Chapter 3

Differential expression of CD97 on human lymphocyte subsets

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ABSTRACT

CD97 is a heptahelical EGF-TM7 receptor broadly expressed on hematopoietic cells as three isoforms with respectively three, four or five epidermal growth factor (EGF)-like domains in their extracellular region. CD97 is constitutively highly expressed on myeloid cells. On resting lymphocytes, CD97 expression is low though cellular activation results in a rapid up-regulation. We here describe in detail the expression characteristics of CD97 on human lymphocytes. We found CD97 to be present on all lymphocytes in blood and lymphoid tissue. Expression of CD97 on B cells was lower compared to T and NK cells and did not differ between B cell subsets. Remarkably, B cells in the circulation were the only lymphocytes that expressed substantial amounts of the largest – five EGF-like domains possessing – CD97 isoform. In contrast, B cells in lymphoid tissue did not express detectable levels of this specific isoform. In CD4+ T cells, CD97 expression was higher on memory cells compared to naïve cells. In CD8+ T and NK cells, we found a down-regulation of CD97 on cytolytic effector cells. Stimulation through CD3 and CD28 resulted in a rapid up-regulation of CD97 in all T
cell subsets within 2 to 4 h. In short, we here demonstrate that human lymphocyte subsets differentially express CD97.

INTRODUCTION

Human CD97 [1;2] is a founding member of the EGF-TM7 family of adhesion class heptahelical receptors [3] further consisting of EMR1 [4], EMR2 [5], EMR3 [6] and EMR4 [7]. Except for EMR1, the EGF-TM7 receptors are characterized by an N-terminal α subunit noncovalently linked to a C-terminal β subunit as a consequence of the cleavage of the polypeptide at a G protein-coupled receptor proteolytic site [8]. The extracellular α subunit consists of a number of tandemly arranged EGF-like domains followed by a large stalk region, while the β subunit consists of seven transmembrane helices. Due to alternative RNA splicing, CD97 is expressed as three isoforms containing three (EGF1,2,5), four (EGF1,2,3,5) or five (EGF1,2,3,4,5) EGF-like domains. The EGF domains of CD97 can mediate binding to cellular ligands. Whereas EGF domain 1 and 2 bind CD55 [9-11], EGF domain 4 interacts with the glycosaminoglycan chondroitin sulfate B, also known as dermatan sulfate [12;13]. All isoforms can bind CD55 while only the largest, EGF domain 4-containing isoform binds chondroitin sulfate B [10;13]. The affinity for CD55 differs between the different CD97 isoforms and is highest for the smallest isoform and lowest for the largest isoform [10]. With respect to the physiological function of CD97, it has recently been reported that CD97 can act as a co-stimulatory ligand modulating the activation of human CD4+ T cells through CD55 [14]. Moreover, studies in mice revealed a role of CD97 in leukocyte migration. Application of CD97-specific antibodies inhibited the recruitment of granulocytes in thioglycolate-induced peritonitis, in dextran sulfate sodium-induced colitis, in collagen-induced arthritis and in Streptococcus pneumoniae-induced pneumonia ([15;16] and Veninga et al., submitted for publication).

Expression of the EGF-TM7 family members is predominantly leukocyte restricted while CD97 is the only member found on lymphoid cells [17]. It has been reported that this expression is generally low on resting lymphocytes and that CD97 is rapidly up-regulated after activation of these cells [18;19]. Up till now, the surface expression of CD97 on the different lymphocyte subsets has not been described. Based on the different functions and compartmentalization properties of lymphocyte subsets,
knowledge on the expression of CD97 could provide additional clues on the function of CD97 in general and of specific CD97 isoforms in particular. By using a panel of monoclonal antibodies (mAbs) recognizing different CD97 epitopes, we here assessed the general surface expression of CD97 and the expression of its largest isoform on human T, B and NK cells in blood and different lymphoid compartments.

MATERIALS AND METHODS

Cell preparation
Heparine-treated blood samples were obtained from healthy volunteers. Bone marrow samples were obtained from patients who were initially suspected of hematological malignancies but in whom no abnormalities could be detected. Tonsils were obtained from patients who underwent tonsillectomy. Small parts of spleen that had been obtained from organ transplant donors were received from the tissue typing laboratory and could be used for scientific research according to paragraph 13 of the Dutch Law for Organ Donation. Splenic parts, containing both red and white pulp, and tonsil parts were minced and then rubbed over a 70-μm gauze. Isolated splenic and bone marrow cells were suspended in IMDM supplemented with 10% FCS, 0.38% (w/v) trisodium citrate, 50 mg/ml gentamycin and 0.00036% (v/v) β-mercaptoethanol before density gradient centrifugation to purify the mononuclear cells. The study was approved by the local medical ethics committee and written informed consents were obtained (where applicable).

Monoclonal Antibodies
The generation of the mAbs against CD97 was described previously [9;12] CLB-CD97/3 and CLB-CD97/1 are mouse mAbs that recognize the stalk region and EGF domain 1 of CD97, respectively. 1B5 is a hamster mAb that recognizes EGF domain 4 of CD97. All CD97 mAbs were biotinylated. Other mAbs used in this study were CD3-FITC, CD4-PECy5.5, CD8-PECy5.5, CD19-PECy5.5, CD38-PE, CD56-PE, IgD-FITC (all Becton Dickinson, San Jose, CA), CD27-FITC (Immunotools, Friesoythe, Germany) and CD45RA-PE (Beckman Coulter, Fullerton, CA). FITC-, PE- and PECy5.5-labeled mouse
IgG1 (Becton Dickinson), biotinylated mouse IgG1 and IgG2a (Caltag Laboratories, Burlingame, CA) and biotinylated hamster IgG (Southern Biotech, Birmingham, AL) were used as isotype controls. Streptavidin-APC (Pharmingen, San Diego, CA) was applied as a second step reagent.

**Flow cytometry**

All flow cytometry was performed on a FACSCalibur (Becton Dickinson) using standard procedures. Leukocyte subpopulations were defined based on their forward and sideward scatter characteristics. To detect the expression of CD97 on different lymphocyte subtypes, cells were first incubated with biotinylated CD97 mAbs. In a second step the samples were incubated with lymphocyte subset specific mAbs along with streptavidin-APC. Prior to cytometry, erythrocytes were lysed using FACS lysing solution (Becton Dickinson). All incubation steps were performed on ice.

**Stimulation assay**

Peripheral blood mononuclear cells (PBMCs) were isolated from heparine-treated blood samples by centrifugation over a Ficoll-Paque PLUS (Amersham Bioscience, Freiburg, Germany) density gradient. Cells were incubated for 4 h in IMDM supplemented with 10% FCS, 200 mM L-glutamate and 50 mg/ml gentamycin at 37°C and 5% CO₂. Agonistic mAbs against CD3 (1XE) [20] and CD28 (15E8) [21] were added at concentrations of 1:1000 and 5 μg/ml, respectively. The expression of CD97 on the CD4⁺ and CD8⁺ T cell subsets was assessed by flow cytometry as described before.

**Statistical analysis**

Except for B cells, CD97 expression on the investigated cells was normally distributed as assessed by the D’Agostino-Pearson test. Consequently, differences between the groups were analyzed using the two tailed t-test or the one way ANOVA test followed by a Turkey post test. Data for the B cell subsets in peripheral blood and tonsil were analyzed with the Kruskal-Wallis test followed by a Dunns post test. A P value smaller than 0.05 was considered statistically significant. All statistical analyses were performed using the GraphPad Prism 5 statistical package (GraphPad Software Inc., San Diego, CA).
RESULTS

All lymphocytes in blood and lymphoid organs express CD97
As a first step, we assessed the expression of CD97 on peripheral blood lymphocytes. This was done by using mAbs binding three distinct epitopes of CD97 (Fig. 1A). Because CD97 is readily up-regulated on lymphocytes, we stained whole blood samples to limit activation resulting from isolation procedures. Cells were analyzed by flow cytometry and lymphocyte populations were defined based on the expression of CD19 (B cells), CD3 (T cells) and CD56 (NK cells), respectively (Fig. 1B). CD97 was expressed on all lymphocytes but expression levels were markedly lower compared to myeloid cells like granulocytes and monocytes (Fig. 1C). And while the expression of CD97 on peripheral NK and T cells was comparable, the expression on B cells was lower, except for the largest isoform detected by the 1B5 mAb (Fig. 1C&D).

Figure 1. Expression of CD97 on lymphocytes in peripheral blood. (A) Schematic structure of the largest isoform of human CD97 possessing five EGF domains. Indicated are the binding sites of the
mAbs CLB-CD97/1, 1B5 and CLB-CD97/3 within EGF domain 1, EGF domain 4 and the stalk region, respectively. (B) Peripheral blood lymphocytes, defined by forward and side scatter analysis, were divided based on expression of CD3 and CD56 into T cells (CD3+CD56−) and NK cells (CD3−CD56+). B cells were defined as the CD19+ cells within the CD3 CD56− population. (C) Expression of CD97 was assessed with CLB-CD97/1 (grey bars), CLB-CD97/3 (black bars) and 1B5 (open bars) for B cells (B), T cells (T), NK cells (NK), granulocytes (G) and monocytes (M). Values are the mean fluorescence intensities (based on the geometric mean) ± SD from eight donors. The respective control antibodies displayed a geometric mean fluorescence no higher than 4 (D) Representative histogram plots for the binding of the CLB-CD97/1 (grey bars), CLB-CD97/3 (black bars) and 1B5 (open bars) on the different lymphocyte subsets.

Next, we tested if the expression of CD97 differs between blood lymphocytes and lymphocytes located in different lymphoid organs. As shown in Figure 2, also in bone marrow, spleen and tonsil all lymphocytes expressed CD97. While expression on B and T cells was largely comparable with the situation in blood, we observed a higher expression on NK cells in all three compartments (Fig. 1C&2B).

**Lymphocyte subsets differentially express CD97**

In the next step of our analysis we investigated if CD97 is differentially expressed on functionally different subsets of B, T and NK cells. To define B-cell subsets in blood, CD19+ cells were co-stained for CD27 and CD38 ([22] and Fig. 3A). No significant difference in expression could be seen between immature, naïve, memory and plasma cells (Fig. 3B). Interestingly, circulating B cells were the only lymphocytes with a considerable expression of the largest CD97 isoform on their surface (Fig. 1D). As shown in Figure 3B, expression of the largest CD97 isoform did not differ significantly between B-cell subsets. Strikingly, B cells derived from lymphoid organs did not express detectable levels of the largest isoform of CD97 (Fig. 2B). To test whether distinct B-cell subsets account for this difference, we co-stained isolated tonsilar CD19+ mononuclear cells for CD38 and IgD ([23] and Fig. 3C). No detectable expression of CD97(EGF1,2,3,4,5) was found on either naïve, germinal center, plasma blast or memory B cells (Fig. 3D). This was in sharp contrast with the higher general expression of CD97 on B-cell subsets in tonsil compared to blood as assessed by the CLB-CD97/1 and the CLB-CD97/3 mAb (Fig. 3C&D). As these mAbs do not discriminate between
isoforms and as the expression of the largest isoform was assessed by the specific mAb 1B5, this implied a specific regulation of the largest isoform of CD97 on B cells.

We pursued our analysis by investigating the expression of CD97 on blood NK cells. NK cells can be subdivided into two subsets based on the expression of CD56 [24] (Fig. 4A). The general expression of CD97 was significantly higher for the

Figure 2. Expression of CD97 on lymphocytes in different lymphoid organs. Lymphocytes were obtained from bone marrow, spleen and tonsil and analyzed for CD97 expression (A) B, T and NK cells were defined as in figure 1. In bone marrow only the CD45<sup>+</sup> cells were analyzed. (B) Expression of CD97 was assessed with CLB-CD97/1 (grey bars), CLB-CD97/3 (black bars) and 1B5 (open bars). Values are mean fluorescence intensities (based on the geometric mean) ± SD for six (bone marrow), two (spleen) and five (tonsil) donors. The respective control antibodies displayed a geometric mean fluorescence no higher than 5.
CD56\textsuperset{bright} population compared to the CD56\textsuperset{dim} NK cells (Fig. 4B). Moreover, the CD56\textsuperset{bright} cells had the highest expression of all lymphocyte subsets (Fig. 4A and Fig. 1D).

Finally, we evaluated the expression of CD97 on the different T cell subsets in blood. These subsets were defined based on the expression of CD4 and CD8 in combination with CD27 and CD45RA ([25] and Fig. 5A). While memory CD4\textsuperset{+} T cells had a significant higher expression of CD97 compared to naïve CD4\textsuperset{+} T cells, no significant difference was found between naïve and memory CD8\textsuperset{+} T cells (Fig. 5 C&D). Interestingly, CD8\textsuperset{+} effector T cells expressed lower amounts of CD97 compared to naïve and memory cells (Fig. 5 D).

**Figure 3. Expression of CD97 on B-cell subsets in peripheral blood and tonsil.** (A) CD19\textsuperset{+} peripheral blood B cells (B) were subdivided, based on the expression of CD27 and CD38, into CD27\textsuperset{CD38+} immature B cells (I), CD27\textsuperset{CD38+} naïve B cells (N), CD27\textsuperset{CD38+} memory B cells (M) and CD27\textsuperset{CD38+} plasma cells (P). (B) Expression of CD97 was assessed with CLB-CD97/1 (grey bars), CLB-CD97/3 (black bars) and 1B5 (open bars). Values are mean fluorescence intensities (based on the geometric mean) ± SD for eight donors. The respective control antibodies displayed a geometric mean
fluorescence no higher than 3. **(C)** CD19⁺ tonsilar B cells (B) were subdivided, based on the expression of CD38 and IgD, into IgD⁺ naïve B cells (N), IgD⁺CD38⁻ germinal center B cells (GC), IgD⁻CD38⁺ plasma blasts (PB) and IgD⁻CD38⁻ memory B cells (M). **(D)** Expression of CD97 was assessed as described above. Values are mean fluorescence intensities (based on the geometric mean) ± SD for five donors. The respective control antibodies displayed a geometric mean fluorescence no higher than 4.

![Figure 4](image)

**Figure 4. Expression of CD97 on NK cell subsets in peripheral blood.** **(A)** CD56⁺ peripheral blood NK cells were divided into CD56bright and CD56dim cells. **(B)** Expression of CD97 was assessed with CLB-CD97/1 (grey bars), CLB-CD97/3 (black bars) and 1B5 (open bars). Values are mean fluorescence intensities (based on the geometric mean) ± SD for eight donors. The respective control antibodies displayed a geometric mean fluorescence no higher than 4. *, P < 0.05 and **, P < 0.01

**CD97 can be rapidly up-regulated on all T-cell subsets**
The rapid up-regulation of CD97 during lymphocyte activation is well established [18;19]. Based on our finding that CD4⁺ and CD8⁺ T-cell subsets in blood express different amounts of CD97, we wanted to know if CD97 up-regulation during cellular activation differs between these subsets. Therefore, we stimulated PBMC with soluble anti-CD3 either alone or in combination with soluble anti-CD28. CD97 expression was assessed after 2 and 4 h using the pan-CD97 mAb CLB-CD97/3 (Fig. 6 A&B). As reported before, CD97 was already up-regulated when the cells were incubated with medium alone [19]. Compared to the respective medium controls, both naïve and memory of CD4⁺ T cells showed a significantly up-regulation of CD97 already after 2 h of stimulation with anti-αCD3. A further up-regulation was achieved after 4 h or when cells were co-stimulated with anti-CD28. Naïve and memory CD8⁺ T cells significantly up-regulated CD97 after 4 h of stimulation with anti-CD3. Interestingly, on effector
cells, a significant increase was already detected after 2 h of stimulation, indicating that these cells up-regulate CD97 more rapidly than naïve and memory cells. Again, in all subsets, CD97 up-regulation increased with time and was accelerated by co-stimulation with anti-CD28.

Figure 5. Expression of CD97 on CD4+ and CD8+ T cell subsets in peripheral blood. (A) and (C) Peripheral blood T cells were divided based on the expression of CD4, CD8, CD27 and CD45RA expression into CD27+CD45RA+ naïve (N) and CD27+CD45RA- memory (M) CD4+ T cells and CD27+CD45RA+ naïve (N), CD27+CD45RA- memory (M) and CD27-CD45RA+ effector (E) CD8+ T cells. (B) and (D) Expression of CD97 was assessed with CLB-CD97/1 (grey bars), CLB-CD97/3 (black bars) and 1B5 (open bars). Values are mean fluorescence intensities (based on the geometric mean) ± SD for eight donors. The respective control antibodies displayed a geometric mean fluorescence no higher than 4. *, P < 0.05 and **, P < 0.01
Figure 6. Up-regulation of CD97 on CD4+ and CD8+ T cell subsets after cellular activation. PBMC were stimulated with soluble anti-CD3 (aCD3) either alone or in combination with anti-CD28 (aCD28) for 2 and 4 h. T cell subsets were defined as described and CD97 expression was assessed with CLB-CD97/3. Values are the means ± SD of the fold change of expression compared to time point 0 (set on 1) for (A) naïve (N) and memory (M) CD4+ T cells and (B) naïve (N), memory (M) and effector (E) CD8+ T cells for five donors in at least three different experiments. Numbers indicate the mean fluorescence intensities (based on the geometric mean) of the CD97 expression at time point 0 for the different T cell subsets. Geometric mean fluorescence of the control antibodies did not change during stimulation. *, P < 0.05 compared to the fold change in CD97 expression in cells incubated with medium alone.

DISCUSSION

CD97 has in the past been described as an early activation marker for human lymphocytes, which display a low surface expression at the resting state. Nevertheless, the surface expression of CD97 on individual lymphocyte subsets has still been elusive. We here show that CD97 is present on the surface of all lymphocytes in blood and lymphoid tissue. In addition, we demonstrate significant differences in expression levels
between lymphocytes. In general, T and NK cells possess higher levels of CD97 than B cells. The highest expression of CD97 was detected on CD56\textsuperscript{bright} NK cells. These cells have predominantly an immunoregulatory role, modulating the activity of activated monocytes and dendritic cells [26;27]. Acquisition of a cytolytic effector cell phenotype correlated with a down-regulation of CD97, as illustrated by the lower expression of the receptor on CD56\textsuperscript{dim} NK cells compared to CD56\textsuperscript{bright} cells and – to a lesser extent – on CD8\textsuperscript{T} effector T cells compared to naïve and memory CD8\textsuperscript{T} cells. Furthermore, memory CD4\textsuperscript{T} but not CD8\textsuperscript{T} T cells, expressed more CD97 than naïve cells. Nonetheless, CD97 expression is readily up-regulated on all T cell subsets between 2 to 4 h of activation.

Taken together this suggests that for T cells, CD97 expression is differentially regulated during the process of activation/differentiation to end-stage memory and effector cells. Moreover, the specific down-modulation of CD97 on subsets with a direct cytolytic capacity appears to be a general phenomenon, common to T cells and NK cells. However, it is still unclear how CD97 surface expression is regulated. CD97 expression might primarily be regulated at the mRNA level as illustrated by the strong correlation between surface expression of CD97 and mRNA expression levels for CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells for instance (data not shown). In addition, our activation data for T cell subsets concord with what has earlier been reported by Eichler and co-workers, who showed that CD97 can initially be mobilized from intracellular stores resulting in a very fast expression on the cellular surface of peripheral blood lymphocytes [19]. So, a future point to address would be to assess if there is a relation between the difference in surface expression of CD97 for the different lymphocyte subsets and their amount of intracellular CD97. If so, it would be important to define under which specific conditions regulation at the level of intracellular protein stores and regulation at the mRNA level impacts on CD97 surface expression. Moreover, the characterization of the regulatory mechanisms responsible for the specific down-regulation of CD97 on subsets with a direct cytolytic capacity might shed a light on other modulation pathways of CD97 expression.

A clue to one of these pathways might be given by the analysis of the expression of the largest -5 EGF-like domains possessing- isoform of CD97 on lymphocytes. We here show that circulating B cells were the only lymphocytes that expressed substantial amounts of this largest isoform. Interestingly, B cells in the lymphoid tissues did not express detectable amounts of this specific isoform. This down-modulation was not due
to the lowering of the overall expression of CD97 isoforms on B cells isolated out of lymphoid tissue, as this overall expression was comparable to that on circulating B cells. This points to a mechanism which specifically would down-modulate the largest isoform of CD97 on B cells in lymphoid tissue. In light of this it is interesting to know that B cells in blood and spleen do express chondroitin sulfate B [14], which is the only known specific ligand for the largest CD97 isoform. Consequently, the down-modulation of the largest CD97 isoform on B cells in lymphoid tissue might be a result of the continuous interaction with chondroitin sulfate B on the surface of neighboring cells in the B cell clusters in this compartment. This would introduce a ligand mediated level of regulation of the CD97 expression.

In summary, our results indicate that CD97 expression can differentially be modulated in lymphocyte subsets and in different compartments. Moreover, this modulation is most likely to take place at different levels. A further definition of these modulation processes and the unraveling of the factors responsible for their induction could shed a light on how and why this occurs on specific subsets and for specific isoforms. Consequently, these answers could give a clue on the specific function of CD97 and its isoforms on certain subsets and under certain circumstances, respectively.

**Reference List**


4. Baud,V., Chissoe,S.L., Viegas-Pequignot,E., Diriong,S., NGuyen,V.C., Roe,B.A., and Lipinski,M., EMR1, an unusual member in the family of


