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Original article

The adhesive protein invasin of *Yersinia pseudotuberculosis* induces neutrophil extracellular traps via β1 integrins

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Abstract

Yersinia pseudotuberculosis adhesive protein invasin is crucial for the bacteria to cross the intestine epithelium by binding to β1 integrins on M-cells and gaining access to the underlying tissues. After the crossing invasin can bind to β1 integrins on other cell surfaces, however effector proteins delivered by the type III secretion system *Y. pseudotuberculosis* efficiently inhibit potential immune responses induced by this interaction. Here, we use mutant *Y. pseudotuberculosis* strains lacking the type III secretion system and additionally invasin-expressing *Escherichia coli* to analyze neutrophil responses towards invasin. Our data reveals that invasin induces production of reactive oxygen species and release of chromatin into the extracellular milieu, which we confirmed to be neutrophil extracellular traps by immunofluorescence microscopy. This was mediated through β1 integrins and was dependent on both the production of reactive oxygen species and signaling through Phosphoinositide 3-kinase. We therefore have gained insight into a potential role of integrins in inflammation and infection clearance that has not previously been described, suggesting that targeting of β1 integrins could be utilized as an adjunctive therapy against yersiniosis.

Keywords: Neutrophil; *Yersinia*; Invasin; Integrin; NET

1. Introduction

Yersinia pseudotuberculosis is closely related to *Yersinia pestis*, the agent of plague. It is a food-borne pathogen that causes gastroenteritis. In the intestine, *Y. pseudotuberculosis* migrates through M-cells to underlying lymphoid tissues known as Peyer's patches. In these patches the bacteria can fight immune cells by expression of the type III secretion system (TTSS) [31]. The TTSS is encoded on a virulence plasmid and codes for several proteins that build up a needle structure reaching through the outer membrane of *Y. pseudotuberculosis* [4]. In close contact with host immune cells bacterial effector proteins, known as Yops (*Yersinia* outer proteins), can be translocated via the needle into the cytoplasm of the immune cells, where they inhibit signaling necessary for phagocytosis [27] and cytokine production or can induce apoptosis in macrophages [31].

The protein responsible for migration through M-cells is invasin, an adhesive protein encoded by the *inv* gene on the bacterial chromosome [17]. Invasin is expressed at the surface of *Y. pseudotuberculosis* and binds to β1 integrins with a much higher affinity than ordinary extracellular matrix ligands that binds to β1 integrins, such as fibronectin [29]. Additionally, invasin has the ability to dimerize, allowing clustering of β1 integrins on the interacting cell [9]. Integrins are expressed as αβ heterodimers that mediates adhesion between cells, extracellular matrix and pathogens. There are 18 α chains and 8 β chains that can be combined into 24 pairs in vertebrates. The β1 integrin is expressed on several cell types around in the body, leukocytes amongst others [16]. Although the binding of invasin and β1 integrin is well-established and has been shown to be essential for infection of *Y. pseudotuberculosis* little is

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known about induction of immune responses triggered by invasin-β1 integrin binding. Recently, it has been shown that invasin from *Yersinia enterocolitica* binds to β1 integrins on macrophages and induces autophagy [10].

We aimed to identify whether invasin-β1 integrin signaling triggers polymorphonuclear neutrophil (PMN) immune responses. Neutrophils are rapidly recruited to sites of infection serving as first line of defense against invading pathogens. They are specialized in killing of microorganisms by different mechanisms, such as for instance phagocytosis and degranulation [20]. An additional antimicrobial mechanism of neutrophils is their ability to produce large amounts of reactive oxygen species (ROS) in contact with microbes which is rapidly produced by the NADPH oxidase complex upon recognition of microorganisms [7]. The importance of ROS in the antimicrobial defense is reflected in patients with chronic granulomatous disease (CGD), a genetic disorder associated with the function of the ROS-producing protein complex in phagocytes. These patients frequently suffer from severe microbial infections [25]. Moreover, the Neutrophil extracellular trap (NET), first shown by Brinkmann and coauthors, is an extracellular mechanism of capturing and killing microbes [6]. NETs are released chromatin coated with antimicrobial proteins. The histones and DNA form the scaffold of the structure which can be dismantled by DNase treatment [3,8].

During NET formation the neutrophil chromatin decondenses, which is dependent on autophagy and signaling through protein phosphoinositide 3-kinase (PI3K) [21]. Subsequently, the nuclear membrane dissolves, releasing the chromatin into the cytoplasm. Intracellular vesicles containing antimicrobial proteins are additionally permeabilized via a hitherto unknown mechanism releasing granular proteins into the cytoplasm and allowing NET components to mix inside the neutrophil. When the plasma membrane later ruptures the NET is released [7]. However, a more recent publication suggests that NETs can be released independent of plasma membrane rupture, with the neutrophil remaining intact [32]. The lytic pathway has been described to be active after 2–4 h post stimulation and to be dependent on the production of ROS. The vesicular pathway in contrast, was shown to be faster with NET release occurring within minutes after stimulation in a ROS-independent manner.

We addressed the interaction of *Y. pseudotuberculosis* and neutrophils in dependence of invasin-β1 integrin signaling. These β1 integrins are expressed on neutrophils and are important in extravasal migration of neutrophils [24], and during the migration the expression is up-regulated [30]. In line with our work a recent publication revealed that NETs can be released through β2 integrin signaling *in vivo* [18], but whether this NET release was ROS dependent remained unclear. Downstream of β1 integrin conveys PI3K the induced signaling, which is important for internalization of *Y. pseudotuberculosis* in HEp-2 cells [19]. In this context, the invasin-β1 integrin interaction is a candidate for signaling that might induce NET release.

We demonstrate in this study that *Y. pseudotuberculosis* invasin induces activation and NET release in human neutrophils in the absence of a functional TTSS. This NET release is mediated through β1 integrins and is dependent on the production of ROS and PI3K-induced signaling [21].

2. Materials and methods

2.1. Neutrophil isolation

Neutrophils were harvested from blood of healthy volunteers according to the recommendations of the local ethical committee (Regionala etikprövningsnämnden i Umeå) and according to the principles expressed in the Declaration of Helsinki.

Neutrophils were isolated as described earlier [1], washed in PBS with 0.5% human serum albumin and resuspended in RPMI 1640 without phenol red substituted with 10 mM HEPES in experiments with *Y. pseudotuberculosis* and HBSS without phenol red in experiments with *Escherichia coli* strains. For serum preparation blood was allowed to clot for 40 min at room temperature and spun at 800× *g* for 10 min. Serum was collected with a pasteur pipette and used for opsonization of bacteria. In experiments with opsonized bacteria serum and neutrophil donors were matched.

2.2. Bacterial strains and growth conditions

All bacterial strains used in this study are listed in Table 1 [22,23]. *Y. pseudotuberculosis* overnight cultures were grown in 2 ml Lucia-Bertani broth (LB) at 26 °C. Subcultures were setup in 2 ml LB and inoculated with 100 µl overnight cultures. The subcultures were subsequently incubated at 26 °C for 30 min and then additionally 60 min at 37 °C in order to induce Yop production in the wild-type strain YPIII pIB1+. *E. coli* overnight cultures were grown in 10 ml LB at 37 °C. Subcultures were inoculated in LB inoculated to an OD_{590} of 0.2 and incubated at 25 °C (*E. coli* Inv+ and *E. coli* Inv−) or 37 °C (*E. coli* YadA+ and *E. coli* YadA−) for 3 h. All bacteria were harvested by centrifugation at 5000 *g* for 10 min, washed once and diluted to appropriate concentration in media. Opsonization was performed by incubating *Y.*

Table 1
Bacterial strains used in this study.

Name	Genotype	Invasin	Description	Reference
YPIII pIB1+	YPIII pIB1	Yes	Virulence plasmid complemented, functional TTSS	[22]
YPIII pIB1−	YPIII	Yes	Virulence plasmid cured	[22]
Δ*Inv*	YP100 pIRR11	No	Virulence plasmid cured, Inv knock-out	[22]
E. coli Inv+	C600 pIRR1	Yes	Invasin expressing strain	[22]
E. coli Inv−	C600 pIRR11	No	Vector control expressing a truncated version of invasin	[22]
E. coli YadA+	C600 pAMS1	No	YadA expressing strain	[23]
E. coli YadA−	C600 pAMS2181	No	Vector control expressing a truncated version of YadA	[23]

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