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POTENTIATION OF FLAGELLIN RESPONSES IN GUT EPITHELIAL CELLS BY INTERFERON GAMMA IS ASSOCIATED WITH STAT-INDEPENDENT REGULATION OF MYD88 EXPRESSION.

Ciara Bannon^{1,2}, Pam J. Davies^{1,3}, Andrew Collett^{1,2} and Geoff Warhurst^{1,3}

¹Infection, Injury and Inflammation Group, Salford Royal NHS Foundation Trust, Salford, M6 8HD, ²Faculty of Medical and Human Sciences, University of Manchester, Manchester, ³Biomedical Sciences Institute University of Salford, Salford

Running head: IFN γ regulation of flagellin-TLR5 responses in gut epithelia

Address correspondence to: Dr Geoff Warhurst, Clinical Sciences Building, Hope Hospital, Salford M6 8HD Tel: 44-(0)161-206-4403 E-mail: Geoffrey.warhurst@manchester.ac.uk

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ABSTRACT

Flagellin acting via toll-like receptor 5 (TLR5) is a key regulator of the host response to the gut microbial flora in both health and disease. This study has investigated regulation of flagellin /TLR5 signalling in human colonocytes (HT29-19A) by interferon-gamma (IFN γ), a cytokine released early in the inflammatory process which has multiple effects on gut epithelial function that may facilitate abnormal responses to enteric bacteria. Flagellin induced a dose-dependent secretion of chemokines CXCL8 and CCL2 in the human colonocyte line, HT29-19A. Exposure to IFN γ did not induce chemokine secretion but markedly potentiated responses to flagellin increasing CXCL8 gene expression and protein secretion by ~4 fold. Potentiation by IFN γ was independent of changes in TLR5 and associated with a rapid, sustained increase in expression of the downstream adaptor molecule MyD88. Knockdown of MyD88 expression using siRNA abolished flagellin-dependent CXCL8 secretion and the potentiating effect of IFN γ . Exposure of non-transformed mouse and human colonocytes to IFN γ also increased MyD88 expression. STAT1 knockdown and use of the broad-spectrum JAK-STAT inhibitor AG490 had no effect on IFN γ mediated upregulation of MyD88. These findings suggest that IFN γ sensitises colonic epithelial cells to bacterial flagellin via a largely STAT-independent up-regulation of MyD88 expression leading to increased secretion of immunomodulatory factors. These data indicate that epithelial responses to flagellin are potentiated by IFN γ , most likely mediated by increased MyD88 expression. This adds to our understanding of the spectrum of effects of this cytokine on gut epithelium that may contribute to bacterial driven inflammation in the gut.

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INTRODUCTION

The gut is colonised by a large and very diverse microbial population that is in a delicate relationship with the host, being tolerated by the immune system under normal circumstances but capable of driving inflammatory disease in susceptible individuals (1). The gut epithelium plays a major role in this relationship, acting as a physical barrier to contain the enteric flora within the gut lumen but also as an immune “sensor” that is poorly responsive to commensals and their antigens while retaining the ability to initiate an appropriate immune response to pathogens (2). However, in conditions such as inflammatory bowel disease (IBD), and for reasons that remain unclear, the epithelium becomes abnormally responsive to the commensal flora triggering a cycle of uncontrolled mucosal inflammation and barrier dysregulation that results in the establishment of chronic inflammation (3).

Evidence suggests that interferon- γ (IFN γ), has a complex role in modulating the barrier function of the gut epithelium in disease. Mucosal levels of IFN γ rise at an early stage in ulcerative colitis (4) and ablation of the IFN γ gene effectively prevents DSS-induced colitis in mice (5). Studies in both *in vivo* and *in vitro* systems have shown that IFN γ compromises the physical barrier by altering the expression and distribution of tight junction proteins (6) and increasing epithelial permeability to luminal factors (7). IFN γ also promotes transcellular transport across gut cells, increasing the adherence and translocation of commensal bacteria (8,9) and the transcellular flux of peptides (10). Evidence also suggests that IFN γ may increase the responsiveness of the epithelium to components of the commensal flora by increasing expression of both cell surface and cytosolic receptors that respond to bacterial antigens by activating pro-inflammatory signalling pathways. IFN γ -mediated activation of the ubiquitin-proteasome system in epithelial cells (11) may also contribute to enhanced antigen presentation, a feature of IBD.

Flagellin is a component of the bacterial flagellum expressed by most motile bacteria in the gut and appears to be a dominant antigen in IBD, particularly Crohn’s disease (CD), with elevated levels of flagellin-specific antibodies found in a high proportion of CD patients (12,13). Monomeric flagellin signals exclusively through Toll-like receptor 5 (TLR5) leading to the activation of a downstream signalling cascade mediated through the accessory protein MyD88 (14). This cascade ultimately leads to the activation of NF κ B and mitogen-activated protein kinases (MAPK) inducing the expression of several proinflammatory genes. Evidence that a dominant negative TLR5 polymorphism (TLR5-stop), associated with impaired responses to flagellin, is negatively associated with the development of CD (15) reinforces the importance of the flagellin-TLR5 interaction as a mediator of pathogenic responses in IBD. Recent studies showing that mice lacking TLR5 develop a spontaneous colitis (16,17) also point to a protective role for flagellin in the homeostatic regulation of immune responses to the commensal gut flora. Despite the importance of flagellin signalling in health and disease, little is known about the regulation of TLR5 responses and, specifically, the influence of IFN γ on TLR5/flagellin signalling.

In this study, we demonstrate that IFN γ enhances flagellin-induced inflammatory responses in gut epithelial cells by a mechanism that is largely independent of TLR5 and mediated primarily by a marked up-regulation of the TLR adaptor molecule MyD88. Evidence is presented that this response to IFN γ is mediated via a largely STAT-independent process. These data provide a molecular basis by which epithelial responses to this key bacterial antigen may be amplified during the early stages of gut inflammation.

EXPERIMENTAL

Materials - Purified LPS from *Escherichia coli* 0111:B4 and purified flagellin from *Salmonella thyphimurium* were obtained from InvivoGen (California, USA). Recombinant human IFN γ was purchased from Sigma-Aldrich (Poole, UK). Human CXCL8 and CCL2 ELISA kits and the JAK inhibitor AG490 were obtained from Biosource International, (Camarillo, CA). HT29-19A cells were kindly provided by Dr C Laboisse, INSERM, Paris. Culture media and its constituents were purchased from Invitrogen (Paisley, UK). All materials for real-time PCR were obtained from Roche Diagnostics (Burgess Hill, UK). Antibodies used included Actin (Sigma-Aldrich), MyD88 (R&D systems, Abingdon, UK), IRAK4 (ebioscience, USA), TLR5 (Santa Cruz biotechnology, California, USA), STAT1 and pSTAT1 (Cell Signalling Technology Inc, Danvers, USA) and Villin (BD Biosciences, UK).

HT29-19A cell culture- The human colonic epithelial line HT29-19A (passages 42-64) was cultured in Dulbecco's Modified Eagles medium (DMEM) containing 4mM glutamine, 50 IU/ml penicillin, 50 μ g/ml streptomycin, 10% FBS and 20mM hydroxyethylpiperazine ethanesulphonic acid (HEPES). Cells were routinely cultured in stock 75 cm² flasks at 37°C in a 5% CO₂ constant humidity environment with media replaced every 2-3 days. For experimental investigations cells were seeded onto 33mm x 10mm culture dishes at 0.4x10⁵ cells/cm² and grown until confluent (6-7 days). In some experiments, cells were seeded onto 12mm TranswellTM polycarbonate inserts (0.4 μ m pore size) at a density of 8x10⁵ cells/cm². Under these conditions, cells form polarised monolayers after ~28 days as judged by the generation of a transepithelial electrical resistance in excess of 100 Ω .cm².

HT29-19A stimulation protocol- For IFN γ studies, HT29-19A monolayers were incubated in fresh DMEM containing IFN γ (1-100ng/ml) for 2-48 hr as indicated. Cells were then washed twice with warmed Hanks' balanced salt solutions (HBSS) containing Ca²⁺ and Mg²⁺ and stimulated with either purified flagellin (1-1000ng/ml) or LPS (1-1000ng/ml) in optiMEM for 4h or 16h as indicated. Supernatants were harvested and stored at -80°C for further analysis and cells were either lysed for protein extraction or placed in Trizol for RNA isolation.

For studies using the JAK inhibitor AG490 (Tyrphostin B42), HT29-19A cells were treated with AG490 (30 μ M) for 20min prior to addition of IFN- γ (100ng/ml).

Isolated mouse and human colonocytes- Mouse colon was removed from 8-12 week old male FVB mice, flushed with Ca²⁺ /Mg²⁺-free Hank's balanced salt solution containing penicillin (100U/ml), streptomycin (100 μ g/ml) and 0.2% dithiothreitol (HBSS-A) and everted onto a glass rod. Segments of human colon were obtained under informed consent (LREC approval 02/082) from a patient undergoing elective surgery for colon cancer, the tissues used being from the macroscopically normal margins of the tumour. Both human and mouse tissues were processed essentially as described previously (18). Briefly, tissues were incubated in cold HBSS-A containing collagenase XIa (60U/ml), dispase I (0.02 mg/ml) and soybean trypsin inhibitor (0.2mg/ml) for 10 min with gentle agitation. This solution was discarded and the tissue incubated for a further 40-50 min with fresh HBSS-A at 36°C. Sheets of intact crypts and epithelial surface cells were dislodged by vigorous mechanical agitation and pelleted. Single cells were removed by repeated (x5) centrifugation at 120g in DMEM containing 2% sorbitol leaving intact crypts and epithelial sheets. Preparations were plated onto 24-well plates (200-300 organoids/cm²), and cultured for 16 hours at 37°C in a humidified 5% CO₂ environment in a 1:1 mixture of DMEM and Ham's F-12 medium (Biowhittaker, Wokingham, UK) containing: 5 μ g/ml insulin, 50nM dexamethasone, 60nM selenium, 5 μ g/ml transferrin, 50nM triiodothyronine, 10ng/ml epidermal growth factor, 20mM HEPES, 2mM glutamine, 100U/ml penicillin, 100 μ g/ml streptomycin, 0.2 μ l/ml normocin and 2% FBS. Following a 4 hour equilibration, 100ng/ml of murine IFN γ or vehicle was added for a further 18 hours, after which the cells were washed and placed in Trizol for RNA isolation.

Leukocyte contamination, as judged by fluorescence detection of CD45 positive cells was routinely <0.5%.

Chemokine immunoassay- Cell supernatants were analysed using commercial ELISA kits for CXCL8 (interleukin-8; IL-8) and CCL2 (monocyte chemotactic protein-1; MCP-1) according to the manufacturer's protocol. Chemokine concentrations were determined from a four parameter curve fit algorithm (DeltaSoft, BioMetallics Inc, Princeton, NJ) of absorbance readings at 490nm.

Gene expression studies - Total RNA was isolated by scraping confluent HT29-19A cultures into Trizol reagent and processing according to the manufacturers instructions. Total RNA (5µg) was reverse transcribed using a commercial first strand cDNA synthesis kit (Amersham Biosciences, Little Chalfont, UK). Relative quantification of gene expression was performed with a Lightcycler 480 real time PCR system (Roche Diagnostics, Lewes, UK) using gene-specific primer pairs combined with detection by either 5'-fluorescein (FAM)-labelled; 3'-dark quencher dye hydrolysis probes (MyD88, IRAK-2, IRAK-4, TRAF-6, STAT1, SOCS3, CXCL8) or SYBR Green (TLR5, TLR4, MD-2). Hydrolysis probes were obtained from the Universal Probe Library (Roche Diagnostics, Lewes, UK). The sequences of the primers along with the number of the Universal Probe Library (UPL) probe used (where appropriate) are; TLR5 (TCAAACCCCTTCAGAGAATCCC and TTGGAGTTGAGGCTTAGTCCCC); TLR4 (TGGAGTGGGAGGACAG and CAAGTGTGGACCCAG); MD-2 (TCTGAAGGGAGAGACTGTGA and AATTAGGTTGGTGTAGGATGAC) MyD88 (CTGCTCGAGCTGCTTACCA and CTTTGGCAATCCTCCTCAA, UPL#33); IRAK2 (CCTCCTCTGAGGCCTGTGT and TGATCTCAATTTGCCACGAA, UPL#1); IRAK4 AGAAGGATGGAAGAAGTTAGCTGT and TGGGACTTTTTCCAGTTTGAAG, UPL#9); TRAF6 (GTCCTCTACCAGCGCCTTG and TCCACAGCTGTTTTACAGTTT, UPL#79); STAT1 (CTGCTCCTTTGGTTGAATCC and GCTGAAGTTCGTACCACTGAGA, UPL#79); SOCS3 (AGACTTCGATTCGGGACCA and AACTTGCTGTGGGTGACCA, UPL#36); CXCL8 (AGACAGCAGAGCACACAAGC and ATGGTTCCTTCCGGTGGT, UPL#72). Assays were run in a total volume of 20µl with 500nM of each primer, 100nM of the UPL probe (where appropriate) and ~0.3µg of cDNA. Gene expression was quantified relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Western blotting- For MyD88, IRAK4 and TLR5 protein analysis, HT29-19A cells were lysed at 4°C by scraping into a buffer containing 150 mM NaCl, 20mM Tris (pH 8.0), 1% (v/v) Triton X-100, 0.5mM phenylmethanesulfonyl fluoride (PMSF) and 5% (v/v) protease inhibitor cocktail (Sigma). For STAT1 and pSTAT1 protein analysis, cells were lysed at 4°C in the following buffer: 150 mM NaCl, 50mM Tris (pH 7.5), 1% (v/v) Triton X-100, 1.0mM PMSF, 2mM EDTA, 1mM sodium orthovanadate, 1% (v/v) protease inhibitor cocktail and 1% phosphatase inhibitor cocktail (Sigma). Samples were left on ice for 30 minutes with occasional mixing and insoluble material removed by centrifugation at 10,000 xg for 10 minutes at 4°C. The supernatant was retained and stored at -80°C until use. Protein concentration of the supernatant was determined using a bicinchoninic acid protein assay (Sigma) and 10-50µg of protein was separated by 10% SDS-PAGE. Separated proteins were electroblotted onto polyvinylidene difluoride membrane (Immobilon-P, Millipore). For STAT1 and pSTAT1, membranes were blocked at room temperature for 1h in TBS-T (50 mM Tris base (pH 7.9), 150 mM NaCl and 0.05% (v/v) Tween 20) containing 5% (w/v) milk protein. All other membranes were blocked at 4°C overnight in the aforementioned buffer. Membranes were washed in TBS-T and probed with polyclonal antibodies to STAT1 and pSTAT1 (both 1:1000 in 50 mM Tris base (pH 7.9), 150 mM NaCl, 0.1% Tween 20 and 5% (w/v) BSA) at 4°C overnight. Membranes were also probed with polyclonal antibodies to MyD88 (1:5000 in 50 mM Tris base (pH 7.9), 150 mM NaCl, 0.1% Tween 20 and 2% (w/v)

milk protein), IRAK4 (1:10000 in TBS-T) or TLR5 (1:1000 in TBS-T) for 2hr. Blots were standardised against either villin or β -actin using commercial antibodies. After incubation for 1h with the appropriate horseradish peroxidase-conjugated secondary antibody, protein bands were visualised using Immobilon HRP substrate (Millipore) followed by exposure to X-OMAT film (Kodak-Eastman). Immunoblot images were scanned and quantified densitometrically using Scion Image software (Scion Corporation, Frederick, USA).

siRNA studies: For knockdown of STAT1, HT29-19A cells were transfected with 125nM of either STAT1 directed siRNA (#42860, Ambion, Cambridgeshire, UK); or control siRNA (Silencer Negative Control siRNA, # 4611, Ambion) using a Nucleofector device according to the manufacturers instructions utilising Program W-017. Each reaction involved resuspending 1×10^6 cells/ml in 100 μ l Nucleofector solution R along with the appropriate siRNA. Reactions without any siRNA were mock transfected under the same conditions. After transfection, cells were immediately seeded onto 12 well plates containing prewarmed medium, allowed to recover for 48h and stimulated with IFN γ (100ng/ml) for 8h. For knockdown of MyD88 expression, cells were transfected with 125nM MyD88 directed siRNA (#9138, Ambion, Cambridgeshire, UK). Protein was extracted to determine STAT1/pSTAT1 or MyD88 protein levels by Western blot and RNA was isolated for analysis of gene expression by real-time PCR.

Statistical analysis- Results are expressed as mean \pm SEM for the indicated number of observations (*n*). Statistical significance was confirmed as a *p* value ≤ 0.05 . All analyses were undertaken using Prism software (Graphpad Prism Inc, San Diego, California, USA).

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RESULTS

IFN γ potentiates the flagellin-dependent secretion of CXCL8 and CCL2 chemokines in HT29-19A colonocytes. HT29-19A cells have been shown previously to express functional TLR5 and IFN γ receptors (19,20) and are therefore a suitable model in which to study the interactions between these pathways. HT29-19A cells secrete low levels of the neutrophil chemoattractant CXCL8 under basal conditions (273 ± 22 pg/mg protein $n=4$) and this was unaffected by incubation with IFN γ for 24h (245 ± 18 ; 262 ± 27 ; 270 ± 29 pg/mg protein for 1, 10, 100 ng/ml IFN γ respectively; $n=4$ in each group; $p>0.05$). Similarly, IFN γ had no significant effect on secretion of the monocyte chemoattractant protein CCL2 (Basal: 12.3 ± 2.1 pg/mg protein; +100ng/ml IFN γ 16.1 ± 1.8 pg/mg protein ($n=5$ in each group; $p>0.05$)).

IFN γ did however have a dramatic effect on the expression of these factors induced by the TLR5 agonist flagellin. Incubation with flagellin derived from *Salmonella thyphimurium* markedly increased secretion of CXCL8 by HT29-19A accompanied by a ~15 fold upregulation of CXCL8 gene transcription (Fig 1A). Activation of CXCL8 secretion was dose-dependent with an EC₅₀ of 26.1 ± 1.3 ng/ml flagellin (Fig 1B).

Pretreatment of HT29-19A colonocytes with 100ng/ml IFN γ for 8 or 24 hours potentiated flagellin-induced CXCL8 secretion by 2.5-4 fold (Fig 1A). This was associated with similar fold increases in CXCL8 mRNA suggesting that the potentiating effect of IFN γ is mediated primarily at the level of CXCL8 gene transcription (Fig 1A). IFN γ potentiation of flagellin-induced CXCL8 expression was dose-dependent. Potentiation by 1ng/ml IFN γ was relatively small with a significant ($p<0.05$) effect with 1000ng/ml flagellin only, however 10ng/ml IFN γ caused significant ($p<0.025$) potentiation at flagellin concentrations of 10ng/ml and above. (Fig 1B). There was however no evidence of a shift in the EC₅₀ for flagellin stimulation of CXCL8 secretion (EC₅₀ with 100ng/ml IFN γ : 27.6 ± 1.8 vs Control: 26.1 ± 1.3 ng/ml). Potentiation by IFN γ was not restricted to CXCL8. Flagellin-dependent CCL2 secretion was increased almost 6-fold by pre-incubation with 100 ng/ml IFN γ for 48h ($p<0.01$) but again there was no significant shift in the flagellin concentration curve (Fig 1C). The potentiating effect of IFN γ on chemokine secretion was also observed in polarised HT29-19A monolayers grown on Transwell filter supports. In common with the data shown for non-polarised cells, application of IFN γ alone (100ng/ml; 24 hr) to the basolateral surface of HT29-19A monolayers had no effect on basal CXCL8 secretion (data not shown) but significantly increased flagellin-dependent CXCL8 secretion into the basolateral compartment (Flagellin 100ng/ml: 1.19 ± 0.27 ng IL-8/cm²; Flagellin + IFN γ : 2.20 ± 0.31 ng CXCL8/cm² $p<0.01$ $n=4$).

IFN γ priming of flagellin responses is not associated with changes in TLR5 expression. Flagellin responses are mediated through the TLR5 receptor in HT29-19A cells (19) and we therefore hypothesised that the priming effect of IFN γ may be mediated by an increase in TLR5 expression. However, as shown in Figure 2A, incubation of HT29-19A colonocytes with IFN γ for up to 48 h at a concentration (100ng/ml) that produced maximal potentiation of flagellin responses did not increase TLR5 mRNA levels. Similarly, there was no evidence of an increase in TLR5 protein expression following exposure to IFN γ (Fig 2B). Indeed there was qualitative evidence that TLR5 protein levels were lower following cytokine treatment although this was not statistically significant (Fig 2B).

Modulation of MyD88 expression by IFN γ in colonocytes. The influence of IFN γ on expression of the major signalling components downstream of TLR5 in human colonocytes was investigated. IFN γ induced a rapid, 3-4 fold increase in MyD88 gene transcription within 2 hr in HT29-19A colonocytes (Fig 3A), which was largely sustained over longer incubation periods up to 48 hours. IFN γ -induced increases in MyD88 mRNA were confirmed at the protein level with a quantitatively similar increase in MyD88 protein levels (Fig 3B).

Comparative studies in polarised HT29-19A monolayers on Transwell supports confirmed a similar increase in MyD88 expression following exposure to basolateral IFN γ (2.48 ± 0.205 fold increase above control; $p < 0.01$ $n = 4$). The IFN γ -mediated up-regulation of MyD88 expression was also observed in isolated mouse colonocytes maintained in short-term culture with exposure to 100ng/ml IFN γ for 24 hours inducing a 2.6 ± 0.6 fold increase ($p < 0.01$; $n = 6$ preparations) in MyD88 mRNA. A single study in colonocytes isolated from human sigmoid colon also showed upregulation of MyD88 (3.8 fold) when exposed to IFN γ under the same conditions. These data suggest that the modulation of MyD88 expression by IFN γ is similar in both transformed and non-transformed cells and is therefore likely to be of physiological relevance.

Studies were also conducted in HT29-19A cells to determine whether IFN γ , in addition to its effects on MyD88 expression, alters expression of other key components in the downstream pathway involved in flagellin signalling, specifically IRAK4, IRAK2 and TRAF6 (Fig 4 A,C). There was no evidence that IFN γ modulated transcription of these components and, in the case of IRAK4, this lack of effect was confirmed at the protein level (Fig 4B).

Knockdown of MyD88 abolishes flagellin-induced CCL8 secretion in control and IFN γ -treated cells. Given recent evidence that flagellin may activate secretion of inflammatory mediators via TLR5- and MyD88- independent pathways (21), the role of MyD88 in mediating IFN γ -induced potentiation of the flagellin response in HT29-19A colonocytes was investigated. Transfection of HT29-19A with MyD88 siRNA effectively silenced expression of MyD88 protein after 48h (Fig 5A). MyD88 gene expression was also reduced by 90% (data not shown). Under these conditions, flagellin-dependent CXCL8 secretion was essentially abolished in both control and IFN γ -treated cells (Fig 5B). These data provide further evidence that flagellin activation of chemokine secretion and its potentiation by IFN γ are dependent on expression of MyD88.

IFN γ upregulation of LPS-dependent CXCL-8 secretion is associated with increased expression of the TLR4-MD-2 complex. As a common component of the TLR signalling pathway, the up-regulation of MyD88 would also be expected to modify LPS signalling via TLR4 and pretreatment with IFN γ enhanced LPS-induced CXCL8 secretion in HT29-19A cells by over 4 fold (Figure 6A). However, in contrast to its lack of effect on TLR5 expression, IFN γ markedly increased expression of both components of the TLR4-MD-2 receptor complex (Figure 6B), consistent with previous findings in colonic epithelial cells (22,23). These data indicate that the synergy between IFN γ and TLR-mediated responses to bacterial antigens in human colonocytes occurs at multiple sites along the TLR signalling pathway and the mechanism of potentiation will vary depending on the TLR agonist involved.

IFN γ -mediated up-regulation of MyD88 expression is primarily STAT-independent. To study the role of STAT signalling in the up-regulation of MyD88 expression in human colonocytes two complementary approaches were used, silencing of STAT1 expression by siRNA and treatment with the AG490, a specific inhibitor of JAK-STAT signalling. Treatment of HT29-19A cells with IFN γ markedly increased levels of STAT1 and its phosphorylated form, pSTAT1 (Fig 7A). Transfection with STAT1-directed siRNA abolished this increase, lowering STAT1 expression by ~90% along with marked reductions in the levels of pSTAT1 compared to mock and control siRNA transfected cells (Fig 8A). However, STAT1 knockdown did not impair IFN γ up-regulation of MyD88 expression with similar increases observed in mock- and STAT1 siRNA-transfected cells (Fig 7B). However, under the same conditions, IFN γ stimulation of SOCS3 expression which is known to be STAT-mediated (24) was reduced by $68 \pm 8\%$ ($n = 4$; $p < 0.01$; Fig 7C). Similar studies with the JAK-STAT inhibitor AG490 were also consistent with the modulation of MyD88 expression by IFN γ being primarily STAT-independent. Treatment of HT29-19A cells with 30 μ M AG490

inhibited IFN γ -stimulation of SOCS3 gene expression by $59.5 \pm 10\%$ (n=4; p<0.01) but had no significant effect on the IFN γ -induced increase in MyD88 expression (Figure 8).

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DISCUSSION

Cross-talk between IFN γ and TLRs involved in LPS and CpG DNA signalling has been identified in several systems including immune cells (25) and the gut epithelium (22,23). However, little is known about how IFN γ modulates flagellin/TLR5 responses in the gut epithelium, despite the potential significance of such an interaction given recent evidence that flagellin plays a central role in gut immunity being involved in both homeostatic regulation of immune responses to the commensal microflora and the pathogenesis of inflammatory bowel disease, a condition associated with loss of epithelial tolerance to enteric bacteria. The present study shows that IFN γ primes human colonic epithelial cells to flagellin resulting in markedly increased secretion of CC and CXC chemokines associated with an upregulation of chemokine gene expression. Unlike the reported interaction with the LPS/TLR4 system in colonocytes (22), the priming effect does not occur at the level of the TLR5 receptor but appears to be linked to a rapid and sustained increase in the expression of MyD88, a key intermediary in TLR signalling.

This data provides further evidence of a concerted effect of IFN γ on microbial-immune signalling in the gut. Evidence suggests that IFN γ is produced in the gut during the early stages of immune activation (23) with recent studies in a mouse model of spontaneous colitis showing that activation of IFN γ -responsive genes are among the earliest molecular changes identified in the gut, preceding the onset of active inflammation (18). Development of an appropriate immune response to both bacterial (26) and parasite (27) colonisation of the gut has also been shown to be dependent on IFN γ release. Increasing the responsiveness of the epithelium to flagellin *per se*, would not necessarily result in immune activation given that TLR5 receptors are localised to the basolateral membrane and, under normal circumstances, would be inaccessible to luminal flagellin (28). TLR5 may be activated more readily in disease situations, where the epithelial barrier is dysregulated allowing translocation of flagellated bacteria or flagellin (29). IFN γ is known to induce gut barrier dysfunction, both in terms of increased transcytosis of bacteria (8,9) and disruption of tight junctions allowing increased diffusion of luminal contents including bacterial antigens (7). The potential synergy between these actions and the sensitisation of flagellin signalling described here provides a basis by which IFN γ may orchestrate bacterial-immune signalling across the gut barrier. While this study has primarily used a transformed colonocyte cell line, the demonstration of a virtually identical up-regulation of MyD88 expression in non-transformed mouse and human colonocytes suggests that this is likely to be a generalisable finding.

In marked contrast to the previously reported sensitisation of LPS responses by IFN γ , where there is clear evidence that up-regulation of the TLR4 receptor complex plays a major role (22,23 and the present study), there was no evidence that IFN γ priming of the flagellin response was mediated through increased expression of the TLR5 receptor. Indeed the trend following IFN γ treatment was towards decreased expression of TLR5 although this did not reach statistical significance. Interestingly, Ortega-Cava and co-workers showed a complex TLR5 response in DSS-induced colitis with a transient increase in colonic TLR5 expression during acute inflammation followed by a decrease during the chronic phase (30). This decrease was ascribed in *in vitro* studies to the action of IFN γ although at concentrations which were towards the higher levels used in the present study. It is interesting to speculate that the loss of TLR5 receptors during chronic inflammation could counteract early sensitisation of flagellin responses associated with up-regulation of MyD88 expression. Preliminary analyses argue against significant IFN γ -mediated up-regulation of signalling components downstream of MyD88 with IRAK4, IRAK2 and TRAF6 being unaffected by exposure to IFN γ , which is again consistent with IFN γ being able to potentiate, but not directly induce chemokine expression in this system. Studies in MyD88 $^{-/-}$ mice have

demonstrated the central role of MyD88 in mediating innate immune responses to flagellin (31,32) and the ability of IFN γ to markedly increase the available pool of MyD88 within gut epithelial cells provides a likely mechanism for upregulation of flagellin responses that is independent of changes in TLR5 expression. Knockdown of HT29-19A MyD88 expression in this study also effectively abolished flagellin-induced chemokine secretion and blocked potentiation by IFN γ . Further investigation of the precise role of MyD88 levels in regulating TLR-mediated responses is clearly warranted but the data presented in this study suggests that the ability of IFN γ to increase MyD88 expression contributes significantly to the amplification of TLR responses in gut cells.

Evidence is presented that IFN γ -mediated up-regulation of MyD88 expression in a human colonic cell line is mediated via a largely STAT1-independent process. JAK-STAT signalling is the principal pathway used by IFN γ to influence gene transcription (33). However, STAT1 knockdown by siRNA had no significant effect on IFN γ activation of MyD88 gene transcription and furthermore, the use of the broad-spectrum JAK-STAT inhibitor, AG490 was without effect. The effectiveness of the two approaches in blocking STAT signalling was indicated by parallel studies on SOCS3 gene expression that has been shown previously to be activated by IFN γ through STAT1/STAT3 dependent processes (24,34). Both STAT1 knockdown and AG490 treatment markedly inhibited IFN γ -dependent increases in SOCS3 gene expression but had no significant effect on the increase in MyD88 expression. We have not elucidated the signalling processes that mediate this STAT1-independent effect in human colonocytes. STAT1-independent effects of IFN γ have been reported in several systems (35) and candidates such as phosphatidylinositol 3-kinase (PI-3K) and protein kinase C have been postulated as mediators (36) although this remains a poorly understood area and it has often proved difficult to delineate the pathways involved in STAT-independent effects of IFN γ . Preliminary studies showing that incubation of HT29-19A colonocytes with broad spectrum protein kinase C inhibitor bisindolylmaleimide and the PI-3K inhibitor, LY294002 did not prevent the IFN γ -dependent up-regulation of MyD88 expression and potentiation of flagellin-induced CXCL8 secretion argue against the involvement of these pathways (results not shown). The involvement of other STAT members is possible although AG490, which inhibits a range of STAT-dependent processes (37,38) was also ineffective in blocking IFN γ -mediated increases in MyD88 expression.

In conclusion, the present study provides evidence that IFN γ modulates gut epithelial responses to bacterial flagellin principally by modulation of MyD88 expression via a largely STAT-independent process. These data add to our understanding of the synergy between IFN- γ and TLR signalling in the gut, showing that IFN γ can interact at multiple sites in the TLR pathway, and that this varies for different TLRs producing different degrees of activation. This extends the spectrum of effects of IFN γ on the epithelial barrier, which may be important in the pathogenesis of bacterial-driven inflammatory disease in the gut.

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FIGURE LEGENDS

Figure 1. Potentiation of flagellin-induced chemokine expression in HT29-19A colonocytes by IFN γ . **A.** CXCL8 secretion (\square) and CXCL8 gene expression (\blacksquare) induced by 100ng/ml flagellin (Flag; 16h) with or without pretreatment for 8hr or 24hr with 100ng/ml IFN γ . CXCL8 gene expression is shown relative to controls; IFN γ alone had no effect on either CXCL8 secretion or gene expression as shown in Fig 1. ** $p < 0.01$ CXCL8 secretion compared to flagellin alone; # $p < 0.01$ fold change in CXCL8 mRNA compared to flagellin alone. Data shows mean \pm SEM for $n = 4-5$ in each group. **B.** Dose dependence of IFN γ potentiation of flagellin-stimulated CXCL8 secretion. HT29-19A were pretreated with IFN γ (\blacksquare 100ng/ml; \blacktriangle 10ng/ml; \blacktriangledown 1 ng/ml) or vehicle control (\circ) for 48hr and CXCL8 secretion measured in response to flagellin (0-1000 ng/ml). Data shows mean \pm SEM ($n = 4$ per group). The IFN γ induced increase in CXCL8 secretion Significant increases ($p < 0.05$) were observed. **C.** Effect of IFN γ on secretion of CCL-2 induced by flagellin. HT29-19A were pretreated with IFN γ (\blacksquare 100ng/ml; \blacktriangle 10ng/ml; \blacktriangledown 1 ng/ml) or vehicle control (\circ) for 48hr and CCL2 secretion measured in response to flagellin (0-1000 ng/ml; 16h). Data shows mean \pm SEM ($n = 4$ per group).

Figure 2. IFN γ does not induce TLR5 expression in HT29-19A colonocytes. **A.** Relative change in TLR5 mRNA levels in cells exposed to 100ng/ml IFN γ for periods of 2-48 hr compared to untreated controls. In each case, cells were subsequently exposed to 100ng/ml flagellin for 4 hours. Data shows mean \pm SEM for $n = 4$ in each group. **B.** Immunoblot shows IFN γ (100ng/ml; 48 hours) effect on TLR5 protein levels in control and flagellin-stimulated HT29-19A cells. Arrows indicate protein bands for TLR5 (110 kDa) and villin (95 kDa) respectively. Immunoblot is representative of 3 separate experiments; the accompanying graph shows the mean change \pm SEM in TLR5 protein determined by densitometric analysis of the 3 experiments.

Figure 3. IFN γ stimulates MyD88 expression in HT29-19A. **A.** Relative change in MyD88 mRNA levels in cells exposed to 100ng/ml IFN γ for periods of 2-48 hr compared to untreated cells. In each case, cells were subsequently exposed to 100ng/ml flagellin for 8 hours. Data shows mean \pm SEM for $n = 4$ in each group. **B.** Immunoblot showing effect of IFN γ (100ng/ml; 48 hours) on MyD88 protein levels in control and flagellin-stimulated HT29-19A cells. Arrows indicate protein bands for MyD88 (35 kDa) and villin (95 kDa) respectively. Immunoblot is representative of 3 separate experiments; the accompanying graph shows the mean change (\pm SEM) in MyD88 protein expression determined by densitometric analysis of the 3 experiments. * $p < 0.05$; ** $p < 0.01$ compared to control.

Figure 4. IFN γ does not alter expression of signalling components downstream of MyD88. Effect of IFN γ (100 ng/ml ; 48hr) on expression of IRAK4 (A), IRAK2 and TRAF6 (C) mRNA expression and IRAK4 protein expression (B) in HT29-19A cells. mRNA data is mean \pm SEM ($n = 6$) shown normalised to control; A representative immunoblot for IRAK4 (50 kDa) is shown standardised against villin (95 kDa).

Figure 5: siRNA silencing of MyD88 effectively abolishes flagellin-stimulated CXCL8 secretion in control and IFN γ -treated HT29-19A colonocytes. **A.** Immunoblot showing levels of MyD88 protein (35kDa) in cells transfected with 125nM MyD88 siRNA compared to control. Blot is standardised against villin (95 kDa). **B.** CXCL8 secretion in response to flagellin (100ng/ml; 8h) with (shaded bars) or without (open bars) pretreatment with IFN γ

(100ng/ml; 24h) in control and MyD88-depleted cells. Data is mean \pm SEM for n=4 experiments ** p<0.01 compared to untreated cells

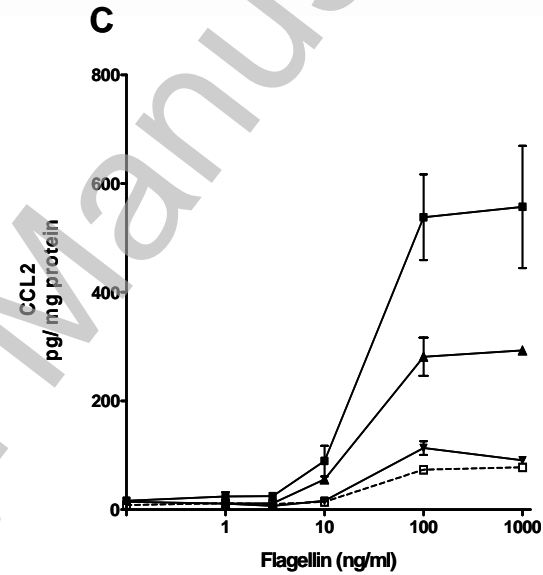
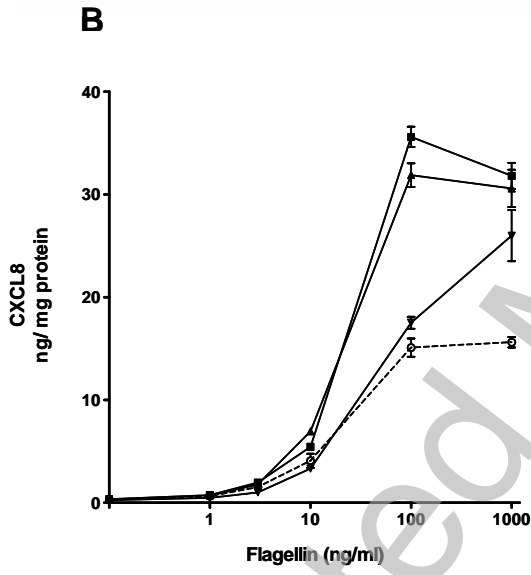
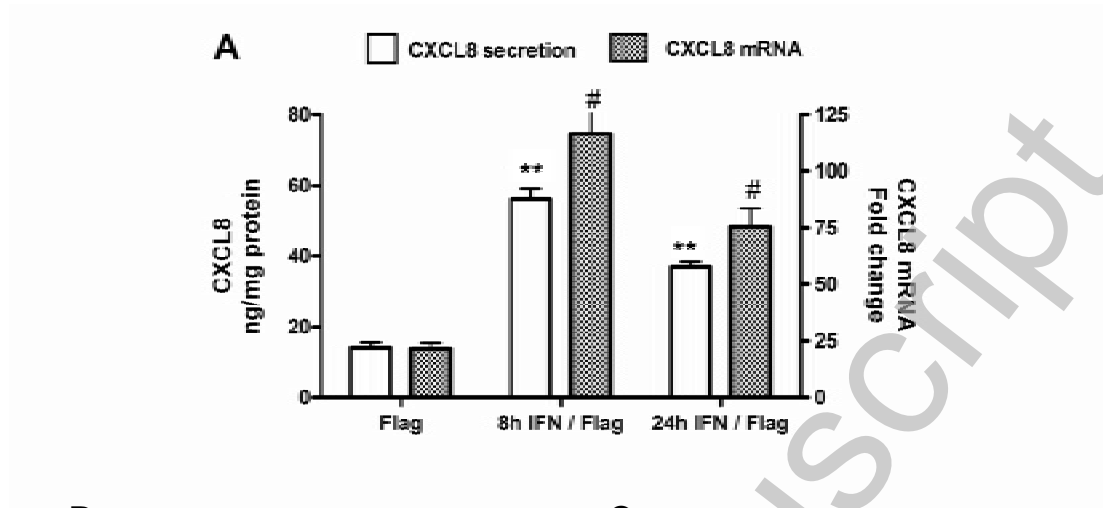
Figure 6: Effect of IFN γ on LPS-induced CXCL8 secretion (A) and mRNA expression of the TLR4 receptor and its accessory protein, MD-2 normalised to control (B). Data shows mean \pm SEM of n=4 experiments * p<0.05; ** p<0.01

Figure 7: STAT1 gene silencing does not prevent IFN γ -mediated increases in MyD88 expression. A. Suppression of STAT1 expression by siRNA. HT29-19A cells were transfected with 125nM control (C-siR) or STAT1 (STAT-siR) siRNA or mock transfected (Mo). After incubation for 48 hr, cells were exposed to IFN γ (100ng/ml) for 8 hr. Data shows knockdown of STAT1 mRNA (left panel) and intracellular levels of STAT1 and phosphorylated STAT1 (pSTAT1) protein (right panel) standardised with β -actin. Two bands are shown for STAT1 and pSTAT1 representing STAT1 α (91kDa; upper band) and STAT1 β (84kDa; lower band). Effect of STAT1 suppression on IFN γ -dependent activation of MyD88 (B) and SOCS3 (C) gene expression. ** p<0.01 compared to mock transfected (Mo) control; # compared to control siRNA (C-siR) transfected cells. Changes in mRNA expression is shown relative to control in each case. Data is mean \pm SEM for n=4-5 in each group.

Figure 8: The broad spectrum STAT inhibitor AG490 prevents IFN γ -mediated increases in SOCS3 expression but has no effect on increases in MyD88 expression. Changes in MyD88 (A) and SOCS3 (B) gene expression in response to incubation with 100ng/ml IFN γ for 24 hours in the presence or absence of 30 μ M AG490 relative to control. Data shows mean \pm SEM from 4 separate experiments.

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FIGURE 1



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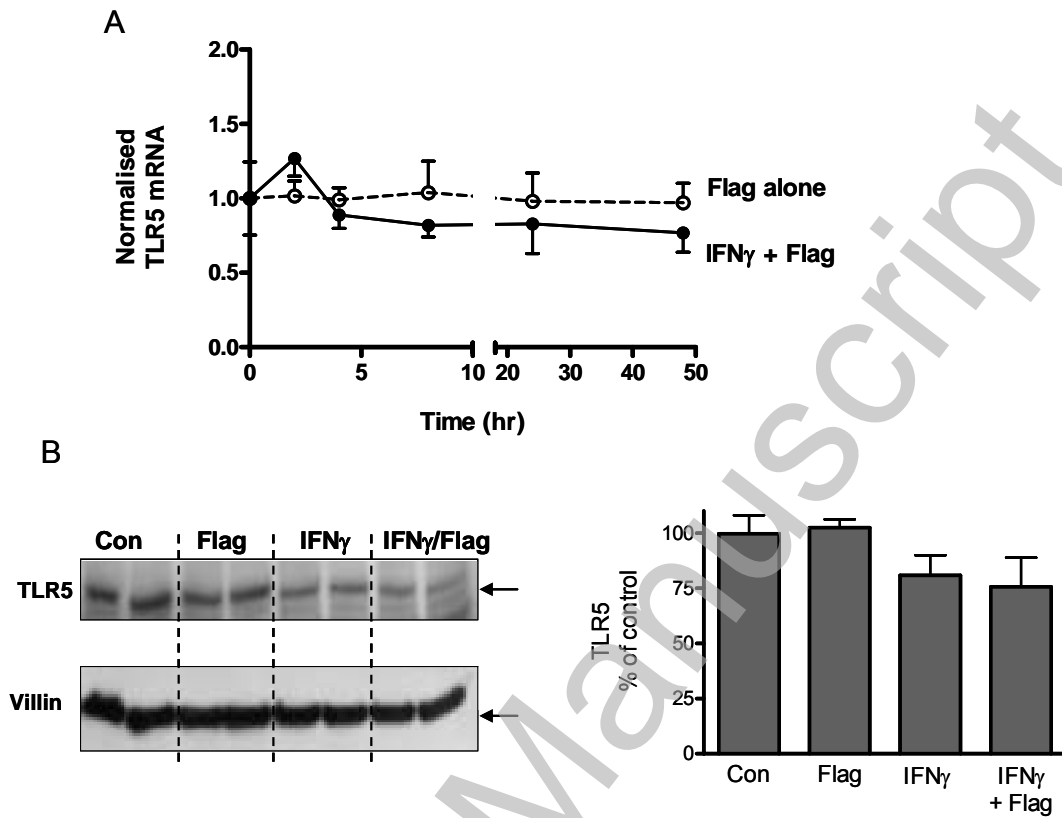
FIGURE 2

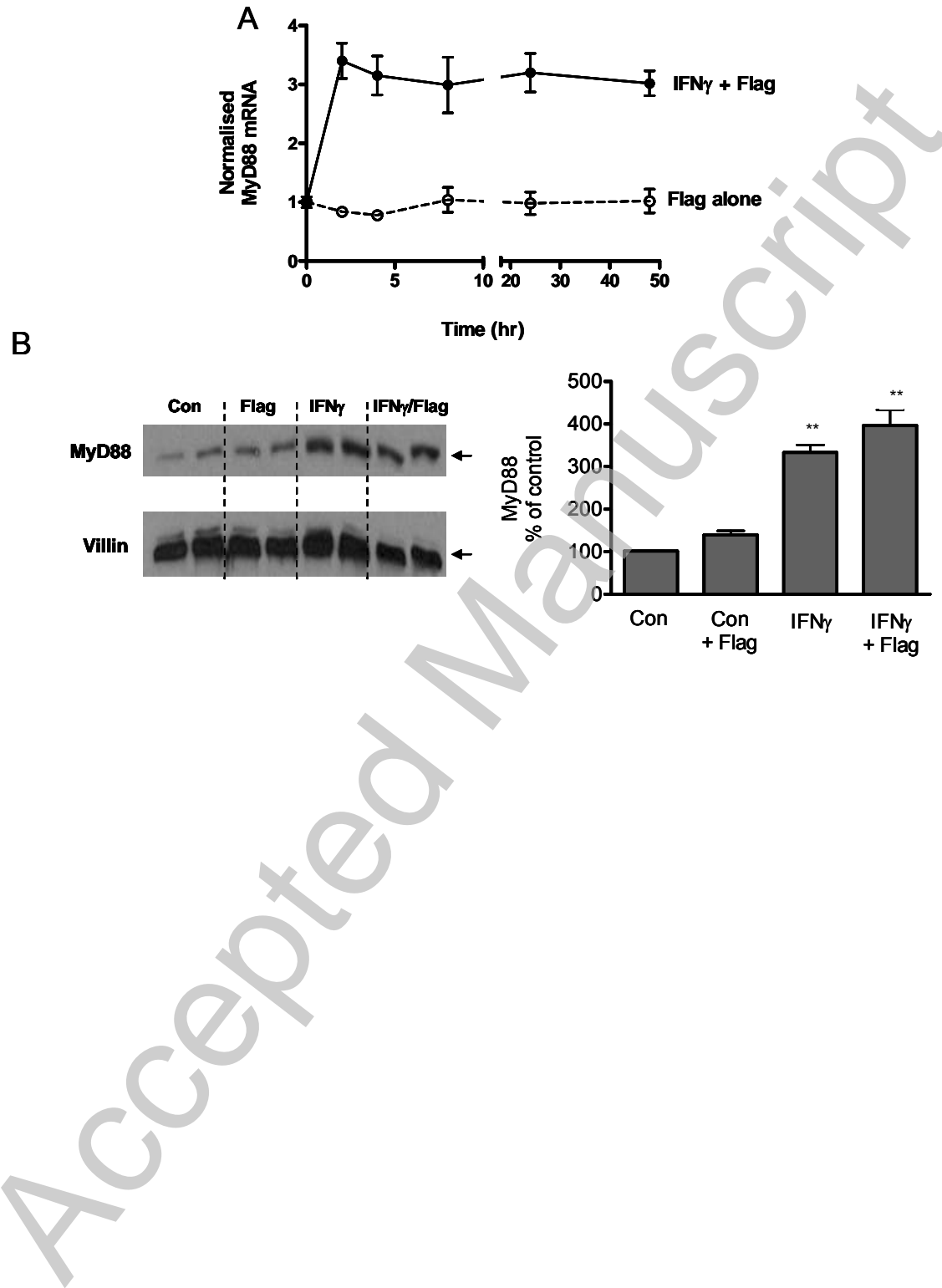
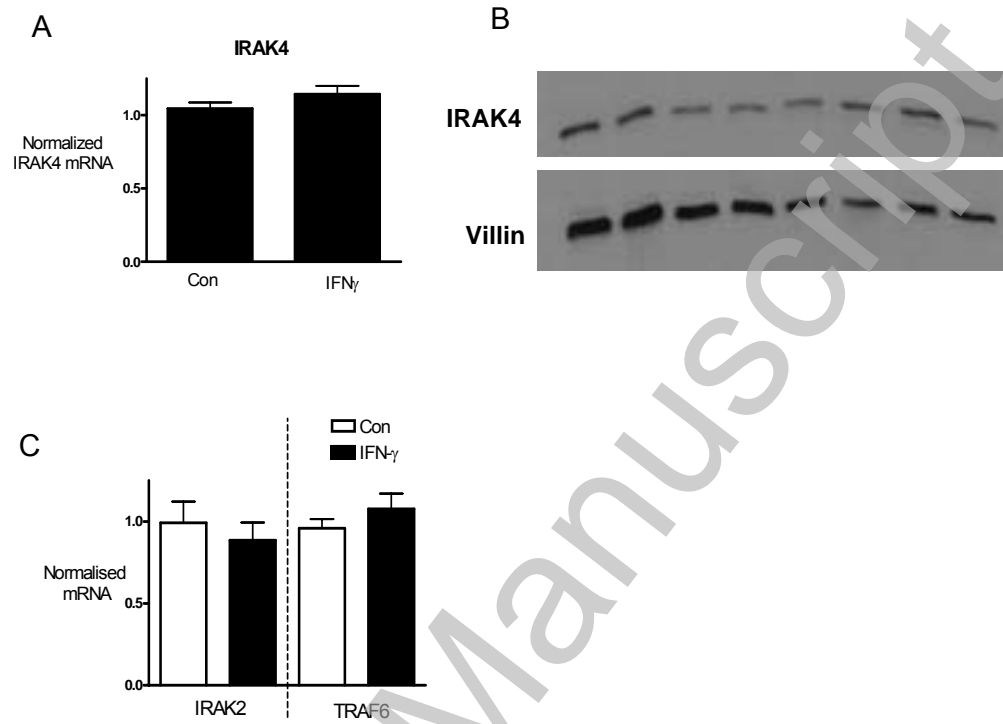
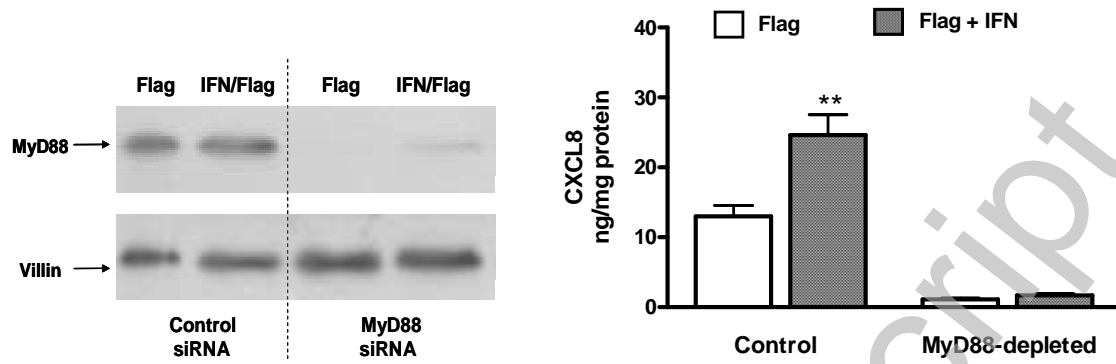
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FIGURE 5

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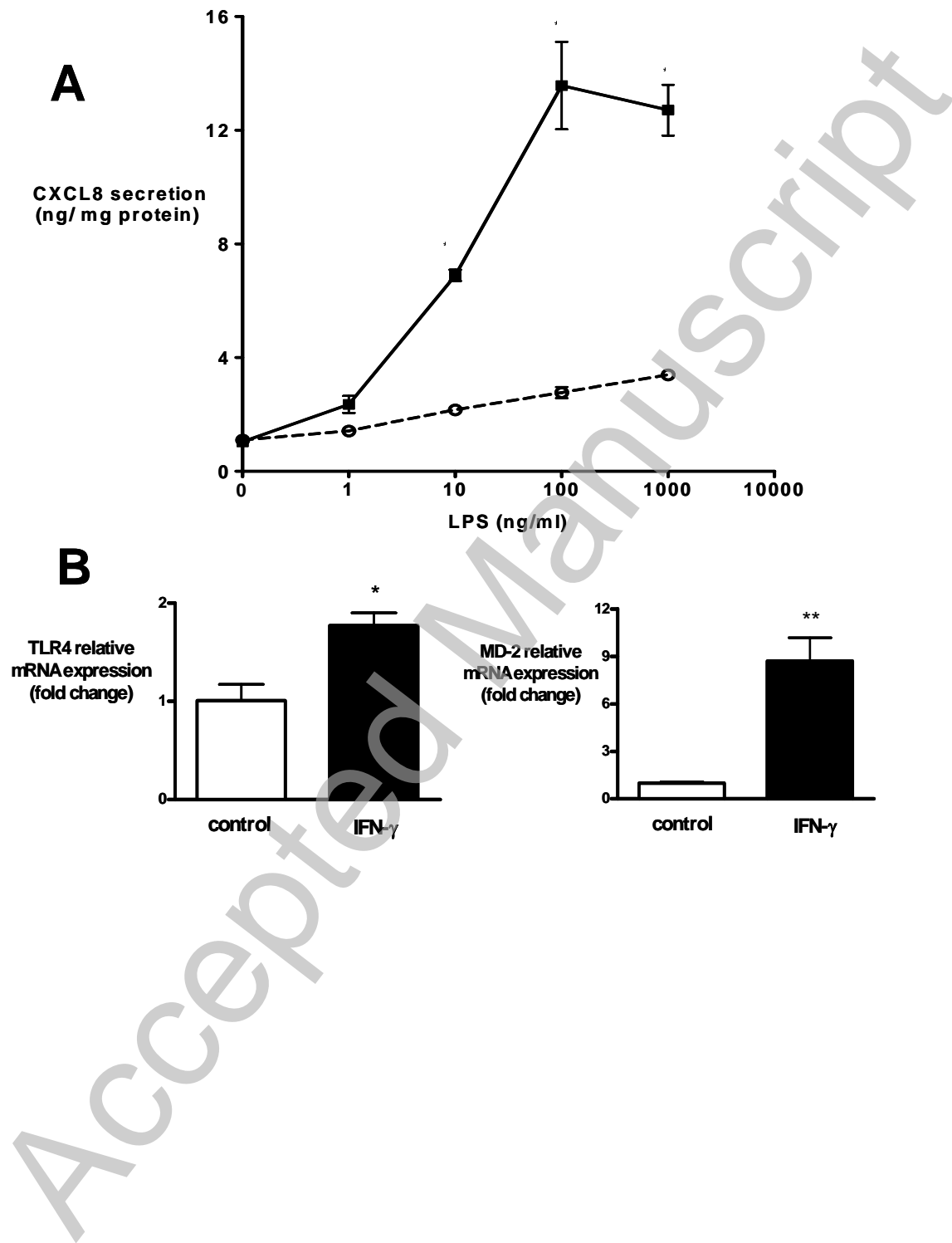
FIGURE 6

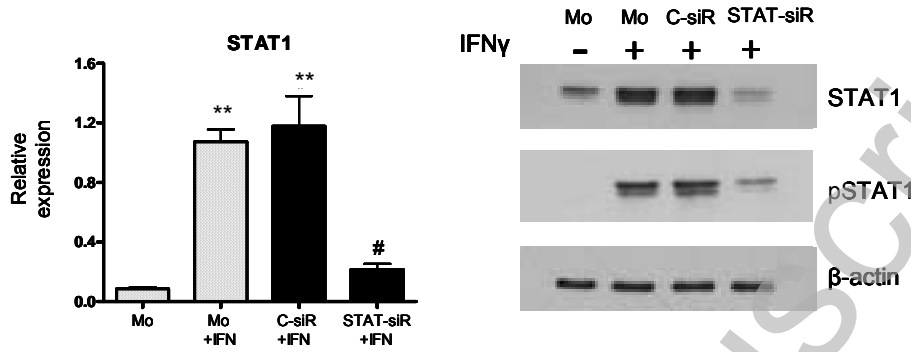
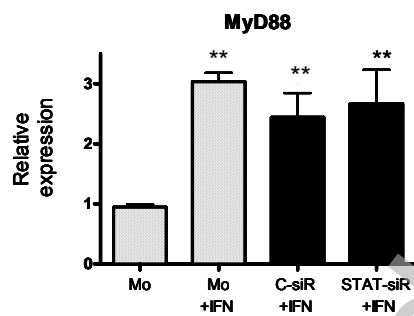
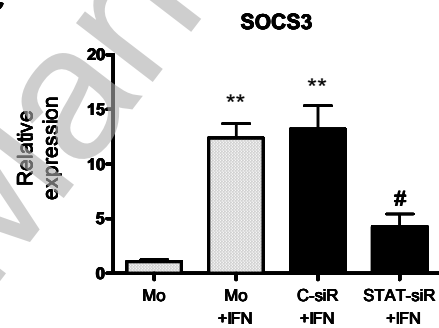
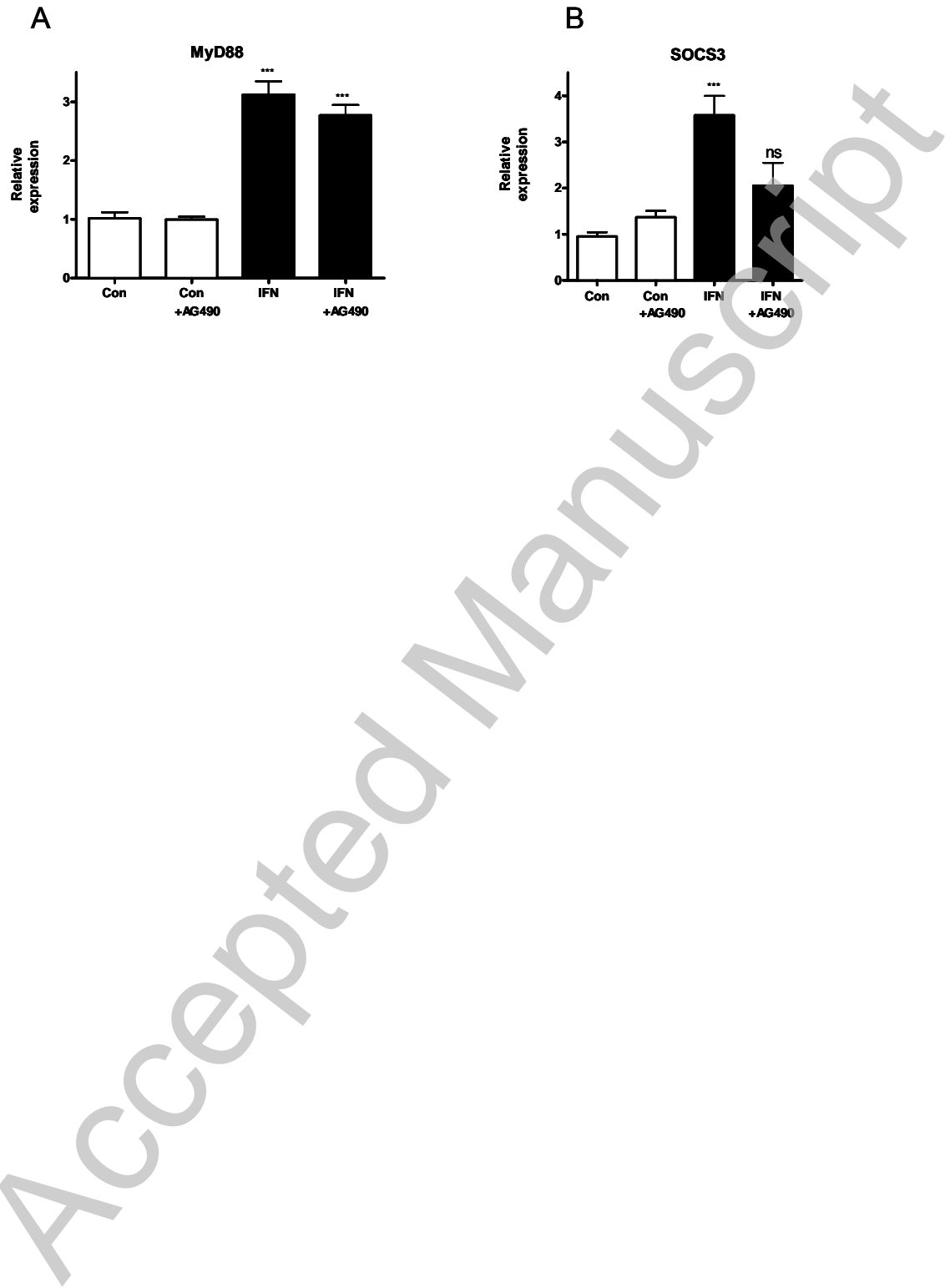
FIGURE 7**A****B****C**

FIGURE 8



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