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(Article begins on next page)

PA28 γ , the ring that makes tumors invisible to the immune system?

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ABSTRACT

PA28 γ is a proteasomal interactor whose main and most known function is to stimulate the hydrolytic activity of the 20S proteasome independently of ubiquitin and ATP. Unlike its two paralogues, PA28 α and PA28 β , PA28 γ is largely present in the nuclear compartment and plays pivotal functions in important pathways such as cellular division, apoptosis, neoplastic transformation, chromatin structure and organization, fertility, lipid metabolism, and DNA repair mechanisms. Although it is known that a substantial fraction of PA28 γ is found in the cell in a free form (i.e. not associated with 20S), almost all of the studies so far have focused on its ability to modulate proteasomal enzymatic activities. In this respect, the ability of PA28 γ to strongly stimulate degradation of proteins, especially if intrinsically disordered and therefore devoid of three-dimensional tightly folded structure, appears to be the main molecular mechanism underlying its multiple biological effects. Initial studies, conducted more than 20 years ago, came to the conclusion that among the many biological functions of PA28 γ , the immunological ones were rather limited and circumscribed. In this review, we focus on recent evidence showing that PA28 γ fulfills significant functions in cell-mediated acquired immunity, with a particular role in attenuating MHC class I antigen presentation, especially in relation to neoplastic transformation and autoimmune diseases.

Keywords: PA28 γ ; proteasome; intrinsically disordered proteins (IDPs); ATP-independent proteolysis; proteasome activator; Ubiquitin Proteasome System

1. Introduction

In both prokaryotic and eukaryotic organisms the proteome is constantly modified by reactions of synthesis and degradation, and the perfect balance between these two opposing processes determines the appropriate steady state levels of every protein in the cell. Although it is known that cells perfectly and quickly adapt the repertoire of proteins to their current physiological needs through regulatory mechanisms that act at both the transcriptional and translational levels, it is now well established that strictly controlled proteolysis is also essential to preserve cellular homeostasis and allow appropriate and efficient adaptation to various forms of stress [1,2]. Not surprisingly, alterations in this delicate network are the cause of various pathologies [3–5], and recently pharmacological approaches aimed at modifying the activity of key components of this proteolytic pathway are emerging to be extremely promising for the therapy of serious diseases, such as different forms of cancer [6–8].

1.1. The Ubiquitin Proteasome System (UPS)

While prokaryotes have at their disposal a rather wide range of proteases, apparently also with redundant functions, in eukaryotes the enzyme responsible for removing damaged or no longer necessary proteins is the proteasome, a self-compartmentalized protease made up of several dozen subunits differing in molecular weight and specific functions. This degradative system, known as the ubiquitin-proteasome system (UPS), is made up of a core with proteolytic activity, the 20S proteasome, and various accessory complexes, which regulate its enzymatic functionality and consequently its biological activities [9–11]. The 20S particle has a molecular weight of ~700 kDa and is made up of the association of two external α -rings and two internal β -rings, each of seven subunits, which join together to form a hollow cylinder, on whose internal surface the enzymatic pockets of hydrolytic sites are housed [12]. Depending on the prevailing specificity of peptide bond cleavage in target proteins, three main peptidase activities are recognized: chymotrypsin-like (carried out by the $\beta 5$ subunit), trypsin-like ($\beta 2$ subunit), and caspase-like (or post-acidic, $\beta 1$ subunit) [13]. The proteasome form that is responsible for the degradation, in a process that requires energy provided by the hydrolysis of ATP, of most intracellular proteins is the 26S particle. This huge (2.4 MDa) and abundantly present (~2% of all proteins) protease consists of the 20S proteasome associated with one or two 19S modulators (which in turn can be divided from both a structural and functional point of view into a base and a lid), which give the degradative machinery the ability to hydrolyze ubiquitinated proteins, after having denatured them through the ATPase activity of some

of its components (Rpt1–Rpt6 subunits) [1,2,14–18]. Only unstructured proteins can, in fact, reach the inner hydrolytic chamber of the 20S, and access to it is strictly controlled by a gate constituted by the N-terminal ends of the α -ring subunits [19–21]. The other 19S subunits perform various functions, including the recognition of the polyubiquitin chain (subunits Rpn1, Rpn10, and Rpn13) and its removal from the substrate protein (subunit Rpn11) [2,14,22–26].

The salient feature of protein degradation carried out by the proteasome is certainly its extreme selectivity. Among the thousands of different proteins present in a eukaryotic cell, the proteasome must be able to specifically recognize the proteins that must be eliminated at any moment (and which often do not even represent the largest fraction of the proteome) and therefore operate a highly selective hydrolysis of these specific substrates [27]. This selectivity is achieved to a large extent (but not exclusively) through the labeling of target proteins with a small globular protein called ubiquitin. Additional ubiquitin molecules are then added sequentially to form what is called a polyubiquitin chain. This polyubiquitin chain is generated by three classes of enzymes acting sequentially: one of the two E1 (Ubiquitin-activating enzyme) enzymes present in eukaryotic cells, one of the several dozen E2 (Ubiquitin-conjugating enzyme) enzymes, and one of the several hundred E3 (ubiquitin-protein ligase), which synthesize the isopeptide bonds that link the first ubiquitin (proximal ubiquitin) to the target protein and the ubiquitin molecules to each other in an ATP-dependent process [28,29]. The 19S regulator has subunits that interact with the multi-ubiquitin tree on the target protein which, together with a segment without three-dimensional structure, represent the two necessary requirements for a substrate to be degraded by the 26S proteasome. However, it should not be forgotten that the 26S proteasome is still capable of degrading substrates that are not classically labeled with ubiquitin, but which use other marker proteins (e.g. antizyme in the case of ornithine decarboxylase) or which are entirely or largely devoid of a tightly folded three-dimensional structure [23,30].

1.2. MHC class I antigen presentation

In jawed vertebrates the adaptive immune system constantly carries out a quality control aimed at identifying as early as possible cells that are synthesizing aberrant proteins, and eliminating them as they represent a potential threat to the organism [31–34]. This process, which is called Major Histocompatibility Complex (MHC) class I antigen presentation, is based on the continuous generation of short peptides of 8-10 amino acids (i.e. MHC class I epitopes or antigenic peptides) derived from all the proteins translated within the cell [5,35–37]. These peptides are largely produced

by the proteasome (although many examples of class I epitopes generated in alternative ways are also known, as will be described in more detail in the next paragraph), imported into the endoplasmic reticulum by transporters associated with antigen processing (TAP) and then exposed on the external surface of the cytoplasmic membrane in association with MHC class I molecules [38–40]. These heterodimeric complexes, which are present on the surface of all nucleated cells, are formed by the association of a highly polymorphic heavy chain with the β_2 -microglobulin, allow class I epitopes to be constantly screened by the T-cell receptors (TCRs) present on the cell surface of a specific class of lymphocytes, the cytotoxic $CD8^+$. In physiological conditions, the MHC class I antigenic peptides derive from normal cellular proteins (self-proteins) towards which $CD8^+$ are tolerant following the negative selection process that occurs in the thymus and is aimed at eliminating the thymocytes with high affinity for self-peptides. At the end of the maturation process, naïve cytotoxic $CD8^+$ T cells emerge from the thymus, which can then be activated in the periphery following the encounter with their cognate antigen [41]. In fact, if the MHC class I-presented epitopes derive from anomalous proteins (non-self, such as, for example, proteins of intracellular pathogens or proteins mutated in their primary sequence) the cytotoxic $CD8^+$ T receptor is activated and, together with a second signal provided by the interaction between co-stimulatory molecules present on the membranes of the two cells involved, gives rise to a cascade of complex events which, in the end, lead $CD8^+$ cells to kill the target cell [42]. Importantly, this immunosurveillance mechanism is active not only against intracellular pathogens (viruses, bacteria, protozoa, fungi), but also against neoplastic cells. In fact, dysfunctions and alterations of the class I antigen presentation mechanism allow tumors to escape the control of the immune system and develop in the body (known as tumor immune evasion) [6–8]. Accordingly, in recent years new pharmacological strategies aimed at blocking one of these immune evasion mechanisms through immune checkpoint inhibitors (ICIs) were shown to be highly useful in the immunotherapy of different neoplastic diseases and are associated with improved survival outcomes [7,43].

1.3. Functions of proteasomes in MHC-I epitope processing

In a pioneering study in 1994 the labs of Ken Rock and Alfred Goldberg convincingly demonstrated that the hitherto unknown protease responsible for the generation from intracellular proteins of class I epitopes was the proteasome [44]. This is now a definitive and consolidated acquisition from many further studies, although it was later highlighted that other proteases, primarily aminopeptidases of the endoplasmic reticulum (ER), can subsequently eliminate some supernumerary residues present at the N-terminal end of the proteasomal products (so-called N-terminal trimming)

[45,46]. Likewise, it has been convincingly demonstrated that some of the class I epitopes are also produced in a proteasome-independent process, which is likely based on the action of different endo- and exopeptidases present in the cytoplasm or ER [47–51]. However, the exact fraction of class-I antigenic peptides that are generated in this proteasome-independent mechanism, and which according to some estimates could be as high as 30% of the total [49], is extremely difficult to quantify in a precise and reliable manner. This difficulty arises above all from the observation that the majority of proteasomal inhibition protocols used in these studies to unveil non-proteasomal peptide generation involve the use of a single inhibitor. Accurate *in vitro* and *in vivo* studies demonstrate, however, that all three proteasomal active sites contribute to protein degradation (moreover with a different relative contribution depending on the chemical properties of the hydrolyzed substrates) and only a strong and simultaneous block of all three active sites (almost impossible with the use of just one of the currently available inhibitors) is able to completely prevent proteasomal degradation [52,53].

In line with the central function of proteasomes in the production of MHC class I epitopes, interferon- γ (INF- γ) (and to a lesser extent other pro-inflammatory cytokines such as type-I interferons and TNF- α), which strongly stimulates the mechanism of class I presentation, induces the ex-novo synthesis of three new proteasomal catalytic subunits. These three new proteins, β 1i/LMP2, β 5i/LMP7, and β 2i/Mec1-1/LMP10, are inserted in place of the constitutive ones in the new proteasomes, which are therefore defined as immunoproteasomes [35,54]. In this way immunoproteasome is formed at the appropriate time in every tissue of the organism, while it is constitutively present (even in the absence of continuous stimulation with INF- γ) in cells of hematological origin such as B and T lymphocytes, monocytes, antigen presenting cells, dendritic cells, macrophages, and medullary thymic epithelial cells [5]. Experiments with fluorogenic peptides have shown that the presence of the INF- γ -induced subunits modified the cleavage properties of the degradative particle. Importantly, it has now been definitively established that compared to constitutive proteasomes, those containing the subunits induced by INF- γ are characterized by increased chymotrypsin-like and trypsin-like specificities, accompanied by a reduction of the caspase-like one [5]. As a result of these modifications in hydrolysis preferences, the epitopes released by INF- γ -induced proteasomes should be enriched in hydrophobic and basic amino acids and poor in acidic ones at their C-terminal end, characteristics that facilitate a high-affinity interaction with both TAPs and MHC-I [39,55]. Although it has been reported that the immunoproteasome also plays an important role in regulating cytokine production and T cell proliferation [56], and may be involved in the response to oxidative stress [57,58] and in promoting degradation of basic proteins [53], the crucial function of the INF- γ -induced proteasomes in processing MHC-I epitopes was

unequivocally demonstrated after transgenic mice lacking all three immunoproteasomal β -catalytic subunits were generated [59].

1.4. The ATP- and Ub-independent PA28 family of proteasomal activators

In addition to the previously described 19S particle, a second class of proteasome regulators is made up of PA28 α , β , and γ , which stimulate proteasomal hydrolytic activity independently of the presence of ubiquitin and ATP [60]. The PA28 proteins form ring-shaped ~ 200 kDa hetero- (α and β) or homo- (γ) heptameric complexes that bind to the 20S core particle and highly stimulates its peptidase, and only for PA28 γ , its proteolytic capacities [61]. Furthermore, PA28 can also associate, in an ATP-independent manner, with the α -ring of a 20S proteasome capped, at the other side, with the 19S regulatory particle. The resulting proteasome particles are known as “hybrid proteasomes” (i.e. PA28-20S-19S) [62–67]. PA28 monomers weigh ~ 28 kDa each and share a high degree of homology in their sequences [68,69]. All three PA28 paralogs retain the same overall secondary structure, which is made up of four α -helices that contribute to stabilizing the tertiary and quaternary structures of the multimeric complex [70–75] (Figure 1).

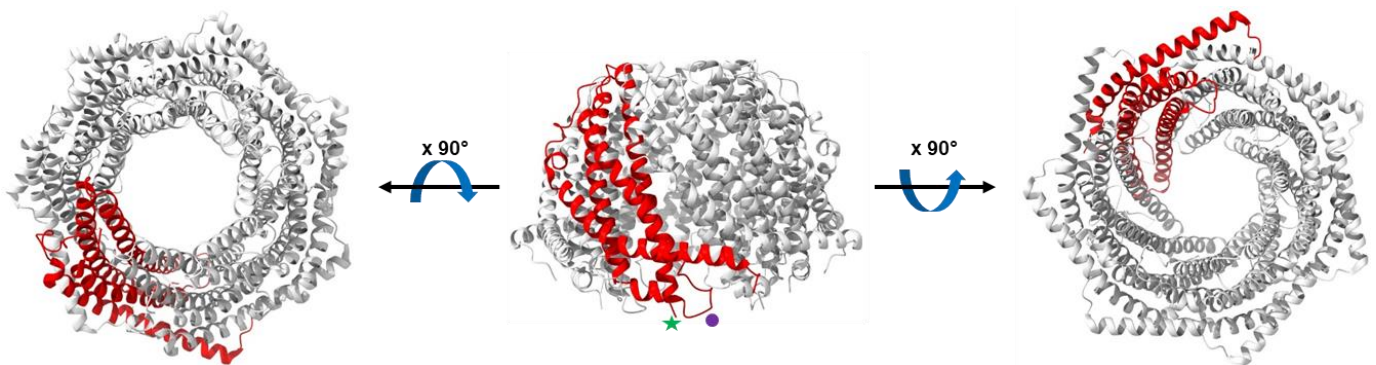


Fig. 1. Structure of the homoheptameric PA28 γ . One subunit is highlighted in red. The star indicates C-terminus, the circle de activation loop. Structures were generated by ChimeraX using the PA28 γ Cryo-EM structure [70].

The highly conserved linker segment connecting helix 2 to helix 3 is referred as “activation loop” since it activates the proteasome thorough opening its gate [76]. Another region of the protein playing a major role in the assembly with the 20S core is the C-terminal end that supplies binding energy for PA28-proteasome complex formation [77]. Finally, the segment connecting helix 1 to helix 2 is called “homolog specific insert” because its sequence differs greatly between the three paralogs [78]. This part of the molecule has not been resolved in structural studies (X-ray and cryo-

EM) of PA28 α , PA28 $\alpha\beta$, and PA28 γ , presumably because it lacks a rigidly folded structure, but is believed to protrude above the PA28 heptamer [60]. Notably, compared to PA28 α and PA28 β the "homolog specific insert" of PA28 γ is composed of a greater number of amino acids, which could underlie the different biochemical properties of PA28 γ (see below). The quaternary organization of PA28 heptamers is formed and stabilized thanks to both polar and hydrophobic interactions [73]. In this way, a "donut-like" structure is delimited around a central aqueous channel which, once aligned with the open pore of proteasome α -annulus, allows the internal proteolytic chamber of the protease to communicate with the external medium. Of extreme interest is the recent demonstration that the central aqueous channel that passes through the PA28 γ homoheptamer has a slightly larger diameter than that of PA28 α and PA28 $\alpha\beta$ [70], which could at least partially account for its unusual ability to activate not only peptidase but also proteolytic proteasomal activity [61]. Although it is now well established that the main molecular mechanism of PA28 activation is through the widening of the proteasomal α -pore [54,77], some important questions still remain. In particular, it seems highly unlikely that the entire molecular action of PA28 is exhausted exclusively in the opening of the gate, since accurate biochemical analyzes indicate that other mechanisms also take place, such as allosteric long-range modifications of the active sites and/or selective filtration on the released products [54]. Furthermore, the capacity of PA28 γ to open the proteasome gate was refuted in a recent study [74] claiming that the stimulatory activity of PA28 γ depends exclusively on allosteric modifications of the proteasomal tryptic site. This conclusion appears surprising because, as previously mentioned, PA28 γ possesses the conserved activation loop that is necessary to displace the N-terminal tails of the proteasome α -subunits and in this manner cause pore dilation. Further studies reporting a high-resolution structure of PA28 γ complexed with mammalian 20S particle are necessary to definitively clarify this important point.

2. PA28 γ

The biochemical and cellular properties of PA28 γ (REG γ or 11S γ) are considerably distinct from those of PA28 α and β [79,80]. In fact, PA28 α and β are highly induced by interferons (and other pro-inflammatory cytokines) and are known to play a major role in class-I presentation, likely through modulation of proteasome peptidase activities (i.e. without affecting total rates of protein turnover) [66]. In accord with a specific immunological role, both paralogs are constitutively expressed only in lymphoid cells and immunological tissues [81,82]. In contrast, PA28 γ is present in every tissue of the body [83–86], and in particular in the central nervous system and spleen [85]. Furthermore, given the lack of INF-responsive elements in its transcriptional control sequences, its levels are INF- γ -

independent [87–89]. Historically PA28 γ has been identified as Ki-antigen, i.e. an autoantigen of subjects affected by systemic lupus erythematosus [90], although its role in the disease has never been clarified in a convincing and definitive manner. Later research showed that PA28 γ is highly homologous with PA28 α and β , and represents a true modulator of the 20S core particle [78,86,91], although only 5% of cellular PA28 γ is bound to the proteasome [92]. This important observation leaves open the possibility that PA28 γ may also perform other biological functions that are not directly attributable to the proteasome. On the other hand, PA28 γ that is not associated with the proteasome could constitute a reserve ready to be used in case the cell has to face increased degradative needs [93]. Up-to-date phylogenetic studies demonstrated that PA28 γ is well expressed in all vertebrates, unlike the PA28 α and β paralogues, which are present only in jawed ones (excluding birds) [94]. Consequently, PA28 γ appears to be a slowly evolving gene, from which PA28 α and β were later derived and rapidly diverged. Accordingly, PA28 γ , the most conserved member of the family, has probably maintained the original function of the ancestor gene, which is probably to stimulate the degradation of proteins devoid of a strongly folded tridimensional structure. Instead, PA28 α and β genes, which appeared more recently, have evolved and differentiated very quickly to play new functions involved in adaptive immune responses [94,95]. Of note, PA28 γ is found mainly in the nucleus, although sometimes its presence in the cytoplasm has also been reported [95–100]. In fact, PA28 γ presents two functional nuclear localization signals (NLS) [87,95], which justifies its prevalent localization within the nucleus [100]. However, it has been demonstrated that PA28 γ can be SUMOylated *in vitro* and *in vivo*, and following this PT-modification be translocated into the cytosol where it shows increased stability and highly enhances the ATP- and Ub-independent proteasomal degradation of the cell cycle regulator p21 [101] and transcription factor CP2c [102]. Therefore, this important observation highlights a new regulatory mechanism through which the intracellular distribution of PA28 γ can be controlled and directed to degradation even of cytoplasmic substrates.

2.1. Multiple biological functions of PA28 γ

Despite numerous studies carried out over the last 30 years, a precise and exhaustive picture of all the biological roles of PA28 γ is still missing. However, it is clear that this proteasome activator, mainly through its capacity to stimulate degradation of loosely folded protein substrates, plays fundamental regulatory functions in many pathways, such as: 1- Stimulation of cell growth and proliferation [103]; 2- Promotion of transition from G to S phase in the cell cycle [97]; 3- Inhibition of apoptosis [104]; 4- Maintenance of centrosome and chromosomal stability [100]; 5- Regulation of

nuclear dynamics through modulation of the number and size of various nuclear bodies, including Cajal bodies (CBs) [105,106], nuclear speckles [107], and promyelocytic leukemia protein bodies [108]; 6- Enhancement of cellular response to DNA double-strand breaks (DSBs) following radiomimetic treatment [109]; 7- Inhibition of autophagy resulting in impairment of lipid metabolism, such as increased steatosis induced by a hyperlipid diet [110]; 8- Promotion of male fertility, by sustaining the maturation process of spermatozoa [111,112]; 9- Improvement of cell viability and motor coordination in some cellular and animal models of neurodegenerative Huntington's disease (HD) [113–115]. Figure 2 shows the main biological functions of PA28 γ , while for a more complete and detailed discussion of the topic we refer to recently published in-depth reviews [79,80].

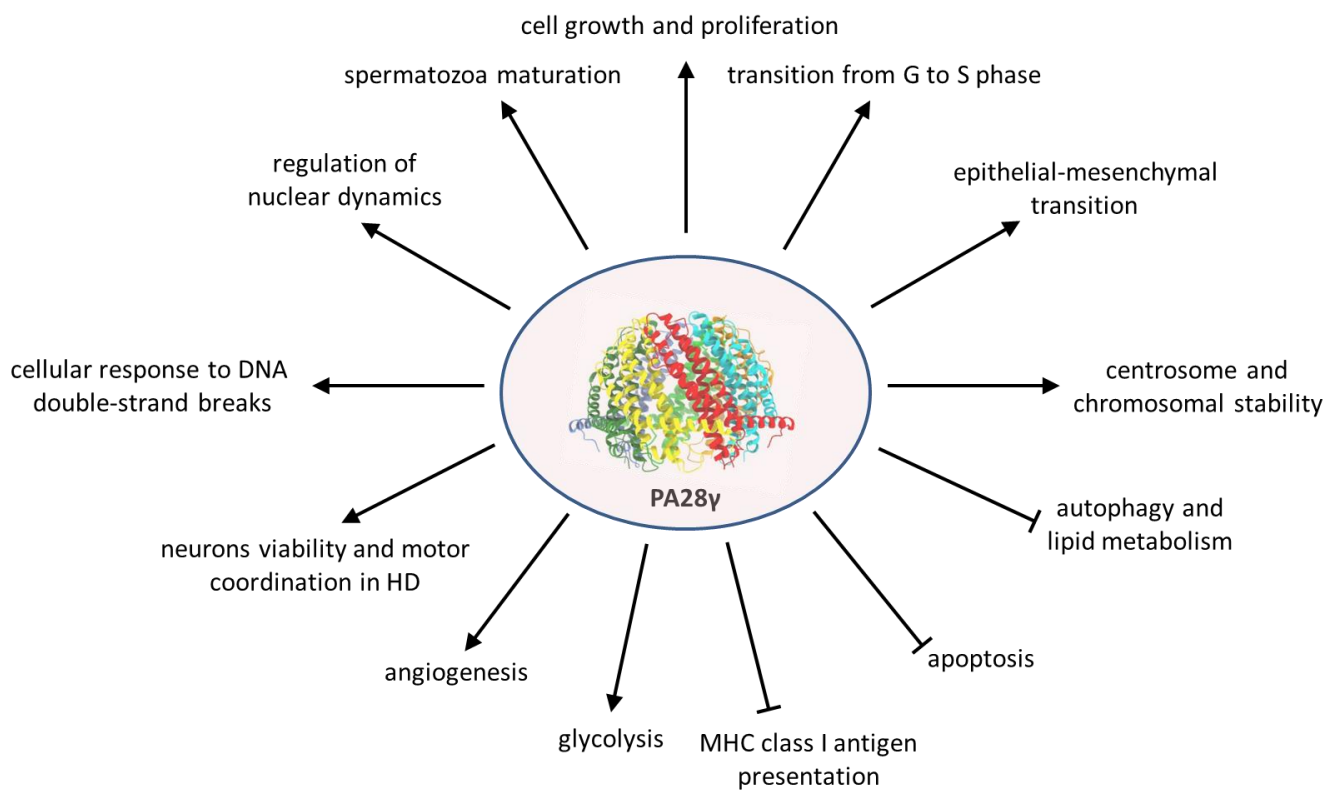


Fig. 2. Biological functions of PA28 γ . $\uparrow$ indicates stimulation, $\perp$ inhibition.

A separate consideration must be made regarding the role played by PA28 γ in the context of neoplastic transformation. In fact, many tumors of different origins express high levels of PA28 γ , so it is commonly considered an oncogene involved in the neoplastic transformation of cells [116–118]. Many of its biological activities (see previous paragraphs), such as its ability to stimulate the growth and proliferation of cells and its anti-apoptotic activity, are well reconciled with a pro-tumor function. Moreover, other crucial roles played by PA28 γ that contribute to justify its pro-oncogenic activity are related to its other functions, such as pro-angiogenic activity [119,120] and stimulation of glycolysis [121] and the epithelial-mesenchymal transition (EMT) [122–124], thereby aimed to adapt cell

metabolism to energy starvation [125]. Furthermore, an important immunosuppressive role of PA28 γ on cytotoxic anti-tumor immune responses, with consequent onset of tumor immune evasion mechanisms, has emerged in recent years and will be described in detail in the next section.

2.2. Immunological functions of PA28 γ

Transgenic mice deficient for PA28 γ expression were independently generated at the turn of the new millennium by two different groups [103,104]. These initial studies showed that genetic elimination of PA28 γ did not lead to increased prenatal mortality; moreover, PA28 $\gamma^{-/-}$ animals appeared in good condition and were able to reproduce. However, in both cases, the growth of knockout mice after birth was delayed, so that their body weight was lower compared to sex-matched WT or heterozygous littermate controls. Of note, this reduced body size seemed to be the consequence of a delay in cell proliferation. In fact, PA28 $\gamma^{-/-}$ MEFs were characterized by a reduced number of actively mitotic cells in both the S and G2/M phases as a consequence of an evident slowdown of the transition from G1 to S phase of the cell cycle. Of note, the levels of PA28 $\alpha\beta$ were not altered in PA28 $\gamma^{-/-}$ mice, corroborating the different biological role of these two proteasomal activators, which evidently do not perform redundant functions and cannot compensate for each other [103,104].

Additionally, the study by Barton et al. specifically focused on the immunological consequences caused by the absence of PA28 γ , with particular reference to MHC class I antigen presentation [104]. As a result, it was demonstrated that PA28 $\gamma^{-/-}$ mice displayed a significant ~15% reduction in CD8 $^{+}$ T cells and do not effectively clear the pulmonary fungal infection caused by *H. capsulatum*. However, surface expression of MHC class I molecules was normal, as was the T cell response to two viral infection models (influenza virus and recombinant vaccinia virus), both in terms of magnitude and hierarchy of antigenic epitopes recognized. Equally unchanged was MHC class I expression in the thymus and the proportions of thymic T cells populations. However, unexpectedly, given the mitotic defects of the knockout MEFs, the proliferative capacity of primary splenocytes from PA28 $\gamma^{-/-}$ mice in response to Con A and LPS stimulation was the same as that of WT controls. In light of all these observations, it was concluded that PA28 γ plays a role in the class I antigen processing pathway, probably through modulation of proteasome cleavage specificities. This role, however, seemed to be limited to only some specific antigens, rather than representing a general mechanism to control class I presentation [104].

In recent years, some studies have re-evaluated the role of PA28 γ in the context of cell-mediated immune responses, and in particular in class I antigen presentation, reaching the conclusion

that it performs important general regulatory functions that had remained largely neglected or at least underestimated until now. In 2019, Yao et al. analyzed the effects of PA28 γ depletion on the processing and presentation of class I epitopes in a mouse model, reporting the onset of autoimmune disorders as a major consequence [126]. Specifically, the authors assessed the populations of different immunological cells present in the spleen of PA28 γ KO mice and observed that 12-month-old transgenic animals presented, compared to WT, a sharp increase in both the percentage and numbers of CD11c⁺ and CD8⁺ T cells (which play a major role in T-cell mediated cytotoxicity), but not of CD4⁺, CD19⁺, or CD11b⁺ cells (which are mostly involved in the humoral immune response). This finding, therefore, strongly suggests that PA28 γ plays a specific function in MHC class I, rather than class II, antigen processing. Furthermore, bone marrow-derived dendritic cells (BMDCs) from PA28 γ ^{-/-} mice stimulate more efficiently proliferation of antigen-specific CD8⁺ compared to BMDCs from WT control littermates. Crucially, this increased activation occurred only if BMDCs were pre-incubated with the full-length antigenic protein (i.e. ovalbumin) but not with a final, presented epitope, strongly indicating that PA28 γ is primarily involved in antigen processing rather than in other steps of the MHC-I process. Remarkably, the molecular mechanism of the immunosuppressive effect of PA28 γ described in the study seems to rely on reduction of the expression levels of immunoproteasomes, consequent to PA28 γ -enhanced 20S hydrolysis of phosphorylated STAT-3 in bone marrow-derived and splenic DC, MEF, and HeLa cells. This is an extremely important observation, documenting a previously neglected function of PA28 γ in counteracting immunity through modulation of the JAK-STAT pathway. However, in several mouse and human cancer cell lines analyzed in another study, no obvious consequences of the presence or absence of PA28 γ on the intracellular levels of the immunoproteasome, in either basal conditions or following the administration of INF- γ , have been found [127]. Additional research is thus required to unveil the precise capacity of PA28 γ to control the amount of immunoproteasome in various cell lines in physiopathological conditions. Furthermore, it is also important to note that the levels of immunoproteasome in different tumors varies greatly, apparently without a conserved pattern in the neoplasms analyzed to date [128,129].

A second study recently analyzed the consequences of manipulating the expression levels of PA28 γ on MHC class I presentation of epitopes derived from PTPs (Pioneer Translation Products) in cancer cells [127]. PTPs represent a particular subset of Defective Ribosomal Products (DRiPs), a group of polypeptides that play an important and much debated role in the generation of class I antigenic peptides, as will be briefly described below. In fact, it is widely accepted that a portion of MHC class I presented epitopes is produced during the physiological turnover of mature cellular proteins, meaning that they have positively passed all the quality controls relating to the processes of

translation, folding, post-translational modifications, and assembly (for components of multimeric complexes), and which therefore represent perfectly functioning proteins [130]. These mature proteins that have reached the "end of their career" and which are also called *retirees*, are characterized by different degrees of stability, although in general their half-life is rather long-lasting and on average has been calculated to be ~46 hours [131]. However, it is now well established that another portion of class I epitopes is also derived from polypeptides that are translated with various errors and anomalies and which together are defined as DRiPs. Importantly, DRiPs have extremely short half-lives (in the order of a few minutes) and in the broadest sense they include prematurely terminated polypeptides and misfolded polypeptides produced from translation of bona fide mRNAs in the proper reading frame, as well as defective polypeptides arising from alternative or defective mRNAs, ribosomal frame shifting, downstream initiation, tRNA-amino acid misacylation, and transcription errors [132–135]. Intriguingly, the reduced half-life of DRiPs would justify the very short time (about one hour) that elapses between the entry of a virus into the cell and the appearance of relevant epitopes on the cell surface, and in striking contrast with the rather high metabolic stability of mature viral proteins [136]. Of particular interest, especially in the context of the topic treated herein, a substantial fraction of DRiPs are degraded by proteasome, but this hydrolytic process was reported to be, at least in part, ubiquitin-independent [133,137]. This important observation clearly reinforces the more general consideration that part of proteasomal processing of class I epitopes does not require the ubiquitination reaction [49,138–140]. Although over the years the exact proportion of MHC class I antigenic peptides deriving from *retirees* compared to DRiPs has been the subject of heated debates and conflicting experimental results [141–143], it appears likely that both sources contribute to the generation of epitopes, with different relative ratios depending on the specific type of antigen and the biological context.

The PTPs assessed in the study by Boulpicante et al. [127] are specific DRiPs most likely produced in the nucleus prior to mRNA splicing that appear to be implicated in eliciting anti-tumor MHC class I immune responses [144–146]. It is well known that mRNAs containing premature termination codons (PTCs), for example as a consequence of a nonsense mutation, are rapidly detected and promptly eliminated by a quality control mechanism called nonsense-mediated mRNA decay (NMD), which takes place during the first round of translation by ribosomes. This surveillance mechanism results in the generation of peptides, but prevents the synthesis of a full-length protein [147]. Initially, it was demonstrated that class I epitopes originating from sequences inserted into genes displaying a PTC were effectively presented, although the complete mRNA, and therefore the corresponding full-length protein, were never produced [144]. Furthermore, preventing the association between the translation initiation factor eIF4E and the cap structure, another way to

impede the generation of complete proteins by the canonical translation ribosomal mechanism, also has a minimal influence on the generation of MHC-I epitopes [144]. Together, these results clearly demonstrate that the synthesis of a complete protein is not essential for the generation of MHC class I epitopes, but in many cases an early and abortive mRNA translation event is sufficient [144], which according to some evidence could also involve a prespliced mRNA [148]. Strikingly, this early ribosomal scanning of nascent mRNAs was later shown to occur in the nuclear compartment [145]. In fact, inserting different antigenic peptide sequences in introns that are spliced out before the mRNAs exit the nuclear compartment results in an equal amount of antigenic peptide products as when the peptides are encoded from the main open reading frame (ORF) [145]. Moreover, intron-encoded nascent peptides and complexes carrying ribosomal proteins S6 and L7 could be clearly detected in the perinucleolar compartment (but not in the nucleoli), further indicating that ribosomes can occupy and scan mRNAs early in the mRNA maturation process, in line with important previous studies documenting nuclear translation [149–152]. Importantly, it has been demonstrated that generation of PTPs is typically increased in tumors, due to errors in mRNA synthesis and maturation mechanisms that characterize neoplastic transformation. Therefore, they represent an important source of tumors-associated antigens (TAAs) that are crucial to elicit an effective anti-tumor immune response [144–146,153]. Intriguingly, PTPs share the same nuclear localization as PA28 γ , leading to the hypothesis that PA28 γ may be involved in regulating their Ub-independent proteasomal degradation.

Given all these premises, the effect of PA28 γ on MHC-I presentation of antigens from exon- as well as intron-derived PTPs in tumors was, therefore, evaluated [127]. As an initial result, a clear inverse correlation between the expression levels of endogenous PA28 γ and the presentation of PTP-derived antigens at the cell surface of multiple cancer cell lines was observed. Crucially, this correlation did not occur if final epitopes of the correct size were directly expressed in cells, unequivocally indicating a role of PA28 γ on antigen processing, rather than some indirect effect on the abundance or functioning of other components of the MHC class I pathway. Even more importantly, overexpression of exogenous PA28 γ clearly resulted in a strong decrease in MHC class I presentation of epitopes derived from PTPs in cancer cell lines (while its reduction leads to the opposite result), which were strictly dependent on the ability of PA28 γ to interact with 20S and modulate its proteolytic activities. In line with a role for PA28 γ in promoting cancer immune evasion, injection in C57BL/6 mice of PA28 $\gamma^{-/-}$ fibrosarcoma cells lead, compared to the parental WT cell line, to the formation of smaller tumors; importantly, this reduced tumorigenicity of the KO line was at least in part attributable to increased activation of MHC class I anti-tumor immune responses.

The molecular mechanism by which PA28 γ negatively interferes with the correct generation of class I epitopes by the 20S proteasome was then investigated by *in vitro* degradation experiments of long polypeptide precursors (with lengths ranging from 45 to 53 residues) containing embedded epitope sequences. Crucially, semi-quantitative tandem mass spectrometry (MS/MS) analysis of all the peptides produced in the degradation of one of these substrates showed no generation of the immunodominant antigenic peptide OVA₂₅₇₋₂₆₄ from ovalbumin when 20S was associated with PA28 γ , while in its absence the epitope was clearly detectable. Apparently, therefore, in this specific case PA28 γ may cause to increased hydrolysis of the antigenic peptide into short fragments, the detection of which, however, is precluded by standard mass spectrometry procedures in order to minimize false-positive identification and signals originating from small chemical compounds. In this regard, however, when in studies complementary to this one the sizes distribution of all peptide products generated during the degradation of the model protein casein by 20S alone or associate with PA28 γ was carefully measured by HP-size exclusion chromatography, only a minimal effect of PA28 γ was detected [61]. Specifically, its unique clear consequence on the chromatographic profile was a reduction of about two-thirds in a minor peak of larger (> 16 residues) products, which could potentially represent possible precursors of antigenic peptides following trimming by aminopeptidases [45]. Furthermore, MS/MS identification of all peptides produced during *in vitro* degradation of two protein substrates (myelin basic protein and chemically denatured IGF-1) by 20S and 20S-PA28 γ complexes, followed by semiquantitative MS/MS analysis of those released in common by both forms of proteasome, demonstrated PA28 γ -induced reduction in the variability of products that are potentially available for class I presentation [61] (Figure 3). Therefore, combining mass spectrometry data with that from HP-SEC, one could conclude that PA28 γ acts primarily by causing a reduction in the variability of peptides longer than 7 amino acids without, however, determining a concomitant increase in the overall quantity of fragments of lower molecular weight. Conceivably, this reduced variability of peptides that are potentially suitable to perform the function of class I epitopes could explain the inhibitory effect of PA28 γ on MHC class I presentation, specifically against nuclear antigens such as, but not limited to, PTPs.

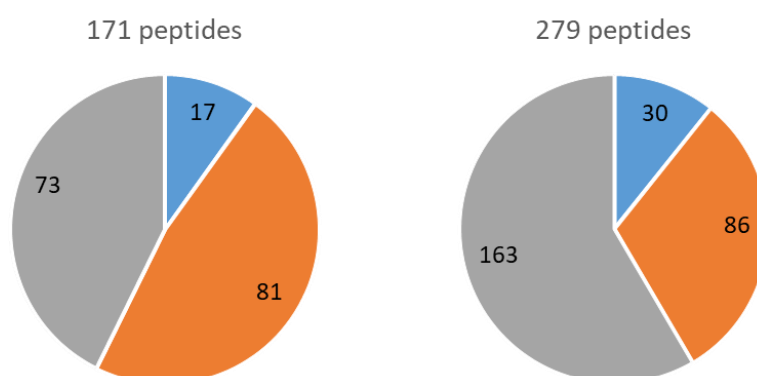


Fig. 3. PA28 γ reduces variability of peptides potentially available for class I presentation produced by 20S proteasome. MS/MS analysis of peptides generated during *in vitro* degradation of reduced and carboxymethylated IGF-1 (left) and myelin basic protein (right) by 20S and 20S-PA28 γ proteasomes. 171 and 279 individual peptides were identified ranging in length from 8 to 25 residues. Peptides released in common by both 20S and 20S-PA28 γ were further analyzed and the relative amount of each product was assessed by comparing the corresponding ion intensities measured in sequential MS/MS analyses. Gray indicates peptides produced exclusively or predominantly by the 20S proteasome, orange indicates peptides produced in similar amounts by both proteasome forms, blue indicates peptides produced exclusively or predominantly by the 20S proteasome associated with PA28 γ [61].

On the other hand, when analyzing the role of PA28 γ in modulating MHC class I antigen presentation, it cannot be overlooked that its prevalent (but not exclusive, as previously discussed) nuclear localization at first sight does not fit well with the generally accepted view that the processing of class I epitopes is an exclusively cytosolic process. However, this notion mainly derives from the general consideration that mature ribosomes carry out proteins translation in the cytosol. Moreover, the endoplasmic reticulum, which contains class I MHC molecules bound on the internal surface of its membrane, and TAPs that import antigenic peptides into the its lumen, is located in this same cellular compartment. However, the experimental data reported over the years by various laboratories that have been summarized in the previous paragraphs, clearly demonstrate that a part of class I epitopes, in particular those which, deriving from self-antigens, play a priority role in anti-tumor immune responses and autoimmune disorders, are generated during early translation events that involve pre-spliced mRNA. Intriguingly, direct and indirect evidence indicates that this process takes place within the nucleus or in the immediate vicinity of the nuclear envelope [146,154]. In this regard, it is noteworthy that the proteasome is abundantly present within the nuclear compartment [155–157], and therefore the possibility of its involvement in the generation of antigenic MHC class I peptides, not only from PTPs but also from mature nuclear proteins (i.e. *retirees*), cannot be excluded a priori. How the antigenic peptides generated in the nucleus then reach the lumen of the ER is currently unknown. However, it must first be observed that the nuclear pore complex (NPC) allows the escape by simple passive diffusion of molecules up to 30 kDa [158], a value well above the molecular weight of the vast majority of proteasomal products [53,61,66,159,160]. Furthermore, a role of molecular chaperones in the export of epitopes from the nucleus to the cytosol can be hypothesized, in light of their involvement in regulating the bidirectional trafficking of proteins through the nuclear pores [161–167], and the function they play in conveying the peptides from the proteasome to the TAPs, which is likely to protect them from abundant cytosolic exo- and endopeptidases [168–171]. Finally, a direct mechanism of passage of peptides from the nuclear envelope (which however lacks TAPs) to

the lumen of the ER has also been hypothesized, although at present this has not been experimentally demonstrated [154].

3. Conclusions and future perspectives

More than 20 years have now passed since PA28 γ knockout transgenic mice were generated for the first time and their phenotype analyzed, reaching the conclusion that PA28 γ plays a rather limited role in acquired immunity and in the pathway of MHC class I antigen presentation. However, some recent studies have questioned these results, providing solid evidence that PA28 γ has important immunological functions, which specifically consist in attenuating class I presentation. In this regard, two different roles of PA28 γ have been described. One is linked to its ability to suppress autoimmune reactions through a molecular mechanism based on reducing the expression of the immunoproteasome, which in the biological context analyzed would be responsible for an excessive generation of autoreactive self-epitopes. The second immunological function of PA28 γ refers to its ability to promote immune evasion of cancer cells as a direct consequence of modulating proteasomal proteolytic activities in the direction of promoting the destruction (rather than the generation) of tumor epitopes. Interestingly, both of these newly described immunological functions result in suppression of class I cell-mediated responses and are likely to be complementary rather than alternative. However, further studies are undoubtedly necessary to more solidly establish the general value of these results and to better clarify their exact molecular mechanisms. Indeed, the role of PA28 γ in regulating immunoproteasome levels via modulation of the JAK-STAT pathway in other cells and in physiological and pathological conditions remains to be definitively established. Similarly, the role of PA28 γ in inducing destruction, rather than processing, of MHC-I epitopes by the 20S proteasome needs to be more broadly confirmed by *in vitro* studies assessing epitopes other than OVA₂₅₇₋₂₆₄. Furthermore, for a more precise understanding of the exact molecular mechanism through which PA28 γ modifies the patterns of 20S proteasome products, size distribution of peptides from other physiological substrate proteins, which are more relevant for the immune system than casein, must be evaluated.

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