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Epithelial cell invasion by *salmonella* typhimurium induces modulation of genes controlled by aryl hydrocarbon receptor signaling and involved in extracellular matrix biogenesis

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ABSTRACT

Salmonella is the only bacterium able to enter a host cell by the two known mechanisms: trigger and zipper. The trigger mechanism relies on the injection of bacterial effectors into the host cell through the *Salmonella* type III secretion system 1. In the zipper mechanism, mediated by the invasins Rck and PagN, the bacterium takes advantage of a cellular receptor for invasion. This study describes the transcriptomic reprogramming of the IEC-6 intestinal epithelial cell line to *Salmonella* Typhimurium strains that invaded cells by a trigger, a zipper, or both mechanisms. Using *S*. Typhimurium strains invalidated for one or other entry mechanism, we have shown that IEC-6 cells could support both entries. Comparison of the gene expression profiles of exposed cells showed that irrespective of the mechanism used for entry, the transcriptomic reprogramming of the cell was nearly the same. On the other hand, when gene expression was compared between cells unexposed or exposed to the bacterium, the transcriptomic reprogramming of exposed cells was significantly different. It is particularly interesting to note the modulation of expression of numerous target genes of the aryl hydrocarbon receptor showing that this transcription factor was activated by *S*. Typhimurium infection. Numerous genes associated with the extracellular matrix were also modified. This was confirmed at the protein level by western-blotting showing a dramatic modification in some extracellular matrix proteins. Analysis of a selected set of modulated genes showed that the expression of the majority of these genes was modulated during the intracellular life of *S*. Typhimurium.

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Salmonella Typhimurium; gene expression; extracellular matrix; aryl hydrocarbon receptor; cell invasion

Introduction

In salmonellosis, a critical step in pathogenesis is the ability of *Salmonella* to adhere to and penetrate host cells. The infection process can be divided into four phases: approach, attachment, entry, and intracellular life. To begin its infection cycle, *Salmonella* needs to reach the cell; the flagellum, a long helical structure, provides the bacterium with a driving force that allows its movement across the extracellular space [1]. Thanks to several adhesins, *Salmonella* can interact with cellular receptors and with components of the extra cellular matrix (ECM). Numerous pathogens exploit the ECM to reach their target cell. For instance, to be limited to *Salmonella*, FimH is recognized by human granule membrane glycoprotein (GP2) expressed at the apical pole of M cells [2] while in pigs, FimH can bind the calreticulin [3]. The Lewis X blood group has been shown to be a receptor for PefA [4]; MisL [5], and SdhA [6] binds the fibronectin, and SiiE uses the transmembrane mucin to promote its entry [7]. Even if it has been clearly established that disruption of the ECM is essential to *Salmonella* entry [8], few data describe the modification induced on ECM after entry.

In non-phagocytic cells, two conceptual frameworks describe the entry of bacteria [9]: the trigger and the zipper entry modes. In *Salmonella*, two major differences characterize these mechanisms. While the trigger mechanism relies on the type III secretion system 1 (T3SS1) that injects effectors directly into host cell, the zipper mechanism depends on the interaction between a bacterial invasion factor and a cellular receptor, which transduces the internalization signal. In addition, while there is cytoskeleton remodeling leading to entry in both mechanisms, the perturbation of the membrane is less pronounced in the zipper mechanism.

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Table 1. Characteristics and designation of the strains used in this study.

Strains	Designation used in the article	References
Salmonella Typhimurium 14028	WT	ATCC
Salmonella Typhimurium 14028 express *rck* and *pagN* in culture	STM- Z T	This study
Salmonella Typhimurium 14028 express *rck* and *pagN* in culture and invalidated for *invA*	STM- Z	This study
Salmonella Typhimurium 14028 invalidated for *rck*, and *pagN*	STM- T	This study
Salmonella Typhimurium 14028 invalidated for invA, *rck*, and *pagN*	STM-3d	Roche et al 2018

However, it has been shown that the T3SS1 can induce both large membrane ruffles and discrete ones [10]. The *Salmonella* pathogenicity island 1 (SPI1) encodes the majority of the components necessary to build a functional T3SS1 that, from a functional point of view, looks like a syringe. The two invasins PagN [11] and Rck [12] are able to induce invasion through a zipper mechanism. The cellular receptor for PagN has not been clearly identified. However, it has been shown that PagN binds a heparinated proteoglycan to promote entry into the cell [13]. The Rck receptor has been clearly identified as the epidermal growth factor receptor [14]. However, the role of Rck and PagN in the pathogenicity of *Salmonella* remains somewhat controversial even though few articles have strongly suggested an *in vivo* role of the invasines PagN [15] and Rck [16].

A large number of studies have established that *in vitro* and *in vivo* infection induces a vast reprogramming of gene expression, particularly of those involved in the regulation of the inflammatory response [17]. As the trigger and the zipper mechanisms are based on very different types of interactions with the host cell, the question arose as to how the cell responds to these two entry routes. We analyzed gene expression in the rat epithelial intestinal cell-line IEC-6, which was unexposed or exposed to: 1/*S.* Typhimurium (STM) expressing constitutively the three invasion factors T3SS1, Rck, and PagN, 2/a STM mutant invalidated for the T3SS1 and expressing constitutively the invasins Rck and PagN, and 3/the STM Rck, PagN double mutant with a functional T3SS1. These three strains can enter the IEC-6 cells through, respectively: 1/both a trigger and a zipper entry, 2/a zipper entry and 3/a trigger entry. For simplicity, hereafter the *S.* Typhimurium strains will be, respectively, denoted as STM- Z T, STM- Z, STM- T, and STM-3d, which expressed none of these entry factors.

Materials and methods

Bacterial strains and plasmids

To study *in-vitro* the influence of the zipper entry on cellular gene expression, from the wild type *S.* Typhimurium (STM ATCC14028) we derived the strain STM- Z invalidated for the T3SS1 by invA deletion and constitutively overexpressing Rck and PagN on a recombinant plasmid (pSUP202:rck-pagN) and strain STM- T invalidated for *rck* and *pagN* that expressed the T3SS1 under our growing conditions. In order to compare isogenic strains, we also derived the STM-Z T strain that is the wild type STM ATCC14028^{TM} strain that expresses the T3SS1 genes under our growing conditions and constitutively overexpresses Rck and PagN on a recombinant plasmid (pSUP202:rck-pagN). As a negative control, we used the wild-type strain deleted of *invA*, *rck* and *pagN* named STM-3d [18]. Characteristics of the strains used in this study are given in Table 1. Deletion of the *invA*, *rck* and *pagN* was performed according to the Datsenko and Wanner [19] method as described in [18]. To derive the STM-Z and STM- ZT strains, we constructed the plasmid pSUP202: *rck* - *pagN*. The plasmid pSUP202: *rck*, where rck is cloned into the cassette encoding tetracycline resistance [20], was used as the recipient plasmid. The plasmid pSUP202: *pagN*, containing the *pagN* gene, cloned into the cassette encoding chloramphenicol resistance [21] was used as the donor plasmid. These two plasmids were digested by the restricted enzymes *Eco*RI and *Nco*I. Restricted fragments were separated into agorose gel (0,7%) by electrophoresis. Fragments of interest were purified from the gel with a QIAquick® Gel Extraction Kit (Qiagen) according to the supplier's recommendations. The purified products were ligated and transformed into *E. coli* MC1061, verified by sequencing and transformed into desired STM.

Cell line

The intestinal epithelial cell-line IEC-6 (ATCC CRL-1592^{TM}) was grown in DMEM (Dulbecco's modified Eagle's medium with glucose 4.5 g/L) supplemented with 5% (v/v) fetal calf serum and 0.1 Unit/mL bovine insulin (Sigma). Cells were maintained in a humidified incubator (90% relative humidity) at 37°C under 5% (v/v) CO_2.

Invasion assays

At confluence, cells were exposed to a multiplicity of infection of 10 for the indicated time, followed by

a measurement of adhesion and a gentamicin protection assay to evaluate the invasion level. These were performed as described before [18]. In brief, the different stages of cell infection were analyzed with cells grown in 24-well tissue culture plates (Falcon) for 5 days to obtain subconfluent monolayers. The cell monolayers were incubated in a culture medium without antibiotics for 24 h, then infected for 1.5 h at 37◦C with 10^7 CFU in 300 µL of serum-free medium (multiplicity of infection = 10). For the adhesion assays, cell monolayers were gently washed six times with phosphate buffered saline and then disrupted with 1 mL of cold distilled water (4°C). Viable intra- and extracellular bacteria were determined on TSA (Tryptic Soy Agar-Difco). For invasion, after infection for 1.5 h at 37◦C, plates were washed with a cell culture medium and incubated in a medium containing 100 µg of gentamicin per mL. After 1.5 h at 37◦C, cells were washed and lysed with 1 mL cold distilled water. Viable intracellular bacteria were assessed by serial dilutions on TSA. Infection and a gentamicin protection assay were also performed in the presence of the drugs. For these assays, cells were pre-treated with chlorpromazine (Sigma) at 10 µg/mL and amiloride (Sigma) at 1 mM for 30 min in a culture medium. Viability of the bacteria was checked in the presence of the drugs. Six biological replicates were performed for unexposed IEC-6 and for cells treated with the drugs. For the microarray experiments, six biological replicates were performed for unexposed cells, for IEC-6 exposed to STM-Z or STM-ZT and five replicates for the infection with STM-T.

Extracellular matrix protein expression profile by western blotting

Protein expression profiling was carried out using extraction of Non-Infected cells (NI) and cells infected by STM-ZT, STM-Z, STM-T or STM-3d strains. Bacteria were deposited on IEC-6 with a multiplicity of infection of 10, for 1.5 h, followed by gentamicin (100 µg/ml) for 1.5 h. Cells were then resuspended in 100 µL of Laemmli buffer and denaturated 10 min at 100°C. Whole-cell protein samples 25 µL were run on SDS- PAGE (100 V) in a 4–15% Miniprotean TGX Precast Protein gels (Bio-Rad) in a Tris-glycine running buffer (25 mM Tris base, 192 mM glycine, 0.1% [wt/vol] SDS [pH 8.31]) and transferred onto a nitrocellulose membrane with Trans-blot Turbo transfer System (Bio-Rad) in Tris-glycine buffer system 15 min at 25 V and 2.5 mA. The blots were probed with the first antibody (1:1000), rabbit anti-laminin (Invitrogen), rabbit anti-fibronectin (Invitrogen) or mouse anti-tubulin overnight at 4°C and detected by chemiluminescence using goat anti-rabbit secondary antibody (1:25000) (Pierce) or goat anti-mouse secondary antibody (1:5000) (Dako) conjugated to HRP for 1 h. Proteins were revealed using the SuperSignal West Dura Extended Duration Substrate (Thermo Scientific).

RNA labelling and microarray processing

Transcriptional profiling was carried out using all of the 23 samples described above in the invasion assay paragraph. All steps were performed by the @BRIDGe core facility (INRAE Jouy-en-Josas, France, http://abridge.inra.fr). Cyanine-3 (Cy3) labeled cRNA was prepared using 100 ng of total RNA using the One-Color Low Input Quick Amp Labeling kit (Agilent Technologies, Santa Clara, CA, USA) following the recommended protocol. Specific activities and cRNA yields were determined using the NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA).

For each sample, 600 ng of Cy3-labeled cRNA (specific activity >6.0 pmol Cy3/µg of cRNA) were fragmented at 60°C for 30 min in a reaction volume of 25 µl containing 25× Agilent Fragmentation Buffer and 10× Agilent Blocking Agent, following the manufacturer's instructions. Subsequently, 25 µl of 2× Agilent Hybridization Buffer were added to the fragmentation mixture and hybridized to three SurePrint G3 Rat Gene Expression v2 8 × 60K Microarrays (Agilent Technologies, AMADID: 074036) for 17 h at 65°C in a rotating Agilent hybridization oven (Agilent Technologies). After hybridization, microarrays were washed for 1 min at room temperature with the GE Wash Buffer 1 (Agilent Technologies), for 1 min at 37°C using the GE Wash Buffer 2 (Agilent Technologies) and then dried immediately.

Immediately after washing, the slides were scanned using a G2565CA Scanner System (Agilent Technologies), which used a scan protocol with a resolution of 3 µm and a dynamic range of 20 bit. The resulting tiff images were analyzed with the Feature Extraction Software v10.7.3.1 (Agilent Technologies), using the GE1_107_Sep09 protocol. The microarray data were submitted to the GEO database and received the accession number GSE151881.

Statistical analyses

Microarrays

The expression of each probe was log2-transformed and normalized by median centering for each array after a filtering step. 58,777 probes were kept to differential analysis. A linear model was performed for each

probe with R package limma version 3.30.7 [22] to identify differentially expressed (DE) genes between the four biological conditions (IEC-6 cells non-exposed, IEC-6 cells exposed to STM- Z, STM- T, or STM- Z T). The model estimated the differences in expression as fold-change (FC) between two conditions by sharing information between samples. Significance of expression changes were determined using moderated t-statistics. The p-values were adjusted for multiple testing by the Benjamini-Hochberg method [23] to control the False Discovery Rate (FDR). Genes with an adjusted p-value below 0.05 were considered DE.

Cellular tests

A Mann Whitney test was performed to compare invasion levels between the different STM strains, using GraphPad Prism version 6.07 for Windows, GraphPad Software, La Jolla California USA, http://www.graphpad.com. Significance is * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

RNA extraction, reverse transcription, and purification of cDNA for qPCR analysis

RNA extraction was performed using the NucleoSpin RNA kit (Macherey Nagel, France). Two hundred ng of total RNA, 0.13 µg of Oligo-d(T_{20}) (Eurogentec) and 0.13 µg of random primer (Promega) were denatured at 75°C for 5 min then incubated on ice for 5 min. The reverse transcription reaction was carried out in a final volume of 50 µl containing: dNTP 1 mM, 30 U/µg RNA of AMV reverse transcriptase (Promega) and 1 U/µl of RNasin (Promega) at 42°C for 60 min. After reverse transcription, cDNAs were purified using the QIAquick PCR purification kit (Qiagen); their concentration was measured using the spectrophotometer ND-1000 Nanodrop and concentrations were adjusted to 50 ng/µl.

Primers and high throughput qPCR

Primer pairs used in the study were designed and produced at Fluidigm; their sequences are indicated in supplementary Table S3. Primer stock solutions at 100 µM were kept at -20°C. Prior to specific target amplification (STA), primers were pooled to the final concentration of 500 nM each. BioMark™, a high throughput PCR platform from Fluidigm, was used to perform the qPCR according to manufacturer's recommendations. Prior to the quantification PCR, the DNA was amplified: sixty ng of purified cDNA was amplified using the pooled primers to a final concentration of 50 nM and a thermal cycling consisting of 5 min at 95°C followed by 18 cycles of 15 s at 95°C and 4 min at 60°C and a final holding step at 40°C. Thereafter, the protocol recommended by the supplier was followed (Fluidigm quick references PN 100–5875 B1, and PN 100–9791 B1). For the quantification of PCR, the following cycling conditions were applied, one Taq activation cycle (95°C, 1 min) and 35 cycles (96°C for 5 sec, 60°C for 20 sec). The software Fluidigm real-time PCR analysis was used to determine the Cq values of each sample/primer pair couple. The Cq was determined by the Auto detector method with a quality control of 0.65 and a linear baseline correction. The fold change (FC) in gene expression was calculated by the 2^{cq} method [24]. An unpaired *t*-test was used to determine the statistically significant differentially expressed (DE) genes. The fold changes (FC) greater than 2 and less than -2, with a p value <0.05 were considered for further analyses.

Bioinformatic analysis

Enriched biological processes were determined and then clustered hierarchically according to Wang's semantic similarity distance using the Bioconductor R package ViSEAGO [25] and Ingenuity Pathway Analysis (IPA) was used to find the potential transcriptional targets of AhR.

Results

S. *Typhimurium can invade IEC-6 cells using both the trigger and the zipper entry processes*

To analyze the cellular response following a trigger or a zipper entry, it was necessary to identify a cell line that was permissive to both entry mechanisms, because in fact *S.* Typhimurium strains often enter cell lines almost exclusively by only one entry mechanism to the exclusion of the other [18]. Moreover, it was necessary for *Salmonella* strain to express all known invasion factors *in vitro*. As Rck and PagN are weakly expressed in standard culture conditions [26], we developed three different modified strains of the wild type STM 14,028: the STM-ZT overexpressing Rck and PagN *in vitro* and expressing the T3SS1 genes (trigger entry and zipper entry), the STM-Z overexpressing Rck and PagN *in vitro* and invalidated for the T3SS1 (zipper entry) and the STM-T expressing the T3SS–1 genes and invalidated for Rck and PagN (trigger entry). In the STM-ZT strain, Rck and PagN are cloned on the same plasmid and expressed under the control of two independent promotors. We selected the IEC-6 cell line

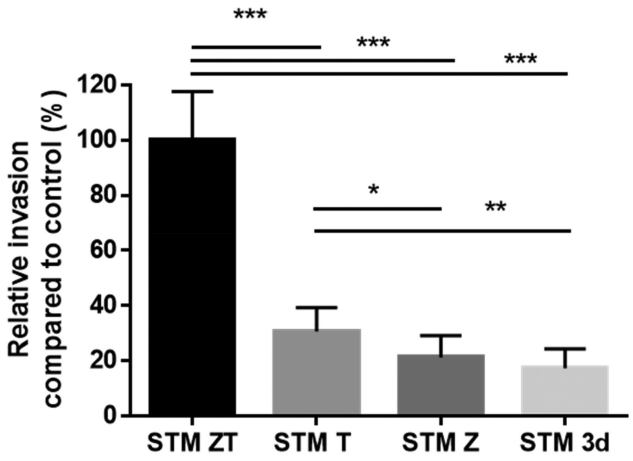

Figure 1. Invasion of IEC-6 cells by *S.* Typhimurium strains entering cells by different entry pathways.

Using a gentamicin assay invasion capabilities of *S.* Typhimurium strains capable of entry by both trigger and zipper mechanisms (STM-ZT), by a trigger (STM-T) or zipper (STM-Z) entry mechanism, or by none of the known mechanisms (STM-3d) were compared. Bacteria were deposited on IEC-6 with a multiplicity of infection of 10, for 1.5 h, then gentamicin was added (100µg/ml) for 1.5 h. Intracellular bacteria levels were assessed after cell lysis. This result corresponds to the mean of 6 independent experiments. Statistical analyses using a Mann Whitney test were performed using GraphPad Prism version 6.07 for Windows. (Significance was *** at $p<0.001$, ** at $p<0.01$, * at $p<0.05$.

because the STM-T and STM-Z strains were still able to enter IEC-6 cells at similar levels but, compared to the STM-ZT, with a reduced efficiency of 30% and 20% respectively (Figure 1). Interestingly, the STM-3d strain, which is unable to express any of these invasion factors has a much lower entry capacity than any of the other strains but retains a small entry capacity, as has been shown previously with other cell lines [18]. This original cellular model allowed us to compare the cellular response to both entry processes in the same cell line.

Cells exposed to the three Salmonella *strains have very similar gene expression profiles*

To determine the influence of the entry mechanisms on the modulation of cellular gene expression, we performed whole-genomic expression profiles of IEC-6 exposed to STM strains invalidated or not for factors responsible for the zipper or trigger entry. Gene expression analyses were performed after 1.5 h of interaction between IEC-6 and STM strains and 1.5 h of gentamicin to kill extracellular bacteria. Gene expression was analyzed using microarrays. Differentially expressed (DE) genes, characterized by the fold change (FC), the ratio between exposed cells and non-exposed cells, indicated that very few significant differences were observed between the STM strains, and no DE genes were found between cells exposed to STM-T and STM-ZT strains (Table 2). When comparing cells exposed to STM- T and STM-Z strains, the expression of *Mlr1*, a transcription factor specific to RNA Pol II, *Nxf3* involved in mRNA transport, *Smpdl3a* a phosphodiesterase of nucleoside triphosphate and *Nxph4*, playing a role in neuronal biology and expressed in cells from colorectal carcinoma [27] was less expressed in STM- T exposed cells. In these latter cells, the expression of *Dclk1* and *Tgfbr1* was lower. DCLK1 is a marker of the intestinal tuft cells involved in intestinal repair [28], whose expression is correlated with that of TGFBR1 in colon cancer [29]. Finally, the expression of *Rhoh*, encoding a Rho GTPase recruited at the *Salmonella* invasion site by SopB, a T3SS1 encoded factor, involved in the activation of Akt [30], was also lower in STM-T exposed cells compared to STM- Z. *Smpdl3a*, *Nxf3,* and *Rhoh* were expressed more in cells exposed to STM- Z than with STM- Z T strain. This low number of DE genes could be related to small differences induced by the entry pathway. Another hypothesis is that the signal is the same and low number of DE genes is related to the slightly different number of intracellular bacteria after the trigger and the zipper entry processes. However, this latter hypothesis is unlikely, as we did not observe any DE genes in the other experiments described below.

Cells exposed to the three Salmonella *strains have different gene expression profiles compared to unexposed ones*

In contrast to the previous observation, and as expected, when IEC-6 cells exposed to one of the three strains were compared to the unexposed cells, numerous DE genes were detected. More than 300 DE genes, with an FC> |2|, were common to the three strains, while around 20 were specific to one strain (Figure 2). Consistent with observations made when comparing *Salmonella* strains with each other, the lists of DE genes between control and IEC-6 cells exposed to STM-Z T, STM-Z, or STM-T were very similar, with only occasional minor differences in the magnitude of the FC (data not shown). We therefore decided to perform the subsequent downstream steps of analysis using the DE gene list obtained from the STM-ZT infection. The list of the 372 significantly DE genes with an absolute FC value greater than 2, between cells exposed to STM-ZT and unexposed cells is given in Table S1.

Table 2. Paired comparisons of gene expression modification induced in IEC-6 cells exposed to the *S.* Typhimurium strains STM- Z T, STM- Z, or STM- T.

STM-T/STM-Z		
Gene Name	FC	GO biological process
XR_589495	11,6	nc RNA
XR_344993	3,7	nc RNA
Mlr1	-2,5	transcription factor RNA polymerase II specific
Dclk1	-2,6	neuronal apoptosis and neurogenesis
Nxf3	-2,6	polyA+ mRNA export
Smpdl3a	-3,0	nucleoside triphosphate catabolic process
Nxph4	-3,0	neuropeptide signalling pathway
Gc	-3,0	vitamin D metabolic process
XM_008775294	-3,9	nc RNA
Rhoh	-4,1	T cell differentiation, negative regulation of I-kappaB kinase/NF-kappaB signalling
Tgfbr1	-5,0	transforming growth factor beta receptor signalling pathway
STM-Z/STM- Z T		
Gene Name	FC	GO biological process
Rhoh	4,7	T cell differentiation, negative regulation of I-kappaB kinase/NF-kappaB signalling
XM_008775294	3,6	nc RNA
Smpdl3a	2,7	nucleoside triphosphate catabolic process
Nxf3	2,6	polyA+ mRNA export
Gc	2,7	vitamin D metabolic process
STM-T/STM-Z T		
Gene Name	FC	GO biological process
XR_589495	10	nc RNA
XR_344993	4,1	nc RNA

DE genes with a p value <0.05 are represented.

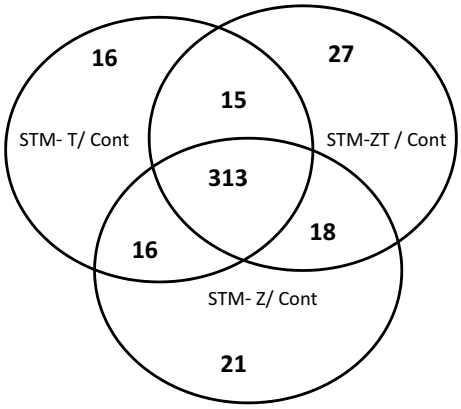

Figure 2. Specific and shared differentially expressed genes between IEC-6 cells exposed to STM-ZT, STM-Z or STM-T strains compared to non-exposed cells (cont).

Venn diagram showing the number of specific and shared differentially expressed (DE) genes (exposed/non-exposed cells) obtained after a whole-expression genomic profiling of IEC-6 cells infected by STM-ZT, STM-Z or STM-T strains. Gene expression analyzes were performed after 1.5 h of interaction between IEC-6 and STM strains and 1.5 h of gentamicin to kill extracellular bacteria. DE genes with a p value <0.05, and a fold change (FC) > |2| are represented.

We then used ViSEAGO [25] to determine the biological processes enriched following infection by the mutant strains. We focused on the common functions enriched; for this reason, we performed the ViSEAGO analysis for genes with a FC > |5|. Briefly, enrichment was carried out by aggregating gene ontology (GO) terms using a semantic similarity distance. The resulting functional enrichment analysis is presented in Figure 3. When compared to unexposed cells, those exposed to STM-ZT, STM-Z or STM-T strains shared identical patterns of enrichment. Immune-related terms such as cellular response to tumor necrosis factor, to interferon gamma and to lipopolysaccharide, as well as neutrophil chemotaxis, chemokine signaling pathway and positive regulation of nitric oxide were among the most enriched. It is noteworthy that wound healing was found to be enriched in cells exposed to the three STM strains. Wound healing consists in a series of interrelated molecular events that work together to restore tissue integrity and cellular function.

Expression of numerous ECM-associated, and of aryl hydrocarbon receptor (Ahr) target genes was modulated during salmonella infection

In a bird's eye view of the list of DE genes, we observed that several of them were linked to the ECM or *Ahr*. The Extracellular Matrix Interaction Database (MatrixDB: http://matrixdb.univ-lyon1.fr/) lists the molecules that are components or regulators of the ECM mole. Of the 372 DE genes from our study, we used this database to find those that were listed in MatrixDB. We found that 126 of our DE genes were in the database (Table S2); with 113 of them upregulated and 13 downregulated.

A literature search allowed us to assign function to some of the 126 DE genes involved in ECM regulation. Table 3 only shows the differentially expressed (DE) genes with a p value <0.05, and a fold change (FC) > |2|. These genes are key regulatory genes of the urokinase

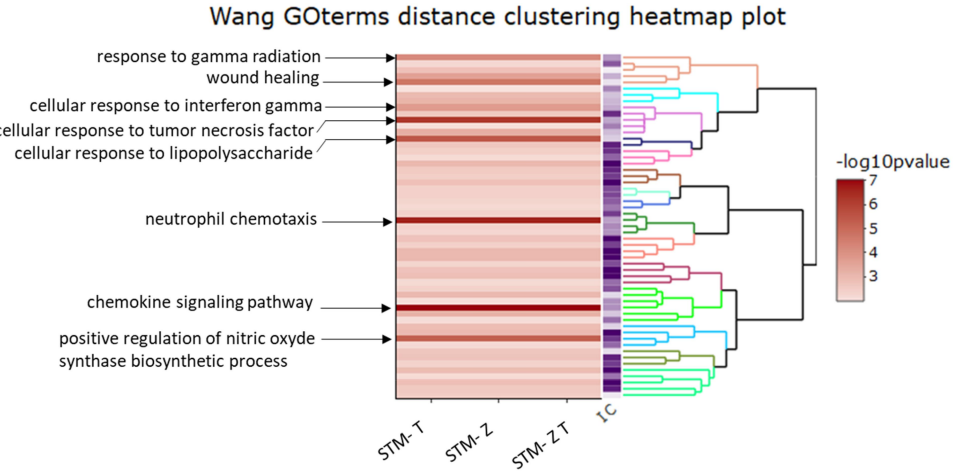

Figure 3. Functional enrichment analysis of biological processes.

Analysis of differentially expressed (DE) genes (exposed/non-exposed cells) obtained after a whole-expression genomic profiling of IEC-6 cells infected by STM-ZT, STM-Z or STM-T strains. Gene expression analyses were performed after 1.5 h of interaction between IEC-6 and STM strains and 1.5 h of gentamicin to kill extracellular bacteria. Clustering heatmap plot of functional sets of gene ontology (GO) terms was obtained using ViSEAGO showing the major biological processes. The plot combines a dendrogram based on Wang's semantic similarity distance and ward.D2 aggregation criterion, a heatmap of -$\log_{10}$(*p*-value) from functional enrichment tests and information content (IC). Focus is made on several major biological processes. For the sake of clarity, we only kept the genes showing high levels of expression $|\log_{10}(FC)| > 5$).

Table 3. Differentially expressed genes in the microarray experiment associated with stimulation or inhibition of the ECM.

Gene	ID	FC	Functional relevance with regard to ECM
Genes associated with urokinase pathway			
SerpinB2	NM_021696	24.8	Inhibition urokinase plasminogen activator (uPA) {Tang, 2013 #2267}
Serpine1	NM_012620	6.1	Inhibition of uPA {Tang, 2013 #2267}
Cyr61	NM_031327	2.0	Enhances Timp1 and Serpine1 production {Chen, 2001 #2282}
Spry1	NM_001106427	2.3	Decreases expression of uPAR at cell surface
Timp1	NM_053819	2.6	Inhibition of MMP {Brew, 2000 #2299}
Plaur	NM_134352	2.3	Receptor urokinase (uPAR) {Mekkawy, 2014 #3033} {Liu, 2014 #2281}
Genes involved in ECM component synthesis/degradation			
Sod2	NM_017051	7.8	Protection of heparan and type I collagen {Petersen, 2004 #2288}
Has1	NM_172323	6.4	Biosynthesis of hyaluronan
Mt2A	NM_001137564	4.0	Upregulation of collagenase expression
Ugdh	NM_031325	3.4	Biosynthesis of glycosaminoglycans
Lrp4	NM_031322	3.2	Induction of ECM genes expression {Asai, 2014 #2289}
Uap1	NM_017259	2.2	Biosynthesis of N-glycans
Adamts4	NM_023959	2.1	Aggrecan degradation {Westling, 2002 #2315}
Genes involved in ECM formation			
Plet1	NM_001014209	13.5	Tissue repair {Zepp, 2017 #2298}
Tnfaip6	NM_053382	3.3	Involved in ECM stability {Lauer, 2013 #2844}
Creb3l1	NM_001005562	2.0	Assembly ECM
Lmo7	NM_001001515	-4.1	Negative regulator of ECM deposition {Xie, 2019 #2847}
Fgf18	NM_019199	-3.8	Positive regulator of ECM deposition
Genes involved in fibrosis			
Tnf	NM_01267	23.3	Pro- anti- fibrotic, contradictory observations {Distler, 2008 #3284}
Tnfsf15	NM_145765	4.5	Pro-fibrotic {Barrett, 2012 #2269}
Arel1	NM_001106744	2.2	Pro-fibrotic {Lear, 2016 #2275}
Wnt10A	NM_001108227	2.8	Pro-fibrotic {Oda, 2016 #2273}
Antxr2	XM_008764602	2.8	Pro-fibrotic {Burgi, 2017 #2276}
Tgfb3	NM_013174	-3.3	Pro-fibrotic {Guo, 2021 #3027}
Fst	NM_012561	4.1	Anti-fibrotic {Patella, 2006 #2317}

Differentially expressed (DE) genes with a *p* value <0.05, and a fold change (FC) > |2| are represented. FC represents the ratio of gene expression between IEC-6 cells exposed to *S.* Typhimurium strain STM-Z T and non-exposed cells.

pathway that control fibrinolysis, like *SerpinB2* and *Serpine1*, which were strongly activated. Genes involved in the ECM formation, synthesis of ECM components or in fibrosis were also upregulated in exposed cells. Conversely, few genes were associated with degradation of ECM (Table 3).

Analysis of upstream regulators with Ingenuity Pathway Analysis (IPA) indicated that AhR was

a significant transcriptional regulator of 101 DE genes from our data (Figure S1). It should be noted that the expression of primary targets of Ahr like *Cyp1a1*, *Cy1b1,* and *Aldh3a1*, was highly increased, while that of *Nqo1*, *Arnt* and *Tiparp* was modestly but significantly increased (Table 4).

Expression of ECM-associated DE genes by quantitative RT PCR

To focus on the modulation of ECM-associated genes, by means of a Biomark Fluidigm ™ platform, we studied the expression of 40 genes (Table S3) that were found DE in the microarray analysis. Samples of cells exposed or not to STM-ZT, were the same as those used for the microarray experiments. Both techniques gave consistent results for 33 genes (82.5%), showing accordance for both the direction of variation and the order of magnitude of the FC. However, for seven genes (17.5%), the FCs were < I2I or not-significant in the Biomark experiment (Table 5).

Modification of ECM gene expression in IEC-6 cells required Salmonella entry

Next, to determine whether the modification of ECM gene expression occurred when *Salmonella* had an extra and/or intracellular location, we inhibited the entry of *Salmonella* and checked the ECM gene expression. As the STM-3d exhibited a small but significant entry, we used a pharmaceutical means to completely prevent *Salmonella* entry. For this purpose, we incubated IEC-6 cells before and during infection with a cocktail of amiloride and chlorpromazine as previously described [18]. Under these conditions, the entry of STM-ZT was reduced to about 3%, compared to the untreated cells for which the entry was arbitrarily set at 100% (Figure 4). Similarly, we observed that the cocktail of amiloride and chlorpromazine also significantly inhibited the entry of the STM-3d strain, reinforcing our choice to use drugs to inhibit *Salmonella* entry. Gene expression measurements in cells with or without intracellular bacteria were performed after 1.5 h of interaction between IEC-6 and *Salmonella* strains. Gene expression was measured by quantitative RT PCR, using the Biomark Fluidigm™ platform for the same set of 40 genes selected for studying the expression of ECM associated genes. When compared gene expression of cells infected by STM-ZT to un-infected cells expression of 27 genes was significantly increased and three whose expression was decreased (Table 6). In contrast, when IEC6 cells, pre-treated with the amiloride/chlorpromazine cocktail, were exposed to the STM-ZT the expression of only 6 genes was modulated after bacteria-cell contact; 2 genes were not found with STM-ZT exposed cells. Four genes (*Angptl4*, *Icam1, Nrg1* and *SerpinB2)* were common to the two conditions but the magnitude of the FC was lower when invasion was inhibited compared to that observed for the STM-ZT exposed cells. These results showed that the majority of the ECM-related genes were induced when STM was intracellular. These results suggest that under the experimental conditions used here, extracellular bacteria do not profoundly modify the expression of ECM-related genes and that ECM modification does not appear to be necessary for entry. In contrast, the presence of intracellular bacteria is required to profoundly modify the expression of ECM-related genes.

IEC-6 cell infection by S. Typhimurium modified glycoprotein composition of ECM

The extracellular matrix is composed of numerous proteins and glycoproteins. It was therefore not possible to carry out a complete screening of the putative modifications induced after cell infection. To confirm that STM-ZT can modify ECM composition, we therefore tested the effect of infection on two major ECM glycoproteins: the fibronectin mainly presents on the apical side and the laminin a major component of the basolateral side. The western blotting presented in Figure 5 clearly shows the dramatic change in ECM composition after cell infection, at least for fibronectin and laminin. However, the fact that the different *Salmonella* strains did not induce the same modifications on these two glycoproteins and that differences between laminin and fibronectin can be observed, even with the STM-3d strain, will lead us to perform a detailed analysis of the consequences of cellular infection on ECM composition. This protein analysis also showed that similar gene expression profiles could induce different effects on the composition of ECM glycoproteins, probably via post-transcriptional modifications.

Discussion

The first and most extensively studied mechanism of entry of *Salmonella* is the trigger one. Epithelial intestinal cell lines HT29, Caco-2 and HeLa, that have often been used in these studies, support an overwhelmingly trigger T3SS1-dependent entry. However, T3SS1-independent entry mechanisms have been described [31] like the Rck-dependent entry, which enables *S.* Enteritidis to enter cells through a zipper mechanism [12,32]. More recently, it has been recognized that in the epithelial cell-line AML12, a wild type *S.* Typhimurium expressing a functional T3SS1, enters through a zipper mechanism [18]. Thus,

Table 4. Differentially expressed genes included in the IPA upstream regulator analysis that may be activated by the AHR pathway.

Predicted up-regulations		Predicted down-regulations		Inconsistent findings		No prediction	
Gene name	Functional category	Gene name	Functional category	Gene name	Functional category	Gene name	Functional category
ACKR3	G-protein Coupled Receptor	ADAM19	Peptidase	C1QB	Other	ALDH3A1*	Enzyme
ACTN1	Transcription regulator	ADAMTS5	Peptidase	CDC25C	Phosphatase	ATOH8	Transcription regulator
ADM	Other	CDKN2C	Transcription regulator	CDH1	Other	BTG2	Transcription regulator
AHRR	Transcription regulator	HEY2	Transcription regulator	CDKN1A	Kinase	Casp8	Peptidase
ALDOA	Enzyme	TGFB2	Growth factor	CEBPA	Transcription regulator	CCNG2	Other
AREG	Growth factor			CERS4	Transcription regulator	CDH7	Enzyme
ARG2	Enzyme			E2F1	Transcription regulator	COL27A1	Other
CCL20	Cytokine			E2F8*	Transcription regulator	CYP2S1	Enzyme
CCL5	Cytokine			FBN2	Other	EBF1	Transcription regulator
CD274	Enzyme			FN1	Enzyme	FAS	Transmembrane receptor
CD3D	Transmembrane receptor			FOSL1	Transcription regulator	GHITM	Other
CERS6*	Transcription regulator			GADD45A	Other	HECTD2	Enzyme
CXCL10	Cytokine			GAS1	Other	JAG1	Cytokine
CXCL2	Cytokine			HES1	Transcription regulator	Mt1	Other
CYP1A1	Enzyme			HIF1A	Transcription regulator	NTM2	Enzyme
CYP1B1	Enzyme			HP90AA1	Enzyme	NNMT	Enyme
CYP2C8	Enzyme			HSPA5	Enzyme	TNFAIP8L1	Other
CCL2	Cytokine			JUN	Transcription regulator	VEGFA	Growth factor
Cdc42	Enzyme			JUNB	Transcription regulator	VEGFC	Growth factor
EDN1	Cytokine			LBP	Transporter		
EGLN3	Cytokine			PLAT	Peptidase		
FMO3	Enzyme			PLK1	Kinase		
HK2	Kinase			Pcp4l1	Other		
IL1R1	Transmembrane receptor			SOX2	Transcription regulator		
IL1R2	Transmembrane receptor			STEAP2	Enzyme		
INSIG2	Other			Saa3	Other		
IRF1	Transcription regulator			TNF	Cytokine		
ITGA5	Transcription regulator			VIM	Other		
KDSR	Enzyme						
MYC	Transcription regulator						
NFE2L2	Transcription regulator						
NOTCH	Transcription regulator						
NQO1	Enzyme						
PDE4B	Enzyme						
PTGS2*	Enzyme						
PTX3	Other						
SDC1	Enzyme						
SERPINB2	Other						
SERPINE1	Other						
SLAMF8	Other						
SLC2A1	Transporter						
SOCS3	Phosphatase						
SOD2	Enzyme						
SPTLC2	Enzyme						
THBS1*	Other						
TIPARP*	Enzyme						
UGCG	Enzyme						
UGT1A6	Enzyme						

The genes are clustered into four categories, which correspond to specific link colours in the Figure S1: "predicted up-regulations" (orange links), "predicted down-regulations" (blue), "inconsistent findings" (yellow) and "no prediction" (grey). The genes are represented in different colours corresponding to the direction of variation (see also Figure S1): strongly up-regulated genes are in red, lightly up-regulated genes are in pink, strongly down-regulated genes are in dark green, and lightly down-regulated genes are in light green. The functional categories of the genes (actually corresponding to different shapes in Figure S1) are also reported in the table.

Table 5. Comparison of gene expression determined by microarray and qRT PC.

Gene	FC qRT PCR	FC microarray
Adamts4	2.7	2.1
Ajuba	-2.4	-2.2
Angptl4	6.4	11.3
Areg	3.1	2.0
Arl4c	-2.2	-2.1
Bdkrb1	4.8	2.5
Bmp4	-5.3	-3.2
Cd302	2.2	2.2
Cd44	2.4	2.0
Ceacam	1.4§	2.0
Creb3l1	2.3	2.0
Cyr61	2.4	2.0
Ephb3	-4.0	-2.2
Errfi1	2.3	2.0
Ets2	2.8	2.5
Fat1	1.9	2.0
Fgf18	-3.8	-3.8
Flrt3	-2.2	-2.2
Gabarapl1	2.3	2.4
Gja1	2.2	2.0
Icam1	6.5	6.5
Lpar1	2.1	2.0
Nrg1	3.6	3.8
Olr1	5.7	5.0
Pla2g2a	1.8	2.0
Plaur	1.1§	2.3
Procr	2.5	2.0
Ptpn12	2.4	3.1
Rhbdf2	4.1	3.3
Rtp4	5.8§	2.2
Sdc4	2.5	2.2
Serpinb2	23	24.8
Serpine1	7.2	6.1
Sfrp2	4.6	3.6
Snx18	1.5§	2.3
Timp1	3.1	2.6
Tnfaip6	4.0	3.3
Tnfrsf11b	5.7	5.1
Tnfsf15	12	4.5
Vegfa	2.4§	2.0

Fold changes (FC) represents the ratio of gene expression between IEC-6 cells exposed to STM-Z T and non-exposed cells. FC with a § have a *p* value >0.05, FC without § have a *p* value <0.05.

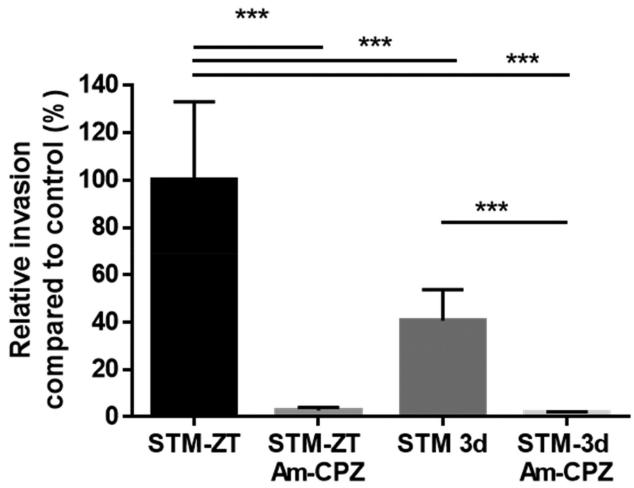

Figure 4. Invasion of the STM-ZT and STM-3d strains impaired for entry by pharmaceutical means.

Invasion ability of the STM-ZT and STM-3d was analyzed using a gentamicin protection assays in the presence of 1 mM amiloride (Am) and 10 µg/mL chlorpromazine (CPZ). IEC-6 cells were pretreated for 30 min with the drugs, or with the appropriate solvent used for the dilution of the drugs as control. Infection was performed in the presence of the drugs; bacteria were deposited on IEC-6 with a multiplicity of infection of 10, for 1.5 h, then gentamicin was added (100µg/ml) for 1.5h. Intracellular bacteria levels were assessed after cell lysis. This result corresponds to the mean of five independent experiments. Relative invasion corresponds to the number of internalized bacteria in cells treated relative to the non-treated cells infected by STM-ZT arbitrarily set at 100%.

Salmonella can use either trigger and zipper entry routes but we were not aware of a cell line that supports both at similar levels. To compare the cellular response induced by bacteria, which entered cells via either the trigger or the zipper entry process, we constructed three genetically modified S. Typhimurium 14,028 strains. The STM-Z is a *ΔinvA* mutant expressing Rck and PagN *in-vitro*, and can enter cells via the zipper entry; the STM-T is a mutant invalidated for the invasins, Rck and PagN, which can enter cells via the trigger entry; the STM- ZT can use both entry processes and the STM-3d is unable to express any of these three invasion proteins. We found that the STM- Z and STM-T strains were both able to enter the intestinal epithelial cell line IEC-6, but to a lower level compared with the STM- ZT strain. To our knowledge, IEC-6 is, to date, the only cell line that can support both entry routes at similar levels. This peculiar characteristic could be linked to the nature of the cell line used in this study. Indeed, IEC-6 were obtained from a normal rat small intestine by serial subcultures and not from a tumor, like most other intestinal cell lines [33]. Profound cellular modifications accompanying cellular transformation could therefore affect the process of *Salmonella* entry into cells of non-tumor or tumor origins differentially [32].

Using microarrays, we carried out a transcriptomic profiling of IEC-6 cells exposed to the STM-ZT, STM-Z, and STM-T strains. As suggested by ViSEAGO analysis, compared to unexposed cells, the three strains presented various significantly enriched processes related to immune response as previously shown in different studies [34–36]. In our study, different chemokine-encoding genes like *Ccl20*, *Cxcl2, Ccl2, C×3cl1, or Cxcl3*, and also *Tnfa*, *Ptgs2,* and the subunits of the NFkB transcription factor *Nfkb1*, *Nfkb2,* and *Nfkbiz*, were among the top upregulated genes with FC higher than 5 and up to 99, indicating that a strong inflammatory response was underway.

Surprisingly, very few differences in gene expression were found when comparing IEC-6 cells exposed to STM-ZT, STM-Z, or STM-T. Modulation of cellular gene expression is a very dynamic process involving different waves of regulation; it is probable that studies at very early time-points could underscore differential

Table 6. Expression profile of selected ECM-associated genes in IEC-6 cells treated or not with chlorpromazine and amiloride before and during infection with the STM-Z T strain.

Gene	STM-Z T/Cont	Ch+Am/Cont
SerpinB2	37	6.5
Angptl4	14	7.2
Icam1	8.6	4.7
Cyr61	4.2	
Rtp4	9.2	
Tnfsf15	50	
Vegfa	4.2	
Ephb3	-4.1	
Olr1	9.2	
Rhbdf2	9.2	
Serpine1	8.4	
Tnfrsf11b	6.0	
Tnfaip6	5.8	
Ceacam1	4.9	
Bdkrb1	4.8	
Isg15	4.0	
Ptpn12	3.5	
Timp1	3.5	
Adamts4	3.3	
Ets2	3.1	
Nrg1	3.0	2.0
Areg	2.8	
Snx18		2.8
Procr	2.7	
Cd302	2.5	
Gja1	2.3	
Creb3l1	2.2	
Plaur	2.2	
Fat1		2.2
Sdc4	2.1	
Bmp4	-2.2	
Fgf18	-3.2	

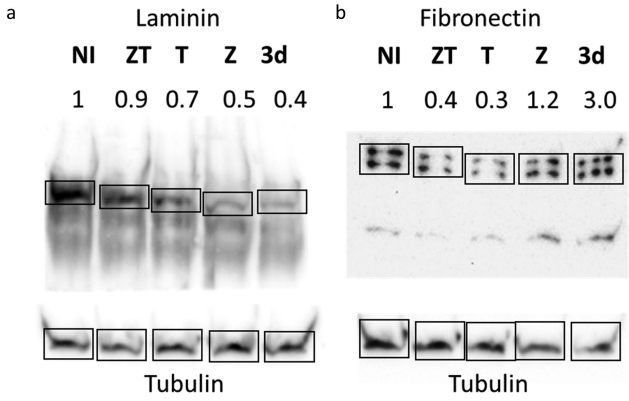

Figure 5. Western blotting analysis of IEC6 cells infected by the different *S.* Typhimurium strains.

Alteration of Extracellular Matrix Proteins (ECM) after bacterial invasion is illustrated by western blotting analysis of laminin and fibronectin. Bacteria were deposited on IEC-6 with a multiplicity of infection of 10, for 1.5 h, followed by gentamicin (100µg/ml) for 1.5 h. Cells were then resuspended in Laemmli buffer and denaturated 10 min at 100°C. Samples from Non-infected cells (NI) and cells infected by STM-ZT, STM-Z, STM-T or STM-3d strains were loaded in a 4-15% Miniprotean TGX Precast Protein gels and then transferred to a nitrocellulose membrane followed by detection of laminin (A) and fibronectin (B). Protein concentrations were normalized to the tubulin-blotting reference and the ratios were expressed relative to non-infected cells. The Regions of interest (ROI) taking into account for this normalization are represented by the rectangles.

responses of the cell to the three STM strains when bacteria enter cells and are not within the cells. On the other hand, during its intracellular life in epithelial cells, *Salmonella* can occupy two niches: the *Salmonella* containing vacuole or the cytosol where bacterial replication is very active. Depending on its location in these niches, *Salmonella* deploys two different transcriptomic programs [37] suggesting that interactions with cellular partners from the two cellular subcompartments are probably different. As we did not detect any obvious differences in cellular response between IEC-6 exposed to the STM strains, this indicates that in our model, STM-ZT, STM-Z and STM-T occupy the same intracellular niche and therefore the entry pathway does not determine intracellular location.

In cells exposed to the three strains, we found that the expression of more than one hundred genes associated with the ECM was modified compared to unexposed cells. This is in line with another study on organoids exposed to *S.* Typhimurium which noticed that among the enriched biological processes, in addition to functions associated with immune response, there was "ECM organization" [36]. ECM, a large network of macromolecules, ensures the morphological and mechanical properties of the tissue and is involved in cell signaling across the membrane. Numerous articles have observed that *Salmonella* interacts with the different proteins of the ECM. Only few have shown that *Salmonella* infection dramatically modifies the ECM gene expression. Berndt *et al.* described the reorganization of fibronectin, laminin, and tenascin in chick cells exposed to *Salmonella* strains with high or low invasiveness capacities [38]. In our data, modulation of the expression of several genes argued for a process of stimulation or stabilization of the ECM and for the establishment of a fibrotic state after 3 h of interaction between the host cell and *Salmonella*. This hypothesis is strengthened by the modifications in fibronectin and laminin observed by western blotting in our model. The urokinase pathway plays a key role in the homoeostasis of the ECM. By hijacking this pathway, pathogens may enhance their invasiveness [39]. In this way, PtgE [40] and the thin aggregative fimbriae [41] from *Salmonella* can interfere with the urokinase pathway, which in turn can favour their entry. A key effector of the urokinase pathway is the plasmin that is involved in the degradation of laminin, fibrin, and fibronectin, some major components of the ECM. On the other hand, plasmin also activates the matrix metalloproteinase-9 also known as collagenase. In our data, several genes related to the urokinase pathway were differentially expressed in exposed cells and in particular, the expression of *Serpine1* and *Serpinb2* was strongly upregulated in exposed cells, suggesting that

plasmin production was inhibited. Indeed, these two proteins are potent inhibitors of uPA [42], a protease that drives the activation of plasmin. Similarly, CYR61, which negatively controls plasmin production, by indirectly increasing SERPINE1 production, was modestly but significantly upregulated in exposed cells [43]. This suggested that the urokinase pathway was repressed and could in turn favor ECM restoration. In addition, the expression of genes that positively influence ECM formation or stability was higher in exposed cells. This was the case for *Timp1* (inhibitor of matrix metalloproteinases) [44], *Plet1* (involved in tissue repair) [45] and *Tnfaip6* (ECM stability) [46]. On the other hand, the expression of *Lmo7*, a negative regulator of ECM deposition [47] was decreased. Furthermore, the expression of genes involved in ECM component synthesis like hyaluronan (*Has1*), glycosaminoglycans (*Ugdh*) or *N*-glycans (*Uap1*) were upregulated in exposed cells. Finally, modulation of genes with increased expression like *Sod2* (protection of heparan from degradation) [48] and *Lrp4* (induction of essential components of the ECM) [49] also supported the idea that an ECM restoration process was ongoing. Taken together these observations suggested that, following perturbations induced by the infection, the expression of master components of the urokinase pathway was repressed and that the formation of ECM was consolidated. Activation of ECM formation could correspond to a consolidation phase of the matrix after degradation by the invading *Salmonella*. However, in different pathological situations, an excess of ECM component deposition induces fibrosis. The latter drives organ damage and most of the time is associated with inflammation. In a model of gut inflammation in mice, it was shown that TNFSF15 (Tl1A) is responsible for the development of fibrosis [50]. In our model, the expression of *Tnfsf15* was also increased and could be related to the development of a fibrotic process associated with inflammation. Similarly, other genes like *Wnt10A* [51], *Arel1* [52] and *Antxr2* [53] which have been shown to be involved in fibrosis, have an increased expression in exposed cells. Conversely, other genes argued for a process of degradation of the ECM. Expression of *Adamts4, which* is involved in degradation of aggrecan [54], was upregulated. These apparent contradictory observations most probably reflect the tight and complex regulation exerted on ECM.

As *Salmonella* can modulate cellular gene expression when they are extra- and intra-cellular, we used drugs (chlorpromazine and amiloride) to completely prevent its entry in order to determine which genes were modulated and at which step. Under our experimental conditions, the expression of far fewer genes was modulated when *Salmonella* cannot enter cells. The four genes, which were induced by adherent bacteria were involved in angiogenesis, a process that is pivotal to tissue repair. Forbester et al. in their study on organoids exposed to *S*. Typhimurium found that "positive regulation of angiogenesis" was another enriched biological process [36]. In our study, *SerpinB2* [55], *Angptl4* [56], *Icam1* [57] and *Nrg1* [58], whose expressions were upregulated in cells treated with the drugs, were shown to have proangiogenic properties suggesting that an angiogenic process was initiated when *Salmonella* is extracellular, whereas the ECM modification is mainly driven by intracellular bacteria. The complex interactions between invading pathogens, host tissues, and immune cells occur in the context of the ECM. Several articles demonstrated that *Salmonella* can modify ECM by a direct interaction in order to improve cell invasion [38]. In our case, we show that *Salmonella* can modify ECM composition when the bacteria is already within the cells. The modifications of the composition and three-dimensional ultrastructure of ECM should have a profound impact on the specific signals that the ECM conveys to immune cells at the forefront of infection and to epithelial cells and consequently to the outcome of infection. This topic could be addressed with enteroids cultured in the presence of immune cells.

In addition to the high number of ECM-related genes, which were modulated by intracellular *Salmonella*, we observed that a hundred DE genes were potential transcriptional targets of AhR. At first, the cytosolic ligand-activated transcription factor AhR was shown to be responsible for a response to xenobiotics, but since then its wide pleiotropic functions have been well documented. Activation of AhR can induce both canonical and non-canonical pathways. In the canonical pathway, binding of one of the numerous ligands on AhR induces its translocation to the nucleus where it binds ARNT. The heterodimer AhR/ARNT interacts with an XRE responsive sequence and activates transcription of many target genes [59]. In our study the expression of key genes, regulated by and involved in AhR pathway like *Arnt* and *Ahrr* [59] was increased. In addition, the expression of *Cyp1a1*, usually used as a readout of AhR activation, was strongly increased as well as that of *Cyp1B1* and *Aldh3a1*, two AhR target genes that are important factors in the detoxification process. The role of AhR in a huge number of physiological functions is well recognized and more particularly in intestinal homoeostasis. AhR influences both gut barrier functions and the activity of different intestinal immune cells, particularly by stimulating production of interleukin 22 by group 3 innate lymphocytes [60]. Given the importance of these functions,

it is not surprising that AhR is implicated in several pathological processes and more particularly in infections. In this context, the role of AhR in response to pathogens is an emerging theme. Its involvement in viral [61,62], bacterial [63,64] and parasitic infections [65,66] has been documented. Bessede et al [67]. have shown, for example, that in mice: the first challenge with LPS protects them from a second challenge with *Salmonella* Typhimurium in an AhR-dependant mechanism. More interestingly, AhR activation reduces mortality in a mouse model of systemic *Salmonella* infection with a concomitant reduced microbicidal capacity of phagocytes and a bacterial burden in surviving mice [68]. Until now, the effect of AhR activation *in vivo* was assessed via its effect on immune cells [68]. Our study suggests that this effect may also be mediated via enterocytes, directly or indirectly. Activation of AhR during *Salmonella* infection raises the question: what bacterial factor could bind this cytoplasmic transcription factor and activate the pathway? Moura-Alves suggested that the AhR is not only an important regulator of the immune response but also represents a novel type of pattern recognition receptor (PRR) [69]. However, the plethora of potential microbial AhR ligands, their different affinities and quantities make it difficult to create general concepts.

In conclusion, the IEC-6 cell line supports both a trigger and a zipper entry. Transcriptomic reprogramming associated with both entry mechanisms is very similar, strongly suggesting that the cellular response does not depend on the entry route used by intracellular bacteria. This study also opens up new avenues of research because we have shown that in addition to modification of expression of numerous immune genes, once intracellular, *Salmonella* modifies in IEC6 cells several factors involved in the structure or regulation of the extracellular matrix and activated the aryl hydrocarbon receptor gene regulation. It is now important to determine the consequences of these modifications on cell invasion and host infection. The ECM plays, indeed, an active role in infection rather than simply providing a scaffold for bacterial adhesion or being a barrier to breach [70]. In addition, it becomes clear that modulation of AhR plays a critical role in the outcome of infections [68]. It is also interesting to note that the modification of both ECM and AhR could have a significant impact on intestinal cell barrier but also on the immune response.

Disclosure statement

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Author contributions

AMC participated in the experimental design, carried out the interpretation of the microarray data, did the quantitative RT PCR on the Biomark Fluidigm™ platform, and drafted the manuscript.

SR participated in the experimental design, performed the cellular experiments, and contributed to the analysis of results.

MM carried out the microarray experiments, and participated in the bioinformatic analysis. CHA participated in the experimental design and did the microarray statistical analysis.

SH participated in the analysis of results, and participated in the discussion.

FK participated in the bioinformatic analysis.

EB participated in the experimental design and performed the cellular experiments.

JT engineered the modified STM strains.

PV conceived the study, participated in its design and the analysis of results, and helped to draft the manuscript.

All authors corrected and approved the final manuscript.

Data availability statement

The data that support the findings of this study are available in the GEO database with the accession number GSE151881.

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