

Cell surface cathepsin G can be used as an additional marker to distinguish T cell subsets

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Abstract. The serine protease cathepsin G (CatG) is involved in numerous processes associated with the innate and adaptive immune system. During an immune response, neutrophils secrete CatG, which can bind to the cell surface of immune cells to provoke the proteolytic processing of cytokines and chemokines in order to stimulate lymphocytes. The present study analyzed peripheral blood mononuclear cells to characterize T cell populations in terms of their CatG content by flow cytometry. It was identified that CatG was exclusively present on the cell surface of a subset of T regulatory cells (Tregs), cluster of differentiation (CD) 39⁺ Tregs, which expressed CatG in contrast to CD39⁻ Tregs. Additionally, CatG was expressed on double positive CD4⁺CD8⁺ T cells, T helper (Th) 9 cells and Th22 cells, implicating CatG as a novel marker to distinguish certain T cell subsets.

Introduction

Organisms are constantly exposed to diverse and harmful factors. As they are exposed to a surrounding environment containing bacteria, viruses and fungi, in addition to multicellular parasites, it is important that complex organisms develop efficient and specialized defense mechanisms. The innate immune system, which includes neutrophils, acts as the first line of contact against potential pathogens. By contrast, B cells, cytotoxic T lymphocytes and cluster of

differentiation (CD) 4⁺ T cells represent the majority of immune cells within the adaptive immune system, which is characterized by different properties, including a variety of antigen-specific receptors (B and T cell receptors) and immunological memory (1). Antigenic peptides loaded to major histocompatibility complex (MHC) class I molecules are detected by CD8⁺ T cells; whereas macrophages, dendritic cells (DCs) and B cells, as professional antigen-presenting cells (APCs), display antigenic peptides to MHC II molecules, leading to CD4⁺ T cell activation when foreign antigens are recognized by these cells (2). CD4⁺ T cells are capable of differentiating into several types of T helper (Th) cells, including Th1, Th2, Th9, Th17 and Th22 cells, and execute distinct effector functions during an immune response (1). For example, Th1 cells detect intracellular pathogen-derived antigens, Th2 and Th9 cells defend against parasites, Th17 cells recognize fungi and extracellular bacteria, and Th22 cells serve as a defense against microbial infections of the skin (3-5).

T regulatory cells (Tregs) are essential for maintaining an immune response, immune homeostasis, and tolerance. Approximately 5% of CD4⁺ T cells are Tregs in normal human peripheral blood. Tregs are divided into thymus-derived natural Tregs, induced Tregs generated by transforming growth factor- β and interleukin (IL)-2 *in vitro*, and peripheral Tregs (6). CD39⁺ Tregs express the ectonucleotidases CD39 and CD73; CD39 hydrolyzes extracellular ATP and ADP to generate AMP, and CD73 further converts AMP to adenosine, which binds to cell surface A2A receptor of effector cells and thereby suppresses a T cell response (7-10). Notably, antigen-specific Tregs express the co-stimulatory molecule CD134 (11,12).

Cathepsin G (CatG) belongs to the family of serine proteases. Due to the structural properties of the active center, which contains a catalytic triad consisting of histidine, aspartate and serine amino acids (13), CatG exhibits chymotrypsin and trypsin-like enzymatic activity with a broad substrate specificity (14,15). CatG and lactoferrin (LF), among other serine proteases, are released by activated neutrophils during an immune response (16). Of note, a previous study by our group identified that LF increased the activity of CatG and lowered its substrate specificity, and the combined action of LF and CatG increased the activation status of human platelets (17). Furthermore, CatG exhibit an antibacterial capacity,

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Abbreviations: APCs, antigen-presenting cells; Cat, cathepsin; CatG, cathepsin G; DCs, dendritic cells; ECM, extracellular matrix; LF, lactoferrin; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; NK cells, natural killer cells; PBMCs, peripheral blood mononuclear cells; SDF-1, stromal cell-derived factor 1; Th, T helper; Tregs, T regulatory cells

Key words: cathepsin G, proteases, T regulatory cells, CD39⁺ Tregs

indicated by the positive charge of sufficient arginine residues within the CatG protein sequence (18) and is a component of the so-called neutrophil extracellular traps, as CatG has, compared with other serine proteases, a notably high affinity towards deoxyribonucleic acid (19,20). In addition to the activation of specific cytokines to modulate an immune response, CatG is able to inactivate cytokines, including IL-2 and IL-6, and the growth and maturation factor CXC chemokine stromal cell-derived factor 1 (SDF1) (21). Additionally, CatG has been detected on the cell surface of different immune cells, namely neutrophils (22), B cells, natural killer (NK) cells (23) and platelets (24), and low levels of CatG have been detected on CD4⁺ T cells (23). Previously, our group demonstrated that distinct NK cell subsets (CD16^{dim}CD56^{dim} and CD16^{dim}CD56^{bright}) possessed proteolytic active CatG on their cell surface in contrast to other NK cell subsets (CD16^{dim}CD56^{bright}, CD16^{dim}CD56^{bright}, CD16^{bright}CD56^{dim}, CD16^{dim}CD56^{dim}, and CD16^{bright}CD56^{dim}) (25). However, which T cell subsets carry CatG, and whether CatG can be detected on Tregs, was not determined. Therefore, the present study analyzed peripheral blood mononuclear cells (PBMCs) for their cell surface CatG content, and identified that CD4⁺CD8⁺ T cells, Th9 cells, Th22 cells and CD39⁺ Tregs, but not CD39⁻ Tregs, harbored CatG at the cell surface, implicating CatG as a novel marker for these cells.

Materials and methods

Sample collection and preparation. Freshly purified or cryopreserved PBMCs from healthy male or female donors, young donors between 18-25 years and elderly donors between 59-70 years (collected between 2015 and 2016; gender ratio: female:male, 50:50), were analyzed. The PBMCs from buffy coats, obtained from the DRK Blood Donation Center, Baden-Württemberg-Hessen; Institute Ulm (Ulm, Germany), were purified using Ficoll density centrifugation (Ficoll-Paque PLUS, GE Healthcare, Little Chalfont, UK). The blood donors were informed with regard to the use of their blood cells for research purposes and their consent was obtained. Briefly, 25 ml of a 1:10 phosphate-buffered saline (PBS, pH 7.4, Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) buffy coat dilution was carefully added to the Ficoll solution (15 ml), which was prepared in a 50-ml centrifuge tube, and centrifuged (750 x g for 20 min at room temperature). Following centrifugation, PBMCs were cautiously collected from the Ficoll interface, washed three times with PBS, and prepared for flow cytometric analysis. In case of later usage of PBMCs, 1x10⁷ cells were stored in fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc.) with 10% dimethyl sulfoxide (DMSO, Serva Electrophoresis GmbH, Heidelberg, Germany) at -80°C.

Detection of CatG on the cell surface of different T cell subsets. The cryopreserved PBMCs were thawed, washed twice with PBS and adjusted to a final concentration of 5x10⁶ PBMCs/ml. Subsequently, the PBMCs were stained with anti-CD25-APC (4 µg/ml; BC96; Thermo Fisher Scientific, Inc.), anti-CD4-APC/Cy7 (4 µg/ml; RPA-T4; BD Biosciences, Franklin Lakes, NJ, USA), anti-CD8-PerCP (4 µg/ml; SK1; BD Biosciences), anti-C-C-chemokine receptor

type 10 (CCR10)-APC (2.5 µg/ml; 6588-5; BioLegend, Inc., San Diego, CA, USA), anti-CD4-Alexa Fluor 700 (4 µg/ml; OKT4; eBioscience; Thermo Fisher Scientific, Inc.), anti-CD39-PerCP/Cy5.5 (2 µg/ml; A1; BioLegend, Inc.), anti-CD127-PE/Cy7 (4 µg/ml; A019D5; BioLegend, Inc.), anti-CD134-PE (4 µg/ml; Ber-ACT35; BioLegend, Inc.), anti-CD183-PE (5 µg/ml; G025H7; BioLegend, Inc.), anti-CD194-PerCP/Cy5.5 (2.5 µg/ml; L291H4; BioLegend, Inc.), anti-CD196-PE/Cy7 (5 µg/ml; G034E3; BioLegend, Inc.), anti-human CatG-FITC (2 µg/ml; ABIN5565299; Acris Antibodies GmbH, Herford, Germany), or isotype control for CatG (2 µg/ml; ABIN96440; Acris Antibodies GmbH). The PBMCs were diluted in PBS (pH 7.4) containing 1% FBS and incubated with the respective antibody set for 30 min at 4°C. Following incubation, the PBMCs were washed, collected by a FACSCanto II cytometer (BD Biosciences), and analyzed using FlowJo software version 7.6.5 (FlowJo LLC, Ashland, OR, USA). The compensation was performed using unstained and single stained PBMCs by using the respective antibody and was calculated using FACSDiva™ software (BD Biosciences). Flow cytometric analysis and antibody selection for the Th subsets were based on resources from Miltenyi Biotec, Inc. (http://www.miltenyibiotec.com/~media/Files/Navigation/Cell%20analysis/resources/App_note-20320_Tcell_subsets_05_WEB.ashx).

Statistical analysis. Statistical analysis was performed with GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as standard error of the median and statistical analysis was performed using one-way analysis of variance and the Bonferroni correction. The use of PBMCs for in vitro experiments was approved by the Ethics Committee of Ulm University (Ulm, Germany; proposal no. 327/14).

Results

CatG is present on double positive CD4⁺CD8⁺ T cells, Th9 cells and Th22 cells. Double positive CD4⁺CD8⁺ T cells are present in small numbers in the peripheral blood (26,27) and their function remains a source of debate; for example, these cells exhibit an antiviral activity (28) but have also been implicated in several pathological conditions, including autoimmunity (29,30) and cancer (31). To determine whether these cells express cell surface CatG, PBMCs were gated for CD8⁺ T cells, CD4⁺ T cells and double positive CD4⁺CD8⁺ T cells, and the levels of cell surface CatG were analyzed. CatG was not detected on the cell surface of CD8⁺ T cells, as illustrated in Fig. 1A and B. This is in accordance with previously published data demonstrating that CatG was absent on CD8⁺ T cells (23,32). In contrast to the CD8⁺ T cells, CatG was identified on the cell surface of CD4⁺CD8⁺ T cells and on CD4⁺ T cells; however, the obtained data for CD4⁺ T cells did not reach statistical significance. These results correlate with previously published data indicating that only low levels of CatG can be detected on CD4⁺ T cells (23). Collectively, these findings suggest that the cell surface expression of CatG differs between CD4⁺ T cell subsets and that only a subpopulation may be positive for CatG. To examine this further, a panel of Th subsets was analyzed. Subpopulations of Th1, Th2, Th9, Th17, and Th22 cells were distinguished by a combination of

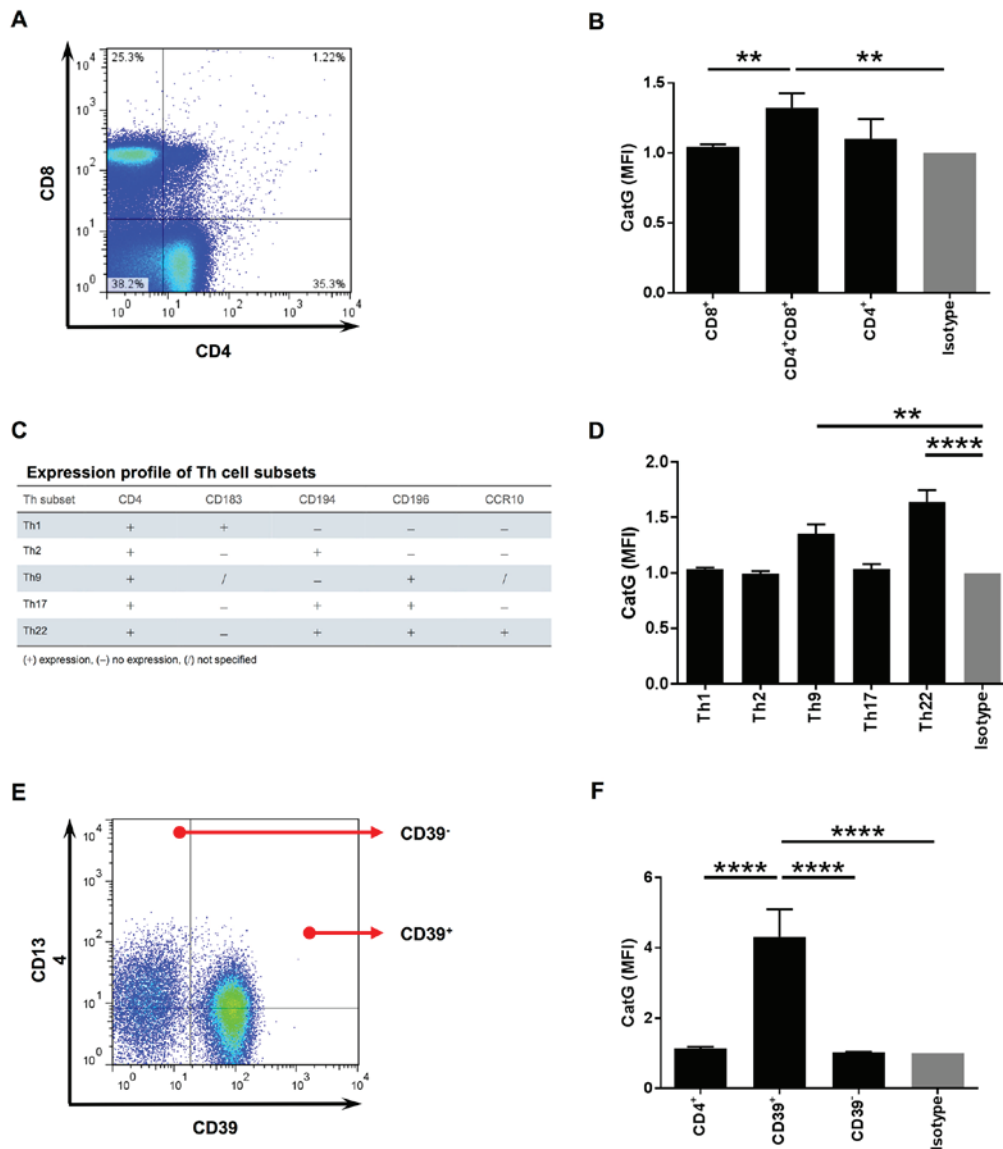


Figure 1. Analysis of CatG on the cell surface of PBMCs. (A) Cell populations were determined by the expression of CD4 and CD8 in combination with the expression of CatG. Double positive CD4⁺CD8⁺ T cells, CD4⁺ T cells, and CD8⁺ T cells were detected and shown as one representative figure. (B) Expression levels of CatG on the indicated cells are summarized in the bar diagram (n=10). (C) Analysis of the activity of CatG on the cell surface of Th cell subsets. (D) Th1, Th2, Th9, Th17, and Th22 cells were detected, and cell surface expression of CatG was compared via flow cytometry. Th1 cells were determined by CD183⁺CD194⁺CD96⁺CCR10⁻ expression, Th2 cells by CD183⁺CD194⁺CD196⁺CCR10⁻ expression, Th9 cells by CD194⁺CD196⁺ expression, Th17 cells by CD183⁺CD194⁺CD196⁺CCR10⁻ expression, and Th22 cells by CD183⁺CD194⁺CD196⁺CCR10⁺ expression (n=10). (E) Detection of CatG at the cell surface of Tregs. CD39⁻ and CD39⁺ Tregs were compared for their cell surface expression of CatG by using flow cytometry. Cells were gated for CD127^{dim}CD4⁺CD25⁺ and the remaining cells (CD39 vs. CD134) were split into Treg cell subsets (CD39⁻ and CD39⁺ Tregs). (F) Levels of CatG on CD39⁻ and CD39⁺ Tregs were analyzed in 37 healthy donors. For comparison, the MFI was used. The data were normalized to the isotype control and MFI values are expressed as the standard error of the median. Significant differences were calculated by one-way analysis of variance and the Bonferroni correction. **P<0.01 and ****P<0.0001. CatG, cathepsin G; CD, cluster of differentiation; CCR, C-C chemokine receptor; Th, T helper; Tregs, T regulatory cells; MFI, median fluorescence intensity.

essential cell surface markers. An overview of the different antibodies used to identify Th subsets, namely CD4, CD183, CD194, CD196 and CCR10 antibodies, is shown in Fig. 1C. Notably, Th9 and Th22 cells carried cell surface CatG, whereas the analysis did not detect any CatG on Th1, Th2, or Th17 cells (Fig. 1D). Taken together, these findings suggest that CatG is present on the cell surface of double positive CD4⁺CD8⁺ T cells, Th9 cells, and Th22 cells.

CatG is expressed on the cell surface of CD39⁺ Tregs. Tregs are a heterogenic cell population. However, two subsets can be distinguished by their CD39 expression status, namely as

CD39⁺ and CD39⁻ Tregs (11). The CD39⁺ Treg subpopulation has a higher immune-suppressing capacity compared with CD39⁻ Tregs (8,9,33). In order to phenotype CD39⁺ Tregs from viable singlets, cells were identified by an antibody panel recognizing cell surface CD4, CD127, CD25, CD134, and CD39 molecules (34). These CD4⁺ T cells, which exhibit a low expression of CD127 and high expression of CD25 (35), were further gated for CD134⁺, and the two groups of CD39⁻ and CD39⁺ Tregs were investigated (data not shown). Notably, CD39⁺ Tregs did express CatG on the cell surface, whereas cell surface CatG was absent on CD39⁻ cells (Fig. 1E and F). In addition, a small demographic donor group of CD39⁺ and

CD39⁻ Tregs was compared; however, no difference in the cell surface expression of CatG was observed when young and elderly donors were compared (data not shown). Taken together, these findings demonstrate that CD39⁺ Tregs harbor CatG on the cell surface, in contrast to CD39⁻ Tregs.

Discussion

During an inflammatory response, neutrophils secrete CatG into the extracellular space to propagate innate and adaptive immune response (16). When simulating an immune response, a previous study indicated that exogenous CatG binds to CD4⁺ T cells, CD8⁺ T cells, NK cells, and B cells (32). Although these findings were not determined for CD8⁺ T cells when excess quantities of CatG were added to the assay (23), the authors identified that the growth and maturation factor CXC chemokine SDF1, being key for an inflammatory response (21), was proteolytically inactivated by CatG present on the cell surface of B cells, NK cells, and weakly on CD4⁺ T cells (23). The aforementioned results are of particular interest as Tregs are responsible for terminating an immune response (36). In turn, the data obtained in the present study suggest that cell surface CatG may support Treg cell function to maintain immune homeostasis.

In our previous study, it was demonstrated that DCs from CatG-deficient mice expressed lower levels of cell surface MHC I molecules compared with their wild-type counterparts. Furthermore, CatG provokes the upregulation of cell surface MHC I molecules on PBMCs and on human glioblastoma cells. Notably, cell surface CatG from PBMCs induces the expression of MHC I on the THP-1 monocytic cell line (37). In consideration of our previously published data and the finding that non-activated CD4⁺CD8⁺ T cells expressed CatG on their cell surface, it may be hypothesized that CD4⁺CD8⁺ T cells provoke an upregulation of MHC I on their target cell in order to monitor the intracellular peptide status via MHC I. In case of an altered MHC I-peptide repertoire, CD4⁺CD8⁺ T cells may act via their specialized function, which is potentially important for an immune response.

Migration and infiltration purposes of CD4⁺CD8⁺ T cells, Th9 cells, Th22 cells, and CD39⁺ Tregs may be an additional reason for the presence of CatG at the cell surface. It is well known that matrix metalloproteinases are not the only essential enzymes in extracellular matrix (ECM) remodeling and cell migration; serine proteases, including CatG, are also capable of degrading the ECM (38). It is possible that CD4⁺CD8⁺ T cells, Th9 cells, Th22 cells, and CD39⁺ Tregs reach their destination more efficiently than non-CatG expressing T cells. The significance of CatG on CD39⁺ Tregs and its biological function can be analyzed by the application of a specific CatG inhibitor, potentially eliciting the modulation of T cell migration. The use of flow cytometry to detect CatG has limitations, additional methods are required in order to further examine CatG and its potential role in the support or regulation of an immune response.

In conclusion, the present study used a direct FITC-conjugated anti-CatG antibody to perform the assay, which was found to be advantageous for the rapid analysis of immune cells compared with the unconjugated anti-CatG antibody used in previous studies (23,32). Furthermore, CatG may

be considered as a novel cell surface marker of CD4⁺CD8⁺ T cells, Th9 cells, Th22 cells, and CD39⁺ Tregs.

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Availability of data and materials

All materials included in this manuscript can be made freely available to any researchers who wish to use them for non-commercial purposes.

Authors' contributions

AP performed the experiments and analyzed the data. TB designed the experiments and wrote the manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all participants and the study was approved by the Ethics Committee of Ulm University (Ulm, Germany, proposal no. 327/14).

Patient consent for publication

Written informed consent was obtained from all participants, which covered publication of the participants' data anonymously.

Competing interests

The authors declare that they have no competing interests.

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