The stromal component in rheumatoid arthritis: CD55 expression, cell death and beyond
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CHAPTER 2

SHEAR STRESS-DEPENDENT DOWNREGULATION OF THE ADHESION-GPCR CD97 ON CIRCULATING LEUKOCYTES UPON CONTACT WITH ITS LIGAND CD55

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ABSTRACT

Adhesion class G protein-coupled receptors (aGPCRs) are two-subunit molecules, consisting of an adhesive extracellular α subunit that non-covalently couples to a seven-transmembrane β subunit. The cooperation between the two subunits and the impact of endogenous ligands on the functioning of aGPCRs is poorly understood. We here investigated the interaction between the pan-leukocyte aGPCR CD97 and its ligand CD55. We found that leukocytes from CD55-deficient mice express significantly increased levels of cell surface CD97 that normalized after transfer into wild-type mice due to contact with CD55 on both leukocytes and stromal cells. Downregulation of both CD97 subunits occurred within minutes after first contact with CD55 in vivo, which correlated with an increase in plasma levels of soluble CD97. In vitro, downregulation of CD97 on CD55-deficient leukocytes, cocultured with wild-type blood cells was strictly dependent on shear stress. In vivo, CD55-mediated downregulation of CD97 required an intact circulation and was not observed on cells that lack contact with the blood stream, such as microglia. Notably, de novo ligation of CD97 did not activate signaling molecules constitutively engaged by CD97 in cancer cells, such as ERK and protein kinase B/Akt. We conclude that CD55 downregulates CD97 surface expression on circulating leukocytes by a process that requires physical forces, but based on current evidence, does not induce receptor signaling. This regulation may restrict CD97–CD55-mediated cell adhesion to tissue sites.
INTRODUCTION

The adhesion class is one of the five classes of G protein-coupled receptors (GPCRs). The human genome encodes 33 adhesion GPCRs (aGPCRs), which are broadly expressed in embryonic and larval cells, cells of the reproductive tract, neurons, leukocytes, and a variety of tumors. Most notable is the molecular structure that sets aGPCRs apart from other GPCRs. Intramolecular processing at a GPCR-proteolytic site (GPS) proximal to the first transmembrane helix gives rise to a seven-transmembrane and an extracellular subunit, which subsequently reassociate non-covalently. Only recently, it became clear that the GPS motif is part of a much larger ~320-residue GPCR autoproteolysis-inducing (GAIN) domain that forms a tightly associated heterodimer upon proteolysis.

The extracellular subunits of aGPCRs can be exceptionally long and contain a variety of structural domains that are known for their ability to facilitate cell-cell and cell-matrix interactions. The first ligand identified was CD55, a widely distributed cell surface molecule regulating complement activity (decay-accelerating factor). CD55 interacts with the N-terminal epidermal growth factor (EGF)-like domains of CD97, an aGPCR broadly expressed by hematopoietic and non-hematopoietic cells. Subsequently identified aGPCR ligands were dermatan sulfate, α5β1 integrin, tissue transglutaminase, phosphatidylserine, lipopolysaccharide, C1q, lasso/teneurin-2, collagen III, and Thy-1/CD90. Evidence was obtained that aGPCRs have a role in cell positioning and tissue organization in various organ systems. Yet, in sensu stricto, aGPCRs are still ‘functional orphans’. The main problem is a lack of understanding of how these atypical GPCRs are activated. For classical GPCRs, ligand binding results in the formation of a transient high-affinity complex of agonist, activated receptor, and G protein. As a consequence, GDP is released from the G protein and replaced by GTP, leading to dissociation of the G protein into an α subunit and a βγ dimer, which both activate several effectors. For aGPCRs, the agonistic potential of most ligands is uncertain. Studies on signal transduction have been done almost exclusively in the absence of agonists and have not unveiled a general mechanism of action. This uncertainty has fostered the idea that aGPCRs may function in a principally different manner from classical GPCRs (5th Workshop on Adhesion-GPCRs, Leipzig 2010).

In this study, we used the CD97–CD55 interaction as a paradigm to explore the consequences of ligation of an aGPCR both in vivo and in vitro. We demonstrate that CD55 contact leads to a continuous downregulation of CD97 on circulating leukocytes that is dependent on physical shear stress and may serve to restrict CD97-mediated cell adhesion to tissue sites. De novo contact of CD97+ leukocytes with CD55 did not appear to induce signaling pathways that were recently shown to be constitutively activated by CD97 in cancers cells. Our findings demonstrate for the first time interaction of an aGPCR with an endogenous ligand in vivo and support the hypothesis that facilitating adhesive contacts may be a prime activity of the extracellular modules of aGPCRs.

MATERIALS AND METHODS

Mice

Mice deficient for CD55 (Cd55<sup>−/−</sup>, synonym: Daf1<sup>−/−</sup>) and CD97 (Cd97<sup>−/−</sup>) have been generated previously by us and backcrossed to C57BL/6 for at least eight generations. Wild-type mice were littermates or were purchased from Charles River (Maastricht, The Netherlands). Congenic
mice expressed \textit{Cd45.1} in the B6.SJL strain. All mice used in this study were matched for age and sex and kept under specific pathogen-free conditions. Experiments were approved by the Animal Ethics Committee of the Academic Medical Center (Amsterdam, The Netherlands).

\textbf{Adoptive transfer}

The equivalent of \(25 \times 10^6\) congenic wild-type (\textit{Cd45.1}) or \textit{Cd55}\(^{-/-}\) (\textit{Cd45.2}) leukocytes, obtained by mashing the spleens under aseptic conditions in sterile PBS through a 70-μm cell strainer, were injected into the tail vein of \textit{Cd55}\(^{-/-}\) or congenic wild-type recipient mice, respectively. To allow tracing of \textit{Cd55}\(^{-/-}\) splenocytes transferred into \textit{Cd55}\(^{-/-}\) mice, cells were labeled prior to the injection with 1 μM 5,6-carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes Europe BV, Leiden, The Netherlands) for 10 min at 37°C. To block coagulation \textit{in vivo}, 20 units/ml heparin (LEO Pharma, Breda, The Netherlands) in PBS was injected into the tail vein of mice 5 min before the injection of \textit{Cd55}\(^{-/-}\) splenocytes. At various time points, blood and spleen from recipient mice were collected and analyzed by flow cytometry and Western blotting for CD97 expression on leukocytes. For Western blot analysis, CFSE-positive transferred leukocytes were sorted on a FACS Aria (BD Biosciences, San Jose, CA, USA) into PBS.

\textbf{Bone marrow-chimeric mice}

Congenic wild-type (\textit{Cd45.1}) and \textit{Cd55}\(^{-/-}\) (\textit{Cd45.2}) recipient mice were γ-irradiated with 5.1 Gy and reconstituted with \(10 \times 10^6\) \textit{Cd55}\(^{-/-}\) or congenic wild-type bone marrow-derived hematopoietic cells, respectively, via tail vein injection. Mice were housed in individually ventilated cages and obtained drinking water containing neomycin during the experiment. After 5 weeks, blood was collected in heparin via heart puncture and analyzed by flow cytometry for CD97 expression on leukocytes.

\textbf{Coculture assays}

To mimic shear stress \textit{in vitro}, 100 μl congenic wild-type or \textit{Cd55}\(^{-/-}\) whole blood was added to \(2.5 \times 10^6\) \textit{Cd55}\(^{-/-}\) or wild-type splenocytes in polypropylene tubes and shaken at RT at 900 rpm or left unagitated. At indicated time points, cells were harvested and analyzed by flow cytometry for CD97 expression. Alternatively, 500 μl congenic wild-type whole blood was mixed with 12.5 \(x\) 10\(^6\) \textit{Cd55}\(^{-/-}\) splenocytes and exposed at 25°C to shear stresses of 1 dynes/cm\(^2\), resembling capillary levels \(^{27}\), or 40 dynes/cm\(^2\), representative for arterial shear stresses in mice \(^{28}\), imposed by a rheometer (MCR 501; Anton Paar, Graz, Austria). After 1 h, cells were harvested and analyzed by flow cytometry for CD97 expression.

To study potential signaling after \textit{de novo} ligation of CD97 by its ligand CD55, 10 \(x\) 10\(^6\) \textit{Cd55}\(^{-/-}\) or wild-type splenocytes were mixed with a leukocyte-free fraction of \textit{Cd55}\(^{-/-}\) or wild-type erythrocytes, obtained by filtering wild-type whole blood diluted in PBS through a filter, containing 2 layers of pre-filter material (#S3AT0006) and 3 layers of intermediate material (#2210) (Fresenius Kabi, Isola della Scala, Italy). After applying shear stress to the mixed cells for 0, 5, 15 or 30 min, erythrocytes were lysed with a buffer containing 155 mM NH\(_2\)Cl, 10 mM KHCO\(_3\), and 1 mM EDTA and the remaining splenocytes were washed in PBS and collected in RIPA buffer (Cell Signaling, Beverly, MA, USA) for biochemical analysis. \textit{Cd55}\(^{-/-}\) splenocytes stimulated with 100 ng/ml phorbol myristate acetate (PMA) for 5 or 15 min were used as positive controls.
Immunological reagents
For flow cytometry, a hamster monoclonal antibody (mAb) against the first EGF domain of CD97 (clone 1B2 29,30) was used, together with an irrelevant hamster control mAb (clone 3C7). Antibodies were biotinylated in house and used in combination with streptavidin-conjugated PeCy7 or APC, and FITC-, PE-, PerCP-Cy5.5-, PeCy7-, APC-, or biotin-conjugated mAbs specific for CD3, CD11b, CD45, CD45.1, CD45.2, CD62L, B220, F4/80, Gr-1, Ly6C, NK1.1 (all eBioscience, San Diego, CA, USA), Ly6G (BD Biosciences), annexinV (IQ Products BV, Groningen, The Netherlands). For Western blot analysis, we used polyclonal antibodies against the α chain of CD97 (R&D Systems, Wiesbaden, Germany), the β chain of CD97 26, phospho-ERK, ERK, phospho-Akt, and actin (all Cell Signaling).

Flow cytometry
Bone marrow cells were harvested from dissected femurs by flushing the bone marrow plug with PBS/0.5% bovine serum albumin (BSA). Peripheral blood was collected in heparin by heart puncture. Single cell suspensions of spleen were obtained as described above. Erythrocytes in all cell preparations were lysed as described above.

For microglia isolation 31, brains were transferred to ice-cold glucose-potassium-sodium buffer (GKN; 8 g/l NaCl, 0.4 g/l KCl, 1.77 g/l NaHPO₄·2H₂O, 0.69 g/l NaH₂PO₄·H₂O, 2 g/l D-(+)-glucose, pH 7.4) with 0.3% BSA and minced through a 70-μm cell strainer. Cells were washed and resuspended in 20 ml of isotonic Percoll (GE Healthcare, Zeist, The Netherlands), diluted in GKN/BSA to a density (ρ) of 1.03 g/ml, then underlain with 10 ml Percoll of ρ = 1.095, overlain with 5 ml GKN-BSA buffer, and centrifuged for 35 min at 2,500 rpm (1,335 x g) and 4ºC with slow acceleration and no brake. After discarding of the myelin layer on top of the ρ = 1.03 Percoll phase, cells were collected from the interface, washed, and resuspended in GKN/BSA buffer.

50 μl whole blood, 5 x 10⁵ bone marrow or spleen cells, or all microglial cells isolated from one brain were used per staining. Non-specific binding of mAbs was blocked by adding 10% normal mouse serum and 2.5 μg/ml anti-CD16/32 (clone 2.4G2; BD Biosciences), together with the appropriately diluted mAbs in PBS containing 0.5% BSA. Cells were incubated for 30 min at 4ºC, followed (where appropriate) by a second incubation step with streptavidin-FITC, -PeCy7, or -APC. For measuring viability, cells were washed in calcium buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2.5 mM CaCl₂) and stained for annexinV in combination with subset-specific cell markers. Flow cytometric analysis was performed using a FACSCalibur (BD Biosciences) and the FlowJo software package (Tree Star, Ashland, OR, USA).

Western blot analysis
Immune cells from spleen and blood or CFSE-positive transferred cells from blood were isolated as described above and solubilized in a lysis buffer consisting of 2% Triton X-100 in PBS, supplemented with Complete Protease Inhibitor Mix (Roche, Mannheim, Germany). Lysates were made by agitation for 30 min at 4ºC, followed by centrifugation in a microfuge at 16,100 x g for 15 min. The resulting supernatant was used for SDS-PAGE. Approximately 3 x 10⁶ cell equivalents were loaded on a protein gel and blotted to an Immobilon-FL polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA) by semi-dry blotting. Membranes were stained overnight using antibodies specific for the CD97 α.
and β chain, phospho-ERK, ERK, phospho-Akt, and actin. For detection, IRDye-tagged secondary antibodies (LI-COR Biotechnology, Lincoln, NE, USA) were used. Analysis and quantification was performed on an Odyssey Infrared Imaging system (LI-COR).

**Soluble CD97 ELISA**

sCD97 in plasma was measured using a recently developed ELISA based on 1B2 as capture antibody, biotinylated 1A2 as detection antibody, and streptavidin-conjugated HRP (horseradish peroxidase) as enzyme. Blood from untreated wild-type, Cd55−/−, and Cd97−/− mice or from recipient wild-type and Cd55−/− mice, 10 min after transfer of Cd55−/− immune cells, was collected in heparin, and plasma was obtained by centrifugation for 10 min at 16,200 × g at 4°C. ELISA plates were coated overnight with 1 μg/ml 1B2 in PBS. Blocking was performed in 5% fetal calf serum (FCS) and 0.01% Tween20 in PBS. 50-μl plasma samples were added to the wells and incubated overnight at 4°C. Subsequently, wells were incubated with biotinylated 1A2 and streptavidin-conjugated HRP for 1 h at room temperature. After each step, samples were washed with 0.05% Tween20 in PBS. Finally, tetramethylbenzidine was added as substrate, and the reaction was stopped with 2 N H2SO4. Absorbance was measured at 450 nm using 655 nm as a reference wavelength. Levels of sCD97 in plasma were calculated from a standard of recombinant mouse sCD97(EGF1,2,4)-mFc fusion protein that was included on each plate.

**Real-time quantitative PCR**

Total RNA was extracted from peripheral blood leukocytes (PBL) and transcribed into first strand cDNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Real-time quantitative PCR was performed with the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using SYBR Green Master Mix (Applied Biosystems) with an input of cDNA equal to 10 ng initial total RNA per reaction. Analysis was performed with Sequence Detection Software (SDS) v 1.2.3 (Applied Biosystems). RNA expression of CD97 (forward primer 5’-CTGCCTCACCAACACTACT-3’, reverse primer 5’-CTCAAGGGCTCTTCCCTTTGT-3’) was normalized to hypoxanthine phosphoribosyl-transferase (HPRT; forward primer 5’-ATGGGAGGCCATCACATTGT-3’, reverse primer 5’-ATGTAATCCAGCGGACGCAA-3’).

**Statistical analysis**

Differences between groups were calculated by unpaired t-test or Mann-Whitney U test. Values are expressed as mean ± SD. A two-tailed p value of less than 0.05 was considered to represent a significant difference.

**RESULTS**

**CD97 expression on immune cells is increased in Cd55−/− mice**

Expression analysis by flow cytometry in wild-type mice revealed a broad distribution of CD97 on almost all leukocytes with, like in humans, highest expression levels found on myeloid cells (Figure 1A). Notably, in mice lacking a functional Cd55 gene, CD97 was significantly upregulated on all leukocytes with the most prominent increase found on cells in the periphery (Figure 1A). This finding suggested that cell surface expression of CD97 is regulated by interaction with its ligand CD55.
Shear Stress-dependent downregulation of CD97 by its ligand CD55

Since flow cytometry only allowed detection of the extracellular part of CD97, the question arose whether expression differences observed in the absence of CD55 affects the whole receptor or only the α chain. For instance, it is conceivable that ligand binding induces release of the α chain specifically, leaving expression of the membrane spanning β chain intact. Western blot analysis with antibodies specific for CD97 α and β chain was performed together with an actin control (upper panel). Bands on immunoblot shown were quantified using Odyssey Imaging software (lower panel). Shown is the relative protein expression of CD97 compared to wild-type cells. (C) Real-time quantitative PCR for CD97 on cDNA samples of PBL of wild-type and Cd55−/− mice. Indicated is the mean and SD of CD97 transcript levels relative to HPRT (n = 4). (D) CD11b+CD45+ microglia from brain were analyzed for CD97 expression as in panel A (n = 5 mice). ns, not significant, *, p < 0.05, and **, p < 0.005

Since flow cytometry only allowed detection of the extracellular part of CD97, the question arose whether expression differences observed in the absence of CD55 affects the whole receptor or only the α chain. For instance, it is conceivable that ligand binding induces release of the α chain specifically, leaving expression of the membrane spanning β chain intact. Western blot analysis with antibodies specific for the α and the β chain of CD97 revealed that expression levels of both chains were increased in Cd55−/− mice (Figure 1B, upper panel). Quantification of the bands on the immunoblot showed that the α and β subunits of CD97 in Cd55−/− mice were upregulated four times in peripheral blood leukocytes (PBL) and two times in splenocytes as compared to wild-type mice (Figure 1B, lower panel). These data imply that CD97 is predominantly expressed
at the cell surface as a dimeric receptor, comprising both the α and the β chain. We next tested if CD55 deficiency leads to increased transcript levels of CD97. By quantitative PCR, we found comparable \( Cd97 \) transcription in wild-type and \( Cd55 \)-/- leukocytes, indicating that modulation of CD97 protein expression through CD55 is regulated at the protein level (Figure 1C).

**Immune cell CD97 is reversibly regulated by CD55 expressed on stromal and hematopoietic cells**

To corroborate the observation that CD55 regulates CD97 cell surface expression, we performed adoptive transfer experiments by intravenous (i.v.) injection of CD55-deficient splenocytes into wild-type recipients and vice versa. One day after the transfer, we assessed the cell surface expression of CD97 on the transferred cells present in blood (Figure 2A) and spleen (data not shown). Wild-type immune cells that had been transferred into \( Cd55 \)-/- mice showed an upregulation of CD97 expression within 24 h to levels comparable with CD55-deficient leukocytes. Conversely, when cells from CD55-deficient mice were transferred into an environment where CD55 is present, expression of CD97 on these leukocytes was downregulated to a level comparable to wild-type cells. Thus, surface expression of CD97 is reversibly regulated by the presence of CD55 in vivo.

Because CD55 is expressed widely throughout the body, we generated bone marrow-chimeric mice to study the role of CD55 on hematopoietic versus non-hematopoietic cells in regulating CD97 expression. Congenic wild-type and \( Cd55 \)-/- recipient mice were lethally irradiated, followed by i.v. injection with \( Cd55 \)-/- or wild-type bone marrow cells, respectively. Five weeks after reconstitution of the hematopoietic compartment, we analyzed the expression level of CD97 on PBL of recipient mice. Levels of CD97 were comparable to wild-type mice in all
conditions where CD55 was present (Figure 2B). We concluded that CD55 of hematopoietic as well as stromal origin can regulate CD97 expression on immune cells.

**De novo** contact with CD55 leads to shedding of the α chain and rapid downregulation of the β chain CD97 from the cell surface *in vivo*

In order to assess the kinetics of CD97 downregulation after ligation by CD55, we measured the expression of CD97 on CD55−/− leukocytes at various time points after transfer to congenic wild-type mice. Within 5 min after the initial contact with wild-type cells bearing CD55 in circulation, expression of CD97 on CD55−/− leukocytes was already reduced by about 50% (Figure 3A). At 20 min after transfer, CD97 expression was normalized to levels found on wild-type leukocytes (Figure 3A).

To examine the fate of both the α and β chain of CD97 after ligation by CD55, we sorted transferred CD55−/− cells from blood of both CD55−/− and wild-type recipients 10 min after transfer. Western blot analysis with CD97 α and β chain-specific antibodies revealed downregulation of both subunits after *de novo* contact with CD55. Quantification of the immunoblot confirmed an almost equal, three-fold reduction in the amount of both chains, indicating that the two subunits of CD97 disappear after ligation with comparable kinetics (Figure 3B).

Due to the two-subunit structure of CD97 and the presence of sCD97 in serum of wild-type mice, we wondered whether the α chain is shed after ligation by CD55, followed by subsequent internalization and degradation of the β chain. To test this possibility, we collected plasma of both CD55−/− and wild-type recipients 10 min after transfer of CD55−/− leukocytes into these mice. Using a sCD97-specific ELISA, we detected a low concentration of sCD97 in wild-type mice.

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**Figure 3. Ligation by CD55 in vivo results in CD97 α chain shedding and β chain downregulation.**

(A) Splenocytes from Cd55−/− mice were transferred into congenic wild-type mice by injection into the tail vein. At indicated time points, blood was collected from recipient mice, and CD97 expression was measured on transferred (CD45.2+) and recipient (CD45.1+) cells by flow cytometry. Indicated is the relative expression of CD97 on transferred cells compared to host cells in the same mouse. One of three independent experiments is shown. (B) Splenocytes from Cd55−/− mice were labeled with CFSE and injected into the tail vein of Cd55−/− and wild-type mice. 10 min after transfer, blood was collected, erythrocytes were lysed, and CFSE+ cells were sorted. Lysates of sorted cells from 9 mice, derived from 3 experiments, were pooled and analyzed by Western blot using antibodies specific for CD97 α and β chain together with an actin control (left panel). Bands on immunoblot shown were quantified using Odyssey Imaging software (right panel). Shown is the relative protein expression of CD97 on cells transferred into Cd55−/− mice compared to cells transferred into wild-type mice. (C) Splenocytes from Cd55−/− mice were injected into the tail vein of Cd55−/− and wild-type mice. 10 min after transfer, blood was collected, and sCD97 was detected in plasma using a sandwich ELISA. Plasma levels of sCD97 in wild-type and Cd55−/− was analyzed for comparison. Shown is the mean ± SD of the amount of sCD97 in one of two independent experiments (n = 3 mice). nd, not detectable, *, p < 0.05
at steady state (Figure 3C). Transfer of Cd55<sup>−/−</sup> leukocytes into wild-type recipients resulted in a significant increase in levels of circulating sCD97, indicating shedding of the α subunit after ligation of CD97 by CD55. In Cd55<sup>−/−</sup> mice, the level of sCD97 was below the detection limit and remained undetectable after transfer of Cd55<sup>−/−</sup> cells, further supporting the idea that ligation by CD55 is needed to process CD97.

**CD55-induced downregulation of CD97 requires shear stress in vitro and in vivo**

To further study the mechanism by which CD55 is regulating CD97 cell surface expression, we tried to set up an in vitro system. We previously demonstrated physical interaction between CD97 and CD55 in coculture systems<sup>5, 6, 29</sup>. Surprisingly, when studying Cd55<sup>−/−</sup> leukocytes cocultured with congenic wild-type blood cells, we observed no downregulation of CD97. We cocultured Cd55<sup>−/−</sup> leukocytes from spleen or blood with wild-type leukocytes or erythrocytes in different ratios and in the presence or absence of wild-type or Cd55<sup>−/−</sup> serum for 30 min up till 48 h, but in none of these conditions was CD97 expression downregulated (data not shown). We concluded that the circumstances needed to regulate CD97 were not recapitulated properly in vitro. The difference could perhaps lie in the circulatory action of the blood, causing shear stress in vivo; this could trigger a conformational change, resulting in CD55-mediated downregulation of CD97.

To test whether this shear stress model is viable, we attempted to recapitulate similar conditions in vitro. We cocultured CD55-deficient splenocytes with wild-type blood while shaking at high speed. Shear stress in this model is heterogeneous, and for the current settings was in the order of

![Figure 4](image_url)

**Figure 4. In vitro regulation of CD97 cell surface expression requires shear stress.** (A) Splenocytes from Cd55<sup>−/−</sup> or congenic wild-type mice were cocultured for 4 h at RT with Cd55<sup>−/−</sup> or wild-type whole blood, applying rigorous agitation (white and black bars, shear around 30 dynes/cm<sup>2</sup>) or not (grey bar). Immediately afterwards, cells were analyzed for CD45.1, CD45.2, and CD97 expression by flow cytometry. Provided is the mean and SD of the relative expression of CD97 on the splenocytes, compared to wild-type splenocytes (0 h) or to Cd55<sup>−/−</sup> whole blood (4 h) (n = 3). (B) Splenocytes from congenic Cd55<sup>−/−</sup> mice were cocultured at RT with wild-type whole blood, applying shear stress as in panel A. At indicated time points, splenocytes were analyzed for CD97 expression, depicted here as mean and SD of relative expression compared to wild-type PBL (n = 3-5). (C) Congenic wild-type whole blood was mixed with Cd55<sup>−/−</sup> splenocytes and exposed to a constant shear stress of 1 dynes/cm<sup>2</sup> or 40 dynes/cm<sup>2</sup> imposed by a rheometer. After 1 h, cells were harvested and analyzed by flow cytometry as described in panel A. One of two comparable experiments is shown. **, p < 0.005 and ***, p < 0.0005
30 dynes/cm². After 4 h, CD97 expression on Cd55−/− cells was strongly reduced and comparable to that on wild-type leukocytes (Figure 4A). Transferred Cd55−/− splenocytes were viable, but slightly activated, as demonstrated by lower CD62L expression on transferred CD45.2+ cells (data not shown). We previously showed that activated leukocytes express higher CD97 levels. Thus, cell activation should not have downregulated CD97 in our experiments. Analysis at earlier time points revealed that although somewhat slower than in vivo, CD97 was downregulated on Cd55−/− immune cells upon contact with wild-type blood cells within 1-2 h (Figure 4B).

To corroborate our findings in a better-defined experimental setting, we used a MCR 501 Couette-type rheometer, where a nearly homogeneous shear stress field occurs in the fluid between two concentric cylinders, generated by their relative movement. We mixed Cd55−/− splenocytes with wild-type blood and applied a constant shear stress for 1 h. A shear stress of 1 dynes/cm², found in capillaries, was not sufficient to induce downregulation of CD97. In contrast at a shear stress of 40 dynes/cm², representative for arteries, we observed that CD97 expression on transferred Cd55−/− cells was efficiently downregulated to wild-type levels (Figure 4C).

Figure 5. In vivo downregulation of CD97 upon CD55 contact requires blood circulation. One group of congenic wild-type mice was injected with Cd55−/− splenocytes by injection into the tail vein, followed by blood collection after 5 or 20 min. Another group of wild-type mice was administered with heparin, injected with Cd55−/− splenocytes, and sacrificed immediately thereafter. Blood was collected 5 and 20 min post mortem. CD97 expression was measured on transferred (CD45.2+) and recipient (CD45.1+) leukocytes by flow cytometry. Provided is the treatment scheme (upper panel) and the mean and SD of the relative expression of CD97 on transferred cells compared to host cells in the same mouse (lower panel). Data was pooled from 3 independent experiments (n = 6 mice). *, p < 0.05
We next investigated whether CD97 downregulation upon *de novo* ligation requires an intact circulation *in vivo*. As shown in Figure 3A, *Cd55*^-/-^ leukocytes rapidly downregulated CD97 after transfer into wild-type mice. To study regulation of CD97 expression in the absence of circulation, we pretreated wild-type recipient mice with heparin in order to prevent coagulation and sacrificed them immediately after adoptive transfer of *Cd55*^-/-^ leukocytes, followed by cardiac puncture after 5 or 20 min. Under these conditions, we found no reduction of CD97 cell surface expression even after 20 min (Figure 5). Erythrocytes express high levels of CD55, ensuring direct contact of CD97 on *Cd55*^-/-^ leukocytes with its ligand even though there is no circulation. Treatment with heparin by itself did not affect downregulation of CD97 expression on *Cd55*^-/-^ leukocytes transferred into wild-type mice (data not shown). Together, these findings indicated that shear stress is necessary for the effective processing of CD97 by its ligand CD55. Consistent with this idea, we found that microglia in the brain that neither circulate nor are in contact with circulating blood cells have comparable levels of CD97 surface expression in wild-type and *Cd55*^-/-^ mice (Figure 1D).

**De novo ligation of CD97 by its ligand CD55 does not induce ERK or PKB/Akt signaling**

In the classical model of agonist-selective desensitization, internalization of GPCRs results from effective receptor activation. Recent studies demonstrated that ectopic overexpression of CD97 in fibroblasts and epithelial cells, and constitutive expression of CD97 in prostate cancer cells stimulates ERK, protein kinase B (PKB)/Akt, and RhoA activation. To explore the possibility that interaction with its ligand CD55 activates CD97 in leukocytes, we incubated CD55-deficient splenocytes with wild-type erythrocytes under rigorous agitation as described above. At 0, 5, 15, and 30 min, we harvested cells and probed lysates for ERK and PKB/Akt activation. We found no phosphorylation of ERK and PKB/Akt in splenocytes that had experienced CD97 ligation.

**Figure 6. De novo interaction of CD97 with its ligand CD55 does not induce ERK or PKB/Akt signaling.** (A) *Cd55*^-/-^ and wild-type splenocytes were mixed with either *Cd55*^-/-^ or wild-type erythrocytes and shaken for 0, 5, 15, or 30 min. After erythrocyte lysis, splenocytes were collected in sample buffer and analyzed by Western blot with antibodies specific for phosphorylated ERK and PKB/Akt. Full ERK or actin was analyzed for comparison of loading. As positive control for ERK phosphorylation, splenocytes in the same experiment were stimulated with 100 ng/ml PMA. As a positive control for the pAkt Western blotting, lysate of platelet-derived growth factor (PDGF)-stimulated synovial fibroblasts was used. One of two independent experiments is shown.
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(Figure 6). Moreover, RhoA was not activated upon de novo ligation of CD97 (data not shown). Thus, CD97 signaling pathways previously identified in tumor cells were not triggered by CD55 binding in leukocytes.

DISCUSSION

The identification of CD55 as binding partner of CD97 in 1996 demonstrated for the first time the ability of aGPCRs to interact with cellular ligands. Specificity and affinity of CD97 for CD55 is closely regulated by composition of the EGF domain region and by individual amino acids that prevent CD55 binding by EMR2, a homolog of CD97 with 97% amino acid identity in the EGF domain region. In spite of cellular and molecular assays proving interaction between CD97 and CD55 in humans, this evidence has been difficult to obtain. Importantly, EGF domain-specific antibodies and recombinant CD55 do not activate known mediators of GPCR signaling. Only recently, studies with gene-deficient mice functionally linked CD97 and CD55. Firstly, mice lacking either CD97 or CD55 had a higher granulopoietic activity, resulting in increased numbers of circulating granulocytes. Secondly, absence of CD97 or CD55 reduced disease activity in two experimental models of arthritis. In both cases, CD97 and CD55 knockout mice developed a highly similar phenotype. Yet, by their nature, these studies could only establish association but not prove causation. Our finding that CD97 surface expression on circulating leukocytes is continuously regulated by contact with CD55 provides the first direct evidence of an aGPCR interacting with its binding partner.

The physiological function of the CD97–CD55 interaction presumably relates to the engagement of adhesive contacts between CD97+ immune cells and CD55+ stromal cells. It seems possible that CD97–CD55 contacts mediate the retention of leukocytes at specific tissue sites. For example, CD55+ synovial lining fibroblasts of rheumatoid arthritis synovial tissue are able to bind CD97-coupled fluorescent beads. A role of CD97 as receiver of local stromal and matrix adhesive codes would fit with its ability to engage with various ligands at low affinity. Besides CD55, CD97 binds the glycosaminoglycan dermatan sulfate, the integrin α5β1, and Thy-1/CD90 through different sites within the extracellular subunit. Similar characteristics have been reported for GPR56, an aGPCR expressed by neurons, various malignant cells, and cytotoxic lymphocytes. GPR56 binds different ligands in man and mouse, including tissue transglutaminase 2 and collagen III. Defective expression of GPR56 on neurons disturbs the integrity of the pial basement membrane and the migration of developing neurons, resulting in a severe human brain malformation called bilateral frontoparietal polymicrogyria (BFPP). Intriguingly, all BFPP-associated missense mutations identified to date are located at the extracellular region of GPR56. Other examples of aGPCRs with a role in cell adhesion are Flamingo and Latrophilin, which coordinate cell orientation and tissue polarity in Drosophila and C. elegans, respectively. The picture that arises from these studies is that aGPCRs facilitate the proper positioning of developing and motile cells in various organ systems via their extracellular modules.

CD97 and CD55 are abundantly expressed by all types of leukocytes. Moreover, many epithelial cells express CD97, and CD55 is found on erythrocytes, endothelial cells, and stromal
cells\textsuperscript{11,26,53}. This wide distribution raised the question how uncontrolled clustering of leukocytes due to homo- or heterotypic cellular CD97–CD55 contacts is prevented. Our data suggest that continuous downregulation of CD97 on circulating leukocytes upon contact with CD55, together with the rather low affinity of their interaction (K\textsubscript{D} of 86 μM\textsuperscript{7}), may prevent clustering or inappropriate binding to the endothelium. In line with this, we previously showed that CD97 and CD55 are dispensable for the extravasation of leukocytes from the blood stream\textsuperscript{26,36}. Once inside the tissue, CD97 expression may increase and facilitate adhesion events through interaction with CD55. A unique aspect of the regulation of CD97 expression upon CD55 contact is the dependency on shear stress, both \textit{in vitro} and \textit{in vivo}. To our knowledge, this mechanism of regulation of expression of a GPCR is without precedent (Figure 7). Whether it relates to the unique two-subunit structure, resulting from autocatalytic processing and reassociation of the protein precursor to almost unchanged appearance at the cell surface, which is a hallmark of most aGPCRs\textsuperscript{3,4}, remains to be shown. Based on our findings, it seems possible that the cleavage site functions as molecular mechanical fracture device that confers a mechanism to terminate activity of the receptor.

It has been proposed that the two-subunit structure of aGPCRs functionally separates the adhesive extracellular subunit from the signaling seven-transmembrane subunit. Recent \textit{in vitro} and \textit{in vivo} studies with epithelial cells overexpressing CD97 have started to shed light on the signaling properties of the receptor. Firstly, Aust and coworkers demonstrated an increase in membrane-associated β-catenin due to PKB/Akt–GSK-3β signaling in transgenic \textit{Villin-Cd97} mice that overexpress CD97 in intestinal epithelial cells\textsuperscript{22}. Secondly, Kelly and colleagues showed that in prostate cancer cells, CD97 signals through ERK, PKB/Akt, and RhoA activation\textsuperscript{24}. Notably, signaling has not been attributed to receptor ligation in these studies, and the Kelly laboratory demonstrated that CD97 in a ligand-independent manner regulates signaling of lysophosphatidic acid receptor 1 (LPAR-1)\textsuperscript{24,54}. We tested whether \textit{de novo} ligation by CD55 would stimulate CD97 signaling, but found no evidence that ERK, PKB/Akt, or RhoA are activated, even though...
our *in vitro* experimental settings mimic *in vivo* events closely. While our experiments do not exclude signaling, perhaps mediated by signaling molecules other than the ones tested, it also seems possible that CD55 binding not at all triggers CD97 signaling but solely mediates adhesive contacts. This question will need to be addressed in future studies using robust and specific readouts for CD97 signaling.

As a consequence of the two-subunit structure of aGPCRs, the two chains can behave as independent proteins as has been shown by studies with transfected cell lines for latrophilin and EMR2. We here failed to demonstrate an independent existence of the transmembrane β chain of CD97 after dissociation from the extracellular α chain. Upon CD55-induced shedding of the α chain *in vivo*, the β chain was downregulated within minutes to the same extent, most likely via internalization and subsequent degradation. How this process is orchestrated remains to be addressed and may further inform us about the underlying working mechanism of aGPCRs.

Another question relates to the possible roles of the CD97 α chain in the circulation. Shedded ectodomains of transmembrane proteins can remain stable and active in solution, thereby regulating various biological processes. Interestingly, the α chain of human CD97 can act as a potent chemoattractant for human endothelial cells and as a pro-angiogenic factor, manifesting its potential biological activity at distant sites. Lack of sCD97 in CD55 knockout mice suggests that CD55 contact-mediated release is the major source of CD97 α chain present in plasma.

In summary, we show here that ligation of the aGPCR CD97 on circulating blood cells results in rapid downregulation of the receptor, probably to restrict cell adhesion to tissue sites. The *de novo* ligation model that we present can facilitate further studies on the working mechanism of aGPCRs.

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**REFERENCES**

SHEAR STRESS-DEPENDENT DOWNREGULATION OF CD97 BY ITS LIGAND CD55

CHAPTER 2


