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**Mammalian peptidoglycan recognition protein TagL inhibits *Listeria monocytogenes* invasion into epithelial cells**

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***Mammalian peptidoglycan recognition protein TagL inhibits  
Listeria monocytogenes invasion into epithelial cells***

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Keywords: innate immunity, PGRP, enteroinvasive pathogens, *Listeria monocytogenes*

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**Abstract**

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6 Peptidoglycan Recognition Proteins (PGRPs) is a family of evolutionary conserved proteins that  
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8 play a basic role in the innate immunity of insects, but their role in the immunity of mammals  
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10 remains unclear. To elucidate its functions a mouse member of PGRP family, TagL was stably  
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12 expressed in colon adenocarcinoma HT29 cells, and its effect on invasion and intracellular  
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14 growth of enteroinvasive pathogenic bacterium *Listeria monocytogenes* was assessed. TagL  
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16 expression substantially impaired bacterial invasion and early intracellular growth. Observed  
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18 effects were partly caused by a loss of viability by intraphagosomal bacteria. Efficient  
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20 phagosome escaping but not efficient invasion helped bacteria to overplay TagL.  
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## 1. Introduction.

The innate immune system recognizes microorganisms by characteristic pathogen-associated molecular patterns (PAMPs), which are characteristic for them and absent from the host (for review see [1]). PAMPs include structural components of bacterial cell wall like peptidoglycan (PGN), lipoteichoic acid (LTA) or LPS. In insects PGN is recognized by special receptors that belong to the family of Peptidoglycan Recognition Proteins (PGRPs) and share a conservative domain homologous to the T7 phage lysozyme (a so-called PGRP domain). The PGN recognition by PGRPs is an upstream event of the two major signaling pathways leading via activation of the Toll and Imd receptors to the immune response in insects ([2], [3]).

The PGRP protein family is evolutionary conserved, and mammalian PGRPs demonstrate a high structural similarity to orthologs from insects (for review see [4-6]). However, the role for mammalian PGRPs in the innate immunity seems to be different in terms of signaling events as mammalian Toll-like receptors (TLRs) are able to recognize PAMPs, and in part PGN, themselves [1]. It is still unclear whether there is any interplay between mammalian PGRPs and TLRs, and the actual role for PGRPs in the innate immunity of mammals has to be elucidated.

Up-to-date four human PGRPs were found, two of which, PGRP-S and PGRP-L, have mouse orthologs named Tag7 and TagL, respectively ([7-9]). PGRP-S/Tag7 and their ovine ortholog OBP are secreted proteins that accumulate in PMN granules and exhibit bactericidal activity *in vitro* and *in vivo* ([10, 11]). In contrast to PGRP-S/Tag7, PGRP-L/TagL has a membrane-spanning domain and is found intracellular and cytoplasmic membrane-bound [9, 12, 14]. PGRP-L/TagL is the only mammalian PGRP that demonstrates N-acetylmuramoyl-L-alanine amidase activity [12, 13]. However, it does not exhibit bacteriostatic or bactericidal activity *in vitro* [12, 13]. Maximal expression of PGRP-L/TagL was detected in liver and fetal liver of both humans and mice [8, 15]. Less abundant expression was observed in thymus, spleen and intestine. Expression of both PGRP-S/Tag7 and PGRP-L/TagL in intestine was shown to be specific for the follicle-associated epithelium (FAE) of Peyer's Patches [14]. PGRP-S/Tag7

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2 expression is co-localized strictly with specialized M cells, which are involved into antigen  
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4 sampling and delivering to underlying lymphoid tissues, whereas PGRP-L/TagL is expressed by  
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6 non-transcytosing FAE cells [14].  
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9 A number of enteroinvasive pathogens use Peyer's Patches to cross through an intestine  
10 mucosal barrier [16, 17]. Some pathogens like *Shigella* or *Yersinia* are unable to pass a brush  
11 border of columnar epithelial cells and exploits M cell transport pathway to be translocated while  
12 others like *Listeria monocytogenes* are able to penetrate both M cells and enterocytes. We  
13 hypothesized that PGRP-S/Tag7 and PGRP-L/TagL might be involved into immune defense  
14 against enteroinvasive pathogens. PGRP-L/TagL was shown to be expressed by non-  
15 transcytosing cells [14], and its effect might be directed against pathogens able to invade this  
16 type of cells.  
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28 Gram-positive bacterium *L. monocytogenes*, which is a food-borne pathogen able to  
29 invade different cell types including epithelial and endothelial cells, and hepatocytes, well suited  
30 as a model to study interactions of enteroinvasive pathogens with TagL-expressing non-  
31 professional phagocytes. *L. monocytogenes* enters the host through intestinal epithelium and is  
32 able to penetrate the apical surface of polarized colonic epithelial cells with a formed brush  
33 border [18, 19]. Upon completion of phagocytosis *L. monocytogenes* disrupts the phagosome and  
34 enters the cytoplasm where it multiplies (for review see [18, 20]). Further dissemination of the  
35 bacterium is due to its ability to move actively through the cytoplasm and to spread from cell to  
36 cell by forming protrusions in the cytoplasmic membrane. Relatively low number of bacterial  
37 factors are involved into the process of intracellular infection that facilitates studying of its  
38 different stages [18, 20]. Active invasion into non-professional phagocytes requires presence of a  
39 set of surface bacterial proteins which composition depends on a cell type. InlA and InlB that  
40 belong to the internalin family and actin-polymerizing factor ActA are the most important for  
41 invasion of epithelial cells [20, 21]. The phagosome disruption is a critical point in the infection  
42 process as a phagosome-entrapped bacterium fails to multiply [21]. The main role in the  
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2 phagosome lysis plays listeriolysin O (LLO), which is assisted by two phospholipases,  
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4 phosphatidyl-inositol specific (PI-PLC) and of broad specificity (PC-PLC). ActA protein is  
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6 absolutely necessary for intracellular movement and cell-to-cell spread. To get evidences on an  
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8 effect exerted by TagL on enteroinvasive pathogens we stably expressed TagL in enterocyte-like  
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10 HT29 cells [22] and studied invasion and early growth of *L. monocytogenes* strains defected in a  
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12 number of important virulence factors.  
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## 18 **2. Materials and methods**

### 19 *2.1. Bacterial strains and growth conditions*

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22 Wild type *L. monocytogenes* strains EGDe (serogroup 1/2a) [23] and P14 (serogroup 4b)  
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24 [24] were used. EGDe $\Delta$ *prfA*, EGDe $\Delta$ *hly* and EGDe $\Delta$ *actA* were genetically modified derivatives  
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26 of the EGDe strain that carried the in-frame deletion of the corresponding genes. The P14A  
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28 strain [24] was a spontaneous derivative of P14 carrying a Gly145Ser substitution in the PrfA  
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30 regulator protein that resulted in PrfA activation and constant virulence gene expression (a PrfA\*  
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32 genotype). Bacteria were routinely cultivated in Brain Heart Infusion (BHI, Difco) broth or agar  
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34 medium at 37°C. Liquid culture was rotated at 185 rpm.  
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### 43 *2.2. HT29 cells stable transfection, maintaining, and analysis*

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45 HT29 cells cultivated in Dulbecco's modified Eagle's medium with 10% fetal bovine  
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47 serum (Life Technologies) were transfected with mammalian expression vector carrying tagL- $\alpha$  -  
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49 splice variant (AF149837) under control of the CMV (human cytomegalovirus) promoter.  
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51 Clones were selected using 400  $\mu$ g/ml G418 (Sigma) and subcloned. After TagL protein analysis  
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53 selected clones were maintained in the presence of 200  $\mu$ g/ml G418. Cellular lysates from G418  
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55 resistant clones were subjected to SDS-PAGE. Proteins were transferred to Hybond P  
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57 (Amersham Bioscience) and probed with rabbit polyclonal anti-TagL antibodies. For detection  
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2 secondary anti-rabbit peroxidase labeled IgG (Life Technologies) and enhanced  
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4 chemiluminescent substrate (Pierce) were used according to manufacturer recommendations.  
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### 9 2.3. *In vitro* invasion and proliferation assay

11 Invasion assay was performed in general as described in [21]. Bacteria from exponential  
12 cultures ( $OD_{600nm} \approx 1.5$ ) were kept frozen in 10% glycerin and thawed at the day of the  
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14 experiment. 0.5 ml bacterial suspension diluted in pre-warmed DMEM so that MOI was about  
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16 100 CFU/cell was added per well of a 24-well plate. After 1h incubation at 37°C cells were  
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18 extensively washed and fresh DMEM supplemented with  $100 \mu\text{ ml}^{-1}$  gentamicin was added to  
19  
20 kill extracellular bacteria. The number of successfully entered bacteria was assessed after 1 h  
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22 incubation with gentamicin by plating of serial dilutions of cell lysates obtained after 5'  
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24 incubation with 1% Triton X-100. The strain invasiveness was evaluated by a ratio of the  
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26 number of entered bacteria to the number of bacteria applied. To assess intracellular proliferation  
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28 the time of incubation with gentamicin was prolonged up to 4 h, and the effectiveness of  
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30 intracellular proliferation was evaluated by a ratio of the number of CFU at this time point to the  
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32 number of entered bacteria.  
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### 42 2.4. *Statistics*

44 Student's unpaired t-test included in the Excel software packet was used and P-values of  
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46  $<0.05$  were considered statistically significant.  
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## 51 3. Results.

### 52 3.1. *Stable expression of TagL in the epithelial HT29 cells*

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54 Human colon adenocarcinoma derived HT29 cells were used to stably express TagL.  
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56 These cells undergo complete enterocytic differentiation in conditions of glucose starvation [22].  
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58 In conditions used they are similar to non-maturated enterocytes. The wild-type HT29 cells do  
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2 not produce noticeable amounts of TagL (Fig. 1) that is consistent with FAE specific TagL  
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4 expression [14].  
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7 Previously it has been shown that the TagL-specific mRNA has 7 splice variants that  
8  
9 differ in their C-terminal parts [9]. All TagL isoforms have an N-terminal signal peptide and a  
10  
11 putative trans-membrane domain but four of them lack the C-terminal T7 lysozyme-like domain.  
12  
13 As soon as a role of this domain might be of importance in interactions with bacterial pathogens  
14  
15 we used the splice variant of maximal length ( $\alpha$  variant) to stably transfect HT29 cells. TagL  
16  
17 expression in transfected cells was checked by Western blotting with TagL-specific polyclonal  
18  
19 antibodies raised against the internal part of the protein [9] (Fig.1). The high level TagL  
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21 expression in transfected cells was maintained by propagation under selective pressure (see  
22  
23 Materials and Methods). The selective antibiotic was removed from the culture medium three  
24  
25 days before infection with *L. monocytogenes*. Prolonged removal of the selective pressure  
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27 returned cells to the wild type phenotype in terms of results described below (data not shown).  
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### 35 3.2. TagL expression impairs *L. monocytogenes* invasion and early growth in epithelial cells

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37 The wild type *L. monocytogenes* strain EGDe [18, 23] (a type strain originally isolated  
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39 from laboratory animals with septicemia) was used to infect the HT29/TagL and the control  
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41 wild-type and mock-transfected HT29 cells. The multiplicity of infection (MOI) was 100:1  
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43 (bacteria to an eukaryotic cell) for all cell types. At the 0 time point gentamicin was introduced  
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45 into the medium to kill extracellular bacteria (see Materials and Methods). The invasiveness was  
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47 evaluated by the ratio of the number of intracellular bacteria after 1 h incubation with gentamicin  
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49 to the initial number of applied bacteria. Little or no difference was observed for the entry into  
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51 the wild type and mock-transfected HT29 cells (Fig. 2). In contrary, expression of TagL  
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53 significantly impaired the invasion efficiency. The number of bacteria successfully invaded  
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55 TagL-expressing cells was about 20-fold less than in control. A similar effect was observed for  
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57 the strain P14 (data not shown).  
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To evaluate a TagL effect on *L. monocytogenes* intracellular proliferation we counted the number of intracellular bacteria after 4 h incubation with gentamicin. This time was demonstrated to be sufficient for phagosome disruption, cytoplasm entrance and performance of one to three rounds of multiplication [21, 25, 26]. Between 1 h and 4 h of gentamicin incubation the number of intracellular bacteria increased 5-fold in control HT29 cells and 2.5-fold in HT29-TagL cells (Fig. 2 B). Thus, expression of TagL impaired both invasion and intracellular bacterial multiplication although the effect on invasion seemed to be much more prominent.

### 3.3. TagL diminishes viability of phagosomally entrapped bacteria

Obtained results suggested that bacteria were subjected to TagL-dependent effects during invasion/phagosome escaping. One possible mechanism of the TagL-dependent drop in the number of successfully entered bacteria might be a bactericidal effect on intraphagosomal bacteria. To test this we studied intraphagosomal survival of the *L. monocytogenes* EGDe  $\Delta hly$  strain, which was incapable to escape from the phagosome due to the absence of phagosome-disrupting factor LLO. TagL expression resulted in the 3-fold decrease in the number of alive intraphagosomal EGDe  $\Delta hly$  bacteria after 4-h gentamicin incubation (Fig. 3). In the control HT29 cells the number of intracellular EGDe $\Delta hly$  bacteria was stable and slightly increased during this period.

The observed increase might result from multiplication of the low percent of bacteria, which escaped from the phagosome due to membrano-lytic activity of phospholipases PI-PLC and PC-PLC [27]. To avoid this mechanism of phagosome escaping we studied the *L. monocytogenes* EGDe  $\Delta prfA$  strain. This strain produces LLO, PI-PLC and PC-PLC in extremely low amounts due to the deletion of the *prfA* gene coded for the transcriptional activator PrfA, which is required for expression of these factors [18, 23, 24]. Indeed, in the control cells 4-hour incubation did not lead to a noticeable alteration in the number of intracellular bacteria (Fig.3). In contrary, TagL expression coincided with a significant decrease

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2 in the number of alive bacteria. These results supported the assumption that intraphagosomal  
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4 bacteria lost viability in TagL-expressing cells.  
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### 9 3.4. The TagL effect on invasion correlates with efficiency of phagosome escaping

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11 Besides its effect on intraphagosomal viability TagL significantly impaired invasiveness  
12 of all strains tested. To analyze a correlation between bacterial abilities to escape from the  
13 phagosome and to invade TagL expressing cells we present the data on TagL relative inhibitory  
14 effect (Fig. 4). A ratio of coefficients of invasion into HT29 and HT29-TagL cells was calculated  
15 for every *L. monocytogenes* strain tested. This ratio for the wild type strain was taken as 100%  
16 and relative values for other strains were calculated. TagL relative inhibitory effect on invasion  
17 of EGDe  $\Delta hly$  was noticeably greater 100% that indicated an advanced reduction in invasion into  
18 HT29-TagL cells (Fig.4). These results suggested a reverse correlation between the TagL  
19 inhibitory effect and the bacterial ability to escape from the phagosome.  
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33 To further address this correlation we studied the TagL effect on invasion of strains with  
34 mutations in the transcriptional regulator PrfA. Two strains were studied: the described above  
35 strain EGDe  $\Delta prfA$ , and P14A that carried highly active PrfA (PrfA\* phenotype)[24]. While the  
36 EGDe  $\Delta prfA$  strain was defected in production of phagosome-destructive factors, the P14A  
37 constantly overproduced them [24]. A TagL relative inhibitory effect for EGDe  $\Delta prfA$  strain was  
38 the most considerable among all strains tested (Fig. 4). For P14A (prfA\*) the TagL relative  
39 inhibitory effect was about two times less than for the isogenic wild type strain P14 (Fig. 4).  
40 These data are consistent with the assumption that the TagL inhibitory effect reversibly  
41 correlates with efficiency of phagosome escaping.  
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54 On the other hand both EGDe  $\Delta prfA$  and P14A strains differed from the wild type in  
55 invasion into the wild type HT29 cells (Table 1). This seems to be due to altered expression of  
56 PrfA-regulated factors Internalin A, InlB and ActA that mediate active invasion into enterocytes  
57 [17, 21]. Changes in expression of these factors as well might affect interactions of entering  
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2 bacteria with TagL-expressing cells. Expression of Internalin A and InlB is only partly  
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4 controlled by PrfA while the *actA* gene is under a very strict PrfA control [18]. If the EGDe  
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6  $\Delta prfA$  strain was impaired in invasion because of defects in ActA expression we could expect a  
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8 similar inhibitory effect for the EGDe  $\Delta actA$  strain. However, TagL expression affected EGDe  
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10  $\Delta actA$  less than EGDe  $\Delta prfA$  and less than the isogenic wild type EGDe strain (Fig.4). Invasion  
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12 of EGDe  $\Delta actA$  into wild type HT29 cells was about 25 % of the wild type strain (Table 1).  
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16 Invasion efficiencies into TagL-expressing cells were similar for wild type and  $\Delta actA$  strains  
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18 (data not shown). Obtained results suggested that the increased TagL inhibitory effect on EGDe  
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20  $\Delta prfA$  was not due to defects in ActA expression and supported the assumption that poor  
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22 effectiveness of phagosome escaping was responsible for it.  
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#### 28 4. Discussion

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30 In this work we addressed the effect that the mammalian peptidoglycan recognition  
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32 protein TagL exerted on *L. monocytogenes* invasion and early growth in epithelial cells. TagL  
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34 expression correlated with a significant decrease in bacterial invasion and reduction of  
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36 intracellular proliferation rates. The most prominent effect TagL exerted on bacterial invasion.  
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40 One could imagine several ways how TagL influenced invasion, among them a loss of  
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42 viability by bacteria during invasion/phagosome escaping and interruption of signaling pathways  
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44 that result in bacterial uptake. We presented here evidences on a TagL associated drop in  
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46 bacterial intraphagosomal viability. TagL and its human ortholog PGRP-L were shown to  
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48 possess *N*-acetylmuramoyl-L-alanine amidase enzymatic activity [12, 13]. Although no a  
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50 bactericidal or bacteriostatic effect has been demonstrated for purified proteins *in vitro* the TagL  
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52 cell wall lytic effect might be harmful for a bacterium entrapped in the phagosome. *L.*  
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54 *monocytogenes* induced phagocytosis does not involve active interruption of the phagosome  
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56 maturation process at early stages [18]. Electron-microscopic study of *L. monocytogenes*  
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58 invasion into bone-marrow-derived mouse macrophages demonstrated that about 80% of  
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2 intraphagosomal bacteria had got injuries noticeable by microphotography in 2 hours after the  
3 start of the experiment [28]. This time corresponds to the time point used in this work to evaluate  
4 an invasion efficiency after gentamicin killing of extracellular bacteria. Although the degree of  
5 injuries might be different in epithelial cells than in macrophages it seems possible that TagL  
6 enhanced the bactericidal effect of intraphagosomal environment, and the TagL amidase activity  
7 non-bactericidal in vitro might be responsible for diminishing of viability of phagosomally  
8 entrapped bacteria. Thus, the observed decrease in invasion efficiency might be at least partly  
9 explained by a direct or indirect bactericidal effect of TagL on bacteria during  
10 invasion/phagosome escaping.  
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23 Injuries received by intraphagosomal bacteria in TagL expressing cells might slow  
24 phagosome escaping or influence the bacterial ability to multiply in the host cytoplasm. Both of  
25 these reasons could lead to the observed two-fold reduction in intracellular proliferation rates  
26 (Fig. 2B). The opposite assumption that TagL could exert the bactericidal effect on cytoplasmic  
27 bacteria seems less feasible. Indeed, previous research demonstrated TagL prevalence on  
28 intracellular and surface membranes rather than in the cytoplasm [14, 15]. In this way, TagL  
29 interactions with a bacterium would preferably occur inside the phagosome where a bacterium as  
30 in the tight contact with membranes than in the cytoplasm where a bacterium was free.  
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42 On the other hand, the TagL repressive effect on invasion might be caused by reasons not  
43 coupled with bacterial viability. Cytoplasmic membrane bound TagL might destabilize contacts  
44 between bacterial surface proteins and eukaryotic receptors that impaired bacterial adhesion.  
45 Alternatively, TagL expression might influence cellular processes that resulted in bacterial  
46 uptake. If TagL impaired *L. monocytogenes* adhesion or invasion defects caused by the absence  
47 of bacterial factors would be suppressed totally or partly in TagL-expressing cells. This is that  
48 we observed: TagL expression partially suppressed invasion defect caused by the absence of  
49 ActA, the *L. monocytogenes* surface protein involved both into adhesion and active invasion [21,  
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29]. These data supported the assumption that TagL might influence bacterial adhesion or uptake although did not allow choosing between these effects.

Recently it has been shown that FAE cells not involved into antigen capturing and delivering express PGRP-L/TagL while M-cells do not express it [14]. The results presented here suggested a possible role for TagL in antibacterial defense against enteroinvasive pathogens to prevent pathogenic bacteria from entering into non-specialized cells.

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20 mammals and *Drosophila*, can recognize gram-positive and gram-negative bacterial cell wall  
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**Table 1.** Invasion efficiency of *L. monocytogenes* strains

Strain	Coefficient of invasion into HT29 wild type cells	Genotype
P14	0,0077±0,0011	wild type
P14A	0,014±0,004	<i>prfA</i> *
EGDe	0,0070±0,0007	wild type
EGDe $\Delta$ <i>hly</i>	0,0067±0,0009	in-frame deletion in <i>hly</i>
EGDe $\Delta$ <i>prfA</i>	0,0011±0,0003	in-frame deletion in <i>prfA</i>
EGDe $\Delta$ <i>actA</i>	0,0018±0,0009	in-frame deletion in <i>actA</i>

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## Figure legends

**Fig. 1.** TagL expression in stably transfected HT29 cells.

Wild type and *tagL*  $\alpha$ -splice variant transfected HT29 cell extracts were separated on 10% SDS-PAGE, transferred onto nitrocellulose membrane and probed with anti-TagL antibodies (see text and Materials and Methods for details).

**Fig. 2.** The effect of TagL on *L. monocytogenes* invasion and early growth.

A. *L. monocytogenes* EGDe strain invasion into wild type, TagL- and mock-transfected HT29 cells. Invasion coefficient is a ratio of the number of entered bacteria to the number of bacteria used for infection. B. Intracellular proliferation is shown by relative numbers of alive intracellular bacteria in HT29 and HT29-TagL cells (see text and Materials and Methods for details). The number of entered bacteria is taken as 100% for each cell line. The data present the mean values and SE of at least two independent experiments made in duplicate. The asterisk indicates the statistically significant difference ( $P < 0.05$ ).

**Fig. 3.** The effect of TagL on intracellular viability of *L. monocytogenes* strains defected in phagosome escaping.

EGDe $\Delta hly$  and EGDe $\Delta prfA$  strains were used to infect HT29 (white columns) and HT29-TagL (black columns) cells. The number of viable intracellular bacteria after prolonged (4h) incubation is shown as a percent of the number of entered bacteria (taken as 100 % for each cell line and each strain and presented by dotted line). The data presents the mean values and SE of two independent experiments made in duplicate.

**Fig. 4.** TagL relative inhibitory effect on invasion of *L. monocytogenes* strains. The number was taken as 100 % that matched the ratio of invasion coefficients into HT29 and HT29-TagL cells for a *L. monocytogenes* wild type strain (the first column shows the data for the EGDe strain, and

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2 for another wild type strain P14 the results were similar). For derivative strains EGD $\Delta$ *hly*,  
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4 EGD $\Delta$ *prfA*, EGD $\Delta$ *actA* and P14A (*prfA*\*) the ratios of invasion coefficients into HT29 and  
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6 HT29-TagL cells are shown as percentage of the ratio for a corresponding wild type bacterial  
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8 strain. The data from at least two independent experiments made in duplicate are shown.  
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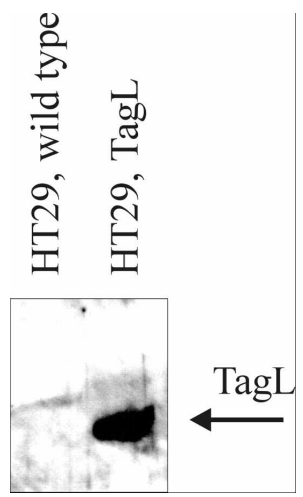
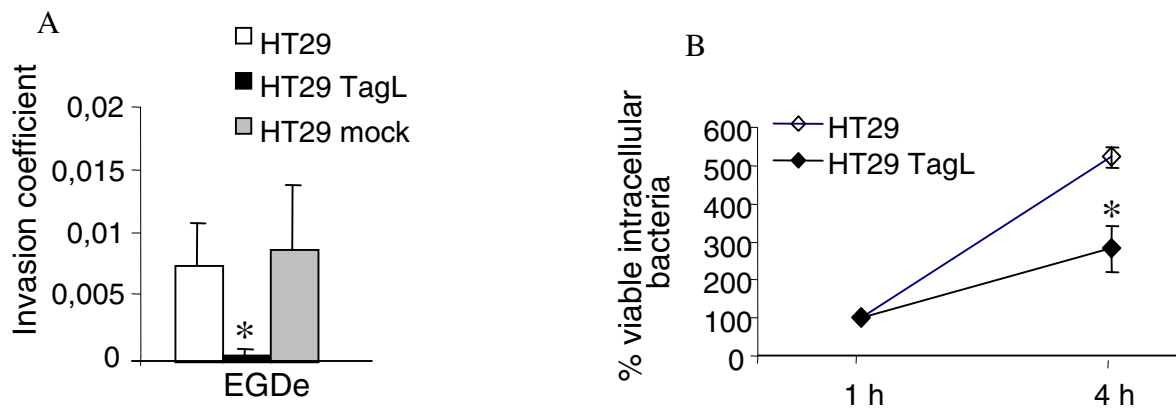


Fig.1

**Fig.2**

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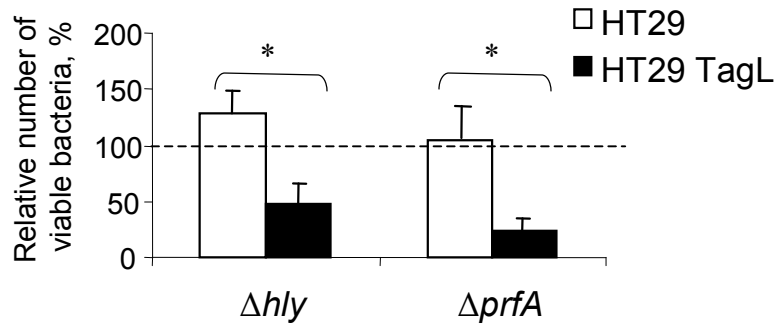
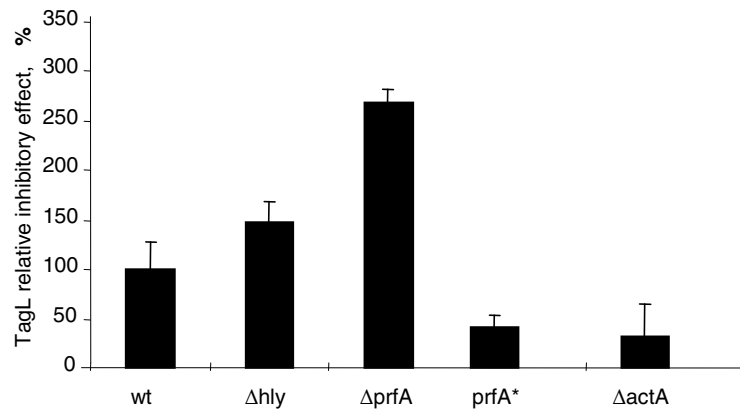


Fig.3

**Fig.4**