Differential expression of gastric MUC5AC in colonic epithelial cells: TFF3-wired IL1β/Akt crosstalk-induced mucosal immune response against *Shigella dysenteriae* infection

Subramaniya Bharathi Raja[1], Malliga Raman Murali[1], Halagowder Devaraj[2] and Sivasithamparam Niranjali Devaraj[1,*]

[1]Department of Biochemistry and [2]Department of Zoology, School of Life Sciences, University of Madras, Guindy Campus, Chennai-600025, Tamilnadu, India

*Author for correspondence (niranjali@yahoo.com)

Accepted 27 July 2011
Journal of Cell Science 125, 703–713

doi: 10.1242/jcs.092148

Summary

An understanding of the signaling mechanism(s) that regulate the differential expression of gastric mucin MUC5AC in colonic epithelial cells would contribute significantly to investigations of its role in colonic mucosa infected with the bacterial pathogen *Shigella dysenteriae*. Here we show that *S. dysenteriae*-induced expression of interleukin-1β upregulates MUC2 expression and the differential expression of MUC5AC. Differential expression of MUC5AC involves crosstalk between interleukin-1β and Akt, whereby the trefoil factor family peptide TFF3 activates Akt by phosphorylation of EGFR. TFF3 also downregulates E-cadherin expression, causing accumulation of β-catenin in the cytosol. Phosphorylation of GSK-3β (inactivated) by activated Akt inhibits ubiquitylation of β-catenin, leading to its nuclear translocation, which then induces the expression of MUC5AC and cyclin D1. Accumulation of cyclin D1 alters the cell cycle, promoting cell survival and proliferation. Human colon HT29MTX cells, which overexpress MUC5AC, were resistant to adherence and invasion of *S. dysenteriae* when compared with other mucin-secreting HT29 cell types. Thus, during infection with *S. dysenteriae*, crosstalk between interleukin-1β and Akt wired by TFF3 induces expression of MUC5AC in colonic epithelial cells. Differentially expressed gastric MUC5AC aids in mucosal clearance of *S. dysenteriae*, inhibiting adherence and invasion of the pathogen to colonic epithelial cells, which protects the host.

Key words: MUC5AC, IL1β/Akt crosstalk, MUC2, Mucosal immunity, *Shigella dysenteriae*, TFF3

Introduction

Mucus plays an important role in protecting non-squamous epithelial surfaces against mechanical damage. It also stabilizes the luminal microenvironment of the cell surface and traps particles, including bacteria and viruses, for mucociliary clearance. The major component of mucus is mucin, a heavily glycosylated glycoprotein (Hollingsworth and Swanson, 2004). Almost 20 different mucin genes have been identified and are named MUC1, MUC2, MUC3A, MUC3B, MUC4, MUC5AC, MUC5B, MUC6–MUC13, MUC15–MUC17 and MUC19–MUC20. MUC2, MUC3 and MUC4 are the prominent mucin genes expressed in normal colonic mucosa, whereas MUC5AC is expressed in airway and gastric epithelial cells and is highly expressed in colorectal carcinomas (Kim et al., 1989; Kim et al., 2000; Weiss et al., 1996; Bartman et al., 1999). Many studies have reported qualitative and quantitative abnormalities of mucin gene expression in several diseases such as inflammatory bowel diseases (IBDs) and shigellosis (Kim et al., 1989; Radhakrishnan et al., 2008; Rajkumar et al., 1998).

Adhesion of enteric pathogens to the mucosa of the gastrointestinal tract was recognized as an important early event in the colonization and development of diarrheal diseases. Previous studies in our lab have demonstrated the high binding specificity of *Shigella dysenteriae* to human colonic mucin compared with human gastric mucin, rat colonic mucin and small intestinal mucin (Rajkumar et al., 1998; Sudha et al., 2001). Later studies in our lab have confirmed the altered expression of MUC2 and differential expression of MUC5AC in response to infection with *S. dysenteriae* using a rabbit ileal-loop model (Radhakrishnan et al., 2008). Reports have stated the role of MUC1 in *Helicobacter pylori* induced gastric carcinoma (Udhayakumar et al., 2007). In the case of *Pseudomonas aeruginosa* infection in cystic fibrosis (CF) patients, overexpression of mucin probably helps the organism to survive (Devaraj et al., 1994). In this scenario, it is important to understand the functional relevance of the differential expression of MUC5AC during *S. dysenteriae* infection and the regulation cascade behind this differential expression.

Expression of cell-surface and gel-forming mucins is mainly upregulated by inflammatory cytokines. Expression of the *MUC2* gene in NCI-H292 human airway epithelial cells is mediated by interleukin-1β (IL-1β) through the activation of protein kinases PKC, MEK, ERK and PI3K (Kim et al., 2000; Kim et al., 2002; Perrais et al., 2002). IL-4 induces gene expression of MUC5AC

and MUC4 in goblet cell metaplasia in vitro and in vivo (Temann et al., 1997; Dabbagh et al., 1999; Damera et al., 2006). TNF-α and nitric oxide induce MUC5AC mucin expression in respiratory epithelial cells through the PKC–ERK-dependent pathway (Song et al., 2007; Smirnova et al., 2000; Smirnova et al., 2001; Song et al., 2003; Iwashita et al., 2003; Koga et al., 2007). Although there are many regulatory mechanisms for upregulation of MUC2 and MUC5AC individually, there are not many studies dealing with differential expression of these mucins in a particular condition. Thus, in this study, we investigated the events of signal transduction involved in differential expression of MUC5AC, in colonic epithelial cells on *S. dysenteriae*

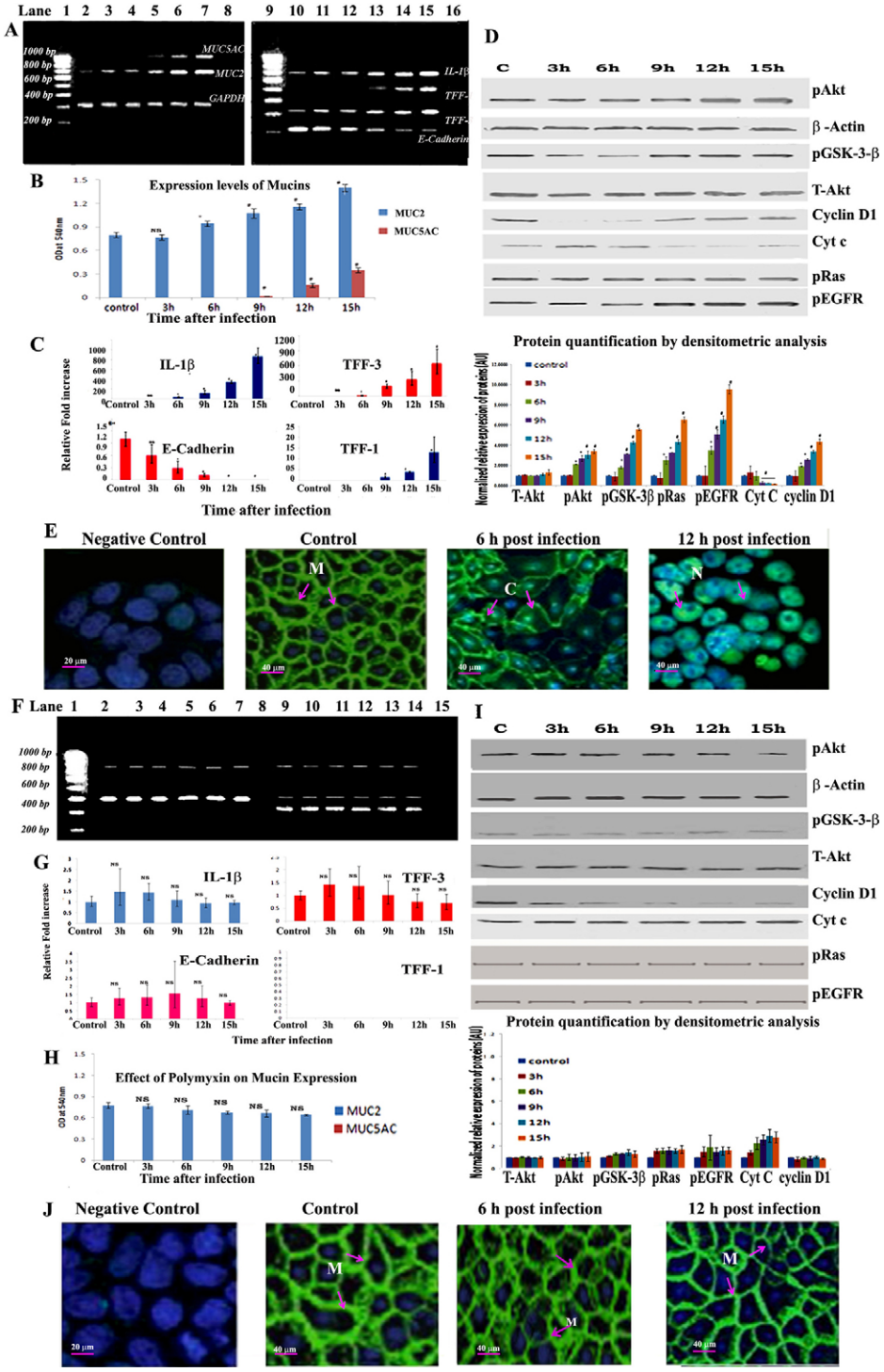

Fig. 1. See next page for legend.

infection and also investigated the protective role of MUC5AC against *S. dysenteriae*.

Results

S. dysenteriae infection upregulates MUC2 and induces MUC5AC expression

To determine whether mucin production is induced by *S. dysenteriae* infection in colonic epithelial cells, we evaluated mucin gene expression by analysing the mRNA expression of MUC2 and MUC5AC in HT29 cells. Time-dependent overexpression of *MUC2* mRNA (697 bp amplified product in multiplex RT-PCR) and differential expression of *MUC5AC* mRNA (902 bp amplified product in multiplex RT-PCR) after 9 hours of *S. dysenteriae* infection was found in HT29 cells (Fig. 1A). The induction of MUC2 and MUC5AC expression by *S. dysenteriae* infection was also confirmed at the protein level by immunoassaying the cell supernatant using specific antibodies. Consistent with the increased gene expression data, the protein levels of MUC2 increased with time and MUC5AC was expressed after 9 hours of infection (Fig. 1B).

Fig. 1. *S. dysenteriae* induces molecular changes in HT29 cells. (**A**) Expression patterns of genes encoding MUC2 and MUC5AC, IL1β, GAPDH, TFF3, TFF1 and E-cadherin in *S. dysenteriae*-infected HT29 cells by Multiplex RT PCR. Lanes 1 and 9, 100 bp marker; Lanes 2 and 10, uninfected control; Lanes 3–7 and 11–15 represent 3, 6, 9, 12 and 15 hours of infection with *S. dysenteriae*, respectively. Lanes 2–8 were amplified with primers specific for GAPDH (312 bp), MUC2 (697 bp) and MUC5AC (902 bp). Lanes 10–16 were amplified with specific primers for IL-1β (812 bp), TFF3, TFF1 and E-cadherin. Lanes 8 and 16, negative control. (**B**) Production of MUC2 and MUC5AC. MUC2 production (blue) was significantly increased in infected HT29 cells in a time-dependent manner. MUC5AC production (red) was observed only after 9 hours of infection. (**C**) Expression profile analysis of IL1β, TFF3, TFF1 and E-cadherin. All expression values were normalized to the value of *GAPDH* gene used as an internal control. Relative amount was calibrated based on the transcript amount of the corresponding gene in uninfected controls. Significant increases in levels of *IL1B*, *TFF3* and *TFF1* mRNA transcripts were observed in *S. dysenteriae*-infected HT29 cells in time-dependent manner, whereas level of mRNA encoding E-Cadherin was significantly decreased with time. (**D**) Immunoblotting analysis (top) and protein quantification (bottom) of phosphorylated EGFR, Ras, Akt, GSK-3β, TAkt, cyclin D1 and cytC. (**E**) β-catenin is found in membrane (M) in uninfected cells (control). At 6 hours post infection, β-catenin is found in cytosol (C); at 12 hours, it is found in nucleus (N). (**F**) Effect of polymyxin B on *S. dysenteriae*-induced gene expression. Lane 1, 100 bp marker; lanes 2–8 were amplified with specific primers for GAPDH, MUC2 and MUC5AC. Lanes 9–15 were amplified with E-cadherin, IL1β, TFF3 and TFF1. Lanes 2 and 9, uninfected control; Lanes 8 and 15, negative control. (**G**) RT-PCR analysis of IL1β, TFF3, TFF1 and E-cadherin in polymyxin-B-pretreated HT29 cells infected with *S. dysenteriae.* All expression values were normalized to the value of *GAPDH* gene used as an internal control. Relative amount was calibrated based on the transcript amount of the corresponding gene in uninfected controls. Changes in expression induced by *S. dysenteriae* are inhibited when cells are pre-treated with polymyxin B. (**H**) MUC2 and MUC5AC production is significantly inhibited in infected HT29 cells upon pretreatment with polymyxin B. (**I**) Immunoblotting analysis and protein quantification of phosphorylated EGFR, Ras, Akt, and GSK-3β and TAkt, cyclin D1 and cytC in polymyxin-B-pretreated HT29 cells infected with *S. dysenteriae*. (**J**) Localization of β-catenin in polymyxin-B-pretreated HT29 cells infected with *S. dysenteriae.* Translocation of β-catenin from membrane to nucleus is not observed 6 hours and 12 hours post infection. Data values were obtained from triplicate analysis and are expressed as the mean ± s.d. $^{*}P<0.05$; $^{\#}P<0.001$.

S. dysenteriae infection regulates expression of IL-1β, TFF3, TFF1 and E-cadherin

S. dysenteriae infection resulted in the gradual induction of mRNA encoding IL-1β and TFF3 in a time-dependent manner, significant changes were observed 9 hours post infection and thereafter. Induction of TFF1 was seen only after 9 hours of *S. dysenteriae* infection, which was similar to the expression of MUC5AC. The levels of mRNA encoding E-cadherin showed a progressive decrease over time (Fig. 1C).

S. dysenteriae infection induces phosphorylation of EGFR, Akt and GSK-3β along with translocation of β-catenin

Western blot analysis showed that phosphorylation of EGFR, Ras, Akt and GSK-3β significantly increased with time up to 15 hours after *S. dysenteriae* infection, after a slight decrease in levels during the first 3 hours of infection (Fig. 1D), without affecting the levels of EGF and TGF-α (supplementary material Fig. S1). Decreased expression of cyclin D1 and increased levels of cytoplasmic cytochrome C (cytC) were observed in the early stages of infection, whereas significantly increased expression of cyclin D1 and decreased levels of cytoplasmic cytC were seen after 6 hours of infection. Immunofluorescence analysis showed the translocation of β-catenin from the membrane to the nucleus in infected HT29 cells after 12 hours of infection, whereas in uninfected control cells, β-catenin was localized in the membrane. At 6 hours after infection, β-catenin was found in the cytosol of HT29 cells (Fig. 1E).

IL-1β regulates of expression MUC2 and MUC5AC during *S. dysenteriae* infection

To confirm that both glycoproteins are induced through IL-1β during *S. dysenteriae* infection in colonic epithelial cells, mRNA expression of MUC2 and MUC5AC were analysed in HT29 cells pre-treated with the antibiotic polymyxin B. Pre-treatment significantly inhibited *S. dysenteriae*-induced MUC2 and MUC5AC expression, even after 15 hours of infection (Fig. 1F,H). polymyxin B pre-treatment markedly suppressed the expression of IL-1β, which was induced by *S. dysenteriae* infection. Overexpression of TFF3 and TFF1, and downregulation of E-cadherin was also inhibited by polymyxin B pre-treatment (Fig. 1F,G). Furthermore, phosphorylation of EGFR, Ras, Akt and GSK-3β, and accumulation of cyclin D1 induced by *S. dysenteriae* infection was repressed (Fig. 1I). Membranous localization of β-catenin was observed in *S. dysenteriae*-infected polymyxin B pre-treated HT29 cells at both 6 and 12 hours, which was similar to that of control HT29 cells (Fig. 1J).

Analysis of *S. dysenteriae*-infected and IL-1β-suppressed HT29 cells

The viability of uninfected cells was almost equal to 100% at all time points. In the case of infected cells, viability decreased to 65% up to 6 hours after infection and thereafter. There was a significant increase in viability to 85% at 15 hours after infection. This increase in cell viability was not observed in infected IL-1β-suppressed (HT29-IL-1β^{sup}) cells (Fig. 2A). Western blot analysis showed that expression of PCNA was increased after 9 hours of *S. dysenteriae* infection in control HT29 cells, whereas only basal level expression of PCNA was observed in

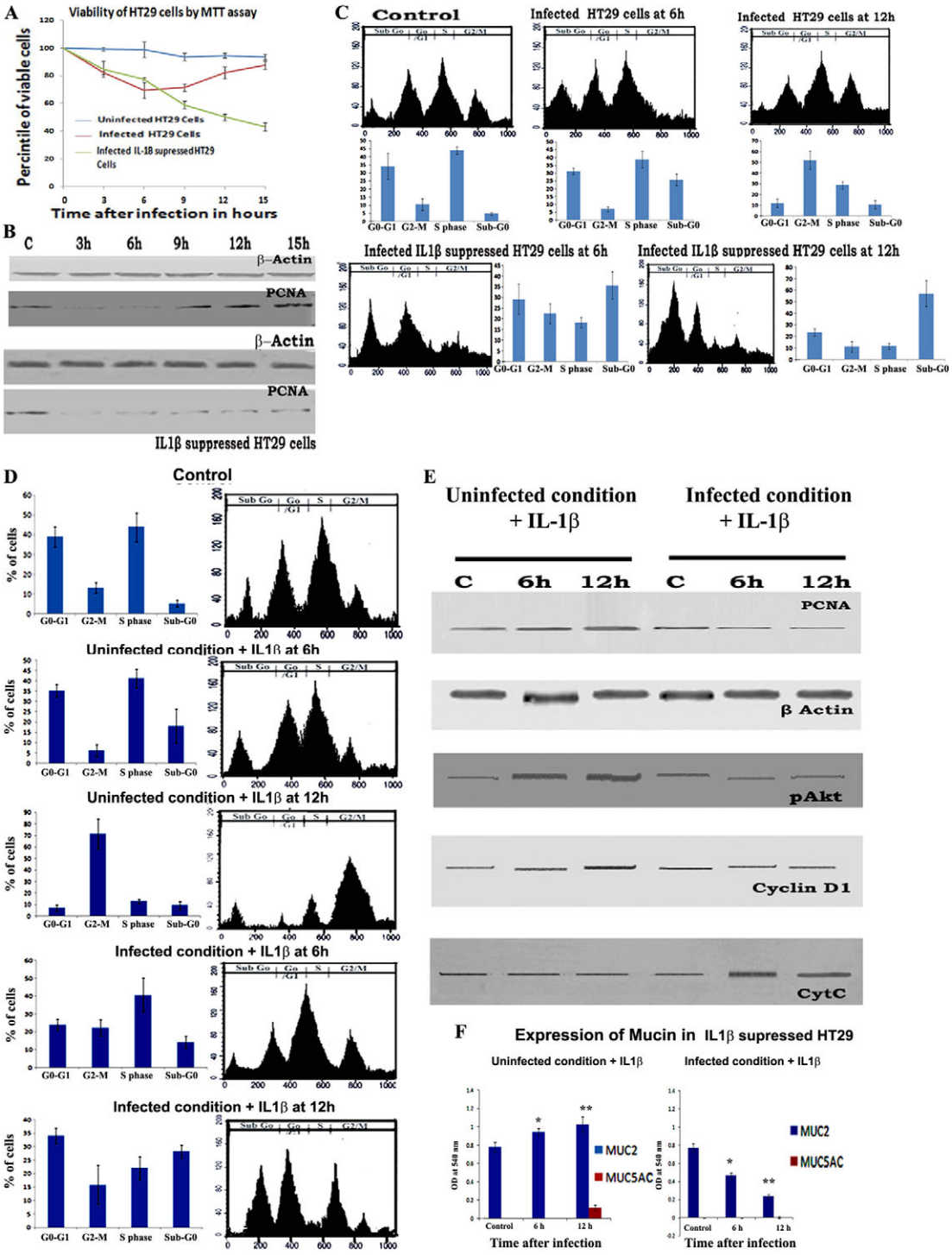

Fig. 2. Differential role of IL1β in uninfected and infected HT29 cells. (**A**) Viability of HT29 cells examined by MTT assay. (**B**) Expression analysis of PCNA in infected HT29 cells and polymyxin-B-pretreated HT29 cells. *S. dysenteriae*-induced expression of PCNA in HT29 cells is inhibited upon pre-treatment with polymyxin B. (**C**) Cell cycle analysis of infected IL1β-suppressed and unsuppressed HT29 cells. Infected unsuppressed HT29 cells show an increased number cells at Sub-G0 levels 6 hours post infection and there is a large increase in the number of cells in G2–M and S phase at 12 hours post infection. In polymyxin-B-pretreated infected HT29 cells, an increased number of cells is observed at both 6 hours and 12 hours post infection. (**D**) Effect of exogenous addition of IL1β on cell cycle regulation in infected and uninfected IL1β-suppressed HT29 cells. Upon exogenous addition of IL-1β to infected IL-1β-suppressed HT29 cells, more apoptotic cells are seen compared with levels in uninfected IL1β-suppressed HT29 cells, whereas S phase and G2–M phase cell numbers are higher in uninfected IL-1β-suppressed cells compared with infected cells after addition of IL-1β. (**E**) Effect of IL1β on *S. dysenteriae*-induced expression of PCNA, cyclin D1 and phosphorylated Akt in infected and uninfected IL1β-suppressed HT29 cells. Significantly increased levels of PCNA, phosphorylated Akt and cyclin D1 are seen upon exogenous addition of IL1β in uninfected IL1β-suppressed HT29 cells compared with infected IL1β-suppressed cells. (**F**) Effect of exogenous addition of IL1β on MUC2 and MUC5AC in *S. dysenteriae*-infected and uninfected IL1β-suppressed HT29 cells. Data values were obtained from triplicate analysis and are expressed as the mean ± s.d. $^{*}P<0.05$; $^{\#}P<0.001$.

S. dysenteriae-infected HT29-IL-1β^{sup} cells (Fig. 2B). Cell cycle analysis of control and *S. dysenteriae*-infected HT29 cells is shown in Fig. 2C.

IL-1β induces apoptosis of infected cells and proliferation of uninfected HT29 cells

Endogenous expression of IL-1β was blocked by pre-treating HT29 cells with polymyxin B. The effect of the addition of exogenous IL-1β on these cells was analysed by FACS and results are shown in Fig. 2D. Increased expression of PCNA, cyclin D1 and phosphorylated Akt was seen in uninfected HT29-IL-1β^{sup} cells after 6 hours and 12 hours of exogenous IL-1β addition; however, cytosolic cytC was unaltered. In *S. dysenteriae*-infected HT29-IL-1β^{sup}, levels of PCNA, cyclin D1 and phosphorylated Akt were decreased, but cytosolic cytC was increased (Fig. 2E). Exogenous addition of IL-1β induced MUC2 overexpression in uninfected HT29-IL-1β^{sup} cells at both 6 hours and 12 hours, whereas differential expression of MUC5AC was seen only at 12 hours. Exogenous addition of IL-1β to infected HT29-IL-1β^{sup} cells, decreased MUC2 expression and MUC5AC differential expression was also not observed (Fig. 2F).

Effect of LY-294002 on *S. dysenteriae*-induced upregulation of MUC2 and differential expression of MUC5AC

We also studied the role of Akt activation in *S. dysenteriae*-induced expression of MUC2 and MUC5AC by pre-treating HT29 cells with LY-294002 (Fig. 3A,C). LY-294002 pre-treatment inhibited *S. dysenteriae*-induced expression of *MUC5AC* and *TFF1* mRNA, although the expression of MUC2, E-cadherin and TFF3 was unchanged (Fig. 3A,B). Phosphorylation of EGFR and Ras was unchanged; however, LY-294002 pre-treatment inhibited *S. dysenteriae*-induced phosphorylation of Akt and GSK-3β (Fig. 3D). Accumulation of β-catenin in the cytosol was observed in infected HT29 cells pre-treated with LY-294002 (Fig. 3E).

Effect of TFF3 knockdown on *S. dysenteriae*-induced upregulation of MUC2 and differential expression of MUC5AC

The time-dependent overproduction of MUC2 was unchanged, whereas MUC5AC synthesis was not observed in *S. dysenteriae* infected TFF3-silenced HT29 cells (Fig. 4A,C). Furthermore, to

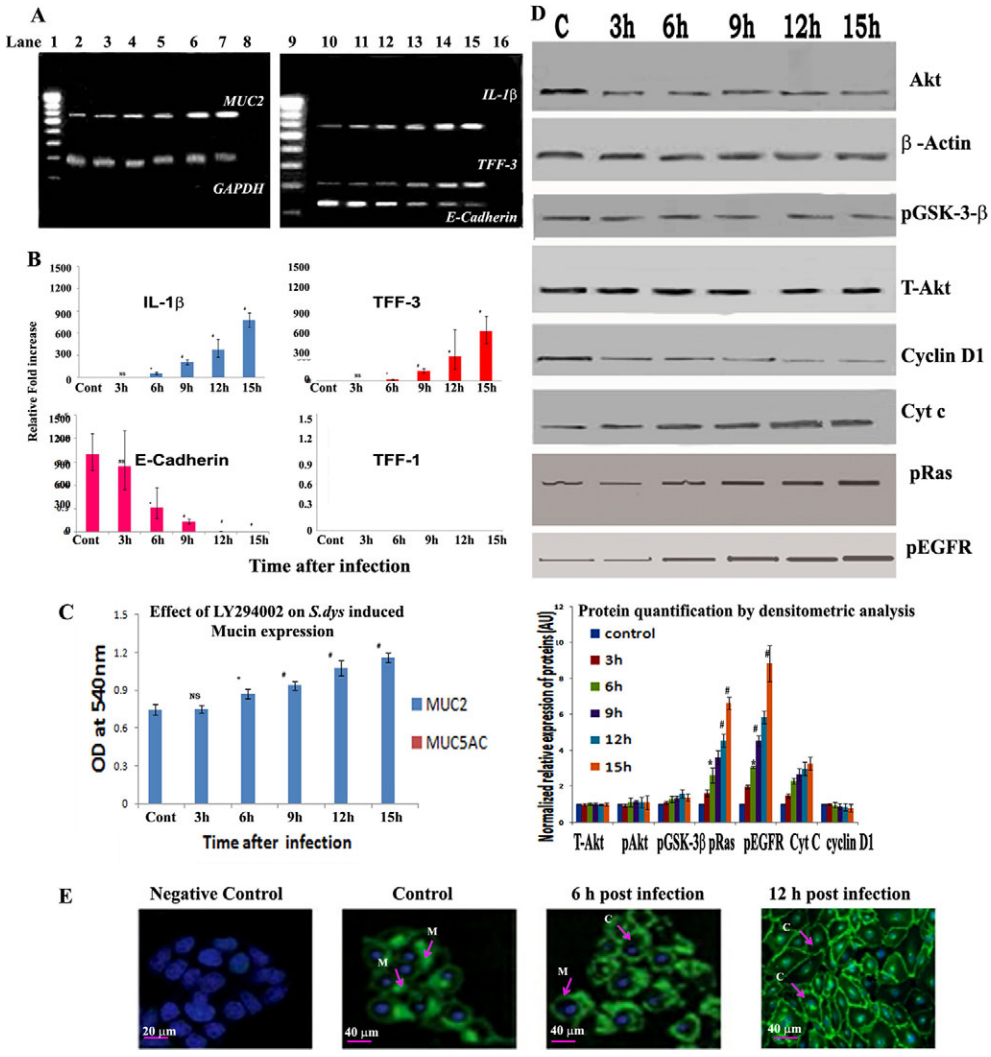

Fig. 3. Effect of Akt inhibition on *S. dysenteriae*-induced molecular changes. (**A**) Expression patterns of mRNA encoding MUC2, MUC5AC, IL-1β, GAPDH, TFF3, TFF1 and E-cadherin. Lanes 1 and 9, 100 bp marker; Lanes 2 and 10, uninfected control; Lanes 3–7 and 11–15 represent 3, 6, 9, 12 and 15 hours post infection with *S. dysenteriae*, respectively; Lanes 2–8 were amplified with primers specific for GAPDH, MUC2 and MUC5AC; Lanes 10–16 were amplified with primers of IL-1β, TFF3, TFF1 and E-cadherin; Lanes 8 and 16, negative control. (**B**) RT-PCR analysis of mRNA encoding IL1β, TFF3, TFF1 and E-cadherin. All expression values were normalized to the value of *GAPDH* gene used as an internal control. Relative amount was calibrated based on the transcript amount of the corresponding gene in uninfected controls. Expression changes of IL-1β, TFF3 and E-cadherin induced by *Shigella dysenteriae 1* in HT29 cells is unaffected by pre-treatment with LY294002, whereas expression of TFF1 is significantly inhibited. (**C**) Results of ELISA for MUC2 and MUC5AC production. MUC5AC production was inhibited significantly in infected HT29 cells upon pretreatment with LY294002. (**D**) Analysis of TAkt, cyclin D1, cytC and phosphorylated EGFR, Ras, Akt and GSK-3β expression. (**E**) β-catenin is localized to the membrane of uninfected cells (control). 6 hours and 12 hours post infection, β-catenin is found in the cytosol. Data values were obtained from triplicate analysis and are expressed as the mean ± s.d. $^{*}P<0.05$; $^{\#}P<0.001$.

confirm that TFF3 is the crosslinking molecule between IL-1β and Akt that leads to production of MUC5AC, we examined the effect of knockdown of TFF3 on infection-induced expression of IL-1β, E-cadherin and TFF1, as well as phosphorylation of EGFR, Akt and GSK-3β. The knockdown of TFF3 inhibited *S. dysenteriae*-induced phosphorylation of EGFR, Akt and GSK-3β. Decreased levels of cyclin D1 and increased levels of E-cadherin were observed in *S. dysenteriae*-infected TFF3-silenced HT29 cells (Fig. 4C,D). However, the expression pattern of MUC2 and IL-1β remained unchanged (Fig. 4A–C). *S. dysenteriae*-induced translocation of β-catenin was not observed upon knockdown of TFF3 (Fig. 4D). The *S. dysenteriae*-induced differential expression of MUC5AC was not seen in HT29 cells transfected with scrambled siRNA, where suppression of TFF3 expression was rescued (supplementary material Fig. S2).

ERK1/2 in IL-1β-induced expression of TFF3, MUC2 and MUC5AC during *S. dysenteriae* infection

As shown in Fig. 4F, ERK1/2 phosphorylation was stimulated by *S. dysenteriae* infection in a time-dependent manner, similar to

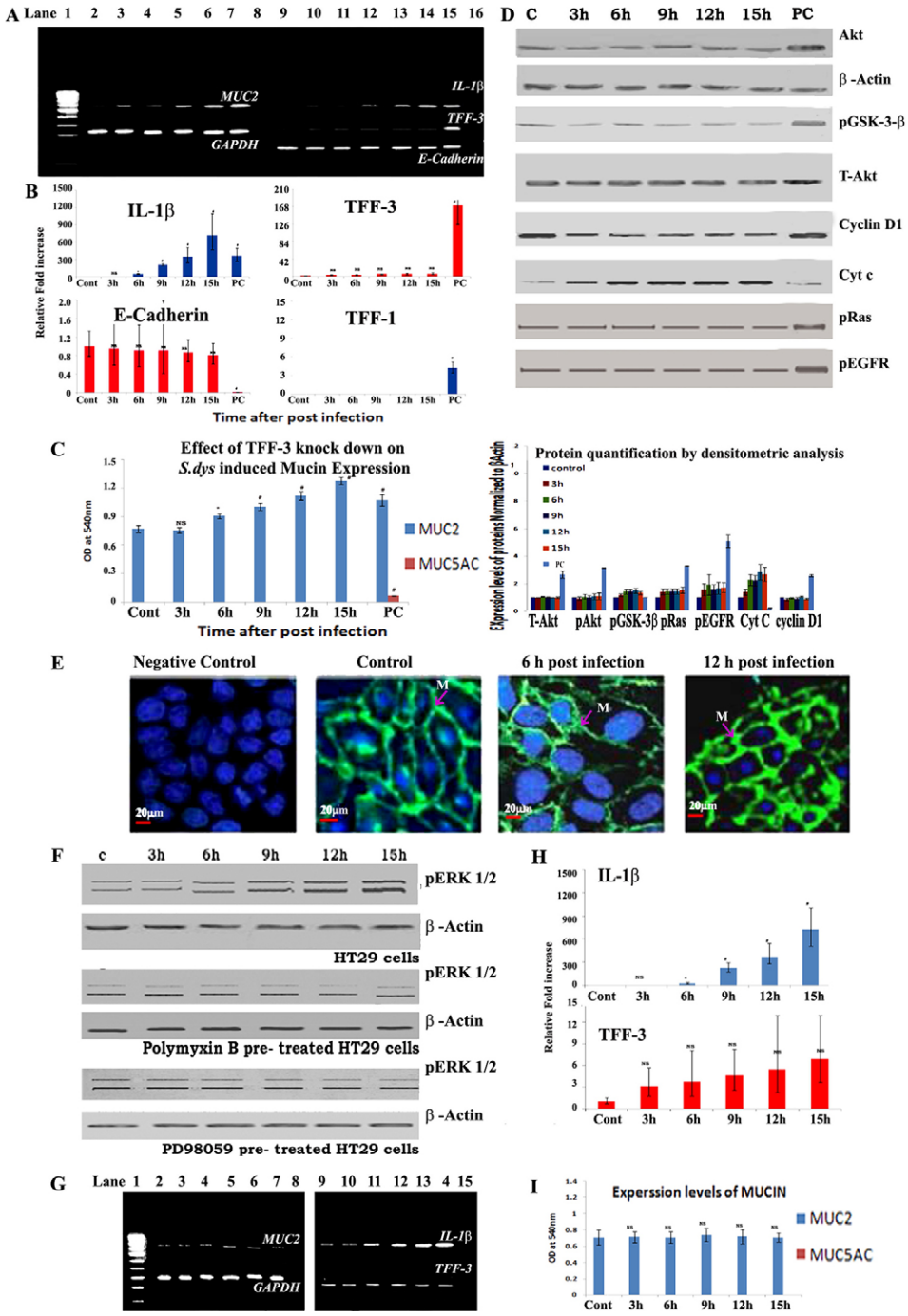

Fig. 4. See next page for legend.

that of TFF3, MUC2 and MUC5AC. Polymyxin B treatment repressed *S. dysenteriae*-induced phosphorylation of ERK1/2. It was also confirmed that the specific inhibitor PD98059 blocked *S. dysenteriae*-induced ERK1/2 phosphorylation as well as expression of TFF3, MUC2 and MUC5AC (Fig. 4G–J).

Adherence and invasion assay of *S. dysenteriae* in HT29 cells

Levels of adherence of *S. dysenteriae* to different mucin-secreting HT29 cells was in the order: HT29p>HT29(NCCS)>HT29-FU>HT29-MTX (highest to lowest adherence; Fig. 5A). At 3 hours post infection, 286,000±28,000, 176,000±12,000, 156,000±19,000 and 67,000±9000 CFU were recovered from HT29p, HT29(NCCS), HT29-FU and HT29-MTX, respectively. At 6 hours post infection, an average of 715,000±26,000, 442,000±49,000, 390,000±19,000, and 176,000±27,000 CFU were recovered, respectively. An increased number of intracellular bacteria were observed in HT29p cells when compared with the other cell lines (Fig. 5B). Viabilities of control and *S. dysenteriae*-infected HT29 cells at 6 hours post infection are shown in Fig. 5C. Viability of HT29(NCCS), HT29p, HT29-MTX and HT29-FU cells was above 95%, which decreased 6 hours after infection. Viability of HT29-MTX cells was high (80%) after 6 hours, but viability of HT29-FU (57%) and HT29(NCCS) (45%) cells was slightly lower.

Fig. 4. Effect of TFF3 knockdown on *S. dysenteriae*-induced molecular changes. (**A**) Expression patterns of genes encoding MUC2 and MUC5AC, IL1β, GAPDH, TFF3, TFF1 and E-cadherin. Lane 1, 100 bp marker; lanes 2 and 9, uninfected control; lanes 3–7 and 10–14 represent 3, 6, 9, 12 and 15 hours post infection, respectively; Lanes 2–8 were amplified with primers specific for GAPDH, MUC2 and MUC5AC; Lanes 10–16 were amplified with IL-1β, TFF3, TFF1 and E-cadherin; lanes 8 and 16, negative control; lane 15, positive control (TFF3 non-silenced uninfected HT29 cells). (**B**) RT-PCR analysis of IL1β, TFF3, TFF1 and E-cadherin. All expression values were normalized to the value of *GAPDH* gene used as an internal control. Relative amount was calibrated based on the transcript amount of the corresponding gene in uninfected controls. Changes in expression of genes encoding IL-1β and E-cadherin induced by *S. dysenteriae* in HT29 cells is unaffected by TFF3 silencing, whereas expression of *TFF1* is significantly inhibited. (**C**) Analysis of MUC2 and MUC5AC production. MUC5AC production alone was inhibited significantly in HT29 cells infected with *S. dysenteriae* upon silencing of TFF3. (**D**) Analysis of TAkt, cyclin D1, cytC and phosphorylated EGFR, Ras, Akt and GSK-3β. PC, positive control. (**E**) β-catenin is localized in membrane in uninfected cells (control) and also in infected HT29 cells 6 hours and 12 hours post infection. (**F**) Role of ERK inhibitor on *S. dysenteriae*-induced MUC2 and MUC5AC. *S. dysenteriae*-induced activation of pERK1/2 in HT29 cells is inhibited upon pre-treatment with polymyxin B and PD98059. (**G**) Expression pattern of MUC2, MUC5AC, IL1β, TFF3, TFF1 and E-Cadherin in PD98059-pretreated HT29 cells infected with *S. dysenteriae.* Lane 1, 100 bp marker; lanes 2–8, GAPDH, MUC2 and MUC5AC; Lanes 9–15, E-cadherin, IL1β, TFF3 and TFF1. Lanes 2 and 9, uninfected control; Lanes 8 and 15, negative control. (**H**) RT-PCR analysis of genes encoding IL1β, TFF3, TFF1 and E-cadherin in PD98059 pretreated HT29 cells infected with *S. dysenteriae.* All expression values were normalized to the value of *GAPDH* gene used as an internal control. Relative amount was calibrated based on the transcript amount of the corresponding gene in uninfected controls. Changes in expression of genes encoding TFF3 and E-cadherin induced by *S. dysenteriae* in HT29 cells is inhibited by pre-treatment with PD98059, whereas expression of IL-1β is unaffected. (**I**) Production of MUC2 and MUC5AC in PD98059-pretreated HT29 cells infected with *S. dysenteriae.* MUC2 and MUC5AC production is significantly inhibited in infected HT29 cells after pretreatment with PD98059. Data values were obtained from triplicate analysis and are expressed as the mean ± s.d. $^{*}P<0.05$; $^{\#}P<0.001$.

Discussion

Overproduction of MUC2 and MUC5AC has been reported in the pathogenesis of cystic fibrosis and bronchial asthma (Levine et al., 1995; Basbaum et al., 1999; Smirnova et al., 2000). A similar mucin expression profile (overexpression of MUC2 and differential expression of MUC5AC) was seen in *S. dysenteriae*-infected HT29 cells, as well as in experimentally induced shigellosis in rabbit (Radhakriahnan et al., 2008). Hence, in the present study the functional relevance and signal transduction involved in differential expression of MUC5AC in *S. dysenteriae* infection was investigated.

At various times post infection, expression of MUC2 steadily increased, whereas expression of MUC5AC was observed only after 9 hours of infection, which is associated with time-dependent increase in levels of IL-1β, TFF3, cyclin D1, pEGFR and phosphorylated Ras, Akt and GSK-3β. Altered expression of MUC2 and differential expression of MUC5AC are also associated with time-dependent downregulation of E-cadherin and nuclear translocation of β-catenin. Entry of β-catenin into the nucleus activates expression of cyclin D1, whereas phosphorylated GSK-3β inhibits ubiquitylation of cyclin D1 (Alt et al., 2000; Barbash et al., 2008; Raven et al., 2008) causing accumulation of cyclin D1. This leads to cell cycle dysregulation, favoring cell proliferation and survival. An increased apoptotic cell population and high cytosolic cytC were found during the first 6 hours of infection, which progressively decreased with time as a result of activation of cell survival. These facts clearly demonstrate that differential expression of MUC5AC involves signaling crosstalk between the inflammatory pathway and cell survival pathway.

IL-1β alone and in combination with LPS, can induce the overexpression of mucins in airway epithelial cells, goblet cells and murine biliary epithelial cells (Kim et al., 2002; Levine et al., 1995; Li et al., 1998; Dohrman et al., 1998; Zen et al., 2002; Perdomo et al., 1994; Zychlinsky et al., 1994; Raqib et al., 1995). *S. dysenteriae* infection causes a severe inflammation in the colon, which is triggered by extensive production of proinflammatory cytokines such as tumor necrosis factor alpha (TNFα) and IL-1β, which have been found in the stool and plasma of patients with acute shigellosis (Perdomo et al., 1994; Zychlinsky et al., 1994; Raqib et al., 1995; Kim et al., 2002). Addition of the IRK-4 inhibitor polymyxin B abrogated the *S. dysenteriae*-induced production of MUC2 and MUC5AC. It also inhibited *S. dysenteriae*-induced change in levels of TFF3, E-cadherin, cyclin D1 and phosphorylated EGFR, Ras, Akt and Gsk-3β, with membranous localization of β-Catenin as a result of suppression of IL-1β expression. Our result shows that IL-1β not only increases MUC2 expression and MUC5AC synthesis but it also increases TFF3 expression and activation of Akt in HT29 cells during *S. dysenteriae* infection. These findings confirm that, during *S. dysenteriae* infection IL-1β induces MUC5AC production through overexpression of TFF3 and activation of Akt.

Most studies state that IL-1β induces apoptosis by activating its target genes (Yasunori et al., 2006; Thirumala-Devi et al., 2007; Dinarello, 2007). Now a question of concern arises: how does IL-1β activate upregulation of *MUC2* and differential expression of *MUC5AC*? To address this, viability of *S. dysenteriae*-infected IL-1β suppressed and unsuppressed HT29 cells were assessed. In infected unsuppressed HT29 cells, viability decreased with decreased expression of PCNA, where

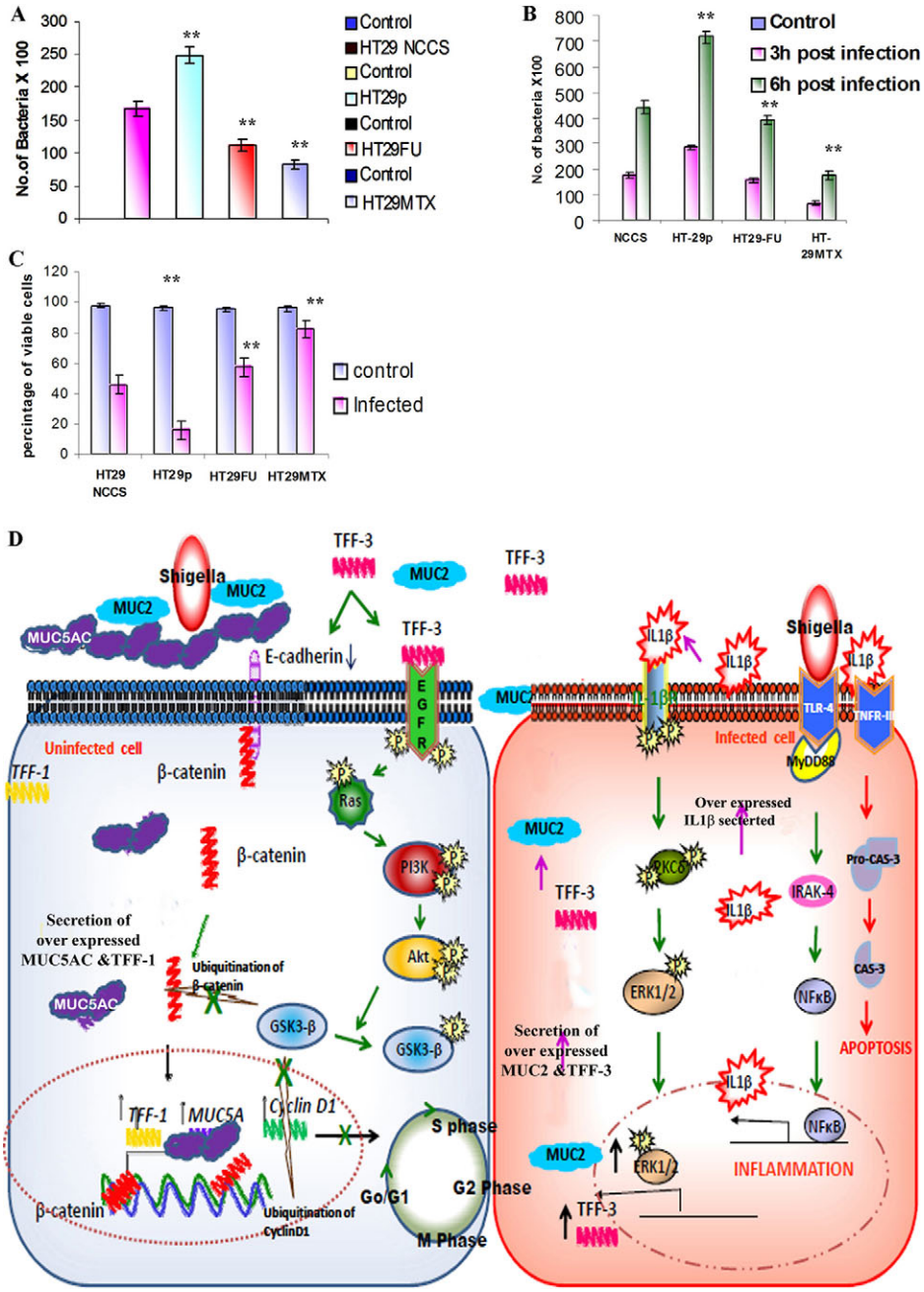

Fig. 5. Adherence and invasion of *S. dysenteriae* to mucous-secreting cells and their viability analysis. (**A**) Adherence of *S. dysenteriae* was significantly (**$P<0.01$) high in HT29 p (non-mucin secreting) when compared with other mucin-secreting cells (HT29 MTX, HT29FU). (**B**) Significant increase (**$P<0.01$) in intracellular *S. dysenteriae* count was observed in HT29 p (non-mucin secreting) when compared with other mucin-secreting cells (HT29FU, HT29 MTX). (**C**) Viability of *S. dysenteriae*-infected HT29 p (non-mucin secreting) was significantly (**$P<0.01$) low when compared with other mucin-secreting cells (HT29 MTX, HT29FU). (**D**) Proposed regulatory mechanism for differential expression of MUC5AC. Interaction of *Shigella* LPS with TLR-4 induces IL-1β overexpression through IRK-4. IL-1β induces apoptosis involving activation of caspase-3 and cytosolic cytochrome C in infected cells (right). IL-1β induces MUC2 and TFF3 overexpression through activation of ERK1/2 in both infected and uninfected cells. In uninfected cells (left), TFF3 induces activation of Akt by interacting with EGFR causing inactivation of GSK-3β. TFF3 also decreases the E-cadherin expression, which disrupts cadherin–catenin interaction leading to cytosolic accumulation of β-catenin. GSK-3β inactivation inhibits ubiquitylation of β-catenin and cyclin D1, causing nuclear translocation of β-catenin and accumulation of cyclin D1. Nuclear translocation of β-catenin induces differential expression of MUC5AC along with cell survival factors (including PCNA and cyclin D1). Accumulation of cyclin D1 induces cell cycle dysregulation, causing proliferation of cells and restitution of damaged cells, where MUC5AC differential expression negotiates further influx of bacteria through mucosal clearance.

a greater percentage of cells were in sub G0 phase until 6 hours post infection. Thereafter, an increase in cell viability, PCNA expression and the percentage of S phase and G2M phase cells was observed, which was not the same in *S. dysenteriae*-infected HT29-IL-1β^{sup} cells. This confirms the involvement of IL-1β in both apoptosis and cell proliferation.

These findings show that IL-1β induces both apoptosis and cell proliferation. However, the specificity of IL-1β in triggering cells to apoptosis or cell proliferation is unknown. To determine the role of IL-1β in inducing cell proliferation, we performed an experiment where endogenous production of IL-1β was blocked using polymyxin B and the effect of exogenous IL-1β on infected and uninfected cells was analyzed. Upon the exogenous addition of IL-1β, *S. dysenteriae*-infected HT29-IL-1β^{sup} cells showed more cells undergoing apoptotis, with increased cytosolic cytC and decreased PCNA levels. Overexpression of PCNA, cyclin D1, activation of Akt and unaltered cytC levels with increased S and G2–M cell populations were observed in IL-1β treated uninfected HT29-IL-1β^{sup} cells. Taken together, these results show that IL-1β induces apoptosis in infected cells and induces proliferation in uninfected cells.

The PI3K selective inhibitor LY-294002 blocked MUC5AC expression induced by *S. dysenteriae*, suggesting that phosphorylation of Akt and Gsk-3β, accumulation of cyclin D1 and nuclear translocation of β-catenin are involved in MUC5AC production. Because LY-294002 pre-treatment did not affect overexpression of IL-1β and TFF3 or phosphorylation of EGFR and Ras, this suggests that they act upstream of Akt. Thus, we

cannot rule out the influence of IL-1β in the activation of Akt by phosphorylation of EGFR and Ras, where TFF3 can act as ligand for EGFR because there were unaltered levels of EGF and TGF-α along with overexpression of TFF3.

Trefoil peptides (TFF1, TFF2 and TFF3) are a group of small proteins belonging to trefoil factor family, which are co-expressed with mucin and play a vital role in restitution of gastrointestinal epithelial cells. TFF3 tends to increase in various pathological conditions including bacterial infection and colon cancer and is also found in brain tumors and breast cancer (Skeen et al., 2006; Wright et al., 1997; Dignass et al., 1994; Kindon et al., 1995). EGFR ligands such as EGF and TGF-α were not altered by *S. dysenteriae* infection; furthermore, TFF3 knockdown inhibited MUC5AC production, with unaltered levels of phosphorylated EGFR, Ras, Akt and GSK-3β. Unaltered expression of E-cadherin, decreased levels of cyclin D1 and membranous localization of β-catenin were observed in *S. dysenteriae*-infected TFF3-silenced HT29 cells. However, TFF3 knockdown did not affect expression of MUC2. These results prove that TFF3 is also involved in activation of Akt through phosphorylation of EGFR and Ras as well as in downregulation of E-cadherin expression.

Recent studies show that TFF3 interacts with the epidermal growth factor receptor (EGFR) and influences its phosphorylation, leading to signal transduction (Guzman et al., 1995; Takeyama et al., 2000; Perrais et al., 2002; Longman et al., 2000), which supports our above findings exemplifying the involvement of TFF3-mediated Akt activation through phosphorylation of EGFR and Ras in MUC5AC synthesis. Thus, for the first time, we report that TFF3 acts as bridging molecule between inflammatory and cell survival pathways, which are involved in the differential expression of MUC5AC. The ERK selective inhibitor blocked the MUC2, MUC5AC and TFF3 expression induced by IL-1β. This suggests that IL-1β influences the overexpression of TFF3 through ERK1/2 in a similar manner to that of MUC2 expression.

It is important to understand the functional relevance of altered and differential expression of mucins during *S. dysenteriae* infection because there are reports that deal with the role of mucins in pathogenesis (Weiss et al., 1996; Udhayakumar et al., 2007; Leteurtre et al., 2004). It is important also to investigate whether the overexpression of MUC2 and differential expression of MUC5AC in shigellosis favors the host or the pathogen. To investigate this, three colon cancer cell lines with different mucin expression profiles, obtained from the heterogeneous colon carcinoma HT29 cell lines were used: HT29p cells are differentiated HT29 cells, selected by hexose deprivation, that do not secrete mucin (Zaretsky et al., 1999); HT29-FU cell line selected by 5-Fluorouracil supplementation shows expression of MUC1, MUC2 and MUC4; HT29-MTX cells selected by methotoxtorate supplementation shows expression of MUC1, MUC3 and MUC5AC (Zweibaum et al., 1985; Lesuffleur et al., 1993; Zweibaum et al., 1995; Lesuffleur et al., 1995).

The binding of *S. dysenteriae* and intracellular bacterial count were higher in HT29p (non mucin secreting) and HT29-FU (MUC2 secreting) compared with HT29-MTX (MUC5AC secreting) cells. This correlates with our earlier reports that showed the specificity of binding of *S. dysenteriae* to colonic mucin (MUC2) and not to small intestinal or gastric mucin (MUC5AC) (Rajkumar et al., 1998; Sudha et al., 2001). A clear zone of mucin lysis was seen around the colonies of *S. dysenteriae*, when they were grown on MUC2, whereas the zone of lysis was not seen when grown on MUC5AC (data not shown) and significantly increased numbers of CFUs were internalized in MUC2-secreting HT29-FU cells compared with MUC5AC-secreting HT29-MTX cells. Differential expression of MUC5AC during *S. dysenteriae* infection is a boon to the host, because it aids in inhibition of its mucosal penetration and invasion of host cells as a result of the lack of mucinolytic specificity to MUC5AC.

We therefore propose a signaling mechanism for the differential expression of MUC5AC in colonic epithelial cells during *Shigella* infection (Fig. 5D). Interaction of *Shigella* LPS with TLR-4 induced overexpression of IL-1β through IRK-4. Overexpression of IL-1β leads to MUC2 and TFF3 overexpression through activation of ERK1/2. Increased TFF3 expression induces activation of Akt by interacting with EGFR, causing inactivation of GSK-3β. TFF3 also decreases E-cadherin expression, which disrupts cadherin–catenin interaction, causing cytosolic accumulation of β-catenin. GSK-3β inactivation inhibited ubiquitylation of β-catenin and cyclin D1, causing nuclear translocation of β-catenin and accumulation of cyclin D1. Nuclear translocation of β-catenin induces differential expression of MUC5AC along with cell survival factors, including PCNA and cyclin D1. This leads to increased accumulation of cyclin D1, causing cell cycle deregulation, inducing cell proliferation and restitution of damaged cells. The differential expression of MUC5AC negotiates further adherence and invasion of bacteria through mucosal clearance and forms part of host immune response.

Materials and Methods

Cell culture and maintenance

HT29 cell lines were obtained from NCCS Pune. Three different HT29 human colon tumor cell lines; HT29-MTX, HT29-FU and HT29p cells were gifts from Thécla Luseferlur, INSERM, Paris, France. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Himedia, Mumbai, India) supplemented with 10% FBS (Himedia), 100 U/ml penicillin, 100 μg/ml streptomycin, 20 μg/ml fungizone (Himedia), pH 7.4 in 25 cm^2 tissue culture flasks (Himedia) at 37℃ under 5% CO_2 and 95% air.

Infection of HT29 cells with *S. dysenteriae*

HT29 cells were seeded into 6-, 12- or 24-well Costar tissue culture plates at a density of 2×10^5 cells/ml in volumes of 2 ml, 1 ml or 0.5 ml per well, respectively. When cells reached confluency, bacteria were infected at 50 CFU per epithelial cell (50:1 ratio) for 30 minutes. The monolayer was washed twice to remove extracellular bacteria. Cultures were incubated for different time intervals in the presence of 50 μg/ml of gentamicin to kill the remaining extracellular bacteria and were used for various assays including western blotting analysis using the following antibodies: human reactive monoclonal antibodies anti-Cyclin D1(1:1000), anti-EGFR (1:500), anti-PCNA and anti-β-actin (1:1000) were from Santa Cruz Biotechnology (Santa Cruz, CA); human reactive monoclonal antibody anti-cytochrome *C* (1:500) was from BD Biosciences (San Diego, CA); and human reactive polyclonal antibody Akt pathway detection kit was from Cell Signaling Technology (Beverly, MA) and was diluted according to manufacturer's instructions.

Inhibitors

Polymyxin B (HiMedia) 50 μM/well (six-well plates) was used to inhibit endotoxin (LPS of gram-negative bacteria) induced IL-1β expression. LY-294002 (Cell Signaling) was used to inhibit PI3K. 50 μM of LY-294002 was added and incubated for 1 hour prior to assay.

siRNA transfection

TFF3 was silenced by transfection with *TFF3* siRNA (Santa Cruz). In a six-well tissue culture plate, 2×10^5 cells per well were seeded in 2 ml antibiotic-free normal growth medium supplemented with FBS. Cells were incubated at 37℃ in a CO_2 incubator until the cells were 60–80% confluent. Cell viability was assessed before transfection. For each transfection, 6 μl of siRNA duplex (i.e. 0.25–1 μg or 20–80 pmols siRNA) in 100 μl siRNA transfection medium (solution A) and 6 μl

of siRNA transfection reagent in 100 µl siRNA transfection medium (solution B) were mixed and incubated for 45 minutes at room temperature. For each transfection, 0.8 ml siRNA transfection medium was added to each tube, mixed gently, overlaid onto washed cells and incubated for 5–7 hours at 37℃ in a CO_2 incubator. After incubation, 1 ml of normal growth medium containing twice the normal serum and antibiotic concentration (2× normal growth medium) was added without removing the transfection mixture. Cells were incubated for an additional 18–24 hours. Medium was replaced with 1 ml of fresh 1× normal growth medium and used for further assay.

Bacterial binding assay

Binding of bacteria to HT29 cells was determined according to published methods (Alwan et al., 1998). Briefly, 100 µl of bacterial suspension (100 bacteria/cell) was added in triplicate to HT29 cells grown in 96-well plates. The plates were incubated at 37℃ for 1 hour. After washing three times with Tris-buffered saline containing 0.01% Tween 20 (TBS-T), the adherent bacteria were fixed by overnight incubation with 0.3% formaldehyde in PBS. Plates were washed three times with TBS-T. 100 µl (1:500 diluted) of *Shigella dysenteriae* 1 antisera was added to each well. Plates were incubated for 1 hour at 37℃ and washed three times with TBS-T. 100 µl (1:500 diluted) horseradish-peroxidase-conjugated anti-mouse immunoglobulin was added to each well and incubated for 1 hour at 37℃. 100 µl of substrate was added to each well and the reaction was stopped by the addition of 50 µl of 2M H_2SO_4. Optical density (OD) at 490 nm was determined using a microtitre plate reader. Negative controls consisted of wells in which normal mouse sera (non-immunized) were used. In some wells, a serially diluted, known number of bacteria alone were coated (in carbonate/bicarbonate buffer, pH 9.6) without epithelial cells to determine the reactivity of the immunized sera and used to calculate the amount of bacteria bound to epithelial cells. Background values for the ELISA, or the amount of non-specific binding of the bacteria to plastic were determined by coating wells with HT29 cells but to which no bacteria were added to ensure that the primary antibody did not crossreact with HT29 cells.

Invasion assay

Invasion assay was carried out according to published methods (Donnenberg et al., 1989). Confluent cells were infected with bacteria in 1:100 ratio and incubated for 3 hours and 6 hours at 37℃ under 5% CO_2 and 95% air. Cells were washed twice with growth medium, followed by two washes with PBS. 0.25 ml of 1% Triton X-100 was added and incubated for 30 minutes to liberate the intracellular bacteria which were counted on LB agar.

Isolation of RNA

For preparation of total RNA, the phenol–guanidinium-thiocyanate-based TriReagent (GeNei, Bangalore, India) was used. To 10^7 cells, 1 ml of TriReagent was added and lysed by repetitive pipetting and allowed to stand for 5 minutes followed by addition of 200 µl of chloroform for phase separation. Samples were vigorously vortexed for 15 seconds and allowed to stand for 15 minutes followed by centrifugation at 12,000 ***g*** for 15 minutes at 4℃. The upper aqueous layer containing RNA was transferred to a fresh sterile DEPC-treated microfuge tube. To this, 500 µl of ice-cold isopropanol was added, gently mixed and allowed to stand for 10 minutes and centrifuged at 12,000 ***g*** for 15 minutes at 4℃. The supernatant was discarded and the RNA pellet was washed with 1 ml of 75% ethanol in DEPC-treated water and again centrifuged at 14,000 ***g*** for 10 minutes at 4℃ to obtain total RNA. This pellet was dissolved in 25 µl of sterile RNase-free water by heating at 55℃ for 20 minutes and stored at −20℃ until use.

RT-PCR

To synthesize cDNA, a reverse transcription reaction solution containing 1.0 µg total RNA in RNase/DNase-free water and 1.5 µl of random hexamer primer (GeNei) were incubated for 10 minutes at 72℃ and chilled immediately. To this, 5.0 µl premixed 10 mM dNTP solution (GeNei), 3.0 µl 10X M-MLV reverse transcriptase buffer (GeNei), 1.0 µl (200 U/µl) M-MLV reverse transcriptase (GeNeiTM, Bangalore) was added and made up to 50 µl using sterile RNase/DNase-free water.

To amplify the cDNA, polymerase chain reaction (PCR) ready mix (GeNei) was used according to the manufacturer's instructions. All PCR samples were denatured at 94℃ for 5 minutes prior to cycling and were extended for 10 minutes at 72℃ following cycling. The PCR assay using primers was performed for 39 cycles at 94℃ for 60 seconds, 60℃ for 60 seconds and 72℃ for 60 seconds. Primers for GAPDH, MUC2, MUC5AC, E-cadherin, IL-1β, TFF3, TFF1 are shown in supplementary material Table S1. Primers were designed using primer3 software and nucleic acid sequence was accessed from the NCBI website (supplementary material Table S1). The primers were purchased from Integrated DNA Technologies (Coralville, IA).

Immunocytochemical analysis

Cells were grown directly on cover slips in six-well plates. Bacterial infection was done as stated above and cells were processed further for immunofluorescence analysis. Cells were washed with PBS and covered with 2–4% formaldehyde in PBS to a depth of 2–3 mm, allowed to stand for 15 minutes at room temperature to fix cells. Fixative was removed and cells were washed with PBS for three times each for 5 minutes. After formaldehyde fixation, cells were covered with ice-cold 100% methanol and incubated for 10 minutes at –20℃. Cells were then rinsed in PBS for 5 minutes and non-specific sites were blocked with Blocking Buffer for 60 minutes. Blocking buffer was replaced by diluted primary antibody [anti-β-Catenin (1:500), Santa Cruz Biotechnology]. Incubated overnight at 4℃ and rinsed three times in PBS for 5 minutes each. Then, cells were incubated with fluorochrome-conjugated secondary antibody (FITC-conjugated secondary antibody from GeNeiTM, Bangalore, 1:1000 dilution) for 1–2 hours at room temperature in the dark. Cells were washed three times with high-salt PBS for 5 minutes each. Coverslips were fixed on glass slides with mounting medium containing antifade reagent. Slides were sealed and viewed under a fluorescence microscope.

Real-time RT-PCR analysis

Real-time RT-PCR analysis were conducted separately in a 10 µl volume containing 50 ng cDNA and 0.25 µM each of forward and reverse primers for GAPDH, IL-1β, TFF3, TFF1 and E-cadherin (supplementary material Table S2). The following cycling conditions were chosen: Pre incubation at 95℃ for 10 minutes, then 45 cycles of 95℃ for 10 seconds, 60℃ for 15 seconds and 72℃ for 30 seconds. Each reaction was measured in triplicate. The relative difference in gene expression was calculated using the cycle time (Ct) values that were first normalised to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the endogenous control in the same sample and the relative control Ct value following the $2^{-\Delta\Delta}$Ct method. The data represent the mean fold changes with s.d.

Statistical analysis

Values were recorded as the mean ± s.d. of three experiments. Experimental results were analyzed by Student's *t*-test. $P<0.001$ was considered highly significant and $P<0.05$ was considered significant for values obtained for treated groups compared with the control group.

Acknowledgements

We gratefully acknowledge Thécla Lesuffleur, INSERM U843, France for the gift of HT29p, HT29-FU and HT29-MTX cell lines.

Funding

This study was supported by a senior research Fellowship from the Indian Council of Medical Research (ICMR) and by the Council of Scientific and Industrial Research, India, to S.B.R.

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.092148/-/DC1

References

Alt, J. R., Cleveland, J. L., Hannink, M. and Diehl, J. A. (2000). Phosphorylation-dependent regulation of cyclin D1 nuclear export and cyclin D1-dependent cellular transformation. *Genes Dev.* **14**, 3102-3114.

Alwan, A., Deignan, T., O'Sullivan, M., Kelly, J. and O'Farrelly, C. (1998). Quantitative assay of Salmonella adherence to intestinal epithelial cells: a new method for assessing novel intervention products. *J. Microbiol. Methods* **33**, 163-170.

Barbash, O., Zamfirova, P., Lin, D., Chen, X., Yang, K., Lu, F., Rustgi, A. K. and Diehl, J A. (2008). Mutations in Fbx4 inhibit phosphorylation-epended dimerization of the SCFF bx4 ligase and contribute to cyclin D1 overexpression in human cancer. *Cancer Cell* **14**, 68-78.

Bartman, A. E., Sanderson, S. J., Ewing, S. L., Niehans, G. A., Wiehr, C. L., Evans, M. K. and Ho, S. B. (1999). Aberrant expression of MUC5AC and MUC6 gastric mucin genes in colorectal polyps. *Int. J. Cancer* **80**, 210-218.

Basbaum, C., Lemjabbar, H., Longphre, M., Li, D., Gencsch, E. and Mcnamara, N. (1999). Control of mucin transcription by diverse injury-induced signaling pathways. *Am. J. Respir. Crit. Care Med.* **160**, S44-S48.

Dabbagh, K., Takeyama, K., Lee, H. M., Ueki, I. F., Lausier, J. A. and Nadel, J. A. (1999). IL-4 induces mucin gene expression and goblet cell metaplasia in vitro and in vivo. *J. Immunol.* **162**, 6233-6237.

Damera, G., Xia, B. and Sachdev, G. P. (2006). IL-4 induced MUC4 enhancement in respiratory epithelial cells in vitro is mediated through JAK-3 selective signaling. *Respir. Res.* **7**, 39.

Devaraj, S., Sheyknazari, M., Warren, W. S. and Bhavanandan, V. P. (1994). Differential binding of Pseudomonas aeruginosa to normal and cystic fibrosis tracheobronchial mucins. *Glycobiology* **4**, 307-316.

Dignass, A. K., Lynch-Devaney, H., Kindon, L., Thim, L. and Podolsky, D. K. (1994). Trefoil peptides promote epithelial migration through a transforming growth factor beta-independent pathway. *J. Clin. Invest*. **94**, 376-383.

Dinarello, C. A. (2007). A signal for the caspase-1 inflammasome free of TLR. *Immunity* **26**, 383-385.

Dohrman, A., Miyata, S., Gallup, M., Li, J. D., Chapelin, C., Coste, A., Escudier, E., Nadel, J. A. and Basbaum, C. (1998). Mucin gene (MUC2 and MUC5AC) upregulation by Gram-positive and Gram-negative bacteria. *Biochim. Biophys. Acta* **1406**, 251-259.

Donnenberg, M. S., Donohue-Rolfe, A. and Keusch, G. T. (1989). Epithelial cell invasion: an overlooked property of enteropathogenic Escherichia coli (EPEC) associated with the EPEC adherence factor. *J. Infect. Dis*. **160**, 452-459.

Guzman, K., Randell, S. H. and Nettesheim, P. (1995). Epidermal growth factor regulates expression of the mucous phenotype of rat tracheal epithelial cells. *Biochem. Biophys. Res. Commun.* **217**, 412-418.

Hollingsworth, M. A. and Swanson, B. J. (2004). Mucins in cancer: protection and control of the cell surface. *Nat. Rev. Cancer* **4**, 45-60.

Iwashita, J., Sato, Y., Sugaya, H., Takahashi, N., Sasaki, H. and Abe, T. (2003). mRNA of MUC2 is stimulated by IL-4. IL-13 or TNF-alpha through a mitogen-activated protein kinase pathway in human colon cancer cells. *Immunol. Cell Biol*. **81**, 275-282.

Kim, K. C., Nassiri, J. and Brody, J. S. (1989). Mechanisms of airway goblet cell mucin release: studies with cultured tracheal surface epithelial cells. *Am. J. Respir. Cell Mol. Biol*. **1**, 137-143.

Kim, Y. D., Kwon, E. J., Kwon, T. K., Baek, S. H., Song, S. Y. and Suh, J. S. (2000). Regulation of IL-1beta-mediated MUC2 gene in NCI-H292 human airway epithelial cells. *Biochem. Biophys. Res. Commun*. **274**, 112-116.

Kim, Y. D., Jeon, J. Y., Woo, H. J., Lee, J. C., Chung, J. H., Song, S. Y., Yoon, S. K. and Baek, S. H. (2002). Interleukin-1beta induces MUC2 gene expression and mucin secretion via activation of PKC-MEK/ERK and PI3K in human airway epithelial cells. *J. Korean Med. Sci*. **17**, 765-771.

Kindon, H., Pothoulakis, C., Thim, L., Lynch-Devaney, K. and Podolsky, D. K. (1995). Trefoil peptide protection of intestinal epithelial barrier function: cooperative interaction with mucin glycoprotein *Gastroenterology* **109**, 516-523.

Koga, T., Kuwahara, I., Lillehoj, E. P., Lu, W., Miyata, T., Isohama, Y. and Kim, K. C. (2007). TNF-alpha induces MUC1 gene transcription in lung epithelial cells: its signaling pathway and biological implication. *Am. J. Physiol. Lung Cell Mol. Physiol*. **293**, L693-L701.

Lesuffleur, T., Porchet, N., Aubert, J. P., Swallow, D., Gum, J. R., Kim, Y. S., Real, F. X. and Zweibau, A. (1993). Differential expression of the human mucin genes MUC1 to MUC5 in relation to growth and differentiation of different mucus-secreting HT-29 cell subpopulations. *J. Cell Sci*. **106**, 771-783.

Lesuffleur, T., Roche, F., Hill, A. S., Lacasa, M., Fox, M., Swallow, D. M., Zweibaum, A. and Real, F. X. (1995). Characterization of a mucin cDNA clone isolated from HT-29 mucus-secreting cells. The 3′ end of MUC5AC? *J. Biol. Chem*. **270**, 13665-13673.

Leteurtre, E., Gouyer, V., Rousseau, K., Moreau, O., Barbat, A., Swallow, D., Huet, G. and Lesuffleur, T. (2004). Differential mucin expression in colon carcinoma HT-29 clones with variable resistance to 5-fluorouracil and methotrexate. *Biol. Cell* **96**, 145-151.

Levine, S. J., Larivee, P., Logun, C., Angus, C. W., Ognibene, F. P. and Shelhamer, J. H. (1995). TNF-α induces mucin hypersecretion and MUC-2 gene expression by human airway epithelial cells. *Am. J. Respir. Cell Mol. Biol*. **12**, 196-204.

Li, D., Gallup, M., Fan, N., Szymkowski, D. E. and Basbaum, C. B. (1998). Cloning of the amino-terminal and 5′-flanking region of the human MUC5AC mucin gene and transcriptional up-regulation by bacterial exoproducts. *J. Biol. Chem*. **273**, 6812-6820.

Longman, R. J., Douthwaite, J., Sylvester, P. A., Poulsom, R., Corfield, A. P., Thomas, M. G. and Wright, N. A. (2000). Coordinated localization of mucins and trefoil peptides in the ulcer associated cell lineage and the gastrointestinal mucosa. *Gut.* **47**, 792-800.

Perdomo, O. J., Cavaillon, J. M., Huerre, M., Ohayon, H., Gounon, P. and Sansonetti, P. J. (1994). Acute inflammation causes epithelial invasion and mucosal destruction in experimental shigellosis. *J. Exp. Med*. **180**, 1307-1319.

Perrais, M., Pigny, P., Copin, M. C., Aubert, J. P. and Van Seuningen, I. (2002). Induction of MUC2 and MUC5AC mucin by factors of the epidermal growth factor family is mediated by EGF-R/Ras/Raf/MAPK signaling cascade and Sp1. *J. Biol. Chem*. **19**, 19.

Radhakrishnan, P., Halagowder, D. and Devaraj, S. N. (2007). Altered expression of MUC2 and MUC5AC in response to Shigella infection, an in vivo study. *Biochim. Biophys. Acta* **70**, 884-889.

Rajkumar, R., Devaraj, H. and Niranjali, S. (1998). Binding of Shigella to rat and human intestinal mucin. *Mol. Cell. Biochem.* **178**, 261-268.

Raqib, R., Wretlind, B., Andersson, J. and Lindberg, A. A. (1995). Cytokine secretion in acute shigellosis is correlated to disease activity and directed more to stool than to plasma. *J. Infect. Dis*. **171**, 376-384.

Raven, J. F., Baltzis, D., Wang, S., Mounir, Z., Papadakis, A. I., Gao, H. Q. and Koromilas, A. E. (2008). PKR and PKR-like endoplasmic reticulum kinase induce the proteasome-dependent degradation of cyclin D1 via a mechanism requiring eukaryotic initiation factor 2alpha phosphorylation. *J. Biol. Chem.* **283**, 3097-3108.

Skeen, J. E., Bhaskar, P. T., Chen, C. C., Chen, W. S., Peng, X. D., Nogueira, V., Hahn-Windgassen, A., Kiyokawa, H. and Hay, N. (2006). Akt deficiency impairs normal cell proliferation and suppresses oncogenesis in a p53-independent and mTORC1-dependent manner. *Cancer Cell* **10**, 269-280.

Smirnova, M. G., Kiselev, S. L., Birchall, J. P. and Pearson, J. P. (2000). TNF-alpha in the regulation of MUC5AC secretion: some aspects of cytokine-induced mucin hypersecretion on the in vitro model. *Cytokine* **2**, 1732-1736.

Smirnova, M. G., Kiselev, S. L., Birchall, J. P. and Pearson, J. P. (2001). Up-regulation of mucin secretion in HT29-MTX cells by the pro-inflammatory cytokines tumor necrosis factor-alpha and interleukin-6. *Eur. Cytokine Netw*. **12**, 119-125.

Song, J. S., Kang, C. M, Yoo, M. B., Kim, S. J., Yoon, H. K., Kim, Y. K., Kim, K. H., Moon, H. S. and Park, S. H. (2007). Nitric oxide induces MUC5AC mucin in respiratory epithelial cells through PKC and ERK dependent pathways. *Respir. Res*. **8**, 28.

Song, K. S., Lee, W. J., Chung, K. C., Koo, J. S., Yang, E. J., Choi, J. Y. and Yoon, J. H. (2003). Interleukin-1 beta and tumor necrosis factor-alpha induce MUC5AC overexpression through a mechanism involving ERK/p38 mitogen-activated protein kinases-MSK1-CREB activation in human airway epithelial cells. *J. Biol. Chem*. **278**, 23243-23250.

Sudha, P. S., Devaraj, H. and Devaraj, N. (2001). Adherence of Shigella dysenteriae 1 to human colonic mucin. *Curr. Microbiol.* **42**, 381-387.

Takeyama, K., Dabbagh, K., Jeong Shim, J., Dao-Pick, T., Ueki, I. F. and Nadel, J. A. (2000). Oxidative stress causes mucin synthesis via transactivation of epidermal growth factor receptor: role of neutrophils. *J. Immunol.* **164**, 1546-1552.

Temann, U. A., Prasad, B., Gallu, M. W., Basbaum, C., Ho, S. B., Flavell, R. A. and Rankin, J. A. (1997). A novel role for murine IL-4 in vivo: induction of MUC5ac gene expression and mucin hypersecretion. *Am. J. Respir. Cell Mol. Biol*. **16**, 471-478.

Thirumala-Devi, K., Lamkanfi, M. and Núñez, G. (2007). Intracellular NOD-like Receptors in Host Defense and Disease. *Immunity.* **27**, 549-559.

Udhayakumar, G., Jayanthi, V., Devaraj, S. N. and Devaraj, H. (2007). Interaction of MUC1 with beta-catenin modulates the Wnt target gene cyclinD1 in H. pylori-induced gastric cancer. *Mol. Carcinog*. **46**, 807-817.

Weiss, A. A., Babyatsky, M. W., Ogata, S., Chen, A. and Itzkowitz, S. H. (1996). Expression of MUC2 and MUC3 mRNA in human normal, malignant and inflammatory intestinal tissues. *J. Histochem. Cytochem*. **44**, 1161-1166.

Wright, N. A., Hoffmann, W., Otto, W. R., Rio, M. C. and Thim, L. (1997). Rolling in the clover: trefoil factor family (TFF)-domain peptides, cell migration and cancer. *FEBS Lett.* **408**, 121-123.

Yasunori, O., Fayyaz, S. and Sutterwala, R. A. F. (2006). The inflammasome: first line of the immune response to cell stress. *Cell* **126**, 659-662.

Zaretsky, J. Z., Sarid, R., Aylon, Y., Mittelman, L. A., Wreschner, D. H. and Keydar, I. (1999). Analysis of the promoter of the MUC1 gene overexpressed in breast cancer. *FEBS Lett.* **46**, 189-195.

Zen, Y., Harada, K., Sasaki, M., Tsuneyama, K., Katayanagi, K., Yamamoto, Y. and Nakanuma, Y. (2002). Lipopolysaccharide induces overexpression of MUC2 and MUC5AC in cultured biliary epithelial cells: possible key phenomenon of hepatolithiasis. *Am. J. Pathol*. **161**, 1475-1484.

Zweibaum, A., Pinto, M., Chevalier, G., Dussaulx, E., Triadou, N., Lacroix, B., Haffen, K., Brun, J. L. and Rousset, M. (1985). Enterocytic differentiation of a subpopulation of the human colon tumor cell line HT-29 selected for growth in sugar-free medium and its inhibition by glucose. *J. Cell Physiol*. **22**, 21-29.

Zychlinsky, A., Fitting, C., Cavaillon, J. M. and Sansonetti, P. J. (1994). Interleukin 1 is released by murine macrophages during apoptosis induced by Shigella flexneri. *J. Clin. Invest*. **94**, 1328-1332.