4: The role of IL-22 in restriction of *Salmonella* invasion of the intestinal epithelium

Collaboration note:

Some of the data in this chapter have been published as: “Interleukin-22 promotes phagolysosomal fusion to induce protection against *Salmonella enterica Typhimurium* in human epithelial cells” (Forbester et al., 2018) on which I am listed as second author.

4.1 Introduction

*In vivo*, IECs play a key role in regulating intestinal homeostasis and may directly inhibit pathogens, although the mechanisms by which this occurs are not currently well understood. The cytokine IL-22 is known to have a role in the maintenance of the gut epithelial barrier and is involved in the induction and secretion of antimicrobial peptides and chemokines in response to infection. It is produced by activated T cells (particularly, CD4+ Th17 cells) as well as by natural killer (NK) cells and binds to a heterodimeric receptor composed of the IL-22R1 and IL-10R2 subunits. The receptor for IL-22 is expressed basally on IECs, meaning that in the iHO model, it is possible to pre-treat organoids with rhIL-22 simply by its addition to the culture medium.

Work on the iHO model by Jessica Forbester prior to the commencement of this project, had established via RNA sequencing that stimulation of iHO with rhIL-22 at 100 ng/mL 18 h prior to infection upregulates antimicrobial genes and other genes previously associated with the barrier defense phenotype (Figure 4.1). Notable examples include the interferon-regulated genes, IFITM1, IFITM2 and IFITM3, which are known to be involved in antimycobacterial and anti-viral response and DUOXA2; an NADPH-oxidase involved in H2O2 production at the epithelial surface, previously noted to have role in defence against enteric *Salmonella* infection. In addition, antimicrobial protein coding genes RegIIIα and RegIIIβ and mucin-producing genes MUC1 and MUC4 were amongst those most highly
upregulated, suggesting an epithelium primed for pathogen defence. The differential expression of some of these genes was also demonstrated via immunostaining of iHO, highlighting for example, increased MUC4 expression following rhIL-22 stimulation.

Alongside maintaining the epithelial barrier, IECs produce and respond to cytokines; of particular importance in this case are cytokines from the IL-10 family (IL-10, IL-19, IL-20, IL-22, IL-26), which have roles both in the innate response to pathogens and in more chronic states of inflammation, such as in inflammatory bowel disease or psoriasis. The receptor complexes for these cytokines overlap, with the surface receptor complex for IL-22 being formed of two subunits; IL-10R2 and IL-22R1, with loss of either subunit causing loss of response to IL-22. Mutations in IL-10R2 can cause severe early-onset inflammatory bowel disease in humans or colitis in mice. IL-22 or IL-22R1 deficient mice were more susceptible to lethal systemic bacterial infection following infection with *Citrobacter rodentium* or chemically-induced colitis. IL-10R2 expression is found across many types of cell, both from the haematopoietic and non-haematopoietic lineages, whereas IL-22R1 expression is more limited, being predominantly located on epithelial cells from the skin, respiratory system, kidney and digestive systems.
iHO produced from hiPSC from a patient with infantile IBD (with a homozygous splice site mutation at the boundary between intron and exon 3 in the IL10R2 gene\textsuperscript{14}) did not display upregulation of the IL-22 regulated genes Lipochalin2 (LCN2) or Dual oxidase 2 (DUOX2) upon stimulation with rhIL-22.\textsuperscript{15} However, this response was restored in an isogenic control line with this mutation complemented via TALEN-based engineering. iHO from healthy volunteers and the isogenic control line demonstrated restriction of intracellular infection with \textit{S. Typhimurium} SL1344 following pre-treatment with rhIL-22 versus those produced from the patient cell line which showed no difference in invasion level.\textsuperscript{7}

Enteric bacteria such as Salmonellae are thought to invade the epithelium through transcytosis via M-cells, or enterocytes.\textsuperscript{16} Once inside the epithelial cell, Salmonellae are able to set up an intracellular niche inside the \textit{Salmonella}-containing vacuole (SCV), which eventually fuses with endolysosomes, producing a drop in pH in the vacuole. \textit{Salmonella} uses a T3SS, encoded on SPI-2 to manipulate the course of phagosomal maturation, delaying acidification and allowing intravacuolar replication.\textsuperscript{17}

Given the restriction of intracellular \textit{S. Typhimurium} infection in iHO pre-treated with IL-22, we hypothesised that IL-22 was able to enhance intracellular defences against \textit{S. Typhimurium}, most likely by altering the environment within the SCV to induce bacterial killing. This chapter investigates the molecular mechanisms behind this hypothesis.

4.2. Phenotyping iHO derived from healthy volunteer cell lines to demonstrate presence of the IL-22 receptor complex

iHO were produced from hiPSCs derived from the healthy volunteer Kolf2 cell line as outlined in Chapter 2 (2.1.2-2.1.4). Once matured, these were immunostained for the IL-22R1 and IL10R2 elements of the IL-22 receptor complex (2.1.5). iHO demonstrated the presence of both subunits, with IL-22R1 being expressed fairly ubiquitously across the basal surface of the iHO, but IL-10R2 appearing clustered to individual cells. This appeared to co-localise with expression of chromogranin A, a protein produced by enteroendocrine cells (\textit{Figure 4.3}). This basal expression of receptors facilitated the delivery of the cytokine rhIL-22 to the basal surface of the iHO during stimulation assays.
RNA was extracted from iHO and iPSC derived from the Kolf2 line and RT-qPCR completed as described in 2.1.5 to check expression of the receptor complex subunits. mRNA was detected for IL-22R1 and IL-10R2 in samples from iPSC and iHO, with significantly increased expression of both receptors in iHO versus iPSC (Figure 4.4).

Figure 4.3: IL-22 receptor immunostaining: (A) Kolf2 iHO displaying diffuse IL-22R1 expression (red), (B) clustered IL-10R2 expression (red) and (C) colocalization of chromogranin A (green) and IL-10R2 (red) expression. DAPI (blue) marks cell nuclei in all panels. (Panel C adapted from Forbester et al, 2018). Images taken on the Zeiss LSM 510 Meta confocal microscope at 20x (Panels A&B) or 40x (Panel C) magnification.

Figure 4.4: Relative gene expression for IL-22R1 and IL-10R2 in iPSC and iHO from Kolf2 cell line. Data presented are from 4 technical replicates, with assays repeated 3 times using paired iPSC/iHO of different batches. Data were analysed using the comparative cycle threshold (Ct) method, with GAPDH as an endogenous control. Unpaired Mann-Whitney test was used to compare results (*p < 0.05).
4.3 Response of IL-22-regulated genes in iHO following rhIL-22 stimulation

Given that LCN2 and DUOX2 are known to be upregulated in response to IL-22, these genes were used as markers of the ability of iHO to respond to IL-22. This response was checked in mature iHO designated for use in experiments. rhIL-22 at a concentration of 100 ng/mL was added to culture medium for 18 hours prior to the harvesting of iHO. RNA extraction and RT-qPCR to monitor expression of LCN2 and DUOX2 were completed using methods described in 2.1.5. Transcripts for LCN2 and DUOX2 were significantly upregulated following rhIL-22 stimulation (Figure 4.5).

![Figure 4.5](Relative gene expression for (A) LCN2 and (B) DUOX2 following rhIL-22 stimulation in Kolf2 cell line. Prior to harvesting, iHO were treated for 18 hours with rhIL-22 at a concentration of 100 ng/mL or left unstimulated. Data presented are from 4 technical replicates, with assays repeated 6 times on separate occasions. Data were analysed using the comparative cycle threshold (Ct) method, with GAPDH as an endogenous control. Unpaired Mann-Whitney test was used to compare results (**p < 0.001).]

4.4 Effect of IL-22 stimulation on S. Typhimurium SL1344 infection of iHO

To establish whether IL-22 stimulation had any effect on the interactions of the iHO with S. Typhimurium, Kolf2 iHO were microinjected with S. Typhimurium SL1344 (henceforth, SL1344) after they had been treated with rhIL-22 for 18 hours (outlined in 2.3). Following modified gentamicin assays measuring intracellular bacterial survival, there were significant differences in the amount of bacteria recovered from cells in each condition, with fewer bacteria recovered from iHO pre-treated with rhIL-22 (Figure 4.6). In order to try to establish where in the infection process this difference was occurring, these invasion assays were also
performed and iHO harvested at 30 minutes to look at early stage invasion. There was a significant reduction in intracellular SL1344 counts in iHO pre-stimulated with rhIL-22, suggesting some inhibition of early invasion. However, in addition to this, infected iHO were incubated for a further 90 minutes following the gentamicin incubation step to investigate longer term intracellular survival, and there was a greater difference in survival seen here, with fewer bacteria recovered from the rhIL-22 treated cells. (Figure 4.6). Gentamicin protection assays were repeated in primary human organoids produced from duodenal tissue and again, significantly fewer bacteria were recovered from cells which had been pre-treated with rhIL-22.7

![Figure 4.6](image.png)

**Figure 4.6: Restriction of intracellular SL1344 infection in Kolf2 iHO pre-treated with rhIL-22.** (A) Modified gentamicin protection assay demonstrates that pre-treatment of Kolf2 iHO with rhIL-22 at 100 ng/mL for 18 hours inhibits SL1344 infection in the iHO at 1.5 hours post-infection. (B) Invasion into cells and survival within cells both appear to be affected, with differences in counts of bacteria recovered both at an early timepoint (30 minutes post-infection) and following a prolonged incubation period within cells (an additional 90 minutes post-gentamicin treatment). Data presented are for at least 3 biological replicates (each averaged from 3 technical replicates), with 30 iHO injected per replicate +/- SEM. Unpaired Mann-Whitney tests were used for all assays (**** p < 0.0001).

To further investigate this phenotype, intracellular invasion assays were performed with *S. Typhimurium* ST4/74 (the ancestral strain from which the histidine auxotroph SL1344 was derived) and ST4/74 with a double gene deletion knockout in the PhoP/Q system (ST4/74 ΔPhoPQ). PhoP/Q is a virulence-associated regulatory system, activated by mildly acidic pH (such as that found inside the endosomes of neutrophils/macrophages following phagocytosis). The PhoP/Q system negatively regulates invasion associated genes and positively regulates genes required for intracellular survival within the SCV. *Salmonellae* with mutations in the genes regulating this system are more sensitive to defensins, with multiple
components of the PhoP/Q system required to resist defensins. ST4/74 exhibited an indistinguishable phenotype compared to SL1344; when injected into iHO that had been pre-treated with rhIL-22; fewer intracellular bacteria were recovered in the IL-22 treated group. There were significantly higher intracellular counts of ST4/74 than ST4/74 ΔPhoPQ. However, for ST4/74 ΔPhoPQ there was no significant difference in the counts of bacteria recovered intracellularly between the treated and untreated groups (Figure 4.7). This data suggested that ST4/74 ΔPhoPQ bacteria were either killed more rapidly in the lumen by their increased susceptibility to antimicrobial peptides, or that bacteria invaded but were unable to survive intracellularly without enhanced survival systems provided by the PhoPQ apparatus. The possibility of luminal bacterial killing is further explored in Chapter 5.

![Figure 4.7: Intracellular bacterial counts at timepoints following injection for ST4/74 and ST4/74 ΔPhoPQ in Kolf2 iHO.](image)

Time course assays for intracellular bacterial invasion were also completed to attempt to further establish the duration of protective effect of rhIL-22 against Salmonella infection. Here, assays were completed using ST4/74 or ST4/74 ΔPhoPQ, with iHO harvested at 1.5, 3, 6 and 8 hours. The initial restrictive effect of rhIL-22 pre-treatment on infection only appeared to hold true until the 3 hour timepoint in iHO injected with ST4/74, and there were no
differences in recovered bacterial counts at any timepoint for iHO injected with ST4/74 \( \Delta \text{PhoPQ} \) (Figure 4.8). Again, recovered intracellular counts were lower for ST4/74 \( \Delta \text{PhoPQ} \) at all timepoints (\( p = 0.02 \)). Beyond the 3 hour timepoint, there was visible disruption of iHO integrity when examined under the microscope, suggesting that bacteria were not restricted to the enclosed lumen at this point and could invade the epithelium from either its apical or basal surface with less concentrated exposure to the iHO luminal contents.

Figure 4.8: Intracellular bacterial counts at timepoints following injection for ST4/74 versus ST4/74 \( \Delta \text{PhoPQ} \) in Kolf2 iHO. iHO were pretreated for 18 hours with rhIL-22 at 100 ng/mL or left unstimulated. Following this, iHO were infected with either ST4/74 or ST4/74 \( \Delta \text{PhoPQ} \) and incubated for 1.5, 3, 6 or 8 hours prior to modified gentamicin protection assay and recovery of intracellular bacteria. Data presented are for 3 biological replicates (each averaged from 3 technical replicates), with 30 iHO injected per replicate, +/- SEM. Unpaired Mann-Whitney tests were used for all assays (n.s. not significant, * \( p < 0.05 \)). Restrictive effect of rhIL-22 on infection appears to only last up to the 3 hour timepoint for ST4/74, and no protective effect is seen with ST4/74 \( \Delta \text{PhoPQ} \). Recovered intracellular bacterial counts are lower in untreated ST4/74 \( \Delta \text{PhoPQ} \) than ST4/74.

4.5 Effect of IL-22 stimulation on S. Typhimurium SL1344 infection in iMO

In order to ensure that the restrictive effect of IL-22 treatment was not an artefact in the iHO system, murine intestinal organoids (iMO) were produced and phenotyped from mucosal small intestine tissue from 2 wild type (WT) C57/B6 mice and 2 equivalent mice harbouring the \( \text{IL-22ra1}^- \) mutation, as described in 2.5. Prior to use of iMO for invasion assays, RT-qPCR was completed to check for the presence of the components of the IL-22 receptor complex, and to assess if the IL-22-regulated genes LCN2 and DUOX2 responded to
IL-22 stimulation. Interestingly, whilst IL-10R2 was expressed in both WT and IL-22ra1−/− mice, it appeared to be expressed at a lower level in IL-22ra1−/− mice. IL-22R1 was expressed at a significantly higher level in WT mice than in IL-22ra1−/− mice and recombinant murine (rm)IL-22 stimulation yielded no upregulation of DUOX2 or LCN2 in IL-22ra1−/− mice, confirming the expected phenotype of lack of response to IL-22 (Figure 4.9).

![Figure 4.9](image-url)

**Figure 4.9:** Relative gene expression for IL-22 receptor complex and response of IL-22 regulated genes to rmIL-22 stimulation. RNA harvested from iMO generated from both WT and IL-22ra1−/− mice underwent RT-qPCR to check for expression of the components of the IL-22 receptor (Panel A). iMO from WT and IL-22ra1−/− mice were treated with rmIL-22 at 100 ng/mL for 18 hours or left unstimulated. RNA was harvested and RT-qPCR completed to compare expression of IL-22 regulated genes DUOX2 (Panel B) and LCN2 (Panel C). Data presented are from 4 technical replicates, with assays repeated 3 times per mouse. Data were analysed using the comparative cycle threshold (Ct) method, with GAPDH as an endogenous control. Unpaired Mann-Whitney test was used to compare results (n.s. not significant, ***p < 0.001). (A) IL-10R2 was expressed in both wild type (WT) and IL-22ra1−/− mice but at a higher level in WT mice; IL-22R1 was expressed at a significantly lower level in IL-22ra1−/− mice. DUOX2 (B) and LCN2 (C) expression was significantly upregulated after treatment with rmIL-22 in WT mice but not in IL-22ra1−/− mice.

Microinjection assays to assess intracellular invasion of SL1344 were then completed (as described in 2.6) in WT and IL-22ra1−/− iMO, either unstimulated or following treatment with rmIL-22 at 100 ng/mL for 18 hours. There were significantly fewer bacteria recovered from WT iMO pre-treated with rmIL-22, and no difference in bacterial counts between the treated and untreated IL-22ra1−/− iMO, indicating that IL-22-induced restriction of infection occurs in the iMO model in addition to the iHO model, and that IL-22R1 mediated signalling is necessary for that protection to occur (Figure 4.10).
Figure 4.10: Restriction of intracellular SL1344 in WT iMO pre-treated with rmIL-22. iMO from WT and IL-22ra1⁻/⁻ mice were treated with rmIL-22 at 100 ng/mL for 18 hours or left unstimulated. iMO were injected luminally with SL1344 and incubated for 1.5 hours prior to modified gentamicin protection assay and recovery of intracellular bacteria. Data presented are for 6 biological replicates (each averaged from 3 technical replicates), with 50 iMO injected per replicate +/- SEM. Unpaired Mann-Whitney tests were used for all assays (** p< 0.01, n.s. - not significant). Pre-treatment of WT iMO with rmIL-22 inhibited intracellular SL1344 infection. This inhibition of infection was not observed in IL-22ra1⁻/⁻ iMO following rmIL-22 treatment.

4.6 Establishing the mechanism of protection mediated by IL-22 treatment in the iHO model

The reduced intracellular bacterial survival in iHO pre-treated with rhIL-22, suggests a difference in intracellular environment induced by IL-22. To further investigate this, TEM images were produced by Jessica Forbester and David Goulding at 90 minutes post-infection for iHO pre-treated with rhIL-22 versus untreated, and intracellular SL1344 populations reviewed at high resolution. Clear differences in the appearance of bacteria were noted between groups, with intracellular bacteria in the rhIL-22 treated group visibly more degraded, and increased numbers of phagolysosomes versus Salmonella-containing vacuoles seen in this cohort (Figure 4.11). Representative images were taken from 30 iHO injected per condition, using 100 μM sections of 1 mm iHO mucosa, and blinded scoring was used to produce counts for bacterial location as listed in Table 4.1. There were more degraded bacteria in Kolf2 iHO pre-treated with rhIL-22 than those untreated (49 vs. 11 respectively), and a significantly higher proportion of phagolysosomes to Salmonella-containing vacuoles in the IL-22 treated group.
It has been reported that IL-22 is able to restrict the intracellular growth of *Mycobacterium tuberculosis* in macrophages and that this effect can be negated by pre-treatment of cells with W7, a synthetic compound which inhibits phagolysosomal fusion. Specifically, W7 (N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride, Sigma-Aldrich) inhibits Ca\(^{2+}\)/calmodulin interactions with their binding partners, which are part of the signalling pathway required for phagosomal maturation. To investigate whether inhibition of phagolysosomal fusion reversed the protective effect of IL-22 treatment on intracellular infection with SL1344, 50\(\mu\)M W7 was added for 6 hours to Kolf2 iHO pre-treated with rhIL-22, prior to microinjecting with SL1344 and completing gentamicin protection assays for intracellular bacterial counts. These assays showed that in the presence of W7, the
restrictive effect of IL-22 on intracellular infection is lost, and recovered bacterial counts are equivalent to those from untreated iHO. In addition, this phenotype was replicated using pre-treatment with 100nM Concanamycin A (Insight biotechnology); a macrolide antibiotic which acts as a vacuolar ATPase inhibitor and has been shown to inhibit acidification of phagosomes and phagolysosomal fusion.\textsuperscript{20,21} In order to show that neither phagolysosomal fusion inhibitor was affecting bacterial invasion via an alternative pathway, intracellular bacterial counts from untreated iHO were compared with W7 and Concanamycin A only treated iHO following injection with SL1344. These assays showed no significant difference in numbers of bacteria recovered between conditions (\textbf{Figure 4.12}).

Rab7, a member of the Rab family of small GTP-ases, acts as a marker of the late endosome and late phagosome.\textsuperscript{22,23} To this end, Rab7 immunostaining and RT-qPCR were used to further validate the mechanism of increased phagolysosomal fusion secondary to IL-22 treatment (methods outlined in 2.1.5). Fold change in \textit{RAB7A} was significantly higher in iHO pre-treated with rhIL-22 then infected versus those left untreated and infected (\textbf{Figure 4.13}). In addition,
increased Rab7 expression via immunostaining was noted in SL1344-injected iHO pre-treated with rhIL-22, with Rab7 staining co-localising with common Salmonella antigen-1 (CSA-1), used to denote the presence of SL1344. Decreased Rab7 staining was observed in iHO which were treated with W7 in addition to rhIL-22. As noted on qPCR, increased Rab7 staining was also seen in uninfected iHO stimulated with rhIL-22 (data not shown).

Figure 4.13: Increased expression of RAB7A in iHO pre-treated with rhIL-22. (A) iHO were either left unstimulated, or treated for 18 hours with rhIL-22 at 100 ng/mL. iHO were then left uninfected or injected with SL1344 and incubated for 3 hours, followed by harvesting and RNA extraction. RT-qPCR with TaqMan gene expression assay for RAB7A was then completed to compare RAB7A expression between treatment groups and unstimulated iHO. Data presented are LOG2 fold change in expression of RAB7A, averaged from 4 technical replicates, with assays repeated 3 times. Data were analysed using the comparative cycle threshold (C\textsubscript{T}) method, with GAPDH as an endogenous control. Unpaired student’s t-test was used to compare results (**p < 0.001). A significant difference in RAB7A expression in SL1344-infected iHO pre-treated with rhIL-22 was noted versus those left untreated. (B) Kolf2 iHO were either left untreated, treated with rhIL-22 (100 ng/mL for 18 hours), or rhIL-22 + W7 (50 µM for 6 hours) prior to injection with SL1344 and incubation for 3 hours before fixing and staining. Immunostaining highlights DAPI (blue), Rab7 (red) and CSA-1 (green), with increased intensity of staining seen in samples pre-treated with rhIL-22 alone, and co-localisation of Rab7 and CSA-1 staining. Images taken on Zeiss LSM 510 Meta confocal microscope at 63x (Untreated, +IL-22 panels) or 40x (+W7 +IL-22 panel) magnification.

4.7 The role of Calgranulin B in IL-22-induced phagolysosomal fusion

It has been suggested that enhanced calgranulin A (S100A8) expression in response to IL-22 is necessary for enhancement of phagolysosomal fusion and inhibition of mycobacterial growth in macrophages.24 This was not noted to be the case in this study by RT-qPCR (data not shown), however, increased expression of calgranulin B (S100A9) was induced by pre-