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REVIEW

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Immunomodulatory Yersinia outer proteins (Yops)–useful tools for bacteria and humans alike

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ABSTRACT

Human-pathogenic *Yersinia* produce plasmid-encoded Yersinia outer proteins (Yops), which are necessary to down-regulate anti-bacterial responses that constrict bacterial survival in the host. These Yops are effectively translocated directly from the bacterial into the target cell cytosol by the type III secretion system (T3SS). Cell-penetrating peptides (CPPs) in contrast are characterized by their ability to autonomously cross cell membranes and to transport cargo – independent of additional translocation systems. The recent discovery of bacterial cell-penetrating effector proteins (CPEs) – with the prototype being the T3SS effector protein YopM – established a new class of autonomously translocating immunomodulatory proteins. CPEs represent a vast source of potential self-delivering, anti-inflammatory therapeutics. In this review, we give an update on the characteristic features of the plasmid-encoded Yops and, based on recent findings, propose the further development of these proteins for potential therapeutic applications as natural or artificial cell-penetrating forms of Yops might be of value as bacteria-derived biologics.

Introduction

In the course of an infection pathogenic bacteria produce a plethora of virulence factors to modulate and undermine the host's countermeasures for their own survival and proliferation. Particularly, secretion systems such as the intriguing type 3 secretion system (T3SS) have been recognized as fascinating nanomachines which inject fine-tuned effector proteins in a one-step or two-step process into the cytosol of targeted host cells.[1] These effector proteins affect nearly all areas of cellular life by modulating, inhibiting or even exploiting essential cellular mechanisms for the benefit of the microbe and in doing so might act in concert.[2-4] Furthermore, bacterial effector proteins might target more than one cellular signaling mechanism so that the currently known targets might not be comprehensive. A mechanistic understanding of these interactions will provide important insights into the strategies of bacterial pathogens for survival in and colonization of the host. Due to the importance of secreted and injected effector proteins during bacterial infection and the spreading resistance against current antibiotics, many studies focused on effector proteins as putative targets for combating bacterial infections. However, exploiting these effector proteins for the development of novel biologics might also offer innovative possibilities for therapeutic interventions in sterile inflammation (reviewed in, refs. 5, 6).

In essence, many secreted bacterial virulence factors are highly adapted regulators of the human immune system. The so-called "Drugs from Bugs" concept aims at taking advantage of these immunomodulatory effects and combines it with the technology of cell-penetrating peptides or proteins (CPPs), which are able to autonomously cross biological membranes. As a result, one would have a self-delivering therapeutic at one's disposal, e.g. to treat diseases dominated by a dysregulated immune system or other failing cellular functions. Surprisingly, over the past years, quite a few bacterial effector proteins were found to harbor already an intrinsic cell-penetrating ability. Consequently, these factors were designated cell-penetrating effectors or CPEs.[5] These include the *Escherichia coli* effectors Tir,[7] TcpC,[89] and NleC,[10] SspH1 of *Salmonella*,[11] IpaH 9.8 of *Shigella* (Norkowski *et al.*, personal communication),[12] and YopM of *Yersinia*.[13,14] The latter three effectors share high sequence homology in their N-terminal regions, which are dominated by an α-helical structure, followed

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by leucine rich repeats.[11,13,15] Besides, a growing number of biologic or synthetic cell-penetrating peptides is available, which can be fused either genetically or chemically to heterologous cargo-proteins for delivery into target cells and also specific target cell compartments of interest (for a review see refs. 16, 17). How these CPEs – or even CPP in general – actually cross eukaryotic membranes is an area of intense research as it appears that translocation mechanisms may also vary according to the particular CPE or the CPP-cargo pair. For CPEs such as YopM endocytic uptake followed by endosomal escape has been shown to be a major uptake route.[5,6,13,14] Employing these technologies we will be able to develop bacterial immunomodulators for therapeutic purposes.

The type III secretion system-associated, plasmid-encoded effectors of the human-pathogenic *Yersinia* species were among the first effector proteins to be molecularly characterized (for reviews see refs. 18-25). Plasmid-encoded *Yersinia* outer proteins (Yops) encompass the following six proteins: YopE, YopM, YopO, YopT, YopP, and YopH. In addition, recent findings indicate that YopQ/K (YopQ in *Y. enterocolitica* and YopK in *Y. pseudotuberculosis* and *Y. pestis*) – a translocated regulator of type-3-secretion – might also have an impact an host cell proteins like Rac1.[26,27] Since there is only very little data available on this Yop, it will, however, not be discussed in any detail in this review. Ongoing research efforts generate novel insights and continuously enhance our understanding of intracellular targets and the mechanisms of action for most Yops (summarized in Fig. 1). Here, we want to give an update of the currently known status of the molecular functions of the individual Yops, their presently known targets, and discuss potential fields of therapeutic applications for recombinant, autonomously cell-penetrating *Yersinia* effector proteins. As the multifunctional 'low-calcium response protein LcrV' shows links to Yop effectors it has also been included in this review.

YopM – A cell-penetrating scaffold protein

Structure and function

The *Yersinia* outer protein M (YopM) is the only known Yop where a catalytic activity appeared to be lacking. In general, YopM belongs to the LPX family of bacterial T3SS-secreted effectors, which is a subgroup of the leucine-rich repeat (LRR) superfamily and includes the *Yersinia* protein YopM, different IpaH (invasion plasmid antigen H) proteins of *Shigella* spp. as well as the *Salmonella* effectors SspH1, SspH2, and SlrP (*Salmonella* leucine-rich repeat protein).[28] These proteins consist of a N-terminal α-helical domain, which is followed by a varying number of LRRs and except for the YopM protein, they all possess a C-terminal NEL domain.[28] However, just recently it has been suggested that the N-terminus of YopM harbors a novel E3 ubiquitin ligase that induces necrotic cell death by targeting NLRP3.[29] This is quite surprising as an E3 ubiquitin ligase domain has not been found previously in YopM and thus this finding needs to be confirmed.

The horseshoe shaped 13–21 leucine rich repeats (LRRs) of YopM act as a scaffold to bind host cell proteins and thus interfere with interactions in which these proteins are involved.[30] *In vitro* tetrameric complexes of YopM were observed, which form a hollow cylinder of yet unknown function.[30] As the number and exact composition of the LRRs varies between *Yersinia* species and strains,[31] YopM proteins vary in size between 41–55 kDa which might also be a reason for the partially contradictory results reported in different studies. Two distinct nuclear localization signals are contained within the highly conserved LRRs 1–3 as well as the C-terminus.[31] The N-terminus consists of two anti-parallel α-helices, which contain not only the signal for secretion and translocation via the T3SS,[32] but harbor also two distinct synergistically active transduction domains which allow for the autonomous translocation of (recombinant) YopM into a huge variety of eukaryotic host cells *in vitro* and *in vivo*.[13,14,33,34] Whether an autonomous cell-penetration of effector proteins might have a direct effect during bacterial infections has remained elusive.
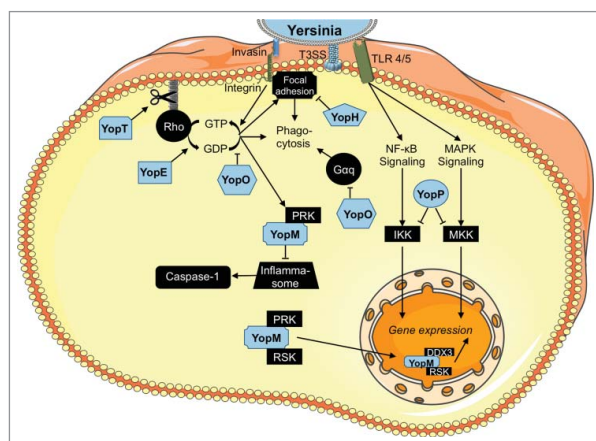

Figure 1. Intracellular functions of Yersinia outer proteins (Yops). YopE, YopT, and YopO inactivate RhoGTPases, thus mainly inhibiting phagocytosis, together with YopH, which dephosphorylates components of focal adhesion complexes. YopP (YopJ in *Y. pestis* and *Y. pseudotuberculosis*) blocks NF-κB- and MAPK-signaling probably by acetylation of important signal transducers. YopM associates with PRK and RSK and regulates pro- and anti-inflammatory gene transcription via a yet unknown mechanism. The figure was generated using Servier Medical Art.

Among the few established interaction partners of YopM, mature α-thrombin was the first to be discovered.[35] When complexed with YopM, α-thrombin is no longer able to induce platelet aggregation.[35] However, this interaction did not contribute to the overall virulence in a mouse infection model of *Y. pestis*.[36] The serine protease inhibitor α_1-antitrypsin is also bound by YopM, yet without altering its inhibitory effect.[37] Nevertheless, it is remarkable that YopM, which is normally directly translocated into the host cell cytosol by the T3SS, specifically interacts with extracellular host proteins indicating a possible extracellular role of this protein. This is supported by the identification of YopM in culture supernatants of HeLa cells infected with *Y. pestis*.[38]

In the cytoplasm, YopM forms a hetero-trimeric complex with the two serine/threonine kinases ribosomal S6 protein kinase (RSK) and protein kinase C-related kinase (PRK, also called PKN), which are subsequently activated.[33,39,40] However, formation of the RSK1-YopM-PRK2 trimer is dispensable for the anti-inflammatory effect of (recombinant) YopM and, furthermore, does not even influence physiological functions of the PRK/PKN and RSK kinases such as cell proliferation and migration.[33] This is also mirrored in the unaltered phosphorylation of the downstream targets BAD, Jun, CREB or Akt.[39,40] On the other hand, interaction with PRK2 seems to be important to dampen pyrin inflammasome activation triggered by YopE- and YopT-induced RhoA inactivation (Fig. 1).[41] Consequently, YopM appears to be less important for colonization by YopE/T-negative mutants.[41] After trafficking from the cytosol to the nucleus of the cells, T3SS-delivered YopM was recently shown to form yet another heterotrimeric complex, namely with RSK1 and the DEAD Box Helicase DDX3.[42] DDX3 mediates nuclear export of YopM, thus controlling YopM activity (Fig. 1).

Furthermore, inhibition of caspase-1 containing inflammasomes by YopM, either through direct binding to caspase-1 or indirectly through blocking the potential caspase-1 activator IQ motif-containing GTPase-activating protein 1 (IQGAP1) has been proposed.[43,44]

Although the underlying mechanism for the observed effects of YopM has remained elusive, YopM has been clearly shown to be an important virulence factor with anti-inflammatory activities. Depending on the *Yersinia* strain, the route of infection, and the examined animal model, YopM-dependent colonization of spleen, liver and lungs,[45,46] depletion of natural killer cells,[47] reduction of pro-inflammatory cytokine secretion (including interleukins 1β, 12, 15, and 18, interferon-γ and tumor necrosis factor-α),[13,47] induction of caspase 3-mediated apoptosis,[48] or inhibition of apoptosis and migration were observed.[49] Additionally, YopM was also suggested to induce elevated levels of the anti-inflammatory cytokine interleukin-10.[46]

Potential therapeutic uses

As YopM is one of the first identified CPEs, possible therapeutic applications for recombinant YopM are currently under investigation. The most promising therapeutic application to date is a topical administration of YopM for the treatment of the auto-inflammatory skin disease psoriasis, with already two granted patents (Fig. 1).[50,51] Like many other inflammatory disorders, psoriasis is characterized by elevated levels of TNF-α, which in turn drives the production of many other cytokines.[52] Consequently, today's mostly used therapeutic strategy relies on the systemic application of anti-TNF-α antibodies.[53] Clear advantages of a cell-penetrating YopM-based topical treatment would be the lack of systemic distribution of the drug (allowing lower dosage and most probably causing less detrimental side effects), a more convenient administration route (creaming instead of injection), a shift toward an earlier and non-stoichiometric intervention (inhibition of the expression of TNF-α, not of the cytokine itself), and a broader target spectrum (inhibition of TNF-α-independent pro-inflammatory cytokine production, with simultaneous induction of the anti-inflammatory cytokine IL-10). However, although these exciting results are very promising, as details of the molecular mechanism are still under investigation, recombinant YopM as a novel biologic has not reached human patients or the clinics yet.

YopE – A GTPase activating protein

Structure and function

Being the first Yop effector protein to be translocated by the T3SS,[54] the 23 kDa YopE plays a major role in the initial bacterial defense against phagocytes. It does so by its GAP (GTPase activating protein) activity targeting the small Rho-GTPases Rac1, RhoG and partially also RhoA, thereby disrupting actin cytoskeleton dynamics (Fig. 1), which is manifested by rounding up of affected cells and their inability to form phagocytic cups.[55-58] Furthermore, YopE is able to activate the GTPase domain of Cdc42 *in vitro*.[59]

Amino acid sequence similarities of YopE to eukaryotic GAPs can only be found in an arginine finger motif, typical for this class of enzymes. However, YopE shares a striking similarity to its eukaryotic orthologues in structure.[60] The first 15 amino acids of YopE contain a secretion and translocation signal, which is necessary and sufficient for translocation into host cells via the T3SS

when YopE is bound to its specific chaperone SycE via aa 15–50.[61] Amino acid residues 50–77 contain an inhibitory sequence for translocation (reversed by SycE binding)[61] which - in the host cell - functions as a membrane localization signal (MLS).[62] Depending on the *Yersinia* serogroup, this MLS harbors two lysine residues which can be ubiquitinated by the host cell, marking YopE for proteasomal degradation.[63,64] This represents an interesting mechanism for fine-tuning not only YopE activity but also the entire *Yersinia* virulence, since YopE also acts as a negative regulator for Yop translocation during infection via a yet unknown mechanism.[65,66] Interestingly, some *Yersinia* strains even secrete a chromosomally-encoded, T3SS-independent A-B toxin, the 'cytotoxic necrotizing factor of *Yersinia*' (CNF-Y), which counteracts YopE-mediated inactivation of RhoA and Rac1 and therefore promotes Yop translocation.[67,68]

By inhibiting the important Rac1 pathway, YopE not only attenuates phagocytosis and Yop translocation, but also contributes to the general immunomodulatory activities of the *Yersinia* outer proteins. In epithelial cells, the response to translocon integration is mainly initiated through RhoA signaling.[69] Following integrin-mediated signaling, Rac1 can activate the MAPKs p38 and JNK, leading to IL-8 production,[70,71] which was found to be inhibited by YopE.[72] Furthermore, Rac1 can trigger caspase-1-dependent IL-1β maturation, which is also inhibited by YopE.[73] In addition, several Rho-GTPases are involved in the production of reactive oxygen species (ROS)[74,75] and by inhibiting these enzymes, YopE was found to reduce ROS levels in macrophages, which appears to be a pre-requisite for splenic colonization by *Yersinia*.[76] Polymorphonuclear neutrophils (PMNs), another important cell type for defense against *Yersinia* infections,[77] are also inactivated by YopE *in vivo*.[78] On the other hand, sustained inactivation of Rho-GTPase function by YopE might be sensed as a danger signal by macrophages, leading to increased killing of intracellular bacteria.[79] Further studies showed that chemical or genetic inactivation of RhoA and Rac1 even leads to higher levels of secreted pro-inflammatory cytokines after additional TLR stimulation,[80,81] again indicating that a prolonged action of YopE might not be favorable.

Potential therapeutic uses

Based on the molecular mechanism described above, potential areas of medical application for a recombinant, cell-penetrating YopE protein are inflammatory bowel diseases (IBD, Fig. 2). This conglomerate of several auto-inflammatory disorders of the gastrointestinal tract is among others characterized by massive infiltration and pro-inflammatory activity of neutrophils.[82,83] By hitting

Rho-GTPases, YopE not only attenuates the production of IL-8 (CXCL8), an important chemoattractant and activator for neutrophils,[84] but should also severely affect neutrophil migration, which is largely dependent on Rho-mediated signaling.[85] An increasing number of research projects is directed toward an inhibition of neutrophil migration and/or activity as well as modulation of Rho-GTPases for the treatment of IBD or auto-inflammatory disorders in general.[85-89]

In line with this, YopE – together with YopT – has been proposed as an inhibitor of caspase-1 activation, since chemical inhibitors of Rho-GTPases also had a negative effect on caspase-1. Indeed, transfected YopE or YopT was able to block IL-1β secretion by HEK293T cells through inhibition of the Rac1-LIMK-1-pathway, leading to impaired activation of caspase-1.[90] This finding led to a patent for the treatment of caspase-1 related diseases, such as inflammatory bowel disease, Crohn's disease, or rheumatoid arthritis, with YopE and/or YopT.[90]

YopT – An irreversible inhibitor of Rho-GTPases

Structure and function

In host cells, YopT, a 36 kDa cysteine protease, induces a very similar phenotype as YopE.[91] Accordingly, YopE and YopT share the same intracellular targets, however, they have different affinities and follow different mechanisms. *In vitro*, YopT cleaves the small Rho-GTPases RhoA, RhoG, Rac1 and Cdc42 directly in front of the C-terminal CAAX motif (C = cysteine; A = aliphatic aa; X = any aa), which carries the isoprenyl membrane anchor (Fig. 1).[92] In contrast to YopE, YopT therefore leads to an irreversible inhibition of Rho-GTPases by inducing their redistribution from the membrane to the cytosol where they do no longer come into contact with their likewise membrane-associated interaction partners.[93] The cleavage occurs regardless of the activation state of the Rho-GTPases,[93] but is dependent on preceding endoproteolytic processing of the CAAX motif by Rce1, which removes the AAX residues.[94] *In vivo*, the preferred target of YopT is RhoA.[95]

YopT is the prototype of a new protein fold subfamily of proteases, which among others encompasses toxin B from *E. coli* or the avirulent protease AvrPphB of the plant pathogen *Pseudomonas syringae*.[96] This classification was based on predictions and homology analysis, although the actual 3D-structure of YopT has not been resolved yet. However, the crystal structure of the subfamily member AvrPphB revealed a papain-like core.[97] Consequently, given the relatedness of YopT to
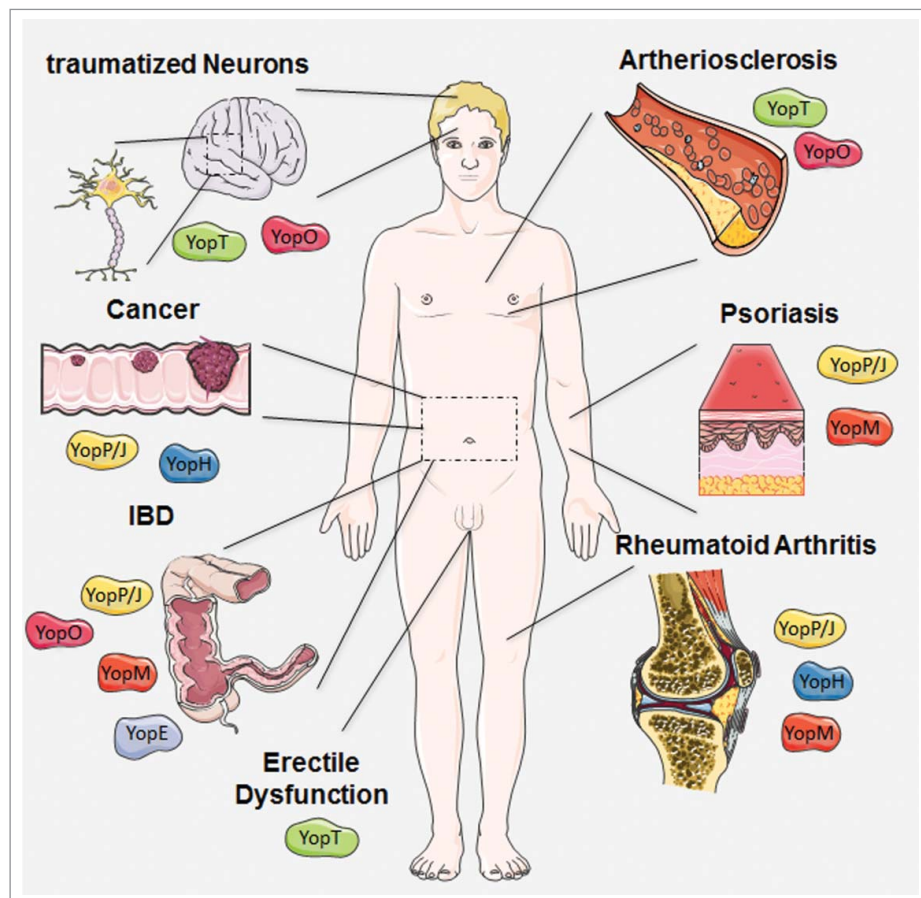
Figure 2. Overview of potential therapeutic uses of Yops. The most promising therapeutic application of YopM is the treatment of the auto-inflammatory diseases such as psoriasis, rheumatoid arthritis (RA), and inflammatory bowel diseases (IBD). Based on the molecular mechanism described before, potential areas of medical application for a recombinant, cell-penetrating YopE protein are IBD. YopT and especially its downstream target Rho-associated protein kinase ROCK are involved in several disease patterns, often within the cardio-vascular field e.g., arteriosclerosis but also erectile dysfunction and traumatized neurons might be a target for a cell-penetrating YopT. A cell-penetrating effector YopO might be beneficial for the treatment of diseases associated with hyperactivated Rho-GTPases similar to YopE and YopT, but also for targeting mediators of auto-immune diseases like inflammatory bowel diseases. YopJ and its impact on signaling cascade displays potential therapeutic potential for inflammatory disorders, such as Psoriasis, RA, and IBD, but also for cancer control. RA also appears to be a promising area of application for recombinant YopH. Moreover, cancer progression also relies on signaling pathways tackled by the effector protein. The figure was produced using Servier Medical Art.

AvrPphB, the *in vitro* activity of YopT can be blocked by E64, an inhibitor of papain-like proteases.[96]

Although YopT inactivates roughly the same pathways and leads to a comparable (yet milder) phenotype as YopE, it cannot fully replace YopE's anti-phagocytic and anti-inflammatory effects. Furthermore, when YopE is present, YopT is even dispensable for colonization *in vivo*.[72] In line with these results, some strains of *Y. pseudotuberculosis* do not even encode a functional copy of YopT.[91]

Potential therapeutic uses

Hyperactivated RhoA – in macrophages and endothelial cells apparently the main target of YopT —[95] and especially its downstream target Rho-associated protein kinase ROCK are involved in several disease patterns, often within the cardiovascular field. A considerable number of studies already investigated small molecule or endogenous inhibitors of RhoA or ROCK for treatment of e.g., arteriosclerosis (Fig. 2). An important signaling axis in this respect is the negative regulation of eNOS (endothelial nitric oxide synthase) synthesis involving RhoA and ROCK. Sildenafil, a standard therapeutic for erectile dysfunction in the context of diabetes mellitus, acts by stabilizing the NO induced second messenger cGMP, resulting in vasodilatation and prolonged penile erection.[98] The same effect was observed in rats treated with a ROCK inhibitor.[99] In contrast to other indications, where a (unfavorable) systemic treatment with a cell-penetrating variant of YopT would be necessary, treatment of erectile dysfunction holds the possibility of

a topical treatment with potentially less systemic side effects than even the established oral therapeutics.

Furthermore, local ROCK inhibition was able to decrease inflammation in inflamed synovial tissues in rheumatoid arthritis.[100] Finally, Tan *et al.* and Lord-Fontaine *et al.* developed a cell-penetrating RhoA inhibitor based on the C3 toxin from *Clostridium botulinum* and demonstrated *in vitro* and *in vivo* its potential use for regeneration of traumatized neurons, which is strongly retarded by active RhoA (Fig. 2).[101,102] Also patients suffering from Alzheimer's disease might eventually profit from a downregulation of the RhoA/ROCK pathway.[103]

A cell-penetrating variant of YopT might thus be a valuable tool for the treatment of pathologies caused by RhoA hyperactivation. Topical treatment of erectile dysfunction, local treatment of traumatized neurons or inflamed synovial tissues would present interesting options for therapeutic applications here. Still, one has to consider that a more specific inhibition of the ROCK pathway would be superior in most cases.

YopO – A multidomain effector protein

Structure and function

The anti-phagocytic effector YopO (also called *Yersinia* protein kinase A, YpkA, in *Y. pseudotuberculosis* and *Y. pestis*) was the first bacterial kinase to be described as a secreted virulence factor.[104] Moreover, it shares striking sequence homologies with catalytic motifs of known eukaryotic Ser/Thr kinases, such as protein kinases A and C (PKA and PKC) or casein kinase 2 (CK2).[104]

YopO is secreted via the T3SS in an inactive state and is redirected by its N-terminal sequence to the host cell plasma membrane, where binding to an actin monomer leads to auto-phosphorylation and activation of the kinase domain.[105-107] To date, the α subunit of a heterotrimeric G protein, Gαq, which controls activation of phospholipase C,[108] as well as several regulators of actin polymerization including the vasodilator-stimulated phosphoprotein (VASP),[109] the Wiskott-Aldrich Syndrome protein (WASP), the Ena/VASP-like protein (EVL), gelsolin, and the formin diaphanous 1 were identified as direct targets of YopO (Fig. 1).[107] By intervening with the regulation of actin polymerization, YopO activity leads to disappearance of stress fibers and rounding of the cells.[110] Disruption of the actin cytoskeleton drastically impairs the phagocytosis of bacteria by macrophages – probably the most important function of YopO during infection.[106]

However, using transfected Henle407 cells as well as yeast cells, it was shown that loss of kinase activity in YopO only attenuates—but not fully abolishes—its ability to disrupt actin polymerization.[110,111] This is due to a second functional domain in YopO that resembles eukaryotic GDP dissociation inhibitor (GDI) domains, which associate with GDP-bound small Rho-GTPases, thereby keeping them in an inactive state.[112] Indeed, YopO was found to interact directly with RhoA and with slightly higher affinity with Rac1 and Rac2, additional regulators of actin dynamics, and this interaction greatly contributes to the actin destabilizing effect of YopO.[112]

Thus, YopO comprises three domains (membrane localization, Ser/Thr kinase, and GDI domain) that act synergistically in order to prevent phagocytosis of the invading bacteria by host cells. The importance of this mechanism was shown in animal experiments, where mutants of *Yersinia pseudotuberculosis* expressing truncated versions of YopO were almost completely attenuated in their virulence.[104]

Taken together, pathogenic *Yersinia* manipulate Rho-GTPase signaling via four different mechanisms: acceleration of GTP conversion (YopE), inhibition of GDP dissociation (YopO), release of Rho-GTPases from the membrane (YopT) and deamidation of a catalytic glutamine residue (CNF-Y). This illustrates the importance of RhoGTPases in the defense against invading *Yersinia*, which includes organization of phagocytosis,[113] activation of MAPK-dependent IL-8 production,[70,71] caspase-1 dependent IL-1β maturation,[73] and the production of reactive oxygen species (ROS) among others.[74,75]

Potential therapeutic uses

YopO targets both Rho-GTPases as well as Gαq. Examples of diseases associated with hyperactivated Rho-GTPases have been mentioned in the YopE and YopT sections above (Fig. 2).[114] Gαq activity in turn has lately been linked to reduced differentiation of Th17 cells and disease progression.[115] Thus, YopO might have beneficial effects regarding neutrophils in this disease background, but adverse effects regarding Th17 cells. Gαq is also known to play a role in hypertension and the formation of thrombi, and inhibitors of Gαq are already under investigation for these indications.[116,117] Being of bacterial origin, a recombinant, cell-penetrating YopO would, however, have to face once more the challenge of systemic application without inducing an acute immune reaction.

YopP – A highly potent anti-inflammatory effector protein

Structure and function

YopJ/P (termed YopJ in *Y. pestis* and *Y. pseudotuberculosis* and YopP in *Y. enterocolitica*) is probably the most effective Yop in terms of suppressing pro-inflammatory signaling pathways in host cells. At the same time, many different isoforms of YopJ/P have been described, which differ in their translocation and/or substrate binding efficiencies and therefore in their contributions to virulence.[118-121] In a mouse infection model, *Y. pestis* EV76 strains which expressed either YopJ or YopP, exhibited remarkable differences in virulence.[122] Hence, general statements concerning the actions of YopJ/P have to be taken with caution. Known targets and effects are listed in Table 1.

Especially in the domains harboring the catalytic center, YopJ/P shares structural (but not sequence) homology to Clan CE cysteine proteases, which encompass many de-ubiquitinating and de-sumoylating enzymes.[123] Indeed, recombinant YopJ was shown to be able to cleave ubiquitin chains (but not SUMO modifications) from an artificial substrate *in vitro*, and cells expressing YopJ contained lower levels of ubiquitinated TRAF2, TRAF6, the inhibitor of κB α (IκBα) as well as the stimulator of interferon genes (STING).[124,125] This observation was

confirmed in two additional studies for TRAF6 (plus adding TRAF3) in transfected as well as *Y. enterocolitica*-infected cells.[123,126] TRAF2 (downstream of the tumor necrosis factor-α-receptor (TNF-R)), TRAF6 (downstream of the toll like-receptor 4 (TLR-4), T- and B-cell receptor (TCR/BCR) as well as interleukin-1 receptor (IL1-R)), and STING (downstream of receptors for cytosolic DNA) get fully activated by K63-linked ubiquitination.[127-129] IκBα, in turn, upon activation is marked for proteasomal degradation by K48-linked ubiquitination, thus unmasking NF-κB, which then consequently can translocate into the nucleus to induce transcription of pro-inflammatory genes (Fig. 1).[130] The outcome of TRAF3 ubiquitination is highly dependent on the circumstances and may be pro- as well as anti-inflammatory.[131]

However, direct de-ubiquitination of cellular proteins by YopJ/P has not been observed. In fact, there is increasing evidence of an intrinsic acetyltransferase activity of YopJ, which is specific for serine, threonine and lysine residues (Table 1). Experiments performed *in vitro* and *in cellulo* demonstrated acetylation of the activation loop of MAPK kinases 2 and 6 (MEKs 2/6).[132,133] In co-transfection experiments, MEKs 4 and 7, transforming growth factor β-activated kinase 1 (TAK1; a MAPK kinase kinase), RICK (a caspase recruitment-domain containing kinase), and IκB kinase (IKK) were also found to be acetylated on critical residues in the

Table 1. Known functions and molecular targets of YopJ/P sorted by host cell types and stimuli. Unless stated otherwise, all listed targets are negatively regulated by YopJ/P. Targets for which direct interaction with YopJ/P was not shown in the respective references, are marked with (?).

Activity	Cell type & stimulus	Direct target	Indirect target	Reference
De-ubiquitinase	Transfected cells (YopJ) / Infected cells (YopP)		TRAF2, TRAF3, TRAF6, IκBα, STING, IL-8 transcription	123,124,125,126
Acetyl-transferase	Transfected & stimulated HeLa cells (YopJ)	MEK 2		132
	Transfected & stimulated HEK cells (YopJ)	IKKα, IKKβ MEK6, IKKβ, not IKKα		132, 133
	Transfected HEK cells (YopJ)	MEK4, MEK7, TAK1, RICK		134,135
	Transfected HEK cells and infected HeLa cells (YopP)	Tab1	TAK1, IL-8 transcription	136
Not determined	Infected HUVECs (YopP)		IL-6 and IL-8 secretion, ICAM1 expression	144
	Infected murine macrophages (YopJ/P)	MEK2, p38(?), JNK(?)	TNF-α secretion	119,145,155
	Infected murine Dendritic cells (YopP)		Antigen uptake, CXCL1, TNF-α, IL-10, IL-12, ICAM1, MHCII, CD80, CD83 expression	146, 151
	Infected + IL-12 & IL-18 stim. murine natural killer cells (YopP)	p38(?), JNK(?),Tyk2(?), STAT4(?)	IFN-γ expression	153
	Infected murine + human macrophages (YopJ/P)		Induction of apoptosis … by RIP1/3 kinase dependent activation of caspase 8	119,147,148,149,150,152,154,155 156,157
	Infected murine dendritic cells (YopP only)		Induction of apoptosis,	119,151

presence of catalytically active YopJ.[132,134,135] Inhibition of TAK1 was likewise demonstrated for YopP, although it was not able to bind TAK1 *in vitro* but rather bound to TAB1, a regulatory protein in active TAK1 complexes.[136] Since YopJ in addition directly binds MEKs 1/3/5,[137] which also contain Ser and Thr residues in their activation loops, these kinases might also be acetylated by YopJ/P. Binding of inositol hexakisphosphate (IP$_6$) to YopJ induces conformational changes resulting in the formation of an acetyl-CoA binding pocket, which greatly boosts acetyltransferase activity *in vitro* as well as inside the host.[138-140]

In summary, it is intriguing to see that YopJ/P apparently is able to block activation of central pro-inflammatory pathways at three consecutive levels: (1) TRAF2 and TRAF6, which are among the first proteins to be activated by their respective receptors; (2) TAK1, a MAPKKK which is activated (directly and indirectly) by TRAF2 and TRAF6; (3) the downstream MAPKKs 4, 6, 7, as well as IKK/IκBα, which in turn control the activation of the MAPKs p38 and c-jun amino-terminal kinase (JNK), or NF-κB respectively – all of which can finally elicit a strong inflammatory response (Fig. 2; pathways reviewed inref. 141). Inhibition of MAPKKs by YopJ was also proposed in studies using transfected yeast cells.[142] On the other hand, not all of these levels might be targeted by all YopJ isoforms and not in all cell types.[143]

In line with these findings on intracellular signaling, key pro-inflammatory cytokines are downregulated by YopJ/P. This includes inhibition of IL-8 transcription in human embryonic kidney (HEK) cells by YopP after MAPKKK overexpression or IL-1 stimulation and by YopJ after TLR-2 or TLR-4 activation,[123,136] inhibition of IL-6 and IL-8 secretion by human umbilical vein endothelial cells (HUVECs) after YopP translocation,[144] as well as reduction of TNF-α secretion by murine macrophages caused by YopP.[145] Furthermore, antigen uptake by dendritic cells and expression of intercellular adhesion molecule 1 (ICAM1) in endothelial cells might also be negatively regulated by YopP.[144,146]

Apart from these strong anti-inflammatory effects of YopJ/P, probably the most captivating feature of YopJ/P is the induction of cell death in macrophages and dendritic cells (YopP only), but not in epithelial or natural killer cells.[119,147-153] Inhibition of the TLR-4, MAPK and NF-κB pathways is necessary for this effect,[154,155] which involves receptor-interacting protein 1 and 3 (RIP1/3) kinase-dependent activation of caspase-8, thus most probably triggering the extrinsic apoptosis pathway.[156,157]

Paradoxically, activation of caspase-8 by YopJ/P also promotes activation of caspase-1, which is responsible for the maturation of the pro-inflammatory cytokines IL-1β and IL-18.[157] Nevertheless, a medium level of macrophage cytotoxicity was proven to be necessary for full *Yersinia* virulence in mouse models, whereas strongly pro-apoptotic YopJ/P isoforms or hypersecretion of YopJ/P impair virulence, just as YopJ/P null mutants do.[119,122] If it turns out that caspase-1 is indeed partially inactivated by (some isoforms of) YopM, this would be another interesting example for the interplay and fine-tuning of different *Yersinia* effector proteins.

Potential therapeutic uses

In rheumatoid arthritis (RA) over-activation of macrophages plays a decisive role. Specialized macrophages, termed osteoclasts, are necessary for bone homeostasis by degrading bone tissue, but sterile inflammation can cause a regulatory imbalance leading to excessive bone destruction.[158] TNF-α was identified as a central driver of these inflammatory reactions and is thus today the main therapeutic target in RA treatment, especially in the form of neutralizing antibodies.[158] In the inflammatory skin disorder psoriasis, macrophages were also suggested to play an important role in maintaining the inflammation status.[159,160] Psoriasis is a particularly interesting option for a treatment with bacteria-derived cell-penetrating proteins, since the site of inflammation can be reached easily by topical application, which means that potential detrimental side effects caused by a systemic distribution of such an exogenous protein are circumvented.

In the context of these diseases, intervention with a cell-penetrating rYopP may have several benefits, as it impairs TNF-α-induced signaling as well as NF-κB- and MAPK-driven TNF-α secretion, and most importantly, it triggers apoptosis in activated macrophages, which are the primary source of TNF-α.[158] Hence, rYopP would cut inflammation at an earlier stage than e.g., neutralizing antibodies, which might be more efficient and especially more sustaining. Furthermore, one would not need a stoichiometric amount of the therapeutic biologic (one neutralizing antibody may only bind two TNF-α molecules), but could use much less, which in turn might be advantageous further in terms of minimizing side effects.

This high therapeutic potential of YopJ/P had attracted already some attention. About 20 years ago, Pettersson and Wolf-Watz filed a patent for the delivery of YopJ by engineered bacteria for the treatment of inflammatory disorders of the gut with extensive IL-8 expression.[161] Wallach and Appel developed a YopP-fusion protein encompassing a TNF-R binding peptide

and an endosomal escape sequence for efficient delivery of YopP into activated macrophages, which express high amounts of the TNF-R.[162] Another group constructed self-assembling YopJ-nanoparticles which readily translocated into and killed human breast cancer cells.[163] Hence, with increasing options for engineering also targeted cell-delivery of YopJ/P, further possible applications will be developed (Fig. 2).

YopH – A versatile phosphotyrosine phosphatase

Structure and function

Phosphorylation of either tyrosine, serine or threonine residues is frequently utilized by eukaryotic cells for signal transduction and thus, pathogenic bacteria have evolved an astonishing arsenal of phosphatases to manipulate these signaling processes in their favor.[164] With YopH, *Yersinia* secrete a highly potent and versatile phosphotyrosine phosphatase.[165] It consists of three major domains: an N-terminal domain (aa 1–129) that includes the secretion and translocation signal as well as a chaperone binding region,[166,167] a proline-rich repeat (aa 130–192), and a catalytic C-terminal domain (aa 193–468), which comprises all invariant features of eukaryotic phosphatases of the PTPB1 family.[168] The catalytic center forms a so-called P-loop (phosphate-binding loop) with the typical $HC(X)_5R(S/T)$ motive, which contains the catalytic active residues C403 and R409, which upon ligand binding get in close proximity to D356 to build a catalytic triad.[169] D356 is part of a second, highly flexible structure termed 'WPD loop'.[168,170] Mutation of C403 or D356 to either serine or alanine renders YopH inactive.[171,172] Residues important for substrate binding were found in the N-terminal as well as the C-terminal domains.[173,174]

Many – yet most probably not all – direct and indirect targets of YopH in several different cell types have been proposed or identified to date (Table 2). As with YopM and YopJ/P, in evaluating these results one has to carefully take into account the particular *Yersinia* species and experimental set-up (*in vitro* vs. *in vivo*, cell types, mouse models, way of YopH delivery, etc.) before reaching more general conclusions. For example, YopH of *Y. enterocolitica* was reported to dampen phagocytosis in murine dendritic cells,[175] while no such effect was observed for YopH of *Y. pseudotuberculosis*.[176] The presumably cell-penetrating construct ANT-YopH, used at a concentration of up to 300 μg/mL by Alonso *et al.*, blocked T-cell activity *in vitro*, but was later also shown to induce the intrinsic apoptosis pathway independent of YopH activity.[177]

Notably, all known interaction partners of YopH are associated with contact-dependent signaling via integrins or the T-cell receptor (TCR), which is mainly transduced by non-receptor tyrosine kinases of the src family.[178] In particular, most of the target proteins (p130cas, FAK, Fyb, Paxillin, SKAP-HOM, SLP-76, PRAM-1) are part of focal adhesion or focal adhesion-like complexes (Fig. 1), which are an important part of integrin and TCR signaling.[179,180] During infection, *Yersinia* tightly bind to β1-integrins via their outer membrane adhesin invasin.[181] Host cells decode this binding as a danger signal that promotes phagocytosis,[182] inflammasome activation,[183] and secretion of several pro-inflammatory cytokines.[184] Therefore, it is of great importance for the invading *Yersinia* to shut down this signaling axis.

In a murine infection model, enzymatically active YopH was found to be sufficient for successful colonization of the spleen by intravenously injected *Y. pseudotuberculosis* mutants.[185] Intranasally administered *Y. pestis* lacking functional YopH effectively colonized the lung, but were not able to spread to the spleen and lungs of infected mice or to prevent early cytokine responses.[186] This observation was mainly linked to the inactivation of neutrophils by YopH, although YopE could fully complement a loss of YopH in one study.[78] A more recent study showed that YopH-deficient *Y. enterocolitica* mutants were not able to block neutrophil recruitment into Peyer's patches of living mice.[187] Currently it is not clear whether an interruption of the T-cell receptor signaling pathway is advantageous for invading *Yersinia*. In intragastrically infected mice, a virulence plasmid-cured *Y. pseudotuberculosis* strain readily colonized lymphatic tissues, where it even associated with T- and B-lymphocytes.[188] On the other hand, $CD8^+$ T-cells were found to be important for the clearance of repeated *Y. pseudotuberculosis* infections.[189]

In times of recurring endemic outbreaks and an increasing awareness of potential bioterroristic attacks, YopH lately became a highly studied target for the treatment of especially *Y. pestis* infections through small molecule inhibitors of YopH.[190-193] Finally, recent data showed that – at least in pathogenic *E. coli* – bacterial proteins involved in the regulation of virulence, including type III secretion, are also activated by tyrosine phosphorylation – a mechanism that was long believed to be completely absent in bacteria.[194] Whether YopH might thus also play a regulatory role within the bacterial cell is an exciting topic for future research.

Potential therapeutic uses

Tyrosine phosphorylation is part of many signaling pathways and thus dysregulation of this mechanism might be

Table 2. Known functions and molecular targets of YopH sorted by *Yersinia* species, host cell types and stimuli. Unless stated otherwise, all listed targets are negatively regulated by YopH. Ag = antigen, DC = dendritic cell, hum. = human, mur = murine, ROS = reactive oxygen species, TCR = T-cell receptor.

Source	Cell type & stimulus	Direct target	Indirect target	Reference
Y. enterocolitica	Infected mur. macrophages		ROS	237
			Akt signaling, mcp1 mRNA, PI3K signaling?	196
	+ zymosan		no inhibition of ROS?	238
	Infected + CD3/CD28-stim. hum. T-cells		IL-2 secretion, proliferation	196
	Infected hum. neutrophils + opsonized zymosan		ROS	239
	Infected hum. granulocytes + fMLP or PMA		Phagocytosis no inhibition of ROS	240
	Infected mur. DCs		Phagocytosis	175
Y. pseudotuberculosis	Infected hum.epithelial cells		IL-8 secretion	241
		p-p130cas, pFAK, pFyb, pPaxillin, focal adhesion complexes	Phagocytosis	173,242,243,244,245,246
	Infected mur. macrophages	p-p130cas, pFyb focal adhesion complexes, SKAP-HOM?, *no binding to FAK*		173,246,247,248,249
	+ opsonized bacteria		ROS	250
			Phagocytosis	251
	Infected + TCR-stim. mur. T-cells		IL-2 secretion	252
			Calcium flux, PI3K activity	200
	Infected + PMA- or ionomycin-stim. mur. T-cells		No effect on IL-2 secretion	252
	Infected + TCR-stim. hum. T-cells	pSLP-76, pLAT, *not pLCK*	Calcium flux, PI3K activity	200
	Infected, Ag-activated mur. B-cells		B7.2 surface presentation	252
	Infected mur. neutrophils	pSKAP-HOM?, pSLP-76?, pPRAM-1? (Fyb homolog), *not Fyb*	SLP-76 signaling, calcium flux, IL-10 mRNA, TNFα mRNA	253
Y. pestis	Infected mur. DCs		No influence on phagocytosis	176
	Transfected hum. epithelial cells co-transfected with target proteins, pervanadate stim.	p-p85 (PI3K subunit), pFyb, pLCK, Vav, Gab1, Gab2	Intrinsic apoptosis pathway	177 254
	Transfected or CPP-YopH treated hum. T-cells		Intrinsic apoptosis pathway	177
	CPP-YopH treated, CD3/CD28- stim. hum. T-cells	pLCK (Y394)	IL-2 secretion	172
	Transfected, CD3/CD28- stim. hum. T-cells	pLCK (Y394), pERK?, pZAP70?, pζ?	NF-κB & NFAT/AP-1 signaling, IL-2 secretion	172,233,254
	Transfected or CPP-YopH treated, Ag-stim. rat mast cells		Degranulation	255

involved in a wide range of diseases. In particular, integrin and growth factor receptor signaling rely on tyrosine phosphorylation, which in turn is partly shut down by YopH. Due to the apparent ubiquitous presence of tyrosine phosphorylation in signaling pathways, two selected possible fields of application for a local treatment with a cell-penetrating form of the phosphotyrosine phosphatase YopH are discussed in the following.

First, rheumatoid arthritis (RA) appears to be a promising area of application (Fig. 2). As already mentioned, the gold standard for treatment of RA is simple blockage of the central mediator of inflammation, tumor necrosis factor α (TNF-α), e.g., by antibodies.[158] Such a therapy was found to effectively reduce bone erosion, but not the underlying inflammation.[195] Therefore, eliminating the source of pro-inflammatory cytokines might be a better approach. In the case of RA, the sources of pro-inflammatory cytokines are mainly hyperactivated monocytes and their differentiated descendants such as macrophages and especially osteoclasts, which in the end cause bone erosion.[158] Due to its versatility, YopH could counteract this series of events on several levels:

- YopH reduces the activation of the Akt pathway in macrophages, probably by blocking the integrin receptor-mediated activation of the phosphoinositide-3-kinase (PI3K) signaling.[196] Recent studies showed improved health when this signaling axis was blocked in murine models of psoriatic arthritis.[197]
- p130cas, probably the best studied direct target of YopH, is essential for actin-remodeling after contact-dependent activation of osteoclasts.[198] This remodeling in turn is essential for the bone destructing activity of osteoclasts.[199]
- Finally, contact-dependent activation of T-cells by monocytes plays an important role in the persistence of inflammation in RA – a fact that is already successfully exploited for therapy.[158] YopH in turn is very effective in shutting down T-cell responses to antigen stimuli.[172,196,200]

Blocking just one of the above mentioned pathways would probably not be sufficient to achieve a satisfactory RA treatment. Unlike most small molecule inhibitors, YopH might however be able to block all of them simultaneously, making it a highly potent candidate therapeutic. Especially interesting could be a combination with YopP, which blocks central inflammatory signaling pathways causing macrophage cell death.

Second, cancer progression also relies on signaling pathways described above (Fig. 2). Integrin signaling via focal adhesions promotes cell survival and can protect so-called cancer stem cells from therapeutic intervention.[201] Focal adhesion kinase (FAK) is found overexpressed and hyper-phosphorylated in many types of cancers, where it facilitates angiogenesis as well as cell migration and proliferation (and thereby metastasis).[202,203] Hyperactivated SKAP-HOM was linked to increased invasion of tumor-associated macrophages, which create a pro-metastatic environment for the tumor cells.[204] Finally, p130cas as a central mediator of integrin and growth factor receptor signaling got into the focus of cancer research recently. Especially in HER2-positive breast cancer, there is increasing evidence that over-activated p130cas plays a major role in promoting cell survival, proliferation and spreading.[205]

As for RA, YopH (targeted by homing sequences or injected directly into the tumor mass) might be able to undermine tumor progression on several levels simultaneously. On the other hand, though, it would also be able to suppress beneficial tumor infiltrating T-cells.[206] This versatility might be seen as a general drawback of YopH for a possible role as a novel biologic. In addition, overshooting PTPase activity has also been linked to cancer progression and other disease patterns,[207] which illustrates the delicate balance that has to be maintained and/or restored by therapeutic interventions.

LcrV (V-antigen) – An essential multifunctional virulence factor with immunosuppressive properties

Structure and function

Low-calcium response protein V (LcrV or V-antigen, 37–39 kDa) has been identified first in *Y. pestis* more than 50 years ago.[208] Subsequently, this plasmid-encoded secreted protein was also identified in all human pathogenic *Yersinia* (for recent review see ref. [209]). Interestingly, homologs of LcrV are also expressed by several other bacteria employing a T3SS such as *P. aeruginosa, V. cholerae, Photorhabdus luminescens* and *Aeromonas* spp.[209] However, quite surprisingly neither any possible intracellular target(s) nor an enzymatic activity has been associated with LcrV yet. Furthermore, LcrV has been intensely studied as part of candidate vaccines against *Yersinia* infections and has been shown to confer protection in animal models.[210-212]

LcrV has been identified as a multifunctional virulence factor exhibiting characteristics of translocator and effector proteins. LcrV is involved in the regulation of Yop production,[213] the translocation of virulence proteins by contributing to the needle tip,[214,215] and to pore formation in the target cell membrane since it facilitates insertion of YopB and YopD in host cell membranes.[216,217] Just recently, LcrV has been described in *Y. pseudotuberculosis* to be required for the early targeting

of YopH *in vivo*.[218] LcrV is secreted by the T3SS and has been detected on the bacterial surface and in the cytosol of target cells.[219] Apparently, LcrV enters host cells independently of T3S and the YopB–YopD pore, however, appears to need direct contact of *Yersinia* with the target cell since extracellularly added LcrV is not able to enter cells.[219]

Nevertheless, extracellularly added, recombinant LcrV has been found to exert also immunosuppressive properties. Treatment of mice with rLcrV leads to suppression of TNFα and IFNγ via amplification of IL-10 and inhibition of neutrophil chemotaxis.[220-223] Further, in murine peritoneal macrophages production of TNFα, IFNγ, IL-12, IL-1β, IL-6, MCP-1, MIP-1α, MIP-1β, and RANTES were inhibited by rLcrV (and rYopB).[224] Interactions of rLcrV with CD14 and TLR2 lead to the secretion of IL-10 in stimulated cells such as macrophages and a general hypo-responsiveness of other TLRs. This effect can also be induced by a short conserved N-terminal peptide of LcrV (VLEELVQLVKDKKIDISIK).[225] The importance of these findings is further corroborated by the reduced susceptibility for oral *Yersinia* infection of TLR2-deficient mice, which in contrast to wild-type mice are capable to resolve an infection.[225]

Potential therapeutic uses

Although unmodified LcrV of *Y. pestis* has been reported to be an extremely unstable protein,[226] it can be produced from *Y. enterocolitica* as recombinant (e.g., His-tagged) protein in sufficient amounts for therapeutic applications.[227] As the effects of LcrV appear to be mainly based on the enhanced production of anti-inflammatory IL-10, possible applications might be directed primarily to the management of infection-associated immunopathology, autoimmunity, or allergy.[228] In fact, IL-10 itself has been tested since its discovery in patients suffering from Crohn's disease, rheumatoid arthritis, psoriasis, hepatitis C infection, HIV infection and for the inhibition of therapy-associated cytokine release associated with organ transplantation.[229] Although systemic applications showed promising results in early clinical trials – e.g., in Crohn's disease patients – other immune pathologies were not as susceptible to IL-10 treatment, which probably might also be caused by IL-10′s role as a regulatory cytokine which is influenced further by the site of expression and the cell type.[230-232] In this respect, topical application of rLcrV might be a suitable strategy to induce targeted, site-specific IL-10 secretion for the treatment of autoimmune disorders. At present, however, studies addressing these potential applications of LcrV have not been reported.

Conclusion

During the long period of coevolution mainly pathogenic bacteria have developed perfectly adapted effector proteins for manipulating cellular responses and the human immune system in their favor. As we are uncovering more and more molecular details of these interactions we might be able to exploit the successful 'research and development' of these bacterial pathogens and produce our own 'biosimilars'. The six plasmid-encoded *Yersinia* outer proteins and LcrV described in this review target several important regulators in different pathways (e.g., Rho-GTPases, MAPKs, or mediators of integrin signaling; Fig. 1), which are dysregulated in major human diseases such as inflammatory bowel diseases, rheumatoid arthritis, psoriasis, or cancer (Fig. 2).

Potentially, the addition of further targeting sequences to either autonomously cell-penetrating effectors (CPE) or effectors combined with cell-penetrating peptides could enable the delivery of recombinant Yops and also of LcrV at specific sites and into specific host cells and, eventually, even host cell organelles of interest. Such targeting might make these novel biologics more efficient and less toxic than conventional drugs, which are often less selective and thus have higher EC$_{50}$. Furthermore, bacterial effector proteins can target intracellular proteins for which no satisfactory chemical inhibitor is available. This would provide a novel, vast pool of innovative candidate therapeutical biologics. Besides, such constructs could be interesting for basic research as well to specifically modulate proteins and pathways of interest. YopH for example has already been suggested as a tool in kinase research.[233]

However, not every level of interaction between Yops and host proteins has been elucidated to date. This bears the problem of potential unwanted side effects due to modulation of yet unknown intracellular targets by cell-penetrating Yops. Moreover, Yersinia outer proteins are very efficient in silencing antibacterial responses of eukaryotic cells, but as they affect many signaling pathways in parallel, their use as a specific therapeutic has to be cautiously explored. On the other hand, as illustrated for the possible role of YopH in the treatment of rheumatoid arthritis, inhibiting more than one pathway can also be an advantage over common standard therapies. Certainly, further thorough and diligent investigations including animal studies are needed to identify and evaluate the severity of probable side effects in relation to the therapeutic benefits of these novel biologics.

Furthermore, bacteria-derived protein therapeutics face similar safety issues as reported for any other drug delivery system.[234,235] In this regard, several limitations

such as poor serum stability, cytotoxicity, and immuno-genicity of conventional CPEs need to be optimized or to be considered in the choice of preferred application routes to expand their usefulness for biomedical applications. Especially the usually pronounced immunogenicity of bacterial CPEs might cause substantial drawbacks for systemic applications and might limit therapeutic options mainly to topically accessible diseases. Concerning serum stability and other safety issues, the field of CPEs can certainly profit from the extensive research on those aspects for other protein therapeutics. For example, Pan *et al.* developed a strategy to enhance serum stability of a CPP-RNA conjugate by coupling it to diethylene glycol (DEGylation),[236] similar to the attachment of polyethylene glycol (PEGylation) to conventional protein therapeutics.

Apart from such obstacles, several patents and ongoing studies on the use of Yops and also other bacterial effector proteins as innovative biologics testify to the appealing nature of this strategy. Further research on the role of Yops during infection will also enhance and strengthen our knowledge base for this translational approach.

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