



Serine Proteases in Immunity

Cathepsin G and its Dichotomous Role in Modulating Levels of MHC Class I Molecules

Timo Burster¹ · Uwe Knippschild² · Ferdinand Molnár¹ · Anuar Zhanapiya¹

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Abstract

Cathepsin G (CatG) is involved in controlling numerous processes of the innate and adaptive immune system. These features include the proteolytic activity of CatG and play a pivotal role in alteration of chemokines as well as cytokines, clearance of exogenous and internalized pathogens, platelet activation, apoptosis, and antigen processing. This is in contrast to the capability of CatG acting in a proteolytic-independent manner due to the net charge of arginine residues in the CatG sequence which interferes with bacteria. CatG is a double-edged sword; CatG is also responsible in pathophysiological conditions, such as autoimmunity, chronic pulmonary diseases, HIV infection, tumor progression and metastasis, photo-aged human skin, Papillon–Lefèvre syndrome, and chronic inflammatory pain. Here, we summarize the latest findings for functional responsibilities of CatG in immunity, including bivalent regulation of major histocompatibility complex class I molecules, which underscore an additional novel role of CatG within the immune system.

Keywords Cathepsin G · Proteases · T regulatory cells · NK cells · MHC · Lactoferrin · Protease-activated receptor

Abbreviations

APCs Antigen-presenting cells

Cat Cathepsin

CatG Cathepsin G

cDCs Conventional dendritic cells

DCs Dendritic cells

HIV Human immunodeficiency virus

HLA Human leukocyte antigen

LF Lactoferrin

MHC Major histocompatibility complex

NE Neutrophil elastase

NETs Neutrophil extracellular traps

NK Natural killer

PAR Protease-activated receptor

PBMCs Peripheral blood mononuclear cells

PMSF Phenylmethylsulfonyl fluoride

SDF1 Stromal cell-derived factor 1

TCR T cell receptor

TGF- β Transforming growth factor beta

Th T helper

Tregs T regulatory cells

Tregs Thymus-derived natural Tregs

T1D Type 1 diabetes mellitus

Introduction

Proteases are proteolytic enzymes that participate in physiological cell metabolism and are mainly responsible for protein degradation by hydrolysis. As a consequence, they are functional in the immune system, for instance, for processing of intracellular antigens within antigen-presenting cells (APCs) (Kramer et al. 2017; Sadeghzadeh et al. 2020). Neutrophils, recruited and activated at the site of inflammation, naturally release different classes of mediators to the surrounding environment and thereby support an immune response. One example is serine proteases, such as cathepsin G (CatG), protease 3 (PR3), neutrophil elastase (NE), and neutrophil serine protease resident in primary granules compared to lactoferrin (LF) located in secondary granules.

✉ Timo Burster
timo.burster@nu.edu.kz

¹ Department of Biology, School of Sciences and Humanities, Nazarbayev University, Kabanbay Batyr Ave. 53, Nur-Sultan 010000, Kazakhstan

² Department of General and Visceral Surgery, Surgery Center, Ulm University Hospital, 89081 Ulm, Germany

All of them harbor the serine amino acid residue at the active center, which is the reason for their classification. Serine proteases comprise a common feature of structural properties of the active center with a catalytic triad consisting of histidine, aspartate, and serine amino acids, which allows the peptide bond between amino acids within the protein sequence to be hydrolyzed (AhYoung et al. 2019; Grzywa et al. 2019; Korkmaz et al. 2018). The proteolytic milieu is critical for the integrity and function of the effective immune system. However, to avoid unintended and continuous proteolytic degradation, immune cells express serine protease inhibitors (serpins) which in turn inhibit serine protease activity (Burgener et al. 2019; Cooley et al. 2001), a mechanism to prevent damage of healthy cells or to block the destruction of the neighboring tissue. These inhibitors are also essential in the advanced life cycle of cells and the process of aging, since the proteolytic activity of proteases under such circumstances is not properly regulated (Gromakova and Konovalenko 2003; Vaughan-Thomas et al. 2000; Wyczalkowska-Tomasik and Paczek 2012). Proteases are not only involved in physiological function, they have the ability to act under pathophysiology conditions. For instance, high proteolytic activity of serine proteases in cancer stem cells supports tumor development (Hillebrand and Reinheckel 2019) and CatG is increased in acute lymphoid leukemia (Khan et al. 2018), indicating a logical rationale for applying pharmacological protease inhibitors to prevent tumorigenesis, which certainly may not interfere with the general proteolytic responsibility of the protease in the organism.

Cathepsins are lysosomal/endosomal proteases responsible for protein degradation within the endocytic compartment. A majority of these proteases are involved in generating antigenic peptides to be loaded into the peptide binding groove of major histocompatibility complex class II (MHC II) molecules and presented to T helper (Th) cells. In addition, cathepsins are found in the nucleus or nuclear membrane, on the cell membrane, and extracellular space in order to activate or deactivate protein- or peptide-based mediators by hydrolysis. Cathepsins belong to three different mechanistic classes of proteases, as noted in the previous section CatG is a serine protease, whereby cathepsin A is also referred to this class, compared to cathepsins B, C, F, H, K, L, O, S, V, W, and X which are classified as cysteine proteases. The third class are aspartic proteases, such as cathepsins D and E, as has been extensively reviewed in Bischof et al. (2017), Cresswell (2019), Holzen et al. (2020) and Zhang et al. (2020).

In this briefing, we will focus on the significance of CatG with respect to its functional role in the immune system. The proteolytic activity of CatG modifies chemokines and cytokines, clearance of internalized pathogens, activation of cell surface receptors, platelet activation, apoptosis, and antigen processing (Burster 2013; Fu et al. 2020;

Zamolodchikova et al. 2020). CatG provokes proliferation of CD4⁺ T cells, and to a lesser extent B cells but not CD8⁺ T cells (Hase-Yamazaki and Aoki 1995). CatG can act in a proteolytic-dependent and -independent antimicrobial manner through the positive net charge of CatG, which is due to arginine residues within the CatG sequence. Furthermore, neutrophils can release extracellular traps (neutrophil extracellular traps: NETs), enclosing DNA and histones as well as cytoplasmic granular proteins, like CatG, to catch and trap microorganisms in the extracellular space, since CatG has a high affinity to deoxyribonucleic acid (Liu and Liu 2020; Ma et al. 2019). However, CatG is involved in autoimmunity, chronic pulmonary diseases, human immunodeficiency virus (HIV) infection, tumor progression, and metastasis (Burster 2013; Meyer-Hoffert 2009; Pham 2008). Furthermore, it was shown that CatG is increased in photo-aged human skin and primary fibroblasts (Zheng et al. 2012); interestingly, specific inhibition of CatG prevents ultraviolet B-induced photo-aging (Son et al. 2012). From a clinical point of view, the absence of active CatG, protease three, and neutrophil elastase in activated neutrophils results in the reduced ability of neutrophils in phagocytosis and NET formation to neutralize microorganisms. Increased generation of reactive oxygen species and release of inflammatory mediators underlying host tissue damage and is responsible for the outcome of Papillon–Lefèvre syndrome, which is characterized by periodontitis followed by a progressive loss of teeth at a young age. A reason for that lays in a mutation in the cathepsin C gene which inactivates the proteolytic capacity of cathepsin C and is thereby not able to convert serine proteases to their active form (Korkmaz et al. 2018; Sorensen et al. 2014). As previously reported, CatG is significantly upregulated in chronic inflammatory pain and inhibition of CatG reduced pain behavior by infiltrating neutrophils in rodents (Liu et al. 2015). Moreover, increased levels of CatG correlate with neutrophil counts in synovial fluid and in rheumatoid arthritis tissue, in contrast CatG-deficient mice as well as mice deficient in CatC are resistant to experimental arthritis (Adkison et al. 2002; Hu and Pham 2005; Miyata et al. 2007). On the one hand, CatG plays a significant role in protecting and defending the organism from invading pathogens; on the other hand, depending on its proteolytic activity, CatG can cause harm when it is active over an extended period of time. Therefore, during inflammation, CatG harbors beneficial effects to fight infections, but after a successful immune response, CatG activity has to be tightly regulated to a steady state level.

In the next sections, we take our recent findings into account to elucidate the function of CatG during an immune response. This includes how LF facilitates the performance of CatG and insight into how CatG regulates intracellular and extracellular cell surface levels of MHC I

molecules, which indicates a novel but dichotomous characteristic of CatG within the immune system.

Regulation of CatG Activity and Functional Consequences in Regulation of MHC Molecules

MHC: The Two Classes

Professional APCs [macrophages, B cells, and dendritic cells (DCs)], granulocytes, natural killer (NK) cells, cytotoxic T lymphocytes (CD8⁺ T cells), and Th cells (CD4⁺ T cells) represent the majority of immune cells of the immune system. CD8⁺ T cells recognize antigenic peptides properly loaded to MHC I molecules, which are expressed by almost all cells of the organism; whereas, antigenic peptides loaded to MHC II molecules and presented by professional APCs are engaged and inspected by CD4⁺ T cells (Jurewicz and Stern 2019).

Modulation of CatG Activity

It is well known that the 235-amino acid covering polypeptide CatG (~ 28 kDa), which was isolated in 1976 from the

human spleen, is highly active at a neutral pH, and exhibits chymotrypsin—and trypsin-like enzymatic activity (Powers et al. 1985; Starkey and Barrett 1976a, b). Surely, this is quite simplified since human CatG has trypsin (hydrolyzes peptide bonds after R and K amino acid residues), chymotrypsin (F, Y, and W), metase (M), and leuase (L) cleavage properties at the P1 subsite within the substrate; however, mouse CatG is limited to chymotrypsin-like activity (F, Y, and W) (Raymond et al. 2010). Strikingly, an additional cleavage site at the P1 position harboring asparagine (N), P2 proline (P), and for P3 position glutamic acid (E) was determined recently by high throughput protease profiling (Nguyen et al. 2018). Focusing on the C-terminal end within a given peptide, human CatG has a cleavage preference for isoleucine (I), alanine (A), and serine (S) at P1' and negatively charged amino acids (D and E) are preferred at the P2' position as was observed in an extended study (Nguyen et al. 2018; Thorpe et al. 2018). According to the three-dimensional structure, CatG has two homologous β -barrels with six antiparallel β -sheets joined with a linker segment for each barrel motif (Fig. 1). The catalytic triad of H⁵⁷, D¹⁰², and S¹⁹⁵ is located between the two-barrel folding pattern, where the active-site cleft is perpendicular to the two β -barrels allowing the hydroxyl group of S¹⁹⁵ to attack the carbonyl carbon of the peptide bond (substrate) in a nucleophilic manner (Hof et al. 1996; Korkmaz et al. 2010). In

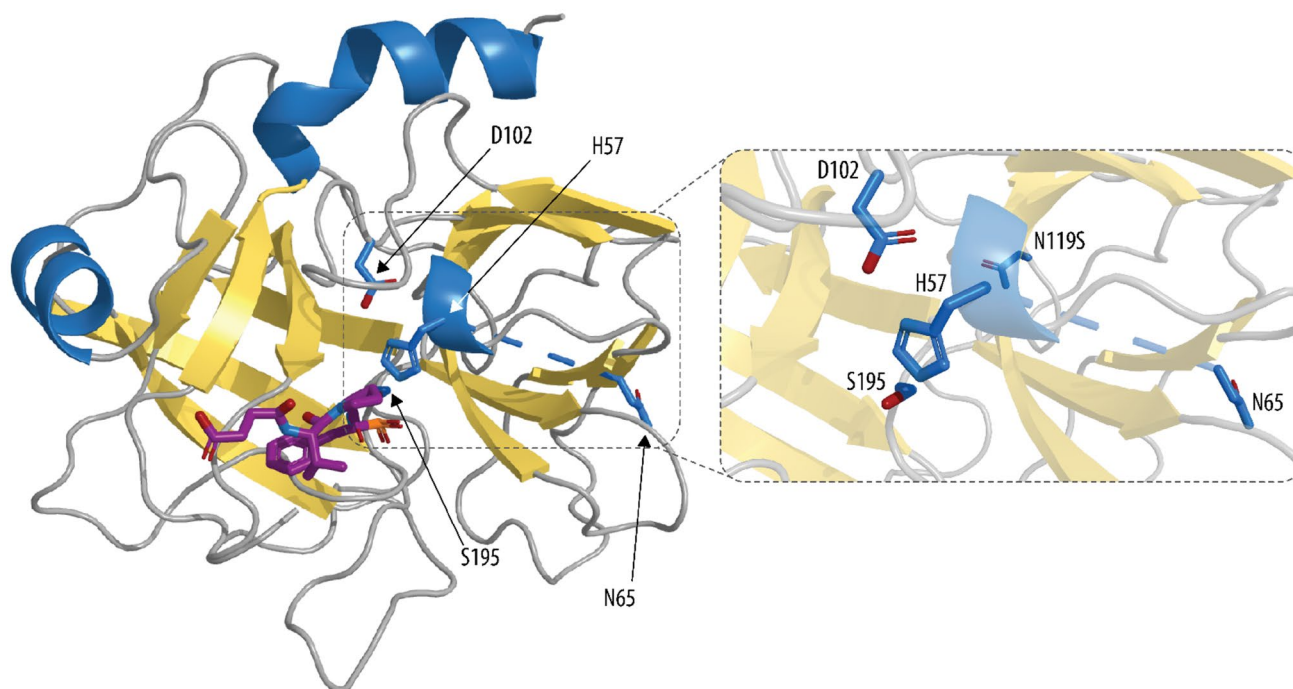


Fig. 1 Three-dimensional structure of human CatG (ribbon plot). The catalytic triad of CatG is indicated by H⁵⁷, D¹⁰², and S¹⁹⁵ which is located between the two asymmetric barrels and depicted in yellow. The C-terminal α -helix is shown on top and presented in blue. The

N-linked glycosylation site is found at N⁶⁵ and the polymorphism in the coding region of CatG is labeled as N¹¹⁹S. The inhibitor Suc-Val-Pro-PheP-(OPh)₂ (violet stick model) is complexed with CatG. PDB: 1CGH (Hof et al. 1996)

humans, four polymorphisms in the 5'-flanking region with no effects regarding the promotor- or transcription activity were observed, opposed to the polymorphism found in the coding region (N¹²⁵S), which is correlated with a lower survival rate after a cardiovascular or cerebrovascular episode due to high levels of plasma fibrinogen in patients with N¹²⁵S polymorphism (Herrmann et al. 2001). The single N-linked glycosylation site at N⁷¹ categorizes CatG for three different glycosylation isoforms, while the fourth isoform is not attached to a carbohydrate side chain (Loke et al. 2015; Salvesen et al. 1987; Watorek et al. 1993). Moreover, it was shown that a monocyte cell line (U937) segregated the ~32 kDa proCatG to the extracellular environment and the ~28 kDa CatG form resides in the lysosome (Lindmark et al. 1990, 1994). In addition, the diverse glycosylation status can destine CatG for secretion or lysosomal storage as suggested by Watorek et al. (1993).

Activated polymorphonuclear neutrophils release LF as well as CatG and other serine proteases during an immune response (Korkmaz et al. 2010); therefore, engagement of LF with CatG was investigated and determined that LF is a natural inhibitor of CatG (He et al. 2003). Having this in mind, we recently investigated the molecular interaction between LF and CatG. LF was incubated with CatG and the catalytic activity of CatG was determined by active-site label and enzyme kinetics; however, we found an increase of CatG activity by LF, proposing that LF interacts with CatG in an allosteric manner (Eipper et al. 2016). These findings were verified by an independent group to obtain a better understanding of the enhancing effect by using a protein digestion assay. Thorpe et al. (2018) found an increase of protein (thioredoxin) turnover rate when an excess of the LF protein was incubated with CatG, even though a specific, direct interaction of LF with CatG might be unlikely. Interestingly, levels of LF was found to be high and the catalytic activity of CatG was significantly increased in serum from osteomyelitis patients consisting of N¹²⁵S polymorphism in contrast to osteomyelitis patients with the N¹²⁵ phenotype. These findings of increased CatG activity can be explained by the interaction of LF and CatG, but more likely by the change of N¹²⁵S within the CatG protein sequence (Perez-Is et al. 2019).

Furthermore, LF broadens the substrate cleavage preference of CatG by lowering the substrate selectivity even for valine, based on the fact that CatG does not normally accept valine at the P1 position (Eipper et al. 2016). The functional consequences can be that LF directs and alters CatG activity for a more efficient performance of degrading pathogen-derived proteins due to the acceptance of an increased number of different amino acid residues at P1. Moreover, invading bacteria lower the pH environment through anaerobic metabolism (Menkin 1956). Interestingly, LF recovers CatG activity even at a lower pH (pH 5) indicating that LF

avoids reduction of CatG activity at the site of inflammation in order to still be able to fight against pathogens (Eipper et al. 2016). Another reason for an LF-mediated increase of CatG activity might be to support the function of platelets during inflammation or wound healing, since it is well known that CatG activates platelets via protease-activated receptors (PARs) (Heuberger and Schuepbach 2019; Selak et al. 1988) and an LF-mediated increase of CatG activity further activates platelets as demonstrated by us just recently (Eipper et al. 2016). Moreover, platelets can coat tumor cells and transfer their MHC I content to tumor cells in such a way that NK cells are not activated when tumor cells reduce levels of MHC I and hide tumor-associated peptide presentation on the tumor cell surface and thereby support an immune evasion of tumor cells (Erpenbeck and Schon 2010; Placke et al. 2012). Based on these findings, it can be speculated that CatG is involved in platelet-mediated tumor progression, since activated neutrophils secrete CatG and LF, which support the performance of platelets, indicating a dual role of CatG.

As we saw in the previous section that LF can increase the catalytic activity of CatG, further components, such as 1,25 (OH)₂D3 (vitamin D), have the potential to modulate immune cells. Indeed, vitamin D provokes a reduction of DCs in the ability to prime T cells (Adorini 2003), suggesting a therapeutic potential of vitamin D for treatment of autoimmunity since auto-aggressive T cells with the potential to destroy healthy cells or tissues are inhibited. Consistent with these findings, it has been revealed that the expression and activity of CatG are elevated in type 1 diabetes mellitus (T1D)-derived peripheral blood mononuclear cells (PBMCs), and inhibition of CatG by a specific inhibitor or by using vitamin D reduce the activation of proinsulin-reactive, so called diabetogenic, T cells. Moreover, freshly isolated conventional DCs (cDCs) from the blood of healthy donors treated with vitamin D showed a reduction of CatG activity determined by the active-site label (Zou et al. 2011). A recent study has suggested that vitamin D is responsible for upregulation of serine protease inhibitors in CD11c⁺ bone marrow-derived mouse DCs (Saul et al. 2019), which gives rise to the speculation that these findings explain why vitamin D decreases CatG activity in cDCs (Zou et al. 2011). Interestingly, cDCs from T1D patients did not show any difference in CatG activity when cDCs were treated with vitamin D, in contrast to cDCs from healthy individuals (Zou et al. 2011). As a result, altered CatG activity has functional consequences during an immune response.

Intracellular Proteolytic Regulation of MHC I

Endocytic proteases, cathepsins, cleave proteins into smaller oligopeptides, and amino acids, thereby controlling the proteome steady state in the cell. The endocytic compartments

within APCs contain cysteine, aspartic, and serine proteases, the concerted action of which generates peptides from self and foreign antigens to be loaded to MHC II molecules. There is crosstalk (cross-presentation) between the MHC II and MHC I antigen processing pathways (Colbert et al. 2020). It is worth noting that cathepsins along with other proteolytic enzymes cooperate with the proteasome with regard to antigen processing and generating antigenic peptides for antigen presentation via MHC I (Jurewicz and Stern 2019).

APCs accommodate CatG. These include primary B cells, monocytes, plasmacytoid DCs, cDCs, cortical thymic epithelial cells, murine microglia, and macrophages; however, CatG is absent in B lymphoblastoid cell lines or monocyte-derived DCs. Interestingly, CatG non-expressing cell lines can take up CatG from the surrounding environment to expand their intracellular protease repertoire for antigen processing (Burster et al. 2004; Stoeckle et al. 2009). Beyond antigen processing, further functional roles of CatG were determined by incubating MHC II molecules with CatG. CatG cleaves MHC II molecules *in vitro* but the CatG cleavage site is sterically inaccessible when MHC II molecules are integrated into the membrane (Burster et al. 2010). Thus, MHC II molecules are stable and resistant to CatG-mediated degradation in the endocytic compartment, which is important for antigenic peptide loading on MHC II molecules and being transferred to the cell surface to display the intracellular peptide status. The question remains as to whether solubilized MHC I molecules are also proteolytically hydrolyzed and whether MHC I molecules resist degradation when integrated into the membrane.

The MHC I molecule is expressed by a heavy chain and β 2-microglobulin on the cell surface. In humans, MHC I molecules are encoded by the three classical loci known as human leukocyte antigen (HLA)-A, HLA-B, and HLA-C. The classical pathway of MHC I-restricted antigen presentation starts with cytosolic antigen processing generally performed by the proteasome-ubiquitin system and transported into the endoplasmic reticulum (ER) lumen. It is here that antigenic peptides are loaded to MHC I molecules with the help of the transporter associated with antigen processing (TAP), tapasin, calreticulin, and ERp57; this MHC I-peptide complex traffics to the cell surface to be inspected by CD8⁺ T cells. The presence of peptide-loaded MHC I molecules on the cell surface can provoke CD8⁺ T cell activation when recognizing, for instance, pathogen-derived antigenic peptides (Santambrogio and Rammensee 2019).

Besides the classical MHC I antigen processing and presentation pathway, cross-presentation of exogenous antigens is possible in DCs, B cells, and macrophages. Eventually, surface MHC I molecules are endocytosed to undergo a new round of antigen loading in the endocytic compartment, and transit back to the cell surface (recycling pathway). Another

pathway comprises the trafficking of MHC I molecules from the ER via the trans-Golgi network to the endocytic compartment (vacuolar pathway). In this pathway, cathepsin S plays an important role in generating peptides for TAP-independent MHC I cross-presentation *in vivo*. Moreover, MHC I molecules can reach the phagosome, where MHC I molecules are loaded with exogenous/endocytic-derived antigenic peptides and the MHC I-peptide complex appears at the cell surface for inspection by patrolling CD8⁺ T cells (Gromme et al. 1999; Grotzke et al. 2017; Hochman et al. 1991; Reid and Watts 1990; Schirmbeck et al. 1995; Shen et al. 2004). MHC I molecules passing the recycling pathway or the phagosome without traveling to the cell surface possibly degraded in the lysosome. In this regard, we addressed the question of which protease is crucial in degrading MHC I molecules and found that CatG, D, and S proteolytically degrade MHC I molecules *in vitro* (Palesch et al. 2016). However, the treatment of PBMCs with selective inhibitors for CatG, D, and S, provoked an increase in levels of MHC I molecules only when PBMCs were treated with the CatG inhibitor. Moreover, introduction of CatG in glioblastoma stem cells and human embryonic kidney 293 cells leads to a significant downregulation of cell surface MHC I molecules, since MHC I molecules are proteolytically digested by endogenous CatG resident in the endocytic compartment, which might be an effective approach to sensitizing NK cells to target cancer cells (Palesch et al. 2016) (Fig. 2).

One immune evasion mechanism of cancer cells includes the downmodulation of cell surface MHC I molecules or the intracellular arrest of MHC I molecules to avoid recognition by CD8⁺ T cells, after which cancer cells instantly maintain a limited set of MHC I molecules and escape destruction by NK cells (Paul and Lal 2017). Viruses have developed a similar strategy to hold back MHC I molecules from the cell surface, for instance, HIV-1 prevents HIV-infected cells from displaying MHC I loaded with HIV-derived antigenic peptides to avoid CD8⁺ T cell-mediated killing. Two models were proposed: At early infection (around 48 h) the HIV-1-derived accessory protein Nef protein forces MHC I molecules to be endocytosed. When the infection progresses, Nef interferes with the transport of nascent MHC I molecules to the cell surface (Dikeakos et al. 2010; Schwartz et al. 1996). In the case of the endocytosis route, Nef binds to the cytoplasmic tail of MHC I during endocytosis. Nef:MHC I transits from the early endosome to the late endosome and finally reaches the trans-Golgi network where Nef:MHC I molecules accumulate. Strikingly, in these experiments, Nef:MHC was not detected in lysosomes (Dirk et al. 2016), which challenges the general statement that Nef:MHC I targets to the lysosomal compartment for degradation (Pereira and daSilva 2016). It is important to note that Nef downmodulates, but not completely, HLA-A, HLA-B, and the non-classical MHC molecule HLA-E (Apps et al. 2015; van

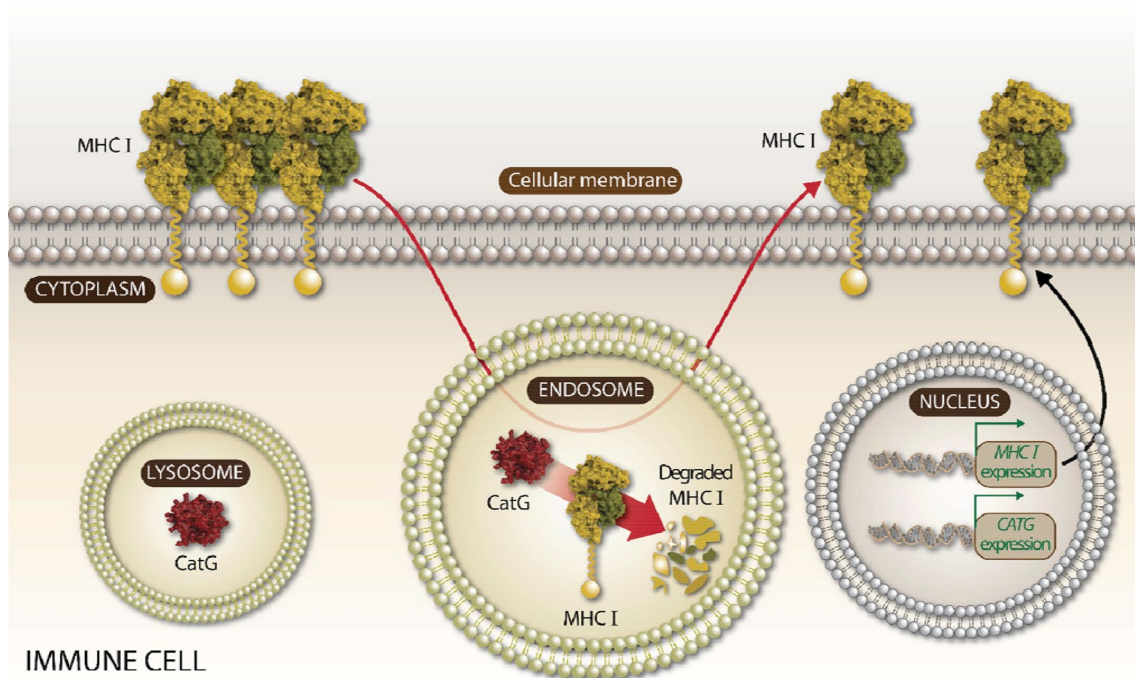
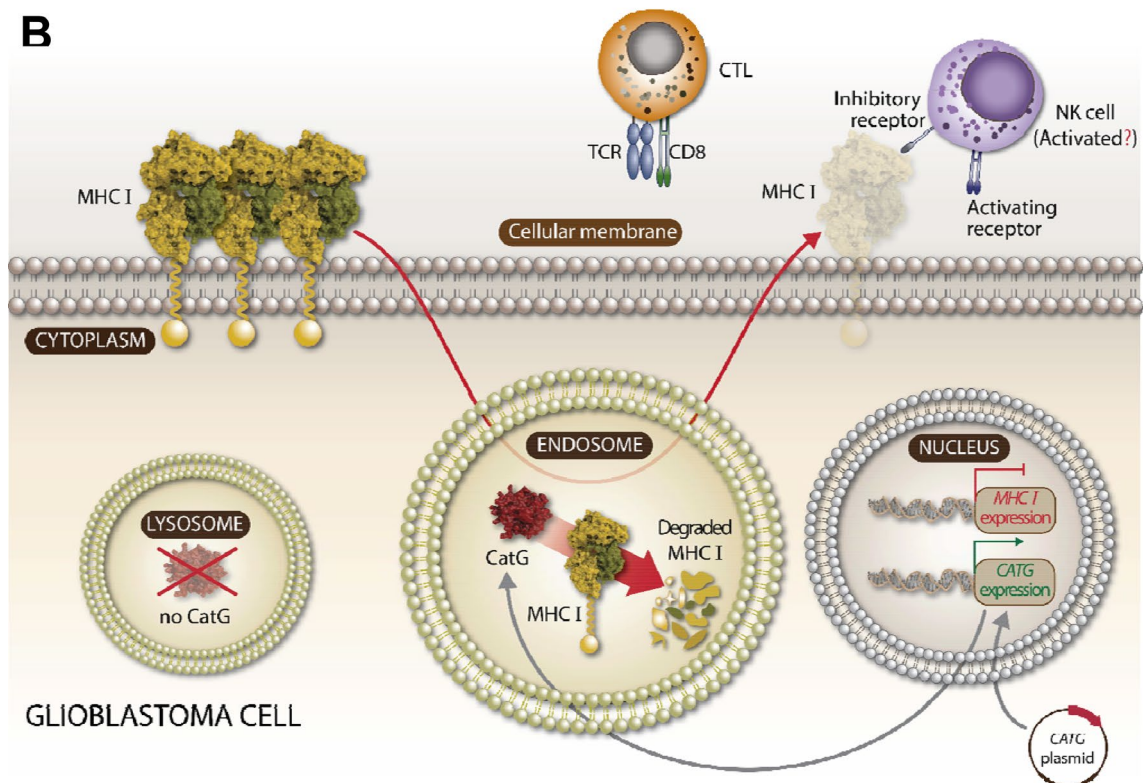
A**B**

Fig. 2 CatG-mediated intracellular proteolytic regulation of MHC I. **a** In general, immune cells express CatG or can take up CatG from the surrounding environment. Cell surface MHC I molecules passing the recycling pathway are degraded by resident CatG in the endosome (or lysosome). **b** Expression of MHC I molecules is impaired in glioblastoma cells (immune evasion). CatG is absent in glioblastoma cells; however, introduction of CatG in glioblastoma cells provokes a significant downregulation of cell surface MHC I molecules. CatG digest MHC I molecules, most likely in the endosome. In this scenario, reduced cell surface MHC I molecules might sensitize NK cells to target cancer cells

Stigt Thans et al. 2019); however, HLA-C cell surface levels are reduced by the viral protein U (Vpu; Vpu has a similar function like Nef) from most primary HIV-1 strains (Apps et al. 2016). Whether MHC I molecules during tracking in the endocytic compartments (and eventually reach the trans-Golgi network) are proteolytically degraded by CatG needs to be experimentally validated.

It has been previously shown that membrane-associated RING-CH-type finger 4 (MARCH4) protein and MARCH9 promote the downregulation of cell surface MHC I molecules by triggering MHC I ubiquitination and subsequently forcing lysosome degradation (Bartee et al. 2004). Recent findings suggest that endogenous MARCH9 mediates MHC I ubiquitination and facilitates delivery of nascent synthesized MHC I molecules loaded with peptides from the trans-Golgi network into the endocytic compartment for efficient antigen cross-presentation in response to specific environmental signals (De Angelis Rigotti et al. 2017). Taken together, endogenous CatG might digest ubiquitin-labeled MHC I molecules and serve as an essential protease for recycling and post-transcriptional regulation of MHC I molecules.

Extracellular CatG and its Functional Role in Controlling Cell Surface Expression of MHC I

By screening the cell surface of different immune cells, it was found that CatG is bound on the cell surface of several cells of the immune system. Such cells contain neutrophils (Owen et al. 1995), B cells, NK cells (Delgado et al. 2001), and platelets (Selak 1994), whereas CatG is only poorly expressed on CD4⁺ T cells (Delgado et al. 2001). It remains an open question as to functional role of CatG on the cell surface.

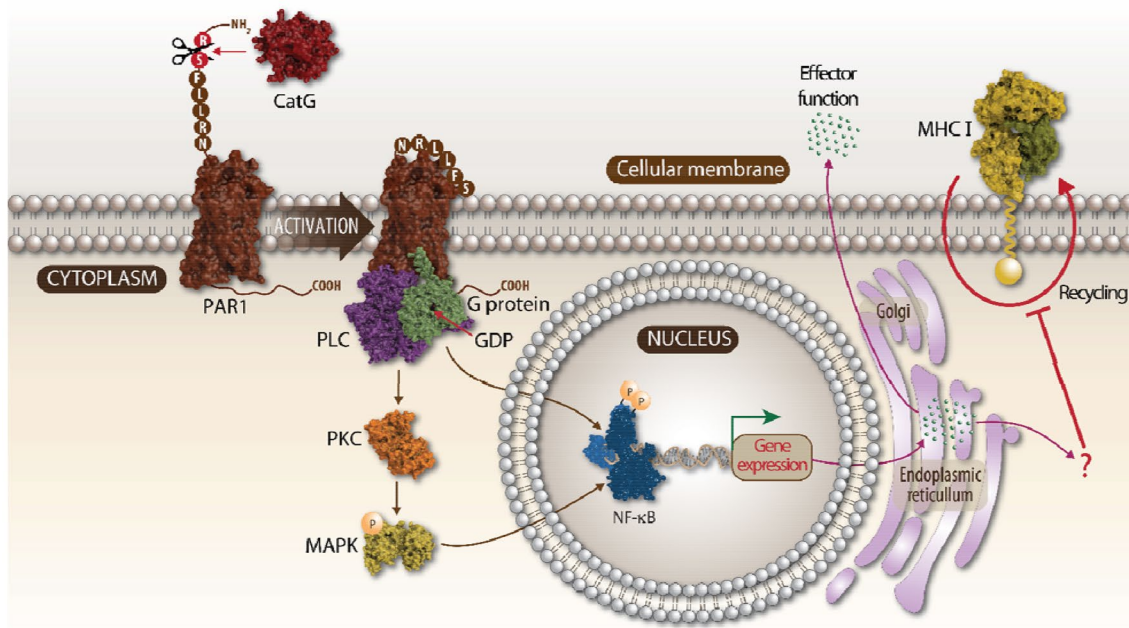
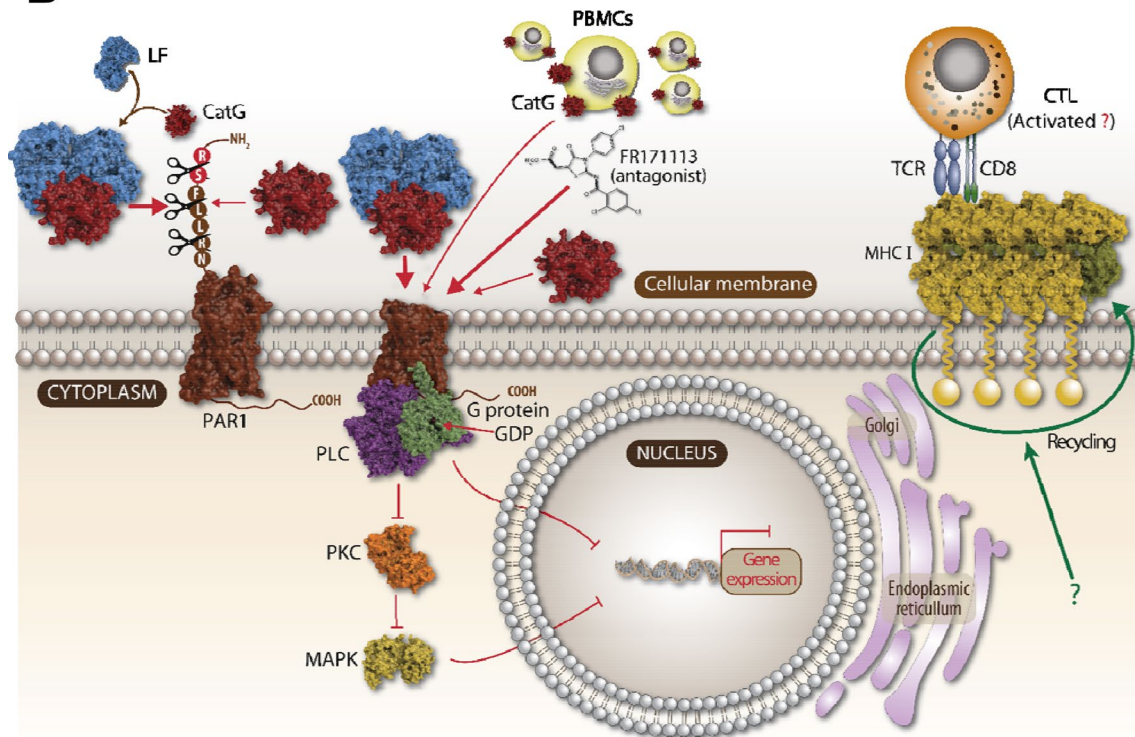
To compare levels of MHC I on DCs from CatG-deficient mice and wild type, we found that DCs from CatG-deficient mice express less cell surface MHC I molecules in contrast to their wild-type counterparts (Giese et al. 2016). This is due to the action of CatG, since exogenous CatG facilitates the upregulation of cell surface MHC I molecules on PBMCs and human glioblastoma cells, most likely via PAR1, as summarized in Fig. 3. Furthermore, LF, which increases

the proteolytic activity of CatG, serves as an enhancer of exogenous CatG-induced upregulation of MHC I molecules on PBMCs and on a B cell line. Not only purified CatG added to the assay increases cell surface density of MHC I molecules of the respective target cell, but also PBMCs, which carry CatG on the cell surface, trigger enhancement of MHC I expression on the cell surface of a monocytic cell line (THP-1) (Giese et al. 2016). The reason for this could be that CatG bound on the cell surface of immune cells provokes directly, or by soluble CatG secreted at the site of inflammation by activated neutrophils, an increase of cell surface MHC I to display the intracellular antigen status of the target cell which can be monitored by CD8⁺ T cells. Some viral strains can hold back nascent MHC I molecules to remain in the cell. Subsequently, antigen-specific CD8⁺ T cells are not activated and viral infected cells evade immune detection.

CatG is found intracellularly (Schroeder et al. 2020) but not on the cell surface of resting CD8⁺ T cells (Delgado et al. 2001; Yamazaki and Aoki 1997), in contrast to double positive CD4⁺CD8⁺ T cells (Penczek and Burster 2019). CD4⁺CD8⁺ T cells show cytotoxic as well as a suppressive capacity depending on the conditions in which they are present. In the case of cytotoxicity towards infection or to recognizing cancer cells, the T cell receptor (TCR) of CD4⁺CD8⁺ T cells interact with MHC I molecules (Overgaard et al. 2015). Therefore, it is most likely that CD4⁺CD8⁺ T cells directly have the possibility to induce an increase of MHC I via CatG to monitor the antigenic peptide repertoire and are able to kill infected cells. This hypothesis and whether viral strains can prevent (or not) CatG-mediated nascent MHC I from reaching the cell surface and whether the viral-derived antigenic peptidome might change during this process remains to be proven experimentally.

The question arises as to why CatG is absent on the cell surface of CD8⁺ T cells (Delgado et al. 2001; Yamazaki and Aoki 1997). A previous study showed that CatG can bind to CD4⁺ T cells, NK cells, and B cells, in addition to CD8⁺ T cells (Yamazaki and Aoki 1997). During inflammation, where CatG is nearby, CD8⁺ T cells can bind CatG and push out intracellular MHC I of the target cell. In this scenario, viral-derived antigenic peptides can be displayed on MHC I and recognized by antigen specific TCR of CD8⁺ T cells. In contrast, when CatG is absent on CD8⁺ T cells, these cells might represent, most likely, resting CD8⁺ T cells. Another reasonable idea could be that CatG bound on the cell surface of CD8⁺ T cells has the capability to protect activated CD8⁺ T cells from secreted perforin and granzyme B and avoids self-destruction (similar to NK cells, discussed below).

The absence or at least decline of MHC molecules, predominantly detected in tumor or virus-infected cells, can provoke NK cell activation by secretion of cytokines/chemokines, granzymes, and perforins for target cell

A**B**

killing purposes (Moretta et al. 2016). Recently, our group has shown that two NK cell subsets ($CD16^{-}CD56^{dim}$ and $CD16^{dim}CD56^{-}$) have active CatG on the cell surface

which is absent on $CD16^{-}CD56^{bright}$, $CD16^{dim}CD56^{bright}$, $CD16^{bright}CD56^{dim}$, $CD16^{dim}CD56^{dim}$, and $CD16^{bright}CD56^{-}$. For these experiments, an active-site label using the

Fig. 3 A model of how extracellular CatG controls cell surface expression of MHC I. **a** PAR1 belongs to a family of G protein-coupled receptors activated by proteases. CatG proteolytically cleaves the N-terminal end of the extracellular domain of PAR1; thereby the tethered activation ligand flips to the extracellular loop two and recruits intracellular G protein. G protein is capable of downstream signal transduction, which results in an effector function, for instance, an inflammatory response. **b** LF increases the proteolytic activity of CatG. CatG cleavage of the extracellular part of PAR1 can also lead to receptor inactivation (“dis-arming”) by degrading the tethered ligand as well as three extracellular loops of PAR1. This process blocks G protein-mediated signaling. CatG-induced upregulation of MHC I molecules might be a result of interference with the MHC I recycling pathway; promoting fusion of the recycling vesicle with the cell membrane to deliver MHC I molecules to the cell surface. As an alternative to being degraded or pausing intracellularly, MHC I molecules can be loaded with a new set of antigenic peptides (viral derived or tumor-associated) and pushed back to the cell surface. PBMCs, which carry CatG on the cell surface, as well as the PAR1 antagonist (FR171113) drive MHC I to the cell surface, which can be inspected by CD8⁺ T cells. CatG-mediated upregulation of cell surface MHC I molecules might be more rapid/efficient by shutting down signal transduction to exert MHC I molecules loaded with a new set of antigenic peptides, instead provoking the expression of nascent MHC I molecules. Additionally, expression of MHC I is prevented in viral infected or cancer cells. Our model suggests how CatG circumvents pathogen or cancer cell-derived immune evasion

activity-based probe MARS116 and avidin-FITC was established to determine the proteolytic activity of CatG on the cell surface of distinct NK cell subsets (Penczek et al. 2016). NK subsets, harboring CatG on the cell surface might proteolytically process perforin and granzyme B and thereby be protected from self-destruction; similar to the proposal for cathepsin B (Balaji et al. 2002); however, cathepsin B-deficient mice did not show impaired CD8⁺ T cell survival during lysis (Baran et al. 2006). This gives rise to the statement that not only one protease might serve as protection from self-lysis, but cell surface CatG might replace the self-protective function of cathepsin B.

An additional functional property of cell surface may be that CatG drives MHC I to the cell surface; when cells are not infected they will survive, in the case of immune evasion of viral infected or transformed cells and nascent MHC I molecules will not appear on the cell surface after CatG engagement. These cells might be recognized by NK cells and are activated for killing.

What about T regulatory cells (Tregs)? Tregs, which maintain immune homeostasis and tolerance, are divided into thymus-derived natural Tregs, which are induced Tregs generated by transforming growth factor (TGF)- β and interleukin two in vitro, and peripheral resident Tregs (Kanamori et al. 2016). A subset of Tregs express the ectonucleotidases CD39 (CD39⁺ Tregs) and the CD73 molecule. While CD39 hydrolyzes extracellular ATP and ADP towards AMP, CD73 is in charge for further conversion of AMP generating adenosine. Adenosine can then bind to the A2A receptor and suppress T cell function (Borsellino et al. 2007; Deaglio

et al. 2007; Longhi et al. 2017; Mandapathil et al. 2010). In terms of CD39⁺ Tregs, CatG was identified on the cell surface by us just recently (Penczek and Burster 2019). Further experiments are necessary to reveal the functional purposes of CatG on CD39⁺ Tregs. One might speculate that cell surface CatG might also force MHC I upregulation or CatG to protect Tregs, which can exocytose perforin (and granzyme B) for cytotoxicity of effector cells (Grossman et al. 2004) from self-destruction by proteolytic processing of perforin and granzyme B, similar to NK cells or CD8⁺ T cells. Moreover, the growth and maturation factor CXCL12 chemokine stromal cell-derived factor 1 (SDF1) is important for an inflammatory response (Luster 1998). SDF1 can be proteolytically inactivated by cell surface CatG present on B cells, NK cells, and CD4⁺ T cells (Delgado et al. 2001); this is of particular interest, since Tregs have the ability to terminate an immune response and maintain immune homeostasis (Whiteside 2014). Thus, cell surface CatG might protect effector cells from friendly fire and supports intercellular communication between immune cells and target cells via regulation of MHC I.

CatG and PARs

PAR1, PAR2, PAR3, and PAR4 belong to a family of G protein-coupled receptors irreversibly activated by different soluble- or cell membrane bound proteases. PARs are substrates for CatG; while CatG proteolytically cleaves the N-terminal end of the extracellular domain of PAR1, PAR2, and PAR4, unmasking a tethered activation ligand which flips to the extracellular loop 2, provokes a conformational change and recruits intracellular G protein. G protein is capable of transducing intracellular signal events via an intricate network of downstream signal transduction, as a result regulating, for instance, an inflammatory response (Adams et al. 2011; Heuberger and Schuepbach 2019). Simultaneously, CatG cleavage of the extracellular face of PAR1, PAR2, and PAR3 can lead to receptor inactivation (“dis-arming” or “off-conformation”) by degrading the tethered ligand or possibly cleaving the three extracellular loops of PAR, thereby blocking G protein-mediated signaling (Ramachandran et al. 2012). Interestingly, it was shown that pharmacological inhibition of PAR1 reduces the progression of several tumor types (Flaumenhaft and De Ceunynck 2017), speculating that inactivated PAR1 in cancer cells might be somehow recognized by immune cells and most likely eliminated by CD8⁺ T cells. Indeed, we found that exogenous CatG as well as the PAR1 antagonist (FR171113) enhanced MHC I expression on the cell surface of PAR1-expressing PBMCs, Epstein–Barr virus-transformed B cell line, and glioblastoma cell line (U87), and sphere-cultured stem cell-enriched cell population from two different patients suffering from glioblastoma (Giese et al. 2016). In the case of glioblastoma

cells, nascent or recycled MHC I bound with tumor-associated peptides might be presented and recognized by CD8⁺ T cells. This mechanism has to be confirmed experimentally.

CatG Can Bind to the Cell Surface and is Also Proteolytic Active

CatG, by means of a cationic composition due to the high number of basic amino acids within the CatG protein sequence, binds to the outer surface of the neutrophil plasma membrane through electrostatic interactions with the anionic sulfate groups of chondroitin sulfate- and heparan sulfate-containing proteoglycans (Campbell and Owen 2007; Korkmaz et al. 2010). Additionally, CatG has been reported to exhibit binding to PAR1-4. Like thrombin which binds first to these receptors and then activates them, CatG might bind to the extracellular part of PARs consisting of a negatively charged amino acid residue pattern (Adams et al. 2011).

Exogenous CatG and NE can bind to the cell surface of cancer cells in a specific and saturable manner and undergo clathrin pit-mediated endocytosis (Gregory et al. 2012). According to previous studies, CatG binds to chondroitin sulfate- and heparan sulfate-containing proteoglycans on the cell surface of recipient cells (Campbell and Owen 2007). Interestingly, it was found that treatment of cancer cells with glycanases to remove respective proteoglycans did not result in diminished cell surface binding or endosomal entry of NE, suggesting the existence of a specific cell surface receptor for NE and presumably for CatG, because both CatG and NE share a significant sequence identity and they compete for cell surface binding sites. Following binding, CatG enters tumor cells via endocytosis in a clathrin- and dynamin-dependent manner (flotillin-1 and caveolin-1-independent) and apparently process tumor-derived proteins that might affect tumor cell behavior, in contrast to NE which induces tumor cell proliferation (Gregory et al. 2012). How can CatG, bonded on the cell surface, still be active at the site of inflammation when natural serine protease inhibitors are present? Matrix metalloproteinases, released by activated neutrophils, proteolytically inactivate α_1 -antitrypsin which is a natural occurring serine protease inhibitor (Vissers et al. 1988). Moreover, low molecular weight serine protease inhibitors, such as phenylmethylsulfonyl fluoride (PMSF), inhibits human neutrophil elastase, but not the bulky natural serine protease inhibitor, α_1 -proteinase inhibitor, postulating that cell surface CatG is still proteolytically active due to steric hindrance of natural serine protease inhibitors which cannot reach the catalytic center when CatG is bonded to the cell surface (Owen et al. 1995). This allows cell surface CatG to be active at the site of inflammation even though natural serine protease inhibitors are in direct proximity.

Conclusion

The summary of our briefing identified a strong association between the proteolytic activity of CatG and cell surface expression of MHC I molecules, indicating a panel of evidence that CatG has a more diverse function than previously recognized. Indeed, our model suggests how intracellular CatG degrades MHC I molecules in the endosomal compartment or when MHC I molecules pass through the recycling pathway. This might sensitize NK cells due to the reduction of cell surface MHC I molecules. On the other hand, exogenous CatG proteolytically cleaves the extracellular domain of PAR1 leading to receptor inactivation and in turn upregulates MHC I molecules. This is a mechanism that involves the MHC I recycling pathway by supporting the fusion of the recycling vesicle with the cell membrane and thereby delivers MHC I molecules to the cell surface to present a new set of pathogen-derived or tumor-associated antigenic peptides. As a result, CatG-mediated upregulation of cell surface MHC I molecules can be inspected by CD8⁺ T and suggests that CatG prevents pathogen or cancer cell-driven immune evasion.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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