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# Differential expression of CD97 on human lymphocyte subsets and limited effect of CD97 antibodies on allogeneic T-cell stimulation

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ABSTRACT

CD97 is a large heptahelical EGF-TM7 receptor broadly expressed on hematopoietic cells as three isoforms with respectively three, four, or five epidermal growth factor (EGF)-like domains. We here describe the expression characteristics of CD97 on human lymphocyte subsets. 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. In $CD4^+$ T cells, CD97 expression was higher on memory cells compared to naive cells. In $CD8^+$ T and NK cells, we found a downregulation of CD97 on cytolytic effector cells. Stimulation through CD3 and CD28 resulted in a rapid upregulation of CD97 in all T-cell subsets within 2–4 h. A link between CD97 expression and lymphocyte proliferation was established in NK cells, which markedly upregulated CD97 in response to IL-2 and IL-15. Mixed lymphocyte cultures revealed a limited ability of the stalk region-specific monoclonal antibody CLB-CD97/3 to inhibit $CD8^+$ and $CD4^+$ allogeneic T-cell proliferation.



## 1. Introduction

Human CD97 [1–3] is a member of the EGF-TM7 family of adhesion class heptahelical receptors [4], further consisting of EMR1 [5], EMR2 [6], EMR3 [7], and EMR4 [8]. At the cell surface, EGF-TM7 receptors are expressed as non-covalently associated dimers, consisting of a large extracellular α- and a seven-transmembrane (TM7) β-chain [9]. The extracellular chain possesses at its N-terminus tandemly arranged epidermal growth factor (EGF)-like domains. 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 extracellular region of CD97 contains binding sites for cellular ligands as first demonstrated for EGF domain 1 and 2, which bind CD55, the decay-accelerating factor for complement [10]. Affinity for CD55 differs between CD97 isoforms and is highest for CD97(EGF1,2,5) and lowest for CD97(EGF1,2,3,4,5) [11,12]. Another ligand, the glycosaminoglycan chondroitin sulfate B, also known as dermatan sulfate, interacts with EGF domain 4, which is only present in the largest isoform CD97(EGF1,2,3,4,5) [13–15]. Finally, the integrins α5β1 (VLA-5) and, possibly, αvβ3 bind a RGD motif in the stalk region of human CD97 [16].

EGF-TM7 receptors are expressed on leukocytes [4]. CD97 was originally identified as an early activation antigen on lymphocytes [1,17]. Later studies detected CD97 on almost all types of leukocytes as well as on normal and malignant epithelial and muscle cells [18–21]. Two recently generated CD97-deficient mice were essentially normal at steady state, except for a mild granulocytosis, which increased under inflammatory conditions [22,23]. Treatment of wild-type mice with monoclonal antibodies (mAbs) in various disease models suggested a role for CD97 in granulocyte trafficking [24–26]. While these *in vivo* studies mainly focused on the role of CD97 on granulocytes, a detailed description of CD97 on lymphocytes is still lacking. Recently, Spendlove and colleagues reported that CD97 can act as a co-stimulatory ligand modulating the activation of human $CD4^+$ T cells through CD55 [27]. In a subsequent study, CLB-CD97/1, a mAb to the first EGF domain of CD97, was shown to block proliferation and IFNγ production of both $CD4^+$ and $CD8^+$ T-cell clones, co-cultured with peptide-pulsed autologous monocytes [28].

Based on the different function and compartmentalization of lymphocyte subsets, knowledge on the expression of CD97 could provide additional clues on the role of CD97 in adaptive immunity. By using mAbs to EGF domain 1, EGF domain 4, and the stalk region, we here assessed the surface expression of human CD97 on subsets of T, B, and NK cells in blood and in different lymphoid compartments. In addition, we evaluated the ability of these mAbs that selectively block CD97-ligand interactions *in vitro* to interfere with

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allogeneic lymphocyte proliferation in mixed lymphocyte cultures (MLCs).

## 2. Materials and methods

### 2.1. Cells

Heparine-treated blood samples and buffy coats were obtained from healthy volunteers. Bone marrow samples were obtained from patients who underwent a diagnostic biopsy and whose bone marrow turned out to be normal. Tonsils were obtained from patients who underwent therapeutic 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 cells from tonsil, spleen, and bone marrow were suspended in IMDM supplemented with 10% fetal calf serum (FCS), before Ficoll-Isopaque PLUS (Amersham Bioscience, Freiburg, Germany) 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.

### 2.2. Monoclonal antibodies

Three CD97-specific mAbs, all biotinylated, were used: CLB-CD97/1 (mouse IgG2a, directed against EGF domain 1), 1B5 (hamster IgG2, directed against EGF domain 4), and CLB-CD97/3 (mouse IgG1, directed against the stalk region) [10,13]. In addition, directly labeled mAbs with the following specificity were used: CD3-FITC, CD4-PE/APC/PECy5.5, CD8-PE/APC/PECy5.5, CD11a-APC, CD19-PECy5.5, CD38-PE, CD49d-PE, CD56-PE, CD103-PE, IgD-FITC (all Becton Dickinson, San Jose, CA), CD11a-APC (Imgen, The Netherlands), CD27-FITC (Immunotools, Friesoythe, Germany), and CD45RA-PE (Beckman Coulter, Fullerton, CA). FITC-, PE-, APC-, 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/PE (Pharmingen, San Diego, CA) was applied as second step reagent.

### 2.3. 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 at saturating concentrations. In a second step, the samples were incubated with lymphocyte subset-specific mAbs along with streptavidin-APC or streptavidin-PE. Prior to cytometry, erythrocytes were lysed using FACS lysing solution (Becton Dickinson). All incubation steps were performed on ice. Data were analyzed using Cellquest software (Becton Dickinson).

To measure apoptosis, cells were harvested and washed in ice-cold HEPES buffer (10 mM HEPES, 150 mM KCl, 1 mM $MgCl_2$, and 1.3 mM $CaCl_2$, pH 7.4) supplemented with 1 mg/ml glucose and 0.5% BSA. Cells were then incubated with APC-labeled Annexin V (ImmunoQuality Products, Groningen, The Netherlands) (diluted 1:200 in HEPES buffer) for 30 min and washed in HEPES buffer. Just before analysis of the samples by flow cytometry, propidium iodide (PI) was added (final concentration 5 μg/ml) to distinguish necrotic cells (annexin $V^-/PI^+$) from early apoptotic cells (annexin $V^+/PI^-$) and late apoptotic cells (annexin $V^+/PI^+$).

### 2.4. Stimulation assay

Peripheral blood mononuclear cells (PBMCs) were isolated from heparine-treated blood samples by centrifugation over a Ficoll-Paque PLUS density gradient. Cells were incubated for 4 h in IMDM supplemented with 10% FCS at 37 °C and 5% $CO_2$. Agonistic mAbs against CD3 (1XE) [29] and CD28 (15E8) [30] were added at concentrations of 1:1000 and 5 μg/ml, respectively. Expression of CD97 on the $CD4^+$ and $CD8^+$ T-cell subsets was assessed by flow cytometry.

NK cells were isolated from PBMC through negative depletion of monocytes and non-NK lymphocytes using the NK cell isolation kit of Miltenyi (Bergisch Gladbach, Germany). Cells were incubated in culture medium in the presence or absence of 50 ng/ml recombinant human IL-2, IL-12, IL-15, or IL-18 (R&D Systems Inc., Minneapolis, MN). After 3 days, expression of CD97 was assessed by flow cytometry.

### 2.5. Mixed lymphocyte culture

Experiments were performed as described previously [31]. In short, frozen PBMCs, isolated from buffy coats by density gradient centrifugation, were thawed, pelleted, and resuspended at a final concentration of $10 \times 10^7$ cells/ml in phosphate buffered saline (PBS). Cells were labeled with 2.5 μM (final concentration) 5-(and 6)-carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes Europe BV, Leiden, The Netherlands) for 10 min at 37 °C. Subsequently, cells were washed in Hank's Balanced Salt Solution (Bio Whittaker, Verviers, Belgium) supplemented with 0.025 M Tris (hydroxymethyl) aminomethane, 100 U/ml sodium penicillin G (Brocades Pharma BV, Leiderdorp, The Netherlands), 100 μg/ml streptomycin sulfate (Gibco BRL, Paisley, Scotland), and 5% FCS (Intero, Zaandam, The Netherlands) (*wash medium*) and resuspended in IMDM supplemented with antibiotics as above, $3.57 \times 10^{-4}$% β-mercaptoethanol (Merck), and 10% heat-inactivated human pooled serum (BioWhittaker) (*culture medium*).

50,000 CFSE-labeled responder cells were incubated with 50,000 gamma-irradiated (3000 rads) allogeneic or autologous stimulator PBMC at 37 °C and 5% $CO_2$ in 96-well round-bottom culture plates in a final volume of 170 μl of culture medium/well. CD97-directed or control antibodies were added at a final concentration of 10 μg/ml. Isotype-matched control antibodies were F23-49 (IgG2a, mouse-anti *Mycobacterium tuberculosis*), F24-2 (IgG1, mouse-anti *M. tuberculosis*), both kind gifts from Dr. A.H. Kolk (Royal Tropical Institute, Amsterdam, The Netherlands), and purified hamster Ig (Tebu-bio, Heerhugowaard, The Netherlands).

After 6 days, the cells were harvested and analyzed by flow cytometry. $CD4^{bright}$ and $CD8^{bright}$ T cells, which retained maximal CFSE fluorescence, were presumed to be non-dividing and therefore designated as non-responding cells. $CD4^{bright}$ and $CD8^{bright}$ T cells that lost any CFSE fluorescence were presumed to have undergone at least one cell division and were therefore designated as alloreactive $CD8^+$ T cells. Calculation of precursor frequencies was performed as described [32]. Since allogeneic proliferation of T cells differs with every stimulator-responder combination, most summary results are expressed as normalized ratios, i.e. results from the allogeneic stimulations in the absence of antibodies were set at 100%.

### 2.6. Granzyme B ELISA

Granzyme B was measured by ELISA (CLB, Amsterdam, The Netherlands) following manufacturer's instructions.