Veterinary Immunology and Immunopathology 196 (2018) 1–4

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Impact of *fliD* and virulence plasmid pSEV on response of chicken embryo fibroblasts to *Salmonella* Enteritidis

Ama Szmolka[a,*], Marta Elsheimer Matulova[b], Ivan Rychlik[b]

[a] Institute for Veterinary Medical Research, Centre for Agricultural Research, Hungarian Academy of Sciences, Hungária krt. 21, 1143 Budapest, Hungary
[b] Veterinary Research Institute, Hudcova 70, 621 00, Brno, Czech Republic

ARTICLE INFO

Keywords:
CEF gene expression
Salmonella virulence
fliD
pSEV
G0S2
ENO2

ABSTRACT

Salmonella Enteritidis is the main serovar of poultry origin in humans, but its complex interaction with certain avian cells is still not fully understood. Previously we identified several genes significantly induced in chicken embryo fibroblasts (CEFs) by the wild-type strain *S*. Enteritidis 11 (SE 11). In the present study, we raised the question whether virulence-attenuated mutants of this strain would induce altered expression of the newly identified fibroblast genes associated with immune and non-immune functions of CEFs. Gene expression was evaluated by real-time PCR following challenge by the parental strain SE 11 and its virulence attenuated mutants lacking flagellin gene *fliD* only or *fliD* and the serovar-specific virulence plasmid pSEV. As a result, deletion mutants induced a lower expression of all immune genes, but an increased expression of the non-immune genes G0S2 and ENO2 relative to the parental strain. Our data indicate the importance of flagella and pSEV in modulation of virulence and host response in this model. We demonstrated, for the first time ever, an increased induction of survival genes G0S2 and ENO2 by virulence-attenuated mutants of *S*. Enteritidis.

1. Introduction

Salmonellosis remains the second most common zoonosis in the EU and *S*. Enteritidis remains the most important serovar in confirmed human non-typhoid salmonellosis. The primary sources of food-borne *Salmonella* Enteritidis outbreaks are poultry and poultry products, mostly eggs (EFSA and ECDC, 2015).

Interaction between non-typhoid *Salmonella* and its host represents one of the central topics in this field. The flagella and the virulence plasmid pSEV of *S*. Enteritidis are among the most important factors influencing *Salmonella* virulence and pathogen recognition as well as the activation of the cascade of host defense mechanisms (Guiney and Fierer, 2011; Wiedemann et al., 2014). Their absence causes reduced invasion and diminished survival in the intracellular environment (Bäumler et al., 1994; Imre et al., 2015) which is what makes such mutants potential vaccine strains. For example, the non-flagellated, virulence plasmid-cured (*fliD*–, ΔpSEV) mutant of *S*. Enteritidis 11 (Imre et al., 2006; Imre et al., 2011) protected chickens against organ invasion, caecal colonisation and faecal shedding by the highly virulent challenge strain *S*. Enteritidis 147 (Imre et al., 2015).

Recent genome-wide technologies enabled the identification of gene expression changes in the chicken caecum, spleen and in professional immune cells (macrophages, heterophils, lymphocytes) in response to *Salmonella* infection (Matulova et al., 2012; Rychlik et al., 2014). Similarly to avian macrophages and epithelial cells, chicken embryo fibroblasts (CEFs) can be efficiently invaded by non-typhoid *Salmonella* serovars (Barrow and Lovell, 1989; Setta et al., 2012; Imre et al., 2015), however the importance of virulence genes in the modulation of the interaction between *S*. Enteritidis and the non-professional immune cells such as CEFs remains unclear.

Fibroblasts play an important role in structural support of the intestinal tissues. They are also considered as sentinel cells inducing immune response against pathogens by production of antimicrobial peptides, proinflammatory cytokines, chemokines and growth factors (Bautista-Hernández et al., 2017). Due to potential immunological functions and the dominance of fibroblasts in the caecal tissue, in our earlier study we performed genome-wide analysis of this cell type response to *Salmonella* infection (Szmolka et al., 2015). In that study, we reported that infection of CEFs with two wild-type strains of *S*. Enteritidis of differing virulence, resulted in a significant upregulation of 19 genes, majority of them involved in immune response, including inflammatory cytokines (IL1β, IL8, IL6) chemokines (CCL17, CCL20) and regulatory factors (NFκBIα, NFκBIζ, IRF1). We also described that CEFs contribute to the overall response to *S*. Enteritidis infection with the induction of a number of metabolic genes such as LOC101750351, BU460569, MOBKL2C, ENO2 (Szmolka et al., 2015). Furthermore, that

* Corresponding author.
E-mail address: szmolka.annamaria@agrar.mta.hu (A. Szmolka).

https://doi.org/10.1016/j.vetimm.2017.12.003
Received 19 June 2017; Received in revised form 8 November 2017; Accepted 5 December 2017

Gene/Locus	Description
IL1β	interleukin 1, beta
CSF3 (cMGF)	colony stimulating factor 3 (granulocyte)
IL8L2 (EMF-1)	interleukin 8-like 2
IL8L1 (K60)	interleukin 8-like 1
LOC422654	similar to K60 protein (chemokine CXC ligand 1-like)
SCYA4 (MIP-1β)	C-C motif chemokine ligand 4
IL6	interleukin 6 (interferon, beta 2)
NFκBIα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IRF1	interferon regulatory factor 1
TNIP2	TNFAIP3 interacting protein 2
NFκBIζ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta
CCL20 (MIP-3α)	C-C motif chemokine ligand 20
CCL17	C-C motif chemokine ligand 17
G0S2	G0/G1 switch 2
ENO2	enolase 2 (gamma, neuronal)
LOC101750351	uncharacterized LOC101750351
K123	K123 protein
MOBKL2C	MOB1, Mps One Binder kinase activator-like 2C
BU460569	uncharacterized

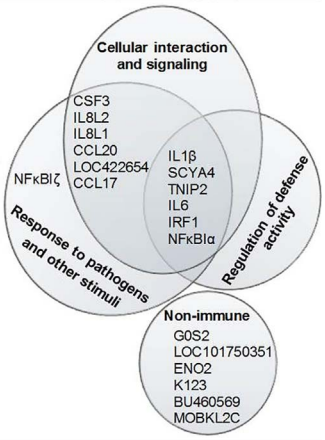

Fig. 1. List and functional classification of the tested chicken genes. Functional classification of the genes is based on results of an earlier report of the authors (Szmolka et al., 2015).

study highlighted that the G0S2 gene, with a potential role in lipid metabolism and survival was more induced by the less virulent strain *S.* Enteritidis 11 (SE 11) in CEFs (Szmolka et al., 2015).

This observation prompted the question whether virulence attenuated mutants of the strain SE 11 lacking the gene *fliD* only or *fliD* and the virulence plasmid pSEV would also be able to upregulate the expression of the same set of genes in CEFs. As a result it was revealed that infection by the deletion mutants of SE 11 characterized by a reduced invasiveness, lead to a moderate induction of immune genes, but to an increased expression of survival genes G0S2 and ENO2, indicating the importance of flagella and of virulence plasmid pSEV as modulators of virulence and of CEF response. We believe that such observations would help to understand the complexity of cross talks between *S.* Enteritidis and non-professional immune cells such as CEFs.

2. Materials and methods

2.1. Bacterial strains

Wild-type strain SE 11 of poultry origin and its virulence-attenuated mutants SE 2102 and SE Δ155 were used in this study. SE 2102 is a non-motile, FliD negative (*fliD*:pFOL1069) mutant of SE 11, containing a 55 kb virulence plasmid pSEV, while SE Δ155 is a non-motile virulence plasmid-cured (*fliD*-, ΔpSEV) derivative of SE 2102 (Imre et al., 2006; Imre et al., 2011). Both mutants of SE 11 have earlier been proven to be significantly ($P < 0.005$) less invasive than the wild-type parental strain in CEF cultures. SE Δ155 was the least virulent mutant but without significant difference from SE 2102 in terms of cell invasion (Imre et al., 2015). Bacteria were grown in Tryptic Soy Broth (TSB, Sigma-Aldrich) for 16 h at 37 °C prior the infection of CEFs.

2.2. Salmonella infection of chicken embryo fibroblasts

Preparation, maintenance and handling of CEFs used for infection have been described by Szmolka et al. (2015). The day before infection, CEFs were seeded into 36 mm Petri dishes (Nunc) and grown for 18 h at 37 °C under 5% CO_2. The purity of the cell population was tested by stereo microscopy, showing that fibroblast cells formed ~90% of the isolated cells. Infection of CEFs was performed on the second day of their growth as described previously (Szmolka et al., 2015). CEFs were infected for 4 h at 37 °C and 5% CO_2 with overnight bacterial cultures at a multiplicity of infection (MOI) equal to 10. After the incubation, CEFs were washed three times with HBSS and lysed directly in the cell-culture vessels by adding 600 µl RLT buffer from the RNeasy Mini Kit (Qiagen). Infection with each of the strains was performed in four replicates.

2.3. Gene expression analysis by real-time PCR

In the present study we have focused on the expression of 19 chicken genes that we have previously found to be significantly upregulated by the infection with wild-type SE 11, based on the Agilent chicken custom 8 × 15 K microarray results confirmed by real-time PCR (Szmolka et al., 2015). For this, 10 ng of total fibroblast RNA was reverse-transcribed into cDNA using an iScript™ cDNA Synthesis Kit (Bio-Rad). The real-time PCR reaction was performed in 3 µl volumes using a LightCycler II (Roche), with an initial denaturation at 95 °C for 15 min followed by 40 cycles of 95 °C for 20 s, 60 °C for 30 s and 72 °C for 30 s. The average Ct value of three housekeeping genes (glyceraldehyde-3-phosphate dehydrogenase, TATA-binding protein and ubiquitin) were used for normalization. Sequences of the real-time PCR primers have been reported previously (Szmolka et al., 2015). Each sample was subjected to real-time PCR in triplicate. The relative expression of each gene of interest was calculated as $2^{-\Delta Ct}$. Finally, fold inductions between the infected and non-infected CEF groups were calculated. Expression levels were analysed using the Studenťs *t*-test.

3. Results and discussion

The tested chicken genes have been selected as being significantly induced in CEFs after the infection with wild-type strain *S.* Enteritidis 11 (Szmolka et al., 2015). In this study we found that CEFs respond to infection by this strain with an upregulation of 13 “immune” genes and 6 “non-immune” genes after 4 h of stimuli. Immune genes were considered all those genes that play role in 1) Response to pathogens and other stimuli 2) Regulation of defense activity or 3) Cellular interaction and signaling based on Gene Ontology (GO) classification of biological processes. Genes that were not assigned to any of these clusters were considered as non-immune genes (Fig. 1).

Using the same experimental setting as before (Szmolka et al., 2015) here we report that CEF genes responsive to the infection with the wild-type strain SE 11 were also inducible by its two virulence-attenuated mutants lacking *fliD* only (SE 2102) or *fliD* and the large serovar-specific virulence plasmid pSEV (SE Δ155). From the 13 immune genes tested (interleukins, chemokines, transcription factor genes) 12 were significantly ($P < 0.05$) upregulated in comparison to their expression in non-infected fibroblasts, but at a lower level than in CEFs infected with parental strain SE 11 (Fig. 2., Table S1). However this reduction was not statistically proven for cytokine genes IL1β and CCL20. Upregulation of gene CCL17 was not confirmed as significant when CEFs were infected with any of the mutant strains and therefore expression of this gene is not presented in Fig. 2.

The absence of pSEV in the double deletion mutant SE Δ155 did not further reduce expression of the immune related genes (Fig. 2, Table