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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.

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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
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 granulomatosus 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 (Regionella 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-Bertianni 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 pBl1+. *E. coli* overnight cultures were grown in 10 ml LB at 37 °C. Subcultures were inoculated in LB inoculated to an OD590 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>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>Invasin</th>
<th>Description</th>
<th>Reference</th>
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<tbody>
<tr>
<td>YPIII pBl1+</td>
<td>YPIII pBl1</td>
<td>Yes</td>
<td>Virulence plasmid complemented, functional TTSS</td>
<td>[22]</td>
</tr>
<tr>
<td>YPIII pBl1−</td>
<td>YPIII pBl1</td>
<td>Yes</td>
<td>Virulence plasmid cured</td>
<td>[22]</td>
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<tr>
<td>E. coli Inv+</td>
<td>C600 pRR1</td>
<td>Yes</td>
<td>YPIII pBl1−</td>
<td>[22]</td>
</tr>
<tr>
<td>E. coli Inv−</td>
<td>C600 pRR1</td>
<td>No</td>
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<td>[22]</td>
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<tr>
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<td>No</td>
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<td>[23]</td>
</tr>
</tbody>
</table>

*pseudotuberculosis* ΔInv strain in RPMI with 5% fresh human serum for 10 min at 37 °C prior to washing and dilution.

### 2.3. ROS production analysis

Neutrophils (5 × 10⁵) were seeded in each well of a white 96 well plate, coated with 2% human serum albumin for 30 min prior to seeding, with 10 µg/ml anti-β1-integrin antibodies (mAB2253z, Millipore), isotype control antibodies (M5284, Sigma–Aldrich) or medium for 30 min together with 50 µM luminol (Sigma–Aldrich) and 1.2 U/ml horseradish peroxidase (Sigma–Aldrich) prior to addition of bacteria (*Y. pseudotuberculosis* MOI 30, *E. coli* MOI 100). Chemiluminescence was measured every other minute over a 3 h time period in a luminometer (Infinite F200, Tecan). Neutrophils treated with 100 nM of the potent NET inducer phorbol 12-myristate 13-acetate (PMA, Sigma–Aldrich) served as positive control. To determine total amount of ROS production the area under curve (AUC) was calculated using Graphpad Prism v5.

### 2.4. Extracellular DNA fluorescence assay

Presence of extracellular DNA was measured as described previously [14]. Briefly, neutrophils (5 × 10⁵) were seeded in each well of a black 96 well plate, coated with 2% human serum albumin. The neutrophils were treated with 10 µg/ml anti-β1-integrin antibodies (mAB2253z, Millipore), isotype control antibodies (M5284, Sigma–Aldrich), 100 nM wortmannin (Sigma–Aldrich), 10 µM DPI (Sigma–Aldrich), 15 mM tempol (Sigma–Aldrich), 4 µM TLR-4 antagonist peptide viper or control peptide CP7 (Imgenex) or medium for 30 min prior to addition of bacteria (*Y. pseudotuberculosis* MOI 30, *E. coli* MOI 100). Extracellular chromatin was detected with 2.5 µM sytox green (Invitrogen). Fluorescence was measured every 10 min over a 16 h time period in a luminometer (Fluostar BMG, Labtech). As 100% controls served neutrophils lysed with 1% Triton x-100. PMNs stimulated with 100 nM PMA served as positive control.

### 2.5. Immunofluorescence microscopy

Neutrophils (10⁵) were seeded in each well of a 24 well plate, where the wells contained a glass cover slip coated with poly-L-lysine. The PMNs were incubated for 30 min at 37 °C with 100 nM wortmannin (Sigma–Aldrich) or with medium prior to addition of bacteria (*Y. pseudotuberculosis* MOI 30, *E. coli* MOI 100) or 100 nM PMA. After 8 h infection samples were fixed with paraformaldehyde (final concentration 2%) and incubated at room temperature for 1 h. The neutrophils were stained with antibodies for elastase (6 µg/ml; #481001, Calbiochem), histone 1 (1.25 µg/ml; #BM465, Acris) and DNA stain DAPI (1 µg/ml). Pictures were taken with a Nikon Eclipse 90i fluorescence microscope.

For NET quantification five pictures were taken and at least 250 neutrophils were analyzed for each condition by measuring the chromatin area (DNA stain) in ImageJ v1.47. Chromatin areas larger than 100 µm² were considered as NETs according to previous studies [14].

### 2.6. Bacterial uptake analysis

Neutrophils (10⁵) were seeded in each well of a 24 well plate, where the wells contained a glass cover slip coated with poly-L-lysine, and incubated for 30 min at 37 °C. *Y. pseudotuberculosis* were added at MOI 30 and incubated for 1 h prior to fixation with paraformaldehyde (final concentration 2%). The specimens were stained with anti-CD66 antibodies (2.5 µg/ml; cat.nr. 551354, BD Biosciences) and a polyclonal anti-Yersinia antibody. Analysis was performed using a Nikon Eclipse 90i confocal microscope with a 60× objective. The number of neutrophils with intracellular bacteria was quantified utilizing Nikon EZ-C1 FreeViewer v3.90.

### 2.7. Data analysis and statistics

Data analysis and statistics were performed with Graphpad prism v5, where significance was calculated by ANOVA and Newman–Keuls method was used as post hoc test. All data is presented as mean +/- standard deviation from experiments with different neutrophils donors.

### 3. Results

#### 3.1. Invasin expressing E. coli induces NET release accompanied by ROS production

Invasin is important for the virulence of pathogenic *Yersinia* [17]. The effect of invasin on human neutrophils in the absence of the TTSS is however not clear. To investigate this effect we infected neutrophils with invasin-expressing *E. coli* for a microscopic analysis. Samples were fixed and stained with DAPI and anti-histone 1 antibody to reveal chromatin structures. Additionally the neutrophils were stained with antibodies directed towards neutrophil elastase, a proteinase located in granules of unstimulated neutrophils and NETs. Neutrophils stimulated with PMA served as positive control, since PMA is a potent inducer of NETs [11]. These neutrophils revealed web-like structures (Fig. 1) positive for DNA stain (A), elastase stain (B) and histone 1 stain (C) which indicates released NETs according to previous studies [14]. Instead, neutrophils infected with invasin-expressing *E. coli* Inv+ the neutrophils reveal chromatin structures similar to those among PMA stimulated neutrophils (Fig. 11–L) showing that this strain induced NETs. These structures were not found in neutrophils infected with *E. coli* Inv− (Fig. 1M–P), which carries a truncated and non-functional Inv gene on its plasmid serving as negative control. Instead, neutrophils infected with *E. coli* Inv− rather resembled non-infected controls. These results indicate that invasin expressed on bacterial surfaces triggers NETs in human neutrophils.
In order to quantify NET release we infected neutrophils with *E. coli* strains in the presence of the cell-impermeable DNA stain sytox green. With this method chromatin release was quantified over time in a fluorescence plate reader. In addition to invasin-expressing *E. coli* strains we used *E. coli* expressing *Y. pseudotuberculosis* adhesive protein YadA, which also binds to β1 integrins, albeit indirectly through ECM proteins [15]. In line with our microscopic investigation we found that invasin-expressing strain *E. coli* Inv+ induced over 20% NET cell death (Fig. 2A) in neutrophils unlike its vector control *E. coli* Inv−, which was not significantly different from non-infected neutrophils. In comparison, expression of the adhesin YadA in strain *E. coli* YadA+ induced similar NET cell death as vector control *E. coli* YadA−, carrying a truncated YadA gene. Both NET cell death signals were not significantly different from the non-infected neutrophils (Fig. 2A). This suggests that invasin, but not YadA, induces chromatin release in human neutrophils. Since it is unknown whether invasin-induced NET release is dependent on ROS we analyzed the neutrophil ROS production in response to our *E. coli* strains in a luminol-based assay. We found that *E. coli* Inv+ induced significantly more ROS production than the vector control *E. coli* Inv− (Fig. 2B). In contrast no difference in the ROS production when neutrophils were infected with *E. coli* YadA+ and its vector control *E. coli* YadA− was observed (Fig. 2B). From these findings we conclude that invasin triggers production of ROS in neutrophils as well as NET release.

### 3.2. The invasin-induced NET release and ROS production is mediated through β1 integrins

To investigate whether the invasin-induced ROS production and NET release is mediated through β1 integrins on the neutrophil surface, we used a β1 integrin blocking antibody. Neutrophils were incubated with blocking antibodies or isotype control antibodies prior to infections and ROS production and NET cell death was quantified in response to invasin-expressing *E. coli*.

By addition of 10 µg/ml blocking antibodies the NET cell death in neutrophils infected with *E. coli* Inv+ decreased to 60% of the antibody free neutrophils challenged with *E. coli* Inv+ (Fig. 2C), a decrease which was absent in neutrophils.
incubated with isotype control antibodies. The blocking antibody had no effect when neutrophils were infected with E. coli Inv−. In line with the NET cell death results, the ROS production was also significantly decreased to 60% when neutrophils were infected with E. coli Inv+ in the presence of the blocking antibody (Fig. 2D). Whereas incubation with isotype control antibody decreased ROS production slightly, albeit to a lower extent than blocking antibodies. Data is presented as mean ± SD from three experiments (A–C) and from four experiments (D). Stars above bars represent significance towards PMNs infected with E. coli Inv+ in (B) and towards untreated PMNs infected with E. coli Inv+ in (C) and (D).

3.3. Invasin-expressing Y. pseudotuberculosis induces NET release and ROS production

We identified invasin as an inducer of NET release when expressed in E. coli. To evaluate its relevance in Y. pseudotuberculosis we analyzed neutrophil response to invasin in a Y. pseudotuberculosis strain background.

Neutrophils were infected with Y. pseudotuberculosis strains, fixed and stained with DNA stain DAPI to visualize DNA structures for a microscopic quantification, similar to a

![Graph A](image1)

**Fig. 2.** Invasin expressed in E. coli induces NET cell death and production of ROS through β1 integrins. (A) PMNs were infected with E. coli in the presence of fluorescent DNA stain sytox green. After 10 h infection strain E. coli Inv+ had induced significantly more chromatin release, i.e. NET cell death, than the vector control E. coli Inv−. (B) The ROS production in response to E. coli was quantified by the chemiluminescent properties of luminol after oxidation. Infections were carried out over a 3 h time period and the total ROS production was normalized to E. coli Inv+ infected PMNs. The E. coli Inv+ strain induced significantly more ROS production in PMNs than the vector control. (C) Addition of anti-β1 integrin antibodies to the conditions in (A) decreased NET cell death for PMNs infected with E. coli Inv+, unlike isotype control antibodies that had no significant effect. (D) Treatment with anti-β1 integrin antibodies decreased ROS production in PMNs infected with E. coli Inv+, as in (B). Isotype control antibodies decreased ROS production, but to a lower extent than blocking antibodies. Data is presented as mean ± SD from three experiments (A–C) and from four experiments (D). Stars above bars represent significance towards PMNs infected with E. coli Inv+ in (B) and towards untreated PMNs infected with E. coli Inv+ in (C) and (D).
previous report [14]. All neutrophils were counted and DNA areas were scored as NET when the area exceeded a threshold area of 100 µm² as calculated by ImageJ analysis. We found that approximately 30% of the neutrophils released NETs 8 h after infection with strain YPIII pIB1−, lacking the virulence plasmid and its encoded TTSS, but expressing invasin (Fig. 3A). In contrast, the YPIII pIB1− derived invasin knockout strain ΔInv did not induce NET release above background levels resulting from non-infected neutrophils. In addition, the wild-type strain YPIII pIB1+, which expresses invasin and a functional TTSS did not induce NET formation. We also utilized the NET cell death quantification on infections with *Y. pseudotuberculosis* strains. We found that 10 h post infection almost 30% of the neutrophils infected with YPIII pIB1− had released their chromatin (Fig. 3B), corresponding very well to the findings in the microscopic quantification (Fig. 3A). Similarly, neutrophils challenged with ΔInv or YPIII pIB1+ strains remained at the level of non-infected neutrophils.

Next, we analyzed whether invasin expressed in *Y. pseudotuberculosis* induces neutrophil ROS production as observed when expressed in *E. coli*. We found that neutrophils infected with YPIII pIB1− induced significantly more ROS than ΔInv.

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**Fig. 3.** Invasin-expressing *Y. pseudotuberculosis* induces NET release in PMNs which is dependent on both ROS production and PI3K signaling. (A) PMNs were infected with *Y. pseudotuberculosis* strains for 8 h prior to DNA staining and microscopic investigation. The DNA stained area of each PMN were measured and scored as a NET if the area exceeded 100 µm². (B) Quantification of NET cell death 10 h post infection in response to infections with *Y. pseudotuberculosis* strains revealed that YPIII pIB1− induced NET cell death in PMNs, confirming the results in Fig. 3A. (C) YPIII pIB1− induced more than double the ROS production than other strains, determined by the ROS production analysis. (D) The amount of NET cell death 10 h post infection in response to YPIII pIB1− decreased significantly upon pre-treatment with 100 nM wortmannin (Wort), 10 µM DPI or 15 mM tempol (Tem). Stars above bars represent significance towards inhibitor free equivalents. (E) Treatment with 100 nM wortmannin significantly decreased invasin-mediated NET release upon infection with YPIII pIB1− determined by microscopic investigation. Data is presented as mean±/− SD from three experiments (A−B), (D−E) or from four experiments (C). Stars above bars represent significance towards YPIII pIB1− infected PMNs.
suggested that invasin-induced NET release might be ROS-dependent. The ROS production in response to the ΔInv strain was though significantly larger than in non-infected neutrophils, indicating that the ROS production in response to *Y. pseudotuberculosis* is not exclusive to invasin. The neutrophil ROS production upon infection with YPIII pIB1+ was lower than upon ΔInv infection with no statistical difference to non-infected neutrophils.

### 3.4. Invasin-induced NET release is dependent on both the production of ROS and PI3K-signaling

We determined that invasin binding to β1 integrin on neutrophils induces NET release. We next focused on the intracellular signaling leading to NET formation. It was recently shown that the generation of NETs is dependent on autophagy [21] and since we observed that invasin induces ROS production, the NET release might be ROS-dependent. Autophagy depends on signaling from the protein PI3K. We used NET cell death quantification to measure chromatin release by neutrophils infected with *Y. pseudotuberculosis* strains and additionally treated with compounds to inhibit PI3K or ROS production. The NADPH oxidase inhibitor DPI and the ROS scavenger tempol depleted ROS production to background levels (data not shown). The same concentrations of DPI and tempol were applied in the NET cell death assay. To analyze the role of PI3K we used inhibitor wortmannin. Addition of wortmannin can block activation of pathways contributing to autophagy, while having negligible effects on NADPH oxidase activation [21]. The inhibitors were added to neutrophils 30 min prior to infection with *Y. pseudotuberculosis* strains. When neutrophils were infected with YPIII pIB1− in the presence of wortmannin they released significantly less chromatin than neutrophils infected with YPIII pIB1− in the absence of wortmannin (Fig. 3D). This was not observed when neutrophils were infected with the strains ΔInv and YPIII pIB1+, indicating that the engagement of the invasin-β1 integrin interaction is essential for PI3K signaling in neutrophils when challenged with *Y. pseudotuberculosis*. PI3K-mediated signaling in turn is completely abrogated by the TTSS. In addition, NADPH oxidase inhibitor DPI and ROS scavenger tempol could efficiently block NET cell death (Fig. 3D). In addition, DPI decreased NET cell death in neutrophils infected with the ΔInv strain. This suggests that other bacterial factors, apart from invasin, might stimulate neutrophils to release NETs, even with an inhibited NADPH oxidase. However, this factor would not be as potent as invasin, since ΔInv induced less ROS and NET cell death than YPIII pIB1− (Fig. 3B and C). The ROS scavenger tempol had a significant effect on all infections (Fig. 3D) suggesting that ROS depletion hampers NET release for all tested conditions and strains. Furthermore, we quantified NET formation by a complementary microscopic analysis using neutrophils infected with *Y. pseudotuberculosis* strains 8 h post infection. These results are very similar to data presented in Fig. 3A which additionally include *Yersinia*-infected neutrophils in the presence and absence of PI3K inhibition (wortmannin). In line with the

![Fig. 4](image-url) Invasin induces NET release, which is abrogated by wortmannin and the TTSS. PMNs were infected with YPIII pIB1− and fixed after 8 h of incubation. Specimens were stained positive for DNA (blue)-, elastase (green)-, and histone 1 (red)- staining and analyzed by immunofluorescence microscopy. Infected PMNs release NETs (A), whereas wortmannin-treated PMNs did not release NETs (B). Pictures were taken with a 40× objective and pictures presented are representatives for three independent experiments. Scale bar is equal to 10 μm.
did not induce NETs and there was no difference between neutrophils with or without addition of wortmannin (Fig. 4C–F). The nuclei of non-infected neutrophils remained lobulated (Fig. 4G) and PI3K inhibition did not affect this morphology (Fig. 4H).

3.5. Invasin-mediated NET release is preceded by bacterial uptake and is not dependent on LPS recognition

As invasin is a protein that gives *Y. pseudotuberculosis* the ability to invade mammalian cells through β1 integrin interactions and, additionally, neutrophils are phagocytic cells it is probable that invasin-mediated NET release is accompanied by bacterial uptake. To determine whether YPIII pB1– is taken up to higher extent than ΔInv, neutrophils were infected with *Y. pseudotuberculosis* strains for 1 h prior to fixation and analysis by confocal microscopy. The neutrophils were quantified and classified with respect to whether they contained intracellular bacteria. When infected with YPIII pB1– more than 80% of the neutrophils had at least one intracellular bacterium (Fig. 5A). In comparison only 25% of neutrophils had intracellular bacteria when infected with ΔInv clearly demonstrating that invasin mediates uptake by neutrophils. Additionally, ΔInv were opsonized with human serum to facilitate uptake and determine whether *Yersinia*-mediated NET release is dependent on internalization. Opsonized ΔInv bacteria were indeed taken up more readily than non-opsonized ΔInv (Fig. 5A). Likewise, ROS production from neutrophils infected with opsonized ΔInv bacteria was higher than from neutrophils infected with non-opsonized ΔInv (Fig. 5B). However, release of NETs upon stimulation with either opsonized or non-opsonized as determined by NET cell death remained at levels below unstimulated neutrophils (Fig 5C). While serum opsonization of *Y. pseudotuberculosis* expectedly leads to increased phagocytosis and ROS production by neutrophils, induction of NET formation was strictly dependent on the presence of invasin. In accordance to this YPIII pB1+ and ΔInv induce comparable amounts of ROS (Fig. 3C) and consequently similarly low amounts of NETs (Fig. 3A and B), whereas neutrophil uptake of YPIII pB1+ is significantly higher than of ΔInv (Fig. 5A). Yet, YPIII pB1+ uptake was 50% lower than compared to neutrophils infected with YPIII pB1– indicating that invasin-β1 integrin interaction induce uptake, which is partly inhibited by the TTSS. In conclusion, this data suggests that *Yersinia*-induced NET release is rather invasin and ROS-dependent, but not directly correlated to phagocytosis.

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**Fig. 5.** Invasin induced NET release is preceded by bacterial uptake and is not dependent on LPS recognition. (A) PMNs were infected with *Y. pseudotuberculosis* strains for 1 h prior to staining of bacteria and PMN membrane marker CD66. Confocal microscopy with a 60/C2 objective was used for analysis. YPIII pB1– was most frequently engulfed as compared to other strains tested. At least 20 PMNs were analyzed per sample. Stars above bars represent significance towards uptake of YPIII pB1–. (B) Strain ΔInv was opsonized with native human serum prior to infection as in Fig. 3B. Opsonization of bacteria increased neutrophil ROS production (C) Opsonization of ΔInv strain affected NET cell death compared to non-opsonized ΔInv, was however below NET cell death from uninfected controls. (D) PMNs were pre-treated with 4 μM TLR-4 peptide inhibitor Viper or control peptide (CP7) prior to extracellular DNA quantification in response to infection with YPIII pB1–. A small, but significant decrease was observed upon pre-treatment with Viper. However, a similar decrease was observed with CP7 as well. Stars above bars represent significance towards PMNs infected with YPIII pB1– without peptide. Data is presented as mean ±/− SD from 3 experiments with different donors for (A), (B) and (D). For (C) one out of three independent experiments in triplicate is shown.
As invasin blockage did not result in complete abrogation of NET release, we aimed to find additional receptors potentially involved in this signaling cascade. One major receptor for recognition of Gram-negative bacteria is Toll-like receptor 4 (TLR-4), which recognizes lipopolysaccharide (LPS) in the bacterial outer membrane. To inhibit TLR-4 recognition of LPS, we used TLR-4 antagonist peptide viper and quantified NETs. When neutrophils were infected with YPIII pB1+ after treatment with viper a small, but significant decrease in NET cell death was observed (Fig. 5D). However, this decrease was not significantly different to the samples treated with the unspecific control peptide CP7. Therefore, it seems as TLR-4 might have a minor role in invasin-mediated NET release.

Taken together, these results suggest that invasin binds to β1 integrins on human neutrophils and mediates NET release which is dependent on NADPH oxidase and subsequent PI3K signaling.

4. Discussion

Human neutrophils are the first line of defense against invading microorganisms. They rapidly migrate to injured tissues from circulation and have several methods to kill and clear infections [20]. *Y. pseudotuberculosis* is a food-borne pathogen that causes the gastrointestinal disease yersiniosis in humans [31]. In order for *Y. pseudotuberculosis* to establish infection migration through M-cells into the underlying Peyer’s patches is essential. This is dependent on the expression of the adhesive protein invasin, which bind to β1 integrins on host cells and allows uptake into epithelial M-cells [17]. The β1 integrin has been previously described to contribute to activation of phagocytosis [2] and cytokine release [13] in response to bacterial invasin, showing its importance in immune responses. To fight these potent immune responses *Y. pseudotuberculosis* has acquired a virulence plasmid encoding the TTSS [31].

More recently, NETs have been described as an immune defense mechanism against microorganisms [6]. To investigate the potential of *Y. pseudotuberculosis* to induce NET formation we used plasmid-cured mutants which, in contrast to wild-type, were unable to inhibit neutrophil responses due to the lack of TTSS. We demonstrated that YPIII strain induced ROS production and NET formation in human neutrophils. The mechanisms behind the triggering of NET formation by plasmid-cured *Y. pseudotuberculosis* were largely unexplored.

We show here that expression of invasin from *Y. pseudotuberculosis* increased neutrophil ROS production, bacterial uptake as well as the release of NETs. Neutrophil ROS and NET release were dependent on invasin and β1 integrin interaction, since antibodies directed against β1 integrins inhibited the resulted in decreased ROS and NET production upon infection with *E. coli* Inv+ (Fig. 2). Isotype control antibodies had no effect on NET formation, however, slightly reduced ROS probably due to protein-related, unspecific ROS quenching. In addition, PI3K acts downstream of the β1 integrin signaling pathway [19] and induces autophagy, which is necessary for decondensation of chromatin and NET release [21]. In line with this, we demonstrated that PI3K inhibition abrogated invasin-triggered NET formation (Fig. 3E–D). The observed induction of invasin-dependent NET formation was dependent on the amount of neutrophil ROS produced. YPIII pB1+ and ΔInv strains triggered comparably low amounts of ROS and consequently similarly low amounts of NETs. Interestingly, neutrophils more readily engulfed YPIII pB1+ as well as complement-opsonized ΔInv as compared to non-opsonized ΔInv (Fig. 5A). In contrast, NET release was not increased upon infection with the opsonized invasin-deficient strain (Fig. 5C). This suggests that the observed induction of invasin-mediated NET formation does not merely depend on the amount of uptake which for instance can be induced by complement opsonization. In agreement, a previous study describes selective release of NETs upon stimulation with filamentous pathogens too large to be phagocytized, whereas inhibition of phagocytosis increased NET formation upon particles small enough to be engulfed [5]. We therefore conclude that amounts of NET release rather correlate to neutrophil ROS. Accordingly, ROS scavenging and inhibition of NADPH oxidase efficiently blocked NET release in response to invasin (Fig. 3E).

Unlike with invasin, we did not observe NET release in response to YadA-expressing *E. coli*. It is known that YadA binds indirectly to β1 integrin through interaction with ECM proteins, for instance fibronectin [15]. Since we did not add ECM components to our experiments, we cannot expect interactions between YadA and β1 integrins. However the YadA ECM bridge binds to β1 integrin with comparably lower affinity than the invasin protein, which should be important for NET stimulation. We did not address whether β1 integrin clustering occurring in the presence of ECM components might contribute to NET release.

The TTSS in *Y. pseudotuberculosis* inhibits NET release in response to invasin-β1 integrin interaction with human neutrophils, and is crucial for both the establishment of infection and outcome [12]. Other enteric bacteria, such as for instance *Vibrio cholerae* evade NET-mediated killing by expressing extracellular nucleases capable in degrading NET structures [26]. *Y. pseudotuberculosis* appears to have intrinsic properties to induce NETs via β1 integrin signaling mediated by invasin. In contrast to nuclease-secreting *V. cholerae*, *Y. pseudotuberculosis* prevents initiation of NET formation by TTSS effector activity. This notion is also supported by our previous finding showing that neutrophil depletion in a mouse model of yersiniosis has no significant effect on *Y. pseudotuberculosis* colonization and dissemination [28].

Moreover, invasin is required for invasion of host tissue and to establish colonization. We therefore hypothesized that the immune system evolved mechanisms to recognize this important virulence factor in order to detect and remove *Y. pseudotuberculosis*. As first line of defense neutrophils likely applied this mechanism. However in the arms race *Y. pseudotuberculosis* adapted by acquiring the TTSS and connected effector proteins to counteract the recognition via invasin-β1 integrin interaction. In agreement with our findings β2 integrins participate in neutrophil immune responses, such as NET release [18]. However, this is the first report showing that β1 integrins induce NET formation in human neutrophils illustrating that the extent of integrin signaling in immune responses is complex and thus
requires further investigation. The bacterial surface component LPS contributed only to a minor extent to Yersinia-mediated NET induction. As heterologously expressed and purified invasin alone did not induce NET formation (data not shown), it seems likely though that other co-stimulatory receptors play a role which yet need to be defined. Remarkably, we report here that singular bacteria equipped with the according ligands, such as invasin, are able to induce NETosis.

Our findings suggest that targeting of β1 integrins could be a potential strategy as adjunctive therapy of yersiniosis, and perhaps other pathogenic Gram-negative bacteria, since activation of β1 integrin signaling could sustain neutrophil functions in situations where they are otherwise inhibited by the invading microbe.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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