression in humans and mice implies that the mouse is a suitable model to study the biological function of CD97 at the different sites of expression. In addition, the evolutionary conserved broad expression suggests that the role of CD97 might not be restricted to immunity.

The Cd97−/− mouse analyzed in this study had no overt phenotype when challenged, except for a mild granulocytosis. Next to basic parameters like weight and size, breeding, and life span, our analyses included a thorough anatomical survey of the architecture of essentially all organs as well as a detailed investigation of the hematopoietic compartment. Our findings confirm and extend results recently obtained by Kelly and coworkers from an independently generated Cd97−/− mouse (27). Reportedly, this mouse was better protected against an acute infection with L. monocytogenes. It was hypothesized that this, at least partly, was due to the mild granulocytosis. Although this slight elevation of granulocyte numbers in blood was also observed in our Cd97−/− mouse, we could not find any evidence of improved antibacterial host defense in pneumococcal pneumonia. Furthermore, Kelly and coworkers showed that in mixed bone marrow chimeric animals, CD97-deficient and wild-type granulocytes accumulated similarly in blood and peritoneum in response to inflammatory stimuli (27). Also, our Cd97−/− mouse showed no altered migration of granulocytes into the peritoneal cavity following application of thioglycollate. Thus, expression of CD97 is not dispensable for leukocyte trafficking, although we cannot exclude at this point the possibility that other EGF-TM7 receptors compensate its function in the Cd97−/− mouse.

In contrast with the normal leukocyte trafficking seen in the absence of Cd97, mCD97 mAbs profoundly diminish the migration of granulocytes in experimental models of inflammation (e.g., colitis, arthritis, and pneumonia) (24, 25) and in IL-8-induced stem cell mobilization (26). This difference led us to directly compare the consequences of CD97 gene deficiency and Ab application in vivo. In thioglycollate-induced peritonitis, we found that mCD97 mAbs, but not a control Ab, significantly inhibited cell accumulation in wild-type but not Cd97−/− mice. In line with this, host defense against pneumococcal pneumonia is affected by mCD97 mAbs (24) but not by CD97 deficiency. Importantly, application of mCD97 mAbs did not affect the granulocyte compartment itself. Although FcR-mediated effects cannot be fully excluded at this point, our data suggest that the in vivo effects of Ab treatment, next to blocking CD97-ligand interactions, reside in actively inducing an inhibitory effect. Presumably, CD97 mAbs have an agonistic effect or regulate localization of the molecule by facilitating dimerization (31) and/or the formation of complexes with other cell surface proteins. Interestingly, a mAb against the related human EGF-TM7 receptor EMR2 that binds outside the ligand-binding EGF domains was recently shown to facilitate human neutrophil function in vitro (32). In addition, an agonistic polyclonal Ab against the adhesion class receptor GPR56 was reported to inhibit the migration of neural progenitor cells in vitro (33).

The observations discussed here underscore the therapeutic potential of manipulating CD97, because different mAbs apparently can result in similar inhibitory effects on neutrophil migration despite the rather broad tissue distribution of the molecule. Exploration of the consequences of Ab treatment at the cellular and molecular level will help to understand the function of CD97 as a prototypical member of the adhesion class of heptahelical receptors.

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Disclosures
The authors have no financial interest of interest.

References
ization of mouse CD97 and study of its specific interaction with murine decay-
dermal growth factor-like short consensus repeat domain-mediated protein-pro-


