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REVIEW

Serine Proteases in Immunity: Guest Editor Timo Burster

Activity-Based Protein Profiling of Serine Proteases in Immune Cells

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Abstract

Multiple types of immune cells utilize serine proteases in their mechanisms of defense against pathogens or altered host cells. Dysregulation of the serine protease activity from these cells underlies different diseases. In the past, the technique of activity-based protein profiling proved to be especially useful for the study of proteases, and various studies have used small-molecule activity-based probes to covalently label and detect serine proteases from immune cells. In this review, we give an overview of the different activity-based probes that have been designed for serine proteases and how their selectivity can be steered. We also discuss how these have been utilized in the detection of various serine proteases from immune cells by different analysis methods (gel electrophoresis, microscopy and flow cytometry) and what biological insights these studies have produced. Overall, activity-based protein profiling has the potential to address functional aspects of serine proteases in the immune system and future efforts may bring translation into clinical application.

Keywords Activity-based probes · Cathepsin G · Covalent protease inhibitors · Neutrophil elastase

Abbreviations

ABP	Activity-based probe
ABPP	Activity-based protein profiling
APC	Antigen-presenting cell
CatG	Cathepsin G
CTL	Cytotoxic T lymphocyte
DCI	3,4-Dichloroisocoumarin
ET	Extracellular trap
FRET	Fluorescence resonance energy transfer
Grz	Granzyme
HyCoSuL	Hybrid combinatorial substrate library
LF	Lactoferrin
MHC-I	Major histocompatibility complex, Class I
MHC-II	Major histocompatibility complex, Class II
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
NETosis	Form of cell death with neutrophil extracellular trap formation
NK cell	Natural killer cell
NSP	Neutrophil serine protease
NSP4	Neutrophil serine protease 4
PBMCs	Peripheral blood mononuclear cells
PMA	Phorbol myristate acetate
PML	Polymorphonuclear leukocyte
PPE	Porcine pancreatic elastase
PR3	Proteinase 3
PS-SCL	Positional scanning synthetic combinatorial library
qABP	Quenched fluorescent activity-based probe
SP	Serine protease

Introduction

Proteases are peptide-bond cleaving enzymes that make up approximately 2% of the human genome. They are divided into seven different groups based on their catalytic mechanism (Rawlings et al. 2018). Serine proteases (SPs) are the most abundant ones, and the majority belongs to the so-called S1 family, whose primary specificity is determined by the P1 position (the amino acid residue N-terminal to the scissile bond) (Di Cera 2009). Depending on their substrate specificity, SPs of the S1 family are divided into three subgroups: trypsin-like SPs, which prefer P1 basic residues,

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chymotrypsin-like SPs, which prefer P1 bulky hydrophobic residues and elastase-like SPs which prefer P1 small aliphatic residues. Activity of SPs is tightly regulated to prevent unwanted, harmful proteolysis. Mechanisms regulating SP activity include the expression as inactive zymogens, the inhibition of activated SPs by endogenous inhibitors such as serpins (van Gent et al. 2003), and compartmentalization of SPs in specialized organelles, such as neutrophil granules (Cowland and Borregaard 2016). SPs are indispensable actors in several physiological processes including blood clotting, digestion and reproduction. Dysregulation of their tightly regulated activity is associated with a variety of diseases, such as cancer, neurodegeneration and diabetes. Various SPs are also involved in the immune system (see section "Serine Proteases in Immune Cells").

Activity-based protein profiling (ABPP), which emerged two decades ago, is a particularly useful technique for the study of proteases (Chakrabarty et al. 2019). ABPP uses small molecules termed activity-based probes (ABPs), which consist of three parts (Fig. 1): a reactive warhead, which covalently reacts with the active site of the target enzyme, a recognition element, which can be utilized to increase the specificity of the probe for its target(s), and a detection tag, which allows the detection of probe-labeled enzymes. Because ABPs exploit the catalytic mechanism to form a covalent bond, ABPP can distinguish between active and inactive proteases. This is a significant advantage over protein quantification and transcriptomics since the activity of SPs is tightly regulated by post-translational mechanisms.

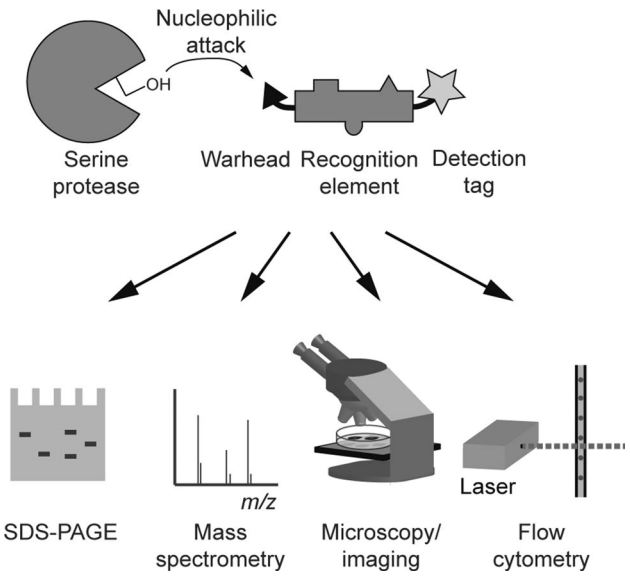

Fig. 1 Schematic representation of an ABP with downstream applications. The warhead (see section "ABPs for Serine Proteases in Immune Cells" for an overview) undergoes a mechanism-based reaction with the active site serine, forming a covalent bond. The recognition element, which can be a peptide or a non-peptidic element such as a heterocycle, influences the selectivity of the ABP. The detection tag may be a fluorophore, (radio)-isotope, biotin or bioorthogonal handle. Analysis takes place by gel electrophoresis, tandem mass spectrometry-based proteomics (after biotin enrichment), fluorescent microscopy or whole-body imaging, or flow cytometry

The selectivity of an ABP is determined by the combination of the warhead and the recognition element. The warhead influences which amino acid side chain is covalently modified (in the case of SPs: the active site serine), whereas the recognition element can be utilized to make an ABP with a higher or lower degree of selectivity. Probes with a broad selectivity can be useful for profiling of many proteases at the same time, while probes with high specificity are particularly useful for fluorescent imaging of a single protease target.

The method of detection of probe-labeled enzymes (Fig. 1) in an ABPP experiment depends on the type of detection tag. Frequently used tags include fluorophores, (radio)-isotopes, biotin or biorthogonal groups such as azides or alkynes (Sadaghiani et al. 2007). Most detection tags can be used for gel-based detection strategies. Fluorophore-tagged probes are also applied in microscopy and flow cytometry, whereas radioisotopes are efficient for probe detection in vivo in less exposed parts of the body. For mass spectrometry-based identification of probe targets, it is required to first separate the targets from the rest of the proteome. For this purpose, most studies apply biotin as a tag, since it allows isolation of even highly diluted probe targets by avidin pull down. Tags can also be installed by means of bioorthogonal chemistry, such as the azide-alkyne "click" cycloaddition reaction (Speers et al. 2003). Equipping probes with an azide or alkyne group instead of a larger detection tag can prevent interference of the tag with the target protein and improve the permeability of probes in cells or organs. Furthermore, it allows variations in the type of tag, since any azide- or alkyne-functionalized detection tag can be bioorthogonally placed onto the probe-bound target protease.

The crucial involvement of multiple SPs in the function of immune cells and the gaps in the knowledge on their exact function has created a critical need for small molecule tools such as ABPs. In this review, we first give an overview of the most important SPs in immune cells (section "Serine Proteases in Immune Cells"). In section "ABPs for Serine Proteases in Immune Cells", we discuss the different reactive groups used in ABPs for SPs and how to influence selectivity in these probes. Section "Applications of ABPP in Immune Cells" highlights some specific applications on various immune cells. We end with some future perspectives of this research area.

Serine Proteases in Immune Cells

Human immune cells utilize a variety of different SPs (Heutinck et al. 2010). A short overview of these proteases and their occurrence is presented in Table 1. A substantial

part of the SPs that play a role in the immune system, are expressed in granulocytes, which are also referred to as polymorphonuclear leukocytes (PMLs), because of their odd-shaped nucleus that often appears in three lobes. The cytoplasm of PMLs contains granules (hence: granulocytes), which are filled with various proteins, including proteases, and other molecules. The content of the granules is secreted upon certain stimuli. Four types of PMLs exist: neutrophils, eosinophils, basophils and mast cells.

The most abundant PMLs are neutrophils, which are amongst the first cells that arrive at the location of an inflammation. It was first thought that neutrophils contain three neutrophil SPs (NSPs): cathepsin G (CatG), neutrophil elastase (NE) and proteinase 3 (PR3). However, a fourth SP called neutrophil serine protease 4 (NSP4) was discovered in 2012 (Perera et al. 2012). Although it has a very similar fold as the other NSPs, its substrate specificity is very different by a unique mechanism (Lin et al. 2014). Neutrophils can act in various ways upon a threat (Kolaczkowska and Kubes 2013): phagocytosis, degranulation or formation of neutrophil extracellular traps (NETs) (Fig. 2a). In the first event, pathogens are internalized and destroyed intracellularly (Lee et al. 2003). In the second event, the contents of the granules are secreted (Lacy 2006), leading to various effects including inactivation of pathogen virulence factors (Stapels et al. 2015) and activation of host proteins such as cytokines and receptors (Meyer-Hoffert 2009). A third event comprises the formation of NETs: a combination of extracellular chromatin and NSPs, which can trap and degrade pathogens (Brinkmann et al. 2004). NETs can also be released in a cell death process other than apoptosis or necrosis, and this is referred to as NETosis (Fuchs et al. 2007).

Eosinophils, mast cells and basophils are all involved in allergic reactions, but also in infections (Stone et al. 2009). Mast cells mainly reside inside tissues. Their granules are filled with tryptase and chymase, which constitute up to 25% of the entire protein content of the cell (Schwartz et al. 1987). Mast cells migrate into the mucosa and connective tissue where they can elicit an inflammatory reaction. During an infection, the content of the granules is released in an IgE mediated process. Because of the involvement of mast cells in allergic reactions such as asthma, tryptase is an attractive drug target, but its overlap in P1 specificity with other proteases complicates the development of highly selective inhibitors.

Natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) defend the body against intracellular pathogens by killing infected cells. The SPs involved in this process belong to the granzyme (Grz) family. NKs and CTLs store their SPs in granules, as PMLs do, but lack a polymorphonuclear character. Upon recognition of an infected cell, degranulation of NKs and CTLs releases granzymes and the protein perforin. Perforin mediates delivery of the released granzymes into the target cell, which leads to cell death (Fig. 2b), part of which proceeds through activation of caspases by GrzB (Voskoboinik et al. 2015).

CatG does not only occur in PMLs but also in different types of antigen-presenting cells (APCs) (Burster et al. 2010). Note that the CatG in these cells may be endogenously expressed in some type of APCs or taken up from the environment by endocytosis in other types of APCs. In the endocytic pathway, CatG takes part in the processing of endocytosed proteins into antigenic peptides, which are subsequently loaded onto MHC-II. Eventually, these complexes are exposed on the cell surface, where they can interact with T-cell receptors (Fig. 2c).

The type II transmembrane SP matriptase, although mostly expressed in epithelial cells, also occurs in various leukocytes. Specifically, its expression has been detected in mast cells (Cheng et al. 2007), monocytes and B lymphocytes (Kilpatrick

Table 1 Serine proteases in immune cells

Serine protease	P1 specificity	Occurrence
Cathepsin G	Large hydrophobic Also Arg/Lys (*H. sapiens)*	Neutrophils, basophils, monocytes, dendritic cells, mast cells
Chymase	Large hydrophobic	Mast cells
Granzyme A	Arg/Lys	NK cells, CTLs
Granzyme B	Asp	NK cells, CTLs
Granzyme H	Large hydrophobic	NK cells, CTLs
Granzyme K	Arg/Lys	NK cells, CTLs
Granzyme M	Large hydrophobic	NK cells, CTLs
Matriptase	Arg	Monocytes, B cells, mast cells
Neutrophil Elastase	Small hydrophobic	Neutrophils, eosinophils, basophils monocytes, mast cells,
NSP4	(modified) Arg	Neutrophils
Proteinase 3	Small hydrophobic	Neutrophils, eosinophils, basophils, monocytes, mast cells
Tryptase	Arg/Lys	Mast cells

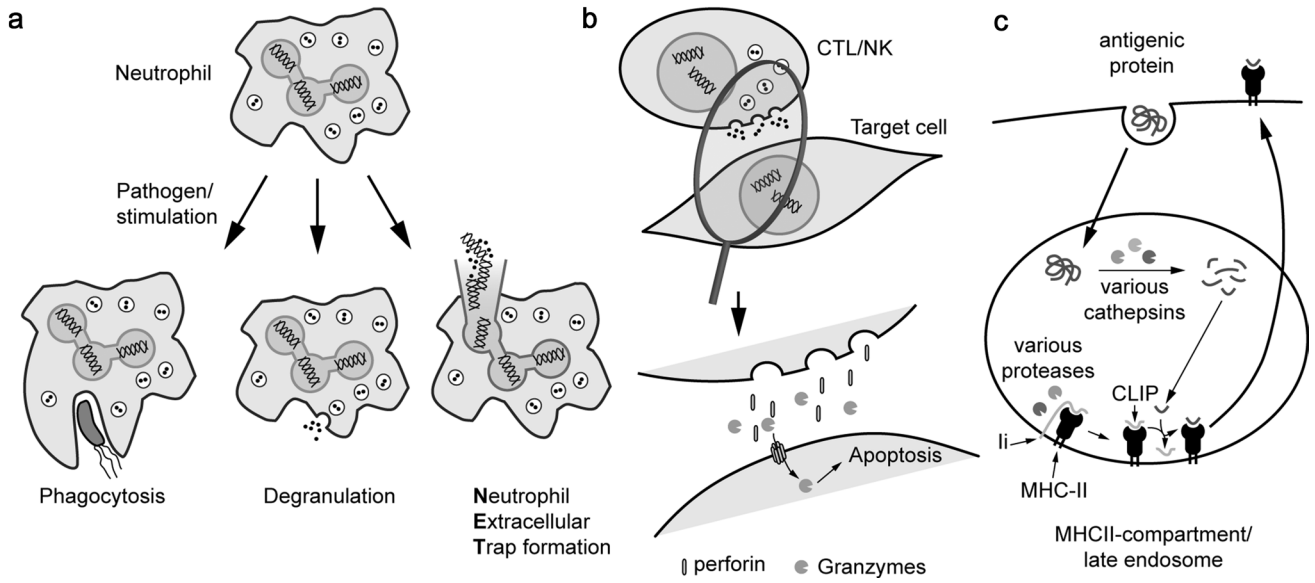

Fig. 2 Selected roles of SPs in immune cells. **a** Activation of neutrophils can lead to three different downstream effects. Left: after phagocytosis of pathogens, fusion of NSP containing granules with the phagosome leads to the destruction of the pathogen. Middle: degranulation releases NSP extracellularly. Right: NETs are formed by various molecules, including chromatin and NSPs. These structures trap and eliminate pathogens. **b** When CTLs or NKs recognize a target cell for the destruction they secrete the contents of their granules, which includes the pore-forming protein perforin and various granzymes. When the granzymes enter the cell, they induce regulated cell death. **c** Simplified summary of MHC-II antigen presentation. When antigenic proteins are endocytosed, they are processed by various cathepsins, including cathepsin G, resulting in multiple peptides. Some of these peptides are loaded onto MHC-II molecules for presentation at the cell surface. Note that premature peptide loading on MHC-II is prevented by the binding of the invariant chain (Ii), which is degraded by several proteases, leaving behind the small peptide CLIP in the MHC-II binding groove until antigenic peptides are ready to be bound. More information about MHC-II antigen processing can be found elsewhere (Unanue et al. 2016)

et al. 2006). Apart from being involved in the activation of plasminogen by monocytes, the functional role of matriptase in leukocytes is unknown.

The complement system represents a part of the innate immune system that protects against pathogens and altered host cells. It harbors several SPs, which are part of a network that is activated by the so-called "classical", "lectin" or "alternative" pathway, respectively (Sim and Tsiftsoglou 2004). Because these proteases do not occur in cells but circulate in the bloodstream and no ABPs have been developed for these proteases, we will not discuss these further within the context of this review.

ABPs for Serine Proteases in Immune Cells

The electrophilic warhead group is a crucial part of an ABP as it covalently reacts with the active site residue. Importantly, the chemical mechanism of the warhead may determine the future applications of the probe, as only some warheads allow for the construction of quenched fluorescent ABPs (qABPs) and therefore, for real-time imaging. It is thus important to choose the right type of warhead and probe architecture for the desired application. Various different warheads have been used for ABPP of proteases from immune cells. Below sections give an overview of these warheads and some examples of probes incorporating these followed by a brief section in which the problem of selectivity with these probes is discussed.

Peptidyl Diphenyl Phosphonates

Peptidyl diphenyl phosphonates are the most widely applied SP ABPs. The reason for their widespread usage might be their ease of synthesis by the three-component Oleksyszyn reaction (Oleksyszyn et al. 1979; Oleksyszyn and Powers 1991). Diphenyl phosphonates are close analogs of amino acids (Fig. 3a) and bind in a substrate-like manner. Therefore, their selectivity can be steered by incorporation of peptides that resemble the substrate specificity of the target protease.

After binding to the active site of a target SP, diphenyl phosphonate warheads are attacked on the phosphorus atom by the active site serine of the target protease, leading to the

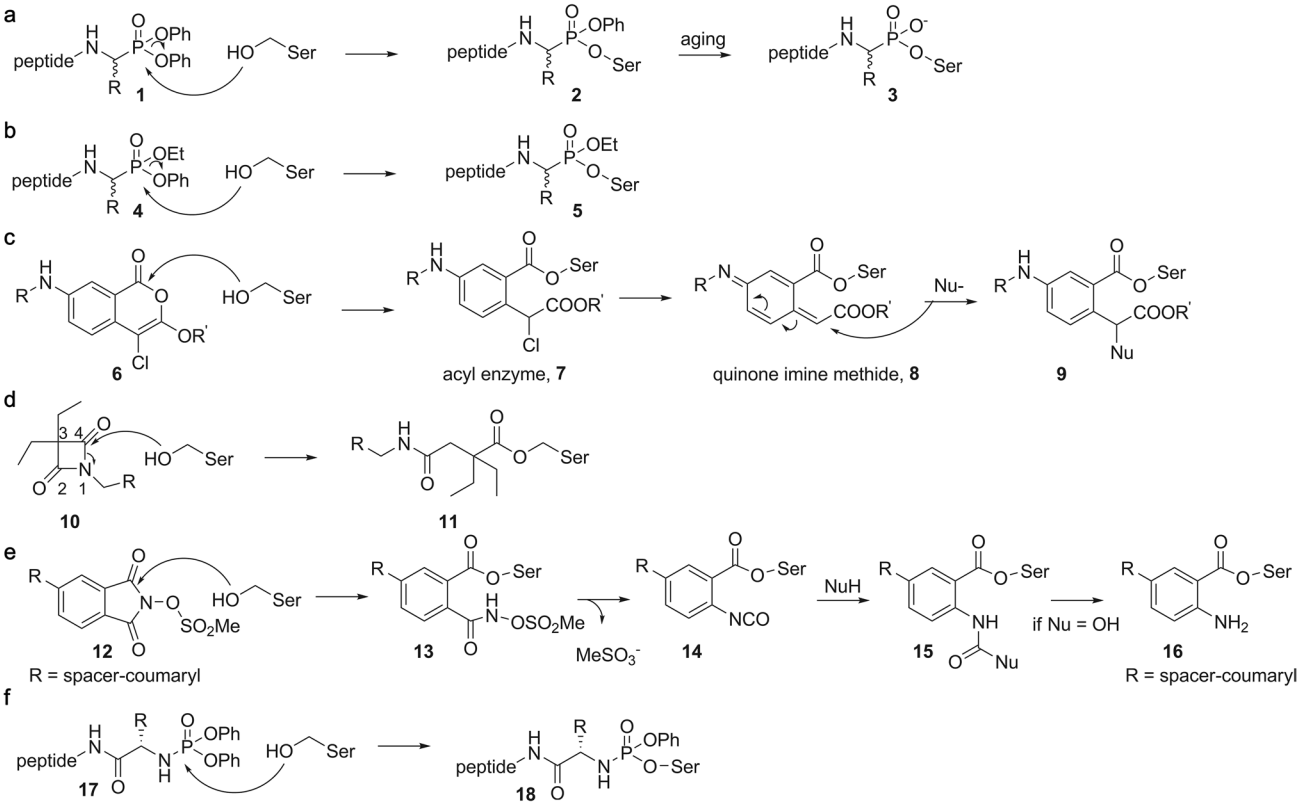

Fig. 3 Overview of the different electrophilic ABPs for SPs and their reaction with the active site serine. **a** Diphenyl phosphonates **1** are generally synthesized as a mixture of isomers at the P1 position. The serine attacks the phosphorus atom, expelling a phenoxy leaving group. Subsequent aging results in the loss of the second phenoxy group. The resulting negatively charged oxygen in **3** interacts with the "oxyanion-hole". **b** Mixed alkyl-aryl phosphonates **4** react in a similar manner as diphenyl phosphonates but do not undergo an aging process due to the lack of a second leaving group. **c** The active site serine attacks 7-amino-4-chloro-isocoumarins **6** on the carbonyl group yielding the acyl-enzyme **7**. If a nitrogen substituent is present in the 7-position, quinone imine methide intermediate **8** is formed and a second nucleophile, such as a solvent molecule or the active site histidine can attack. **d** 4-Oxo-β-lactams **10** are attacked on the carbonyl carbon and the four-membered ring opens. **e** Reaction of the active site serine with the carbonyl carbon of sulfonyloxyphthalimides **12** gives rise to an acyl-enzyme intermediate **13**. This undergoes a Lossen rearrangement yielding a reactive isocyanate **14** which in turn reacts with a second nucleophile. **f** Diphenyl phosphoramidates **17** react in a similar manner to diphenyl phosphonates. The active site serine attacks the phosphorus atom thereby expelling a phenoxy moiety

expulsion of one of the phenoxy leaving groups (Fig. 3a). The phosphonate then stays permanently attached to the serine side chain, but generally loses the second phenoxy leaving group in an "aging" process after which the negatively charged oxygen occupies the oxyanion hole of the protease active site (Fig. 3a).

The reactivity of diphenyl phosphonates towards the nucleophilic serine residue in a protease active site is related to the electrophilicity of the phosphorous atom, which in turn is influenced by the leaving group. Therefore, introducing electronegative substituents at the phenoxy leaving group may enhance reactivity. For example, changing the leaving group from phenyl to chlorophenyl led to an almost five times higher potency against chymotrypsin (Abuelyaman et al. 1997). In NE, the introduction of a para-S-methyl group increased the potency of the probe fourfold compared to a phenoxy leaving group (Grzywa et al. 2014).

Additionally, the stereochemical configuration of the phosphorus atom strongly influences the activity of diphenyl phosphonates. The (R)-enantiomer of diphenyl phosphonates typically exhibits superior inhibitory properties (Walker et al. 2000). Schulz-Fincke et al. (2018a) separated the diastereomers of a peptidyl diphenyl phosphonate-based NE inhibitor and showed that only one of them strongly inhibits NE. The inhibitor was then conjugated to a BODIPY fluorophore, resulting in an ABP that selectively labeled NE spiked into a HEK cell lysate (Schulz-Fincke et al. 2018a).

Peptidyl Mixed Alkyl-aryl Phosphonates

Mixed alkyl-aryl phosphonates have only one leaving group attached to the phosphorus atom (Fig. 3b). This leaving group can be coupled to a fluorescent quencher to generate qABPs which enable real-time imaging. Mixed alkyl-aryl

phosphonates have been synthesized from diethyl phosphonates by non-hydrolytic mono-dealkylation and then coupling to a substituted phenol leaving group (Serim et al. 2015). Mixed alkyl-aryl phosphonates are thought to react with active site serine residues in a similar manner as diphenyl phosphonates do, although they probably do not undergo an aging process since they lack the second leaving group.

Relatively general qABPs for SPs, incorporating only the warhead with a P1-residue and no further peptidic linkers, have been synthesized. One of these probes contained a valine phosphonate as the warhead and has been used to label purified NE (Serim et al. 2015). However, no qABPs have been used for real-time imaging of SPs in immunity so far.

Isocoumarins

Isocoumarins are non-peptidic, heterocyclic inhibitors of SPs. 3,4-dichloro-isocoumarin (DCI) has been applied as a general SP inhibitor and is often used to show that a SP ABP is active site directed. In that case, preincubation of a protease with DCI prevents labeling during subsequent incubation with the probe.

The inhibitory mechanism involves an attack of the active site serine onto the carbonyl group of the isocoumarin, thereby opening the lactone ring and forming an acyl-enzyme. The acyl-enzyme can regain its activity by hydrolysis or if it is treated with a nucleophile such as hydroxylamine (Kam et al. 1993). However, depending on the substitution at the 7-position, a quinone imine methide (**8**) is formed as a second electrophile. This species can then react with nucleophilic side chains, for example, the active site histidine, to establish a stable, permanent linkage to the protease (Fig. 3c).

Isocoumarins have been equipped with detection tags to obtain ABPs. Powers and co-workers showed that these react with various SPs including CatG and NE (Kam et al. 1993). Haedke et al. (2012) synthesized several isocoumarin probes functionalized with an alkyne handle at either the 3- or the 7-position of the isocoumarin scaffold and different substituents at the other position respectively. These ABPs showed that CatG accepts hydrophobic substituents at either of these positions.

Oxolactams

The 4-oxo-β-lactam scaffold has been used as an inhibitor scaffold to develop elastase inhibitors. Various structurally diverse molecules have been synthesized to obtain a structure-activity relationship (Mulchande et al. 2008, 2010). Glutaraldehyde-crosslinked crystals of PPE used as a model for NE have been soaked with a 4-oxo-β-lactam to confirm a covalent mode of action. These crystals showed that the scaffold reacts with active site serine, S195, in a ring-opening reaction, covalently linking the inhibitor to the protease (Fig. 3d). Moreover, the crystal structure as well as in silico docking experiments on human NE revealed that the small substituent at the 3-position interacts with the S1-pocket of the protease, explaining the observed selectivity (Hofbauer et al. 2015; Mulchande et al. 2010).

Ruivo et al. (2016) used the oxolactam scaffold as warhead to synthesize selective ABPs for NE. They kept the diethyl substituent at the 3-position in place to dock into the small hydrophobic S1 pocket of NE and, therefore, to preserve the previously observed selectivity. Fluorescent and biotin tags were introduced as substituents on the nitrogen atom by means of click chemistry. Interestingly, despite the relatively simple structure, these probes showed good potency and very good selectivity for NE over the closely related protease PR3. Eventually, one ABP detected as little as 5 ng NE and selectively labeled NE in neutrophil cell lysates (Ruivo et al. 2016). This highlights the possibility of obtaining selective non-peptidic ABPs.

Sulfonyloxyphtalimides

Sulfonyloxyphtalimides are inhibitors of SPs (Neumann and Gutschow 1994) and, therefore, can be used as a warhead in the construction of an ABP. Their reaction with the active site serine involves a nucleophilic attack on one of the carbonyl carbons and subsequent opening of the heterocyclic ring yielding an acyl-enzyme (Fig. 3e). The formed hydroxamic acid derivative **13** subsequently undergoes a Lossen rearrangement yielding a reactive isocyanate species (**14**). Intermediate **14** can either be trapped by water or covalently react with another nucleophile in the vicinity, such as a side chain of the target protease, leading to an irreversible bond (Fig. 3e).

Schulz-Fincke et al. (2018b) synthesized a probe for NE incorporating a sulfonyloxyphtalimide warhead linked to a coumarin fluorophore by a PEG linker. The probe showed selectivity for NE over various cysteine proteases and SPs including PPE, although its selectivity over PR3 still needs to be determined. The probe was able to detect NE in neutrophil cell lysates.

An interesting extra feature of probe **12** (Fig. 3e) is the possibility to perform fluorescence resonance energy transfer (FRET) between the anthranilic acid moiety which is formed after reaction of the isocyanate intermediate with water and the coumarin fluorophore (conversion of **15** to **16**; Fig. 3e). Since this moiety is only formed in case of reaction with the target SP, non-reacted probe will not fluoresce, resulting in a reduction of background fluorescence. Eventually, a tryptophan of PPE in close proximity to the anthranilic acid moiety is used as a FRET donor for the anthranilic acid and excited at 285 nm. Anthranilic acid, in turn, acts as donor for the

coumarin fluorophore, emitting fluorescence at 490 nm. In this system, only bound probe gives a signal at 490 nm and it could be an interesting alternative approach for quenched ABPs (Schulz-Fincke et al. 2018b).

Diphenyl Phosphoramidate Peptides

Phosphoramidate peptide probes are similar to peptidyl aminophosphonates, but their peptide backbone is reversed (Fig. 3f) and the electrophilic phosphoramidate warhead is bound to the N-terminus. Conveniently, these molecules can be made by solid-phase peptide synthesis. However, the reactivity of the warhead is substantially reduced compared with diphenyl phosphonates, which can be attributed to the lower electrophilicity of the phosphoramidate and a less optimal recognition of the inversed peptide backbone. Despite these differences, they probably interact with the active site in a similar way, because the amino acid adjacent to the phosphoramidate determines the target protease. Accordingly, NE was labeled by a probe with a valine recognition element (Haedke et al. 2014).

Influencing Probe Selectivity

Heterocyclic warheads such as isocoumarins, 4-oxo-β-lactams and sulfonyloxyphtalimides do not closely resemble polypeptide substrates of a protease. Therefore, it is difficult to rationally design probes with improved selectivity. Nonetheless, extensive work on the isocoumarin scaffold as SP inhibitor (Powers et al. 2002) and as ABP revealed that substituents at the 3- and 7-positions can both dock into the S1-pocket of a target protease. Therefore, a certain degree of ABP selectivity can be obtained by introducing the appropriate side chain mimic in these positions (Haedke et al. 2012). Additionally, if biotinylated probes are used, a longer spacer that increases the distance to the isocoumarin core gives more potent probes (Kam et al. 1993).

For 4-oxo-β-lactams, the substituent at the 3-position of the lactam-core binds to the S1-subsite of a protease. Substitutions at the nitrogen can be used to introduce residues that can dock into more distant subsites (Mulchande et al. 2008, 2010).

Contrary to non-peptidic ABPs, probes incorporating phosphonates do resemble protease substrates. To further improve the selectivity of phosphonates, the warheads are often coupled to peptides of varying length that bind to the respective subsites of the target protease.

One possibility to obtain information about subsite specificity is the screening of positional scanning synthetic combinatorial libraries (PS-SCLs) (Backes et al. 2000). In PS-SCLs peptides are varied in one of the subsite positions with all the natural amino acids. An isokinetic mixture of natural amino acids is used for the not-varied positions to obtain equal amino acid distribution in the resulting peptides (Fig. 4). Using such a PS-SCL, Mahrus and Craik (2005) found distinct substrate preferences for the individual granzymes and were able to obtain remarkably selective probes for GrzA and GrzB. However, a common limitation of this strategy is the restriction to natural amino acids and, therefore, to a very limited chemical space, complicating the design of selective ABPs.

Drag and coworkers have developed a strategy that expands the chemical space of these libraries with non-natural amino acids. These so-called hybrid combinatorial substrate libraries (HyCoSuL) (Kasperkiewicz et al. 2014; Poreba et al. 2014, 2017) have used more than 100 non-natural amino acids and eventually led to much more selective probes. Because of the very strict specificity of the S1 pocket in many SPs, such as NSPs and granzymes, the sublibraries are usually synthesized with a fixed P1-residue suitable for the protease of interest. After cleavage from the resin, the library is tested in a fluorescent kinetic assay. Peptides incorporating the best amino acids in the individual positions are then synthesized and evaluated for their activity to rule out any negative subsite cooperativity. Once very selective substrates for a given protease are obtained, they

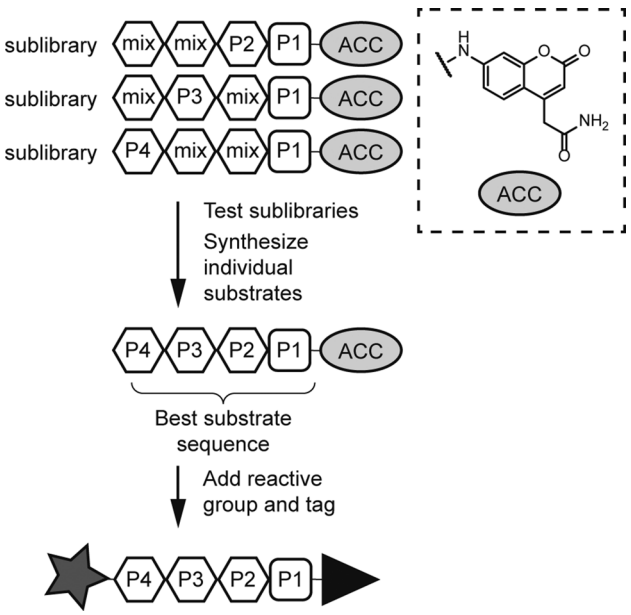

Fig. 4 Principle of PS-SCL and HyCoSuL library screening. The P1 amino acid is fixed and sublibraries of fluorogenic substrates carrying individual amino acids in the P2, P3 or P4 positions, respectively, and a mixture of amino acids in the remaining ones are synthesized. Their activity is tested and individual substrates carrying the most active amino acids in the respective positions are subsequently made. A selective ABP can then be made by adding a reactive group and a tag to the optimal peptide sequence. Note that the amino acids with the highest activity are not necessarily the ones that result in the highest specificity over related proteases. This should be taken into account when selecting the amino acids in each individual position

can be used to synthesize ABPs. To accomplish this, the scissile peptide bond is replaced by a phosphonate warhead and the desired detection tag is introduced, leading to translation of a selective substrate into a selective probe. A set of peptidyl-diphenyl phosphonate ABPs selective for each member of the NSPs has been developed using this approach (Kasperkiewicz et al. 2014, 2015, 2017).

Gütschow and coworkers synthesized diphenyl phosphonates with selectivity for the matriptase family by introducing two benzguanidine groups as non-natural recognition elements. The benzguanidine groups mimic arginine residues and are assumed to bind in the S_1 and S_2 pockets of matriptase (Haussler et al. 2016). Although the probes show no selectivity for matriptase over matriptase-2, they can be applied for selective matriptase labeling, since tissue distribution of matriptase-2 is limited (Haussler et al. 2017).

Phosphonate warheads have also been coupled to peptides that dock into protease S3 and S4 subsites via Cu(I)-catalyzed 1,3-dipolar cycloaddition on solid support, evading the liquid phase synthesis usually necessary for final construction of peptidyl phosphonate ABPs. Some of these probes were then used to label NE and CatG amongst other proteases (Serim et al. 2013).

Applications of ABPP in Immune Cells

Probing NSPs in Neutrophils

Because neutrophils release NSPs during degranulation and can form NETs at the site of inflammation, ABPs represent attractive tools to gain more knowledge about the implication of NSPs in inflammatory processes and NETosis. In 2014, the Drag laboratory reported a biotinylated diphenyl phosphonate ABP with the P4-P1 position all comprising of non-natural amino acids (**19**; Fig. 5a),

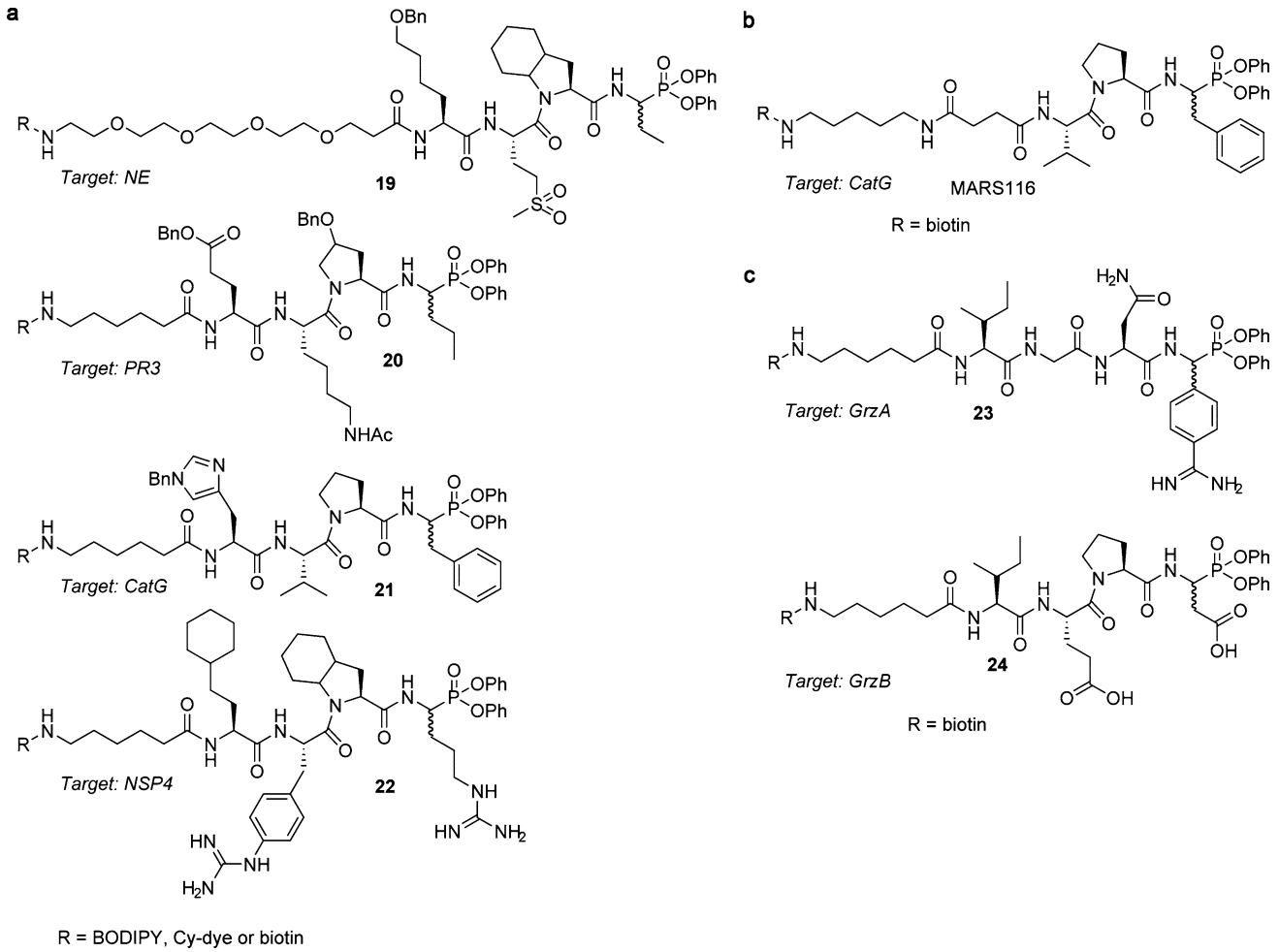

Fig. 5 Structures of ABPs for different SPs. **a** Structures of probes for the individual NSPs, **b** structure of MARS116 targeting CatG, **c** ABPs for GrzA (**24**) and GrzB (**25**)

giving rise to highly sensitive detection of NE (Kasperkiewicz et al. 2014). This probe was used in combination with phorbol myristate acetate (PMA) treatment of isolated human neutrophils, which induces the formation of NETs. These NETs were associated with very little NE activity, although antibody staining revealed that NE was present. It must be noted, however, that NE activity in NETs is donor dependent: very low activity was seen in 7 out of 10 donors, whereas in only 3 out of 10 donors NE was found to have higher activity in NETs (Barrientos et al. 2013). Recently, a sulfoCy5 analog of probe **19** was applied for detection of NE in inflammatory bowel disease. In experimental mouse models of colitis, active NE was found in lysates of distal regions of inflamed colons, but not in samples from healthy mice. More importantly, active NE was found in mucosal biopsies from patients with ulcerative colitis. In contrast, control samples from healthy donors only showed very low NE activity, highlighting the potential use of this probe as a diagnostic tool for ulcerative colitis (Anderson et al. 2019).

Continuous efforts using HyCoSuL have led to the development of selective ABPs for CatG, PR3 and NSP4 (**20–22**; Fig. 5a), all equipped with different fluorophores or biotin (Kasperkiewicz et al. 2015, 2017). Although the probe optimized for NE still showed cross-reactivity with PR3, selective labeling of all four NSPs could be achieved by labeling PR3 first and subsequently applying the less selective NE probe (Kasperkiewicz et al. 2017). Kasperkiewicz et al. (2017) found that the reactivity of the NSP probes was very high, some with kinetic $k_{obs}/[I]$ values larger than 10^6 M^{-1} s^{-1}. Hence, the reaction between the probes and the enzymes is fast and can be assumed to be complete. As a result, they were able to titrate the individual NSPs with the biotinylated probes acting as inhibitors, with measurement of residual NSP activity by subsequent addition of the fluorescent probe. With this experimental setup, the concentration of biotinylated probe that prevents binding of fluorescent probe equals the concentration of the active NSP. These experiments allowed determination of the concentration of each individual NSP in lysates of neutrophil cells. It was found that active NSP4 is only present in low amounts in comparison with the other three NSPs, which is in agreement with previous findings of NSP4 only being 5–10% of the amount of NE (Perera et al. 2012). Most importantly, Kasperkiewicz et al. (2017) found that the different NSPs were localized separately into different azurophilic granules. The percentage of individual colocalization could be determined by flow cytometry showing that only 0.2% of granules display colocalization of all four NSPs, suggesting a discriminatory packaging system (Kasperkiewicz et al. 2017).

Neutrophils do not only contain azurophilic granules (also referred to as primary granules), but also secondary and tertiary granules, and secretory vesicles, all filled with different types of content (Lacy 2006). Eipper et al. (2016) investigated whether lactoferrin (LF), a content of the secondary granules, influences the activity of CatG using MARS116 (Fig. 5b). MARS116 is a biotinylated ABP that was previously developed for CatG (Zou et al. 2012). Contrary to earlier findings (He et al. 2003) LF was found to enhance CatG activity at physiological concentrations. Interestingly, the enhancement was more pronounced at low pH suggesting a role for LF in maintaining CatG activity at acidic inflammatory sites. Moreover, in supernatants of zymosan stimulated granulocytes LF was present and CatG activity was higher than in the supernatant of PMA stimulated granulocytes, where LF was absent. Remarkably, CatG became more promiscuous when treated with LF, as it became reactive against ABPs with leucine and valine in the P1 position in the presence, but not in the absence of LF (Eipper et al. 2016).

Probing CatG in Peripheral Blood Mononuclear Cells

The NSP CatG is not only present in neutrophils, but also plays a role in antigen processing (see also section "Serine Proteases in Immune Cells" and Fig. 2c). The Burster group, therefore, sought to apply the concept of ABPP to peripheral blood mononuclear cells (PBMCs) using the biotinylated MARS116 ABP (Fig. 5b). MARS116 was able to detect CatG activity in low amounts of PBMC lysate. Next, the MARS116 ABP was applied to compare CatG activity in PBMCs with or without infection by Epstein-Barr virus, the main causative agent of infectious mononucleosis ("kissing disease"). This virus is particularly persistent as it evades the immune system by several mechanisms including influencing antigen presentation (Ressing et al. 2015). However, Epstein–Barr virus infection of PBMCs did not result in different CatG activity, as monitored with the MARS116 ABP, revealing that this is not one of the underlying mechanisms of immune evasion (Zou et al. 2012).

Another strategy of immune evasion used by infected cells and tumor cells is downregulation of MHC-I molecules on the cell surface, leading to a decrease of the CTL response. A complete lack of MHC-I molecules on the surface, however, is not favorable for the infected cells since this would trigger NK cell response. The Burster group showed that soluble as well as membrane-tethered CatG is able to cleave MHC-I (Palesch et al. 2016). Therefore, they applied MARS116 to investigate the role of CatG in MHC-I degradation in glioblastoma and found no CatG activity in the human glioblastoma cell lines A172 and U87 as well as in cells derived from glioblastoma patients. Transfection of the glioblastoma cell line U87 with CatG led to the expression of active CatG, as verified with the MARS116 ABP, and to a reduction of cell surface MHC-I as shown by flow

cytometry. Hence, the authors speculate that the absence of CatG in glioblastoma may serve the purpose of preventing a complete reduction of cell surface MHC-I to avoid clearance by NK cells (Palesch et al. 2016).

MARS116 has also been applied in flow cytometry analysis to measure active cell surface CatG. To this end, PBMCs were incubated with MARS116 along with different NK cell markers. Fluorescein isothiocyanate-conjugated avidin was then used to detect bound MARS116 probe at the cell surface. Flow cytometry analysis revealed that CatG was only present in specific subtypes of NK cells (using anti-CatG antibody), but not in all of these subtypes CatG was found to be active (as demonstrated by reactivity towards MARS116). Overall, this indicates that CatG protein levels do not accurately reflect activity and highlights the need for ABPP flow cytometry analysis (Penczek et al. 2016). To be able to further assess CatG activity within the cell, MARS116 was conjugated to fluorescein instead of biotin. This probe was then used in flow cytometry experiments of permeabilized PBMCs for the detection of CatG in specific subsets of T cells by costaining with different cell surface markers. Whereas the biotinylated MARS116 showed non-specific fluorescence, presumably originating from background staining of endogenously biotinylated proteins by the used fluorescein-conjugated avidin, use of the new probe omits these background signals and is better suited for flow cytometry applications (Schroeder et al. 2020).

Probing Granzymes in NK-cell Mediated Cell Death

Granzymes along with perforin are released by NK cells to kill infected target cells (see Fig. 2b). Mahrus and Craik (2005) set out to develop probes for GrzA and GrzB to investigate the role of these granzymes in the NK cell-mediated apoptosis of target cells. They determined the substrate specificity of each of the five humans granzymes using PS-SCLs and eventually obtained partially cell-permeable biotinylated probes (**23** and **24**; Fig. 5c) with selectivity for GrzA and GrzB. Using the probes as inhibitors they determined that GrzA is a minor and GrzB a major effector of target cell lysis. This is further underlined by the high and low levels of GrzB activity which correlate to high and low levels of lytic potential in NK-92 and NKL cell lines, respectively (Mahrus and Craik 2005).

Summary and Future Directions

Since the advent of ABPP, a multitude of ABPs applicable in immune cells has been synthesized. A range of different warheads is available to tailor the probe structure towards a specific target protease, enabling applications such as flow cytometry or fluorescence microscopy. Although non-peptidic probes can be used to selectively target SPs, optimization of probe selectivity is easier for peptidyl phosphonates, because they bind in a substrate-like manner. Here, it is now possible to gain considerable selectivity towards a desired protease by first performing substrate specificity profiling with natural and non-natural amino acids and subsequently converting these substrates into diphenyl phosphonate probes. These efforts have led to ABPs with high selectivity towards CatG, PR3, NE, NSP4, GrzA and GrzB. However, for other SPs in immune cells, such as the other granzymes, chymase and tryptase, highly selective ABPs still need to be developed. Recent advances in the use of non-natural amino acids may lead to the future design of recognition elements to gain selectivity for these SPs.

Although various lines of biochemical evidence exist for the selectivity of above-mentioned ABPs, mass spectrometry-based proteomics experiments that confirm the identity of the probe targets and the selectivity profile of the probes, have not yet been performed. We expect that these experiments will be included more frequently in future studies, because of the various proteomics protocols available for ABP studies.

Despite the development of many probes for SPs, their application to immune cell biology has been limited so far. Most studies until now have made use of gel-based detection, whereas only a selected number of reports used flow cytometry detection, which is an otherwise widely used technique within immunology research. Flow cytometry experiments using MARS116, for instance, helped to reveal that only particular subsets of NK cells display active cell surface CatG albeit being present in other subsets as well. This underlines the power of ABPP flow cytometry: it allows detection of the activity state, which is an advantage over antibody-staining methods. qABPs could be of particular interest here, as they would reduce background signal and allow staining of active intracellular SPs as well. Naturally, processes such as degranulation cannot be studied in this manner: the cells themselves would not be marked, because labeled proteases would diffuse away from the cell. This limitation may be overcome by fluorescence microscopy. To date, very few studies have used fluorescence microscopy on immune cells. As ABPs with fluorophores of different colors have only recently become available for SPs, we expect that these will be utilized more extensively in the future. The power of ABP-based microscopy is, for example, illustrated by their application to the NSPs, where it was key in revealing that there is little overlap in the occurrence of NSPs in azurophilic granules, suggesting a tightly regulated granule packing system.

Future applications of fluorescent ABPs may lie in the elucidation of the mechanism of formation and effect of extracellular traps (ETs) and their protease content. NETs are a commonly known type of ETs but also other PMLs

(eosinophils, basophils and mast cells) as well as macrophages are known to form ETs (Doster et al. 2018). The role of proteases in pathogen clearance and toxicity of these ETs, however, remains to be elucidated.

Although no real-time imaging experiments on immune cells have been performed using ABPs, the use of mixed phosphonate warheads will allow the future design of qABPs which enable this type of experiments and would form a valuable future tool.

We expect that the application of ABPP in isolated immune cells will be followed by application in vivo. In these type of experiments, new challenges will include serum stability, half-life and probe toxicity. Once overcome, ABPs would be applicable to localize activated immune cells in inflammatory processes, such as neutrophils in chronic inflammation or mast cells in allergic reactions. Importantly, not only would detection of the inflammatory sites be possible, but also monitoring of the efficacy of treatment. These strategies may eventually be translated into clinical settings.

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