Salmonella exploits HLA-B27 and host Unfolded Protein Responses to promote intracellular replication

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ABSTRACT

Objectives; *Salmonella enteritica* infections can lead to Reactive Arthritis (ReA), which can exhibit an association with Human Leucocyte Antigen (HLA)-B*27:05, a molecule prone to misfolding and initiation of the Unfolded Protein Response (UPR). This study examined how HLA-B*27:05 expression and the UPR affects the *Salmonella* life cycle within epithelial cells.

Methods; Isogenic epithelial cell lines expressing two copies of either HLA-B*27:05 and a control HLA-B*35:01 heavy chain (HC) were generated to determine the effect on the *Salmonella* infection lifecycle. A cell line expressing HLA-B*27:05.HC physically linked to the light chain beta-2-microglobulin and a specific peptide (referred to as a single chain trimer, SCT) was also generated to determine the effects of HLA-B27 folding status on *S.enteritica* life cycle. XBP-1venus and ATF6-FLAG reporters were used to monitor UPR activation in infected cells. Triacin C was used to inhibit *de novo* lipid synthesis during UPR and confocal imaging of ER tracker stained membrane allowed quantification of glibenclamide associated membrane.

Results; *S.enteritica* demonstrated enhanced replication with an altered cellular localisation in the presence of HLA-B*27:05.HC but not in the prescence of HLA-B*27:05.SCT or HLA-B*35:01. HLA-B*27:05.HC altered the threshold for UPR induction. *Salmonella* activated the UPR and required XBP-1 for replication, which was associated with endoreticular-membrane expansion and lipid metabolism.

Conclusions; HLA-B27 misfolding and an UPR cellular environment is associated with enhanced *Salmonella* replication, whilst *Salmonella* itself can activate XBP-1 and ATF6. These data provide a potential mechanism linking the life cycle of *Salmonella* with the physicochemical properties of HLA-B27 and cellular events that may contribute to ReA pathogenesis.

INTRODUCTION

Misfolding of the Human Leucocyte Antigen B27 allele (HLA-B27) is known to induce the unfolded protein response (UPR),[1 2] has been proposed to explain why HLA-B27 drives the inflammatory arthritic disorder Ankylosing Spondylitis (AS). Protein misfolding within the ER can disrupt ER homeostasis and trigger the UPR as measured by activation of the XBP-1[2] and ATF6 pathways[1] and in AS patients, by upregulating the UPR effector gene, HRD1. [3]

The clinical manifestation of Reactive Arthritis (ReA) after *Salmonella enterica* (*S. enteritica*) infection, has a mixed association with HLA-B27.[4]. Some studies suggest that HLA-B27-positive individuals exhibit increased susceptibility to ReA[5 6 7]or increased risk to *Salmonella* infection [8] whist others have found no strong association. [9 10 11] *S. enteritica* grows within a specialized membrane-bound compartment termed the *Salmonella* containing vacuole (SCV), prior to cellular escape and dissemination.[12-14] The mechanistic link between intracellular bacteria and HLA-B27 is unknown, but mammalian cell lines expressing HLA-B27 can harbour higher numbers of internal *Salmonella*.[15-17]

We have previously shown that fusing the HLA-B27 HC, beta-2-microglobulin (β2m) and presented peptide together within a single chain trimer (SCT) significantly reduces misfolding relative to the orginal HC.[18] Here we have used the SCT to test the hypothesis that HLA-B27 misfolding impacts on endocellular growth and localisation of *S. enteritica* in infected cells. We also tested the extent to which *S. enteritica* influences activation of both the XBP1 and ATF6-mediated UPR pathway.

MATERIALS AND METHODS

Unfolded Protein Response induction

UPR responses were induced with tunicamycin (TUN), thapsigargin (TPG), MG-132, or Calcimycin (A23187) from Calbiochem, with appropriate vehicle (DMSO alone) controls.

Transfection of UPR reporter constructs

Polyethylenimine (PEI, JetPrime) was used to transfect cells with the UPR reporter plasmids ΔDBDXBP-1venus(v) and ATF6-FLAG [19 20] following manufacturer's conditions. Cells were fixed at the desired post-infection (p.i) time points for 10 mins with 3.8% paraformaldehyde (PFA: pH 7.4) and fluorescence measured using LSR2 and LSR Fortessa flow cytometers (BD Biosciences), and data analyzed using FlowJo 8.7.3 software.

S. enteritica colony forming unit enumeration and microscopy

Colony forming unit (cfu) enumeration was performed by lysing cells in 1% Triton X-100/PBS. Lysates were serially diluted into 1% bovine serum albumin (BSA)/0.1% Tween-80% and plated on LB agar at room temperature for ~16hrs. Each experimental condition was performed in triplicate and each plating in duplicate. For microscopic analysis, coverslips containing infected cells were

washed with 1x DPBS, fixed for 10 mins with 3.8% PFA (pH 7.4), washed twice with 1x DPBS and stored at 4ºC.

UPR-mediated membrane expansion during infection

Glibenclamide BODIPY-FL (green, Invitrogen) was used to quantitate endo-reticular membrane size and localization. HeLa cells were treated with UPR inducing drugs and labelled with Glibenclamide according to manufacturers protocol. Labelled cells were analyzed by FACS. Cell nuclei were counterstained with DAPI and visualised by fluorescence microscopy. For control and drug treated cells equivalent exposures were collected.

To determine endoreticular derived membrane expansion during infection, HeLa cells were grown either on sterile glass, infected with *S. enterica* Typhimurium expressing mCherry (see supplementary materials and methods) and stained with Glibenclamide Green. Cells were fixed, washed and counterstained with DAPI followed by fluorescence microscopy or automated confocal analysis. Images were acquired by an Opera LX (PerkinElmer) plate reader with a confocal microscope (NA=0.6, 40x air objective). Exposure times were; 100ms for the DAPI channel (365 nm), 2000 ms for the ER channel (488 nm), 2000 ms for the *Salmonella* channel (561 nm). Camera pixels were binned by 2 resulting in a pixel size of 0.323 x 0.323µm. 4800 images were acquired per 96-well plate (50 images per well) that was processed in one batch using the same image analysis pipeline, algorithms and parameters. See supplementary materials and methods for analysis of Glibenclamide mean fluorescence intensity (MFI).

RESULTS

XBP-1 and ATF6 activation following *Salmonella* infection. We utlised our previously described epithelial cells with identical sites of transgene integration (and therefore isogenic) expressing physiological levels of HLA-B27[3](**Figure S1A-E**). Control cell lines encoding HLA-B*35:01 HC (HLA-B35.HC) transgene or the FRT vector alone (referred as Empty (E)84)_were generated (integration at the same two loci). HLA-B27 and –B35.HC lines were incubated with or without the influenza nucleoprotein peptide 383-391 ($NP_{383-391}$). The ability of HLA-B27 to present cognate peptide to $NP_{383-391}$-specific $CD8^+$ cytotoxic T lymphocytes (CTL) was confirmed by an increase in CD107a cell surface expression (**Figure S1F, top right panel**). Two dimensional immunoblotting of cell lysates revealed HLA-B27.HC can form HC-dimers under these experimental conditions (**Figure 1A, arrow top panel)** but only monomeric HLA-B35.HC was detected (**Figure 1A, bottom panel**).

HLA-B27 expression can activate XBP-1,[2 21] therefore we determined whether our cell lines had altered sensitivity to UPR induction. Using the XBP-1vΔDBD reporter, which on splicing the 26bp intronic sequences leads to GFP expression[19] we observed no induction of the UPR within these cell lines under normal culture growth conditions. Reasoning the expression of HLA-B27 could alter the threshold at which the UPR could be activated, we monitored XBP-1vΔDBD activation with increasing concentrations of the UPR inducing agent thapsigagrin (TPG). HLA-B27 positive cells had an altered threshold for UPR induction, whilst the E84 and B35 lines did not exhibit significant differences (**Figure 1B**).

Next, we determined whether *Salmonella* affected the UPR in the absence of HLA-B27 expression. Cells were infected with *S.enterica* expressing mCherry (referred to as *ST*.mCherry) and activation of ΔDBDXBP-1v was monitored following various multiplicity of infections (MOI) including 3:1, 50:1 and 100:1 over 1, 4, 8 and 24hrs. XBP-1s activation was determined by the fold increase in MFI above the uninfected ΔDBDXBP-1v transfected cells. Infection led to enhanced XBP-1s protein levels 8-24hrs post infection (p.i.) (**Figure 1C**). TLR ligands LPS and flagellin did not activate the ΔDBDXBP-1v reporter and heat killed bacteria had minimal impact (**Figure 1D).** Endogenous XBP-1s mRNA levels were then quantified and QPCR of XBP-1s transcript levels showed increased expression 8–16hrs p.i. demonstrating activation of the IRE-1 pathway (**Figure 1E**). We also examined the impact of infection on the ATF6 pathway. Cells were transfected with ATF6.FLAG and the cleaved cytosolic-FLAG tagged domain was quantified by immunoblotting p.i. Peak cleavage of the ATF6 cytosolic domain was observed 12hrs p.i. (**Figure 1F-G**) with a 1.5-2.5 fold increase in activation in infected compared to non-infected cells. HeLa.B27.HC and -B35.HC expressing cell lines were then tested for their ability to support bacterial growth. Cells were infected with *S.enterica* for 24hrs prior to enumeration of cfu. A 4-5 fold increase in cfu from HLA-B27.HC expressing cells was observed compared to cells expressing HLA-B35.HC or E84 controls (data not shown) (**Figure 1H**).

XBP-1 pathway is required for efficient *Salmonella enterica* Typhimurium replication. Our observations suggested that activation of the XBP-1 pathway plays a significant role in *S. enterica* growth and replication. To support our hypothesis, we analysed *S. enterica* replication in XBP-1 deficient mouse embryonic fibroblasts (MEF). XBP-$1^{+/+}$ and XBP-$1^{-/-}$ MEF cell lines were infected with *ST.*mCherry, harvested and analyzed by flow cytometry. Importantly the MFI, which is a measure of the average number of bacteria per cell, for both cell types were similar at 1 and 4hrs p.i., indicating no significant differences in either bacterial invasion or early infection stages. However, when the bacteria began replication, the XBP-$1^{-/-}$ MEF cell line exhibited significantly lower MFI values than the XBP-$1^{+/+}$ cell line indicating reduced bacterial growth (**Figures 2A**). XBP-$1^{+/+}$ cells had approximately three fold more bacteria than in XBP-$1^{-/-}$ cells during the replication phase (8-24hrs p.i.) (**Figures 2B-C**).

Enhanced bacterial replication is linked to UPR induced lipid metabolism. Next we wished to test the hypothesis that UPR promotes *Salmonella* replication. To mimic UPR conditions, HeLa cells were pretreated with the following UPR inducing pharmacological agents; A23187 (0.5mM), MG132 (0.5μg/mL), tunicamycin (TUN), (0.5μg/mL) and TPG (200nM) (see **Figure 3A for mode of action)**. HeLa cells were treated with DMSO (vehicle control) or the respective UPR inducer 16hrs prior to infection with *ST.mCherry* and the fold increase in infected cells over the control was determined 24hrs p.i. Overall the data indicate that an UPR environment leads to an increase in intracellular *Salmonella* (**Figure 3B**).

TPG appeared to have a more pronounced effect on *Salmonella* replication and also has the most direct effect on ER homeostasis (**Figure 3A**). We employed TPG further to determine the effect of a pre-existing ER stress environment. HeLa cells were treated with TPG at 25, 100 and 400nM 16hrs prior to infection. TPG pretreated cells showed significant increases in the MFI of infected cells when compared to the controls (**Figure 3C**). A 4-7 fold increase in TPG treated cells was recorded 24hrs p.i. (**Figure 3D**). Interestingly, flow cytometry revealed no significant difference in the percentage of HeLa cells infected with increasing TPG concentrations (**Figure S2A**). Direct quantification of bacterial replication by cfu recovery also demonstrated significant increase in bacterial numbers in TPG treated cells at 8 and 24hrs p.i. **(Figure 3E)**. Depending on the signals and duration, UPR can lead to production of pro or anti-apoptotic factors. The observed increase in bacterial counts 8hrs p.i. maybe due to the production of anti-apoptotic factors. To assess the level of cell death, activation of caspases was followed by staining with the FITC conjugated pan caspase detection reagent FLICA and the live dead dye 647. Treatment of cells with UPR inducing drugs did not alter activation of caspases or induction of cell death during infection indicating that the observed enhanced bacterial counts post-UPR is not linked to increased cellular death (**Figure S3**).

Salmonella replication and intracellular niche development require a source of membrane which would depend in part on lipid biosynthetic pathways, which can be activated and/or enhanced by

the UPR. We therefore inhibited *de novo* synthesis of long chain fatty acid synthesis during the induction of the UPR with Triacin C (TRC), a potent inhibitor of long fatty acyl CoA synthetase isoforms 1, 3 and 4. HeLa cells were treated with DMSO, TRC (200 nM), TPG (200 nM), or co-treated with TRC (200nM) and TPG (200nM) 16hrs prior to infection. TRC treatment prior to infection did not significantly alter the percentage of cells infected either in the presence or absence of TPG (**Figure S2B**). Cells treated with TPG alone showed significant increases in MFI values of the infected cells when compared to the DMSO or TRC controls (**Figure 4A**). However, co-treatment of cells with TRC and TPG significantly reduced the effect of the UPR induction on the levels of intracellular bacteria 24hrs p.i. (**Figure 4A-B**). These observations were confirmed by recovery of viable bacteria from similarly infected HeLa cells (**Figure 4C**) indicating inhibition of lipid metabolism during UPR induction reduces the increase in intracellular bacteria observed at late time points.

***Salmonella* infection leads to endoreticular-membrane expansion** To determine whether the increase in intracellular bacteria from cells undergoing the UPR was dependent on replication within the SCV, we utilised the isogenic *S. enterica* Typhimurium ΔsifA mutant which can infect but is impaired in intracellular growth and can escape the SCV.[22-24] HeLa cells were treated with DMSO or 200nM TPG 16hrs prior to infection with *ST*.mCherry or the isogenic Δ*sifA* mutant. *ST*.mCherry exhibited significant increases after TPG treatment as already described. In marked contrast, UPR induced cells infected with the Δ*sifA* mutant, demonstrated no increase in bacterial numbers (**Figure 5A)**. Thus, intracellular localization within the SCV is required for UPR mediated effects on bacterial replication.

As UPR activation is associated with endoreticular membrane expansion and lipid metabolism,[25 26] we determined whether *Salmonella* infection could lead to expansion of endoreticular membrane during the replication/growth phase of its lifecycle using glibenclamide green ER tracker dye as a marker for endoreticular membranes. HeLa cells were infected with either wild-type or Δ*sifA* *ST*.mCherry as a control strain. At 4 and 24hrs p.i., cells were stained with glibenclamide green ER tracker dye and analysed by automated confocal microscopy. The levels of glibenclamide labelled membrane in infected cells was quantified and compared to uninfected cells. An increase in glibenclamide labelling in cells infected with wild-type but not *sifA* deficient bacteria was observed (**Figure 5B**) which supports our observation that XBP-1 is not activated by Δ*sifA* bacteria (**data not shown**).

The folding status and expression of HLA-B27 alters *Salmonella* replication and cellular localisation. To address the role of the folding status of HLA-B27, we generated an HLA-B27 molecule fused to the light chain β2m and an HLA-B27 specific peptide derived from the influenza nucleoprotein (referred to as HLA-B27.Single Chain Trimer (HLA-B27.SCT)) As a control we also generated a similar fusion protein for HLA-B35 with EBNA1 peptide (**Figure 6A**). The HLA-B27 and –B35.SCTs were transfected into the original FLIPIN HeLa founder line. Functional activity of the

HLA-B27.SCT was determined by incubation ±$NP_{383-391}$ peptide. The HLA-B27.SCT line was an effective CTL target in the absence (**Figure 6B, top left panel**) and presence (**Figure 6B, top right panel**) of exogenously added peptide, whereas the HLA-B35.SCT line did not activate HLA-B27-NP restricted CTL lines (**Figure 6B, bottom left and right panels**). We next determined whether HLA-B27.SCT could form dimeric conformations. Both cell lines were treated with NEM and lysates were seperated by charge and Mw as described previously. Immunoblotting with the anti-V5 tag antibody pK revealed that neither the HLA-B27 SCT or –B35 SCT lines form high Mw conformers (**Figure 6C, top and bottom panels respectively**).

Next, we wished to know whether HLA-B27.SCT cells could support enhanced *Salmonella* replication. HLA-B27.SCT, along with HLA-B35 and B27.HC lines were infected with *ST.*GFP. CFU recovery, determined 24hrs p.i. demonstrated enhanced numbers of bacteria in the B27.HC but not in the B27.SCT line (**Figure 6D**).

As *Salmonella* survival can correlate with their intracellular localisation,[27] we tracked *ST.*GFP within the different HLA-class I expressing cell lines using confocal microscopy. Following infection, cells were co-stained for the trans Golgi specific marker giantin (red) and the nucleus with DAPI (blue) (**Figure 6E**). In the E84 and HLA-B35.HC cell lines, we detected *ST.*GFP concentrated in juxtaposition to the Golgi apparatus, which reflects *Salmonella* within the SCV. Surprisingly, in the presence of HLA-B27.HC, we noted that *Salmonella* markedly do not reside in close proxiimity to the Golgi, but instead were located more within the periphery (**Figure 6E, panel ii**). In contrast, in infected B27.SCT cells (**Figure 6E, panel iv**), the bacteria resided in similar locations to the B35 and E84 cells (**Figure 6E, panel iii**), suggesting that bacterial location is associated with enhanced replication, in a process influenced by the folding efficiency of HLA-B27.

DISCUSSION

Why *Salmonella* exhibits an association with ReA and HLA-B27 remains undetermined. Here we have analysed *Salmonella* growth where HLA-B27 misfolding and the UPR are limiting parameters. Our study demonstrates that it is not expression of HLA-B27 alone that results in enhanced bacterial replication, but HLA-B27 misfolding, which influences the ER stress environment. Our demonstration that HLA-B27 expression can reduce the threshold of ER stress induction and that *Salmonella* can induce the UPR provides key additional insight as to why such bacteria manipulate and exploit ER stress pathways to their benefit.

In the presence of misfolding HLA-B27, *Salmonella* predominantly reside in an altered peripheral cellular localisation. Thus HLA-B27 may alter SCV biogenesis and intracellular movement. Maturation of the SCV and bacterial cellular localisation can determine survival and replicative capability of *Salmonella*.[14 22 27 28] During the early stages of infection, the SCV migrates by following an endosomal maturation route, to a juxtanuclear location associated with the microtubule organizing center (MTOC) and the Golgi apparatus in epithelial cells.[13 14] It is possible that bacteria fail to form an SCV or exit the SCV more rapidly in the presence of misfolding HLA-B27 or escape from the SCV and randomly redistribute throughout the cytoplasm. Alternatively, *Salmonella* could initially occupy SCVs in juxtaposition to the Golgi and the movement to the periphery is enhanced in the prescence of HLA-B27.

HLA-B27.HC expression and endocellular *S. enteritica* growth both independently cause activation of the XBP-1 ER-stress pathway. XBP-1 activation peaks at 8-16hrs p.i. (**Figure 1D-E**), which correlates with enhanced bacterial numbers in cells undergoing ER stress and/or expressing HLA-B27.HC with a propensity to misfold. Activation of both XBP-1 and ATF6 coincides with the replication/growth phase of *S.enterica* Typhimurium. During this phase *Salmonella* membrane modifications have been reported to be at their peak[29] which might therefore lead to UPR activation. We do not currently know the complete cellular stress response to *Salmonella* in our model cell system, but plan to address this by RNA-Seq to map other significant differences in how HLA-B*27:05 and –B*35:01 expressing cells respond after infection. However, taken together, data reported here suggest the ER-stress consequences of HLA-B27 misfolding provide a favourable environment for replication of *S. enteritica* within HeLa cells. Interestingly, *Salmonella* infections can affect IL-23 production,[30] a cytokine that has been implicated to be important in the disease phenotype of the spondyloarthropathies.[31] It is possible that the co-occurrence of *Salmonella* and HLA-B27 could have a cumulative or multiplicative effect on the UPR which could explain the enhanced risk of ReA and/or an increased risk of symptomatic *Salmonella* infection in HLA-B27+ individuals in a population exposed to infection.[5 6 8] However, the effects of HLA-B27 on *Salmonella* may depend on the temporal activation of the UPR.[32] Our observations with pre-existing UPR activation do suggest that these aforementioned factors could indeed influence *Salmonella* replication.

The origin of the membrane that makes up the growing SCV remain poorly defined but ER membrane markers have been reported in SCVs. The ER membrane bound markers calnexin and PDI were demonstrated to co-distribute with SCVs and up to 20% of intracellular bacteria.[33] [34] Proteome analysis of host cell membranes modified by *Salmonella*, indicated that ER membranes can be redirected to their intracellular niche.[29] UPR activation can regulate ER membrane by increasing phospholipids and ER protein levels as well as modulating fatty acid, sphingolipid, phospholipid and sterol metabolism, which ultimately lead to expansion of ER membranes.[25] [26] [26] [35] [36] Our experiments with Triacin C, suggest that the long chain fatty acid CoA synthase (ACSL) family of proteins which are involved in fatty acid degradation, phospholipid remodelling and production of long chain acyl-CoA esters could well be involved in this pathway. [37] [38]

Several intracellular bacteria such as *Brucella* and *Legionella* interact directly with ER membrane.[39] Recently it has been reported that *Chlamydia,* which is also associated with ReA[40] can also induce ER stress responses for the purposes of exploiting host lipid metabolism.[41] Interestingly both *Chlamydia* and *Salmonella* have been reported to associate with and/or recruit ER derived membranes.[29] [42] Bacterial species such as *Salmonella* that depend on expansion of membrane compartments to accomodate their growth would benefit from the enhanced lipid production that results from UPR activation. Together, along with observations that HLA-B27 can induce or alter the ER stress environment, UPR induction may be a common feature of intracellular bacteria that reside in vacuoles and may link with the pathology associated with ReA.

FIGURE LEGENDS

Figure 1; *Salmonella* exhibit enhanced replication in the prescence of HLA-B27 and can activate XBP-1 and ATF6. (**A**) 2D isoelectric focusing of lysates from B27.HC cells and immunoblotted with anti-V5 pK demonstrate dimer formation (arrow) (top panel) whilst B35.HC does not form dimers. (**B**) HLA.B27.HC expressing cells transfected with the XBP1ΔDBD-venus reporter construct (UPR leads to a 26nt intronic sequence excision, leading to the expression of the venus fluorescent protein) and treated with increasing concentrations of thapsigargin (TPG) exhibit a reduced UPR threshold. (**C**) The fold increase in ΔDBDXBP-1v activation following infection of HeLa cells with *ST.*mCherry at MOI 3:1, 50:1 and 100:1. (**D**) The fold increase in ΔDBDXBP-1v activation following infection of HeLa cells with *ST.*mCherry. Non-infected cells (mCherry-) from the same well expressing ΔDBDXBP-1v were used as non-infected controls to calculate the fold increase in XBP-1 activation in the infected cells (mCherry+). No induction of XBP-1v activation was seen in cells treated with heat killed *S. enterica* Typhimurium (HTST), LPS and flagellin. (**E**) Activation of endogenous XBP-1s was demonstrated by qPCR. All cycle threshold (Ct) values were normalised to GAPDH and data plotted as a fold induction of XBP-1s mRNA compared to levels detected at baseline. (**F**) The fold increase in ATF6 activation as measured by the cleaved cytosolic-FLAG tagged domain, following infection of HeLa cells with *ST.*mCherry. (**G**) Immunoblot with anti-FLAG tag antibody of ATF6 following infection in 2 separate experiments (INF 1 & 2) compared to non-infected (NI) cells. Inactivated (i) and activated (a) ATF6 are denoted by arrows. (**H**) Relative fold increase of bacteria recovered from four individual experiments. Fold increases in *Salmonella* recovered were calculated based on cfu recovered from control E84. There in an average 4.2 fold increase in bacteria recovered from *Salmonella* infected HeLa.B27.HC cells relative to the E84 and HLA-B35.HC control lines 24hrs p.i. The Mann Whitney test was used to compare cfu recoveries between HLA-B35 and HLA-B27 expressing cell lines and exact p values calculated (*P=0.0286).

Figure 2: *Salmonella* require an intact XBP-1 pahway for efficient replication (**A**) Graph comparing the MFI of infected cells. The MFI of both cells types were similar at early time points (1 and 4hrs p.i.). When the bacteria enter log phase replication, the XBP-$1^{-/-}$ cells show much lower MFI values than XBP-$1^{+/+}$ cells. Mean mCherry MFI values ±SEM are shown (n=3) and ANOVA was performed ($p < 0.0001$) with Tukey's multiple comparision post-test to determine if there were significant differences between individual groups $P < 0.001$(***). (**B**) The fold difference in the MFI of the XBP-$1^{+/+}$/XBP-$1^{-/-}$ cells at the various time points p.i.(**C**) Recovery of viable bacteria from infected cells show similar differences between the XBP-$1^{+/+}$ and XBP-$1^{-/-}$ cells at 8 and 24hrs p.i. Mean fold differences in cfu values (n=5 or 6) ±SEM are shown. The Mann Whitney test was used to compare cfu recoveries.

Figure 3: *Salmonella* exhibit enhanced recovery from cells undergoing UPR. (**A**) Pharmacological UPR inducing agents and their mode of action. A23187 is a Ca^{2+} ionophore that disrupts intracellular Ca^{2+} levels. Thapsigargin is a non-competitive inhibitor of the SERCA pump

leading to reduced Ca^{2+} ER concentrations. Tunicamycin inhibits the addition of carbohydrate (CHO) moities to newly synthesised proteins. MG132 inhibits proteasome mediated degradation leading to accumulation of misfolded proteins within the ER. Triacin C is also shown and inhibits *de novo* lipid synthesis. (**B**) Treatment of cells with UPR inducing drugs increase the numbers of intracellular bacteria 24hrs p.i.. Cells were treated with A23187 (0.5mM), MG132 (0.5μg/mL), TPG (200nM) and TUN (0.5μg/mL). Mean fold increases in mCherry MFI values ±SEM are shown (n=3). ANOVA was performed on mCherry MFI values ($p < 0.0001$) with Tukey's multiple comparision post-test to determine significant differences between individual groups $P<0.05$ (*) and $P< 0.001$ (***). (**C-D**) TPG treated cells exhibit significant increases in MFI values of the infected cells when compared to DMSO controls (**C**) and in the fold difference in MFI values between the DMSO and TPG treated cells (**D**) in a concentration dependent manner. Mean MFI values ±SEM (n=3) are shown and ANOVA was performed ($p < 0.0001$) with Tukey's multiple comparision post-test used to determine if there were significant differences between DMSO and all drug treated groups at 24hrs p.i. $P< 0.001$ (***). (**E**) Recovery of viable bacteria from infected cells exhibit similar fold differences between the DMSO and TPG treated cells infected at 24hrs p.i. Mean fold differences in cfu values (n= 6) ±SEM are shown. The Mann Whitney test was used to compare cfu recoveries between DMSO and TPG treated cells and exact p values calculated (*P=0.004 and **P=0.015).

Figure 4: *Salmonella* require *de novo* lipid synthesis during enhanced recovery from cells undergoing UPR (**A**) HeLa cells were treated with DMSO, TRC (200nM) and TPG (200nM) or co-treated with 200nM TRC and 200nM TPG at 16hrs prior to infection with *ST.*mCherry. TPG treated cells exhibit significant increases in the MFI values of the infected cells when compared to DMSO or TRC controls. Co-treatment of cells with TRC and TPG significantly reduces the effect of the TPG on levels of intracellular bacteria at 24hrs p.i. Mean mCherry MFI values ±SEM are shown (n=3) and ANOVA was performed ($p<0.0001$) with Tukey's multiple comparision post-test to determine significant differences between individual drug treatment groups $P<0.001$ (***). (**B**) The fold difference in the MFI values of the DMSO, TRC, TPG and TRC/TPG treated cells. (**C**) Observation by FACS were confirmed by recovery of viable bacteria from similarly treated and infected cells. Colonies were counted and number of bacteria present per cell in each sample calculated (n=12). The Mann Whitney test was used to compare cfu recoveries between DMSO and TPG or TPG and TRC/TPG co-treated cells and exact p values calculated (***P=0.0001).

Figure 5: *Salmonella* infection can increase endoreticular membranes and exhibit altered cellular location in the presence of HLA-B27 (**A**) Increases in numbers of intracellular bacteria in cells treated with UPR inducing drugs is dependent on intracellular replication of bacteria within the SCV. HeLa cells were treated with DMSO or 200nM TPG at 16hrs prior to infection with *S. enterica* Typhimurium 12023 or ΔsifA strains expressing mCherry. Wild type bacteria which replicate within the SCV, show increases in intracellular bacteria in the TPG treated samples while those infected with the ΔsifA mutant show no increase in the TPG treated cells. (**B**) HeLa cells were infected with wild-type (WT) or ΔsifA *ST.*mCherry, stained with ER tracker (green) at 4 and 24 hrs p.i. and

analyzed by confocal microscopy using a Opera LX plate reader. Quantification of endoreticular membrane content in infected cells. MFI of glibenclamide staining in infected (INF) and non-infected (NI) cells were compared at 4 and 24hrs p.i. after infection with either WT or Δ*sif*A 12023. MFI values ±SEM are shown (n=135-1476). For **B** statistical analysis was performed using the Kruskal-Wallis test (p <0.0001) and was performed with Dunn's multiple comparision post-test to determine significant differences between individual groups P< 0.001(***).

Figure 6: ***Salmonella* exhibit altered cellular location in the presence of folding and misfolding HLA-B27** (**A**) Schematic of the MHC class I SCT used in this analysis. (**B**) HLA-B27.SCT (top left and right panel) can activate NP specific T cell clones and act as efficient targets in the absence of exogenous NP peptide (top left panel). HLA-B35.SCT does not activate NP-B27 specific CTLs (bottom left and right). (**C**) 2D isoelectric focusing of lysates from B27.SCT and B35.SCT cells and immunoblotted with anti-V5 pK demonstrate no dimer formation. (**D**) Expression of HLA-B27 in the context of the SCT molecule reverses the enhanced bacterial recovery phenotype observed in HLA-B27 heavy chain expressing cells. ANOVA was performed (P=0.0033) with Tukey's multiple comparison post test to determine significance between individual groups P<0.05 (*, **). (**E**) Differences in bacteria recovered at later time points is not due to increased adhesion or invasion of HeLa.B27.HC by *St.*GFP. Flow cytometric analysis of cells infected with *St.*GFP over time shows no observed difference in the number of cells infected or relative number of bacteria per cell (as determined by MFI) until later infection time points i.e. >8 hrs p.i. Data is presented relative to results from control E84 cells. Two way ANOVA was performed (P<0.0001) with multiple t-tests to determine significance between individual groups P<0.0001 (****). (**F**) *Salmonella* (green) reside in SCVs associated with the Golgi apparatus, stained with Giantin (red) in HeLa.FLP (E84, control i) and HeLa.B35.HC (ii). HeLa.B27.SCT (iv) expressing cells exhibit no altered localisation of *Salmonella* when compared HLA-B27.HC (iii) Nuclei are stained with DAPI (blue).

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DATA SHARING; all data is available on request to antony.antoniou@northumbria.ac.uk