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Abstract: Flagellin is the principal component of bacterial flagellum and a major target of the host immune system. To provide new insights into the role of flagellin in fish immune responses to flagellated microorganisms, a recombinant flagellin from Y. ruckeri (rYRF) was produced and its bioactivity investigated in the trout macrophage cell line RTS-11 and head kidney cells. rYRF is a potent activator of pro-inflammatory cytokines, acute phase proteins, antimicrobial peptides and subunits of the IL-12 cytokine family. This and the synergy seen with IFN-γ to enhance further expression of specific IL-12 and TNF-α isoforms may suggest that flagellin could be a useful immune stimulant or adjuvant for use in aquaculture. Gene paralogues were often differentially modulated, highlighting the need to study all of the paralogues of immune genes in fish to gain a full understanding of the effects of PAMPs or other stimulants, and the potential immune responses elicited.

Suggested Reviewers:

Highlights

1. *Yersinia ruckeri* flagellin is a potent activator of fish pro-inflammatory cytokines
2. *Y. ruckeri* flagellin also activates acute phase proteins and antimicrobial peptides
3. Paralogues of trout inflammatory genes are differentially activated by flagellin
4. IFN-γ pretreament modulates flagellin-mediated inflammatory responses
5. IFN-γ pretreament promotes the expression of specific IL-12 isoforms in rainbow trout

Re-examination of the rainbow trout (*Oncorhynchus mykiss*) immune response to flagellin: *Yersinia ruckeri* flagellin is a potent activator of acute phase proteins, anti-microbial peptides and pro-inflammatory cytokines *in vitro*

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Abstract

Flagellin is the principal component of bacterial flagellum and a major target of the host immune system. To provide new insights into the role of flagellin in fish immune responses to flagellated microorganisms, a recombinant flagellin from *Y. ruckeri* (rYRF) was produced and its bioactivity investigated in the trout macrophage cell line RTS-11 and head kidney cells. rYRF is a potent activator of pro-inflammatory cytokines, acute phase proteins, antimicrobial peptides and subunits of the IL-12 cytokine family. This and the synergy seen with IFN-γ to enhance further expression of specific IL-12 and TNF-α isoforms may suggest that flagellin could be a useful immune stimulant or adjuvant for use in aquaculture. Gene paralogues were often differentially modulated, highlighting the need to study all of the paralogues of immune genes in fish to gain a full understanding of the effects of PAMPs or other stimulants, and the potential immune responses elicited.

Key words: Flagellin, *Yersinia ruckeri*, rainbow trout, cytokine, acute phase protein, antimicrobial peptide

1. Introduction

The initiation of immune responses in a host exposed to infectious agents depends on the recognition of pathogen-associated molecular patterns (PAMPs) present on the pathogens by specific pattern recognition receptors (PRRs) expressed by phagocytic cells such as macrophages (Kawai and Akira, 2010; Chettri et al., 2011). Flagellin is the principle structural protein of flagellum, a whip-like filament appended to the bacterial surface to provide the major force for bacterial motility in Gram positive and negative bacteria (Zhao and Shao, 2015). Due to its wide presence in diverse bacterial species and extreme abundance in each bacterial cell, flagellin is a powerful PAMP and a major target of the host immune system.

Monomeric flagellin (30–60 kDa, dependent upon the taxa of the bacterium) contains four distinct globular domains, D0, D1, D2, and D3, shaped into a 'boomerang'. About 40 amino acids from each terminus of the flagellin molecule constitute the D0 domain. The D1 domain contains about 100 residues from the N-terminus and 50 residues from the C-terminus. The D0 and D1 domains are crucial for assembly of the helical filamentous structure and therefore highly conserved among different species of bacteria, and contain primarily α-helical structures, whereas the D2 and D3 domains exhibit high sequence diversity and are composed largely of β-sheets (Yoon et al., 2012; Akira et al., 2006; Zhao and Shao, 2015). In the extracellular space, flagellin is recognized by Toll-like receptor 5 (TLR5) expressed by antigen-presenting cells and T cells. Mammalian TLR5 is a plasma membrane-localized PRR (TLR5M) that contains an extracellular domain possessing leucine-rich repeats (LRRs), a transmembrane region, and a cytoplasmic signaling domain denominated the Toll/interleukin-1 receptor homology (TIR) domain. The LRR domain in TLR5 directly binds to the conserved D1 domain of flagellins. The activation of TLR5 mediates the production and secretion of pro-inflammatory cytokines, chemokines and co-stimulatory molecules for development of effective immunity (Hayashi et al., 2001; Jacchieri et al., 2003).

TLR5, though effective in detecting extracellular flagellin, is powerless to detect flagellin that has reached the host cytosol, a situation that often occurs during infection. Nucleotide binding domain and leucine rich repeat containing proteins (NLRs) are a functionally diverse protein family. The NLR family of apoptosis inhibitory proteins (NAIPs) are encoded within a small cluster of genes in the mouse but only one gene in humans and have a critical role in host defence against bacterial infection (Vance, 2015). Mouse NAIP5/6 and human NAIP are cytosolic receptors for bacterial flagellin (Zhao et al., 2011; Kofoed et al., 2011; Kortmann et al., 2015). Upon ligand-binding, NAIPs co-oligomerize with a downstream adaptor protein called NLRC4 that recruits and activates Caspase-1 (CASP-1) protease. CASP-1 orchestrates innate anti-bacterial responses by inducing a rapid lytic cell death,

called pyroptosis, and also mediates the processing and release of the pro-inflammatory IL-1β and IL-18 (Zhao and Shao, 2015; Vance, 2015).

In rainbow trout (*Oncorhynchus mykiss*) and other teleost species, two TLR5 genes are present in the genome (Tsujita et al., 2004; Tsoi et al., 2006; Baoprasertkul et al., 2007; Hwang et al., 2010; Munoz et al., 2013). One (TLR5M) encodes for an extracellular LRR, a transmembrane region, and a cytoplasmic TIR domain as seen in mammalian TLR5. The other encodes only the LRR in the extracellular domain and thus produces a soluble form of TLR5 (TLR5S). Trout TLR5M is ubiquitously expressed in all tissues whereas TLR5S is predominantly expressed in liver (Tsujita et al., 2004). Both the TLR5M and TLR5S recognize flagellin from the Gram negative bacterium *Vibrio anguillarum*. The immune responses to flagellin have been examined in salmonids and other fish species recently (Chettri et al., 2011; Hynes et al., 2011; Scott et al., 2013; Gonzalez-Stegmaier et al., 2015), where up-regulation of IL-1β and IL-8 expression is seen to flagellins from *V. anguillarum* and *B. subtilis*. Curiously the response was one order of magnitude lower in rainbow trout than in gilthead seabream (*Sparus aurata*) (Gonzalez-Stegmaier et al., 2015), although this may be related to species-origin of the flagellin. The fish responses to flagellin have only been examined in terms of a limited numbers of pro-inflammatory genes (IL-1β1, IL-6, IL-8 and TNFα) and the responses of adaptive cytokine genes are largely unknown (Chettri et al., 2011; Hynes et al., 2011). IL-12 was previously reported to be down-regulated by flagellin *in vivo* since a p40 gene, that encodes one of two peptides that form IL-12 (along with p35), was found to be down-regulated (Hynes et al., 2011). This conclusion needs to be re-evaluated in light of the multiple paralogues now known, with three genes of p35 and p40 present in salmonids that potentially make 9 heterodimeric IL-12 isoforms with different functions (Wang and Husain, 2014; Wang et al., 2014). Indeed, the recent identification of multiple paralogues of many cytokines in fish (eg in salmonids there are three each of IL-1β and TNFα) (Husain et al., 2012; Hong et al., 2013) means the cytokine response to flagellin stimulation is far from complete. Moreover, a flagellin from the pathogenic bacterium *Yersinia ruckeri*, the causative agent of enteric redmouth disease (ERM) that primarily affects farmed salmonids (Harun et al., 2011), has been shown to induce non-specific protection against a variety of bacterial pathogens *in vivo* in rainbow trout (Scott et al., 2013). However, the mechanism(s) of flagellin-mediated non-specific protection in fish is largely unknown.

In this study, we first produced a recombinant flagellin from *Y. ruckeri* (rYRF) and then investigated the host cell responses to rYRF using the monocyte/macrophage like cell line, RTS-11 and *in vitro* cultured head kidney cells. We found that rYRF was a potent stimulant of pro-inflammatory cytokines but had no effect on adaptive cytokine expression *in vitro*. Different paralogues of pro-inflammatory cytokines were found to be differentially modulated in terms of their sensitivity to flagellin stimulation and kinetics of the response. We also found that genes for several acute phase proteins

(APPs) and anti-microbial peptides (AMPs) were rapidly upregulated and provide a potential mechanism for flagellin mediated non-specific protection to bacterial infection. Furthermore, we found that IFN-γ, a cytokine of type 1 immune responses, modulated flagellin-mediated up-regulation of cytokines, APPs and AMPs, and synergized with flagellin to up-regulate the expression of specific IL-12 isoforms. This study provides new insights into the role of flagellin in immune responses to flagellated microorganisms, and suggests that flagellin may be a useful immune stimulant or adjuvant for use in fish aquaculture.

2. Materials and methods

2.1 Experimental fish

Healthy rainbow trout were purchased from the Mill of Elrich Trout Fishery (Aberdeenshire, Scotland, UK) and maintained in 1-m-diameter fibreglass tanks with recirculating freshwater at 14 °C at the Scottish Fish Immunology Research Centre, University of Aberdeen, UK. Fish were fed twice a day with a commercial diet (EWOS) and were given at least two weeks for acclimation prior to use.

2.2 Recombinant flagellin production

The coding region of flagellin was amplified from a pathogenic strain of *Y. rucheri* (MT3902, Harun et al., 2011a) using primers (forward: GCGGTCATTAACACTAACAGCCTG; and reverse: ACGCAGCAGAGACAAGACAGT) designed against AGL46983, and the Q5 high fidelity enzyme (New England Biolabs, UK). The amplified product was cloned to a pTriEX-6 vector (Novagen). The construct (pTri-YRF) encodes an identical amino acid sequence to AGL46983 except for an insertion of N after R14 and a mutation (M409 to L) at the D0 domain, and a his-tag (ASSAHHHHHHHHHH) added at the C-terminus for purification. Thus, the recombinant *Y. ruckeri* flagellin (rYRF) was 439 aa, with a calculated molecular weight of 45.4 kDa and a theoretical pI of 6.21. Following transformation of the pTri-YRF plasmid into BL21 Star (DE3) competent cells (Invitrogen), the induction of recombinant protein production, purification under denaturing conditions, refolding, re-purification under native conditions, SDS-PAGE analysis of proteins and quantification of protein concentration were as described previously (Costa et al., 2011; Wang et al., 2011a; 2015b). The wash buffer used under denaturing conditions contained 1% Triton X-100 and 40 mM imidazole, that effectively remove membrane proteins eg lipopolysaccharide (LPS). The refolding buffer contained 50 mM Tris-HCl (pH7.5), 10% glycerol, 0.6 M arginine monohydrochloride and 0.5% Triton X-100. The purified protein was desalted in desalting buffer (DSB) (50 mM Tris-HCl, pH7.5, 140 mM NaCl, 10 mM arginine and 50% glycerol) using PD-10 Desalting Columns (GE Healthcare). After sterilization with a 0.2 μm filter, the rYRF (0.75 mg/ml) was aliquoted and stored at -80^{o}C ready for stimulation of cells.

2.3 Stimulation of RTS-11 cells

The monocyte/macrophage-like cell line, RTS-11, from rainbow trout spleen (Ganassin and Bols, 1998), was used for bioactivity studies. The cells were maintained in Leibovitz (L-15) medium (Invitrogen, UK) containing 30% foetal calf serum (FCS; Labtech International, UK) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; P/S; Invitrogen, UK) at 20 °C, and passaged as

described previously (Ganassin and Bols, 1998). For experiments, cells were collected by centrifugation (200 × g, 5 min), washed once with L-15 medium containing 0.5% FCS, diluted in L-15 containing 10% FCS to 1× 10^6 cells/ml, and seeded into 12-well cell culture plates at 2 ml/ well. Overnight cell cultures were stimulated with 0.01-1000 ng/ml flagellin for 4 h, or 100 ng/ml flagellin for 1, 2, 4, 8, 12, 24 h. RTS-11 cells were also cultured with/without 20 ng/ml rIFN-γ (Wang et al., 2011b) overnight (~20 h) and re-stimulated with/without 100 ng/ml flagellin for 4 h.

2.4 Stimulation of primary head kidney (HK) cells

Primary HK leukocytes from freshly killed rainbow trout were isolated following the method previously described by Wang et al. (2011a). Briefly, fish were anaesthetised, killed, and the anterior kidney removed aseptically and passed through a 100 μm nylon mesh using L-15 Medium supplemented with P/S, heparin (10 units/ml), and 1% FCS. The primary HK cells were resuspended in L-15 medium containing 10% FCS at 2 × 10^6 cells/ml and then stimulated with rYRF (100 ng/ml) for 1, 2, 4, 8, 12, 24 and 48 h.

2.5 Total RNA extraction, cDNA synthesis and real-time PCR analysis of gene expression

The treatments were terminated by dissolving the cells in TRI reagent (Sigma, UK). Total RNA extraction, cDNAs synthesis and real-time PCR analysis of gene expression were as described previously (Wang et al., 2011a, 2011b, 2014). The expression of cytokines, antimicrobial peptides (AMPs) and acute phase proteins (APPs), as well as the house keeping gene elongation factor-1α (EF-1α), was examined. The primers for real-time PCR are detailed in Table 1, with at least one primer of a pair designed to cross an intron so that genomic DNA could not be amplified under the PCR conditions used. The expression of each gene was first normalized to that of EF-1α, and presented as a fold change by calculating the average expression level of the rYRF stimulated sample divided by that of the controls at the same time point.

2.6 Statistical analysis

The data were statistically analyzed using the SPSS Statistics package 22 (SPSS Inc., Chicago, Illinois). The analysis of real-time PCR data was as described previously (Wang et al., 2011a,b). To improve the normality of data, real-time quantitative PCR measurements were scaled, with the lowest expression level in a data set defined as 1, and log2 transformed. One way-analysis of variance (ANOVA) and the Bonferroni post hoc test were used to analyse expression data derived from RTS-11 cells, with $P < 0.05$ between treatment and control groups considered significant. For data from HK cells that consisted of sample sets from individual fish, a Paired-Samples T-test was applied. The induction of gene expression was first normalised to the highest induction level (defined as 100) during the time course and used for clustering analysis using XLSTAT software (Addinsoft).

3. Results

3.1 Production of recombinant *Y. ruckeri* flagellin (rYRF) in *E. coli*

A protein of the expected size of 45.4 kDa was induced by IPTG stimulation of transformed BL21 cells, and purified under denaturing conditions with extensive washing in 1% Triton X-100 buffer to remove LPS (**Fig. 1**). The purified rYRF was refolded *in vitro* and re-purified under native conditions, and denaturants and other contaminants were removed by extensive washing of the purification column. Although flagellin and LPS share common bioactivities (eg up-regulation of IL-1β and TNF-α in RTS-11 cells), IL-17C1, known to be upregulated by LPS (Wang et al., 2010), was not induced with up to 1000 ng/ml rYRF (**Fig. S1**), confirming that LPS contamination in the recombinant preparations was negligible.

3.2 Dose dependent modulation of gene expression by rYRF in RTS-11 cells

Initial tests of rYRF mediated gene expression in RTS-11 cells and HK cells found that rYRF was bioactive at concentrations as low as 0.01 ng/ml and peaked around 4 h for most genes tested. Thus a dose-response analysis of gene expression was conducted in RTS-11 cells stimulated for 4 h with 0.01 ng/ml to 1000 ng/ml of rYRF. The sensitivity to rYRF stimulation was gene specific and could be categorised into three groups. The most sensitive genes were IL-8, TNF-α3 and SAA, where expression was up-regulated at 0.01 ng/ml ($p<0.05$) and reached the highest fold induction at 1 ng/ml rYRF (Fig. 2). Many genes were induced at 0.1 ng/ml ($p<0.05$) and also reached the highest fold induction at 1 ng/ml rYRF (eg IL-6, IL-11, SAP1, hepcidin, CATH2, subunits of the IL-12 family (p19, p28B, p35A1, p35A2, p40B1 and p40B2), IL-34 and M17) (Fig. 2, S1). The less responsive genes, including IL-1β1, IL-1β2, nIL-1Fm, IL-17C2, TNF-α1 and TNF-α2, were induced in a dose dependent manner from 0.1 or 1 ng/ml and reached the highest fold induction at 100 ng/ml rYRF (Fig. 2, S1). In terms of fold induction, the expression of IL-6 and IL-8 was increased over 1000-fold, and that of IL-1β2, IL-17C2, p35A1, p19, SAA, hepcidin and CATH2 over 100-fold (Fig.2, S1). Of the paralogues examined, IL-1β2 was more inducible than IL-1β1; TNF-α3 was the most sensitive, followed by TNF-α2, and then TNF-α1; IL-17C2 expression was induced by rYRF but IL-17C1 was refractory; and finally, IL-12 p40B1 and p40B2 were inducible by rYRF but p40C was refractory (Fig.2, S1).

3.3 Time dependent modulation of gene expression by rYRF in RTS-11 cells

Dose-responses of rYRF revealed that the induction of inducible genes was highest at ≤ 100 ng/ml. To understand the kinetics of rYRF modulated gene expression, a time course of rYRF stimulation was

conducted at this concentration for 1-24 h in RTS-11 cells. A total of 42 selected cytokines, APPs and AMPs genes known to be expressed in RTS-11 cells were analysed (Figs. 3-5, S2-3). TLR5M was highly expressed but refractory to rYRF stimulation from 1 h to 24 h in RTS-11 cells (Fig. S2A), whilst the expression of TLR5S was non-detectable. Most of the genes responsive to rYRF stimulation reached their highest levels at 4 h with the exception of IL-1β2 and TNF-α3 that reached their highest level at 1 h after stimulation (Figs. 3-5, S2-3).

The expression of IL-1 family cytokines: Five IL-1 family members, three IL-1β paralogues (Zou et al., 1999; Pleguezuelos et al., 2000; Husain et al., 2012), a novel IL-1 family member (nIL-1Fm) (Wang et al., 2009) and IL-18 (Zou et al., 2004) are known in rainbow trout. IL-1β2 expression was highly responsive to rYRF and reached the highest induction level at 1 h (279-fold), which was maintained to 4 h and then decreased but remained higher than unstimulated controls to 24 h (Fig. 3B). IL-1β1 expression was induced from 1-24 h and peaked at 4 h (57-fold) (Fig. 3A). nIL-1Fm expression was induced from 2-24 h but peaked later at 12 h (23-fold) (Fig. 3D). In contrast, IL-1β3 expression was only marginally induced, at 4 h (3-fold, Fig. 3C), and IL-18 expression was refractory (Fig. S2F).

The expression of TNF-α paralogues: Three TNF-α paralogues are known in rainbow trout (Laing et al., 2011, Zou et al., 2012, Hong et al., 2013). The expression of both TNF-α1 and TNF-α2 was up-regulated from 1-24 h and peaked at 4 h (57-fold for TNF-α1 and 91-fold for TNF-α2) (Fig. 3E-F). TNF-α3 expression was also induced from 1-24 h but peaked at 1 h, the earliest time point examined (405-fold, Fig. 3G).

The expression of IL-6 family cytokines: Four IL-6 family members, IL-6, IL-11, M17 and CNTF-like, have been cloned in rainbow trout (Iliev et al., 2007; Wang et al., 2005; Wang and Secombes, 2009). IL-6 expression is highly induced from 1-24 h and peaked at 4 h, with 1,324-fold increase (Fig. 3H). The expression of IL-11 and M17 was also induced from 1-24 h and peaked at 4 h but with lower fold-induction (17-fold for IL-11 and 16-fold for M17), but CNTF expression was refractory (Fig. S2B-D).

The expression of other inflammatory cytokines: IL-8 was the first known chemokine and attracts neutrophils, T lymphocytes and basophils *in vitro* (Laing et al., 2002). Its expression was rapidly induced at 1 h (198-fold), dropped at 2 h (55-fold) and then peaked at 4 h (653-fold) (Fig. 3I). Fish IL-17C is phylogenetically related to mammalian IL-17C and IL-17E. Two trout IL-17C paralogues, IL-17C1 and IL-17C2 are present in rainbow trout (Wang et al., 2010). IL-17C2 expression was induced from 1-24 h and peaked at 4 h (117-fold) (Fig. 3K), however IL-17C1 expression was refractory to rYRF. IL-34 is a macrophage growth factor and regulates the mononuclear phagocyte system (Wang et al., 2013). IL-34 expression was also induced from 1-24 h and peaked at 4 h (16-fold) (Fig. 3L).

The expression of IL-12 family cytokines: Genes for 6 active α-chains (p19, p28A, p28B, p35A1, p35A2 and p35B) and 4 β-chains (p40B1, p40B2, p40C and EBI3) are known to be present in rainbow trout (Wang and Husain, 2014, Husain et al., 2014; Jiang et al., 2015). The expression of the α-chains is low in RTS-11 cells, with p28A and p35C expression undetectable in controls and not described further. The induction of the α-chain expression was transient. The expression of p19 and p35A2 was induced at 2-8 h and peaked at 4 h (112-fold for p19 and 7-fold for p35A2). The upregulation was only detectable at 4 h (93-fold) and 8 h for p35A1, and at 4 h for p28B (6-fold) (Fig. 4A-D). Whilst the expression of EBI3 and p40C was refractory, a moderate induction of p40B1 was seen from 1 h to 12 h and peaked at 4 h (8 fold), and that of p40B2 from 4 h to 24 and peaked at 8 h (6-fold) after stimulation with rYRF (Fig. 4E-H).

The expression of anti-inflammatory cytokines: The expression of four anti-inflammatory cytokines, IL-10A and IL-10B (Harun et al., 2011b), and TGF-β1A and TGF-β1B (Maehr et al., 2013) was also examined during the time-course of rYRF stimulation. Both TGF-β1 paralogues are highly expressed in RTS-11 cells but were refractory to rYRF stimulation. The expression of IL-10 was low and could be induced to some degree at 4 h (2-fold) for IL-10A, and at 4h to 12 h (up to 3 fold) for IL-10B (Fig. S3A-D).

The expression of other cytokine genes: Several other cytokine genes, including IL-4/13A, IL-4/13B1, IL-4/13B2 (Wang et al., 2015b), IL-15 (Wang et al., 2007), IL-21 (Wang et al., 2011), IL-22 (Monte et al., 2011) and IFN-γ2, are known to be expressed in RTS-11 cells. Their expression was not modulated by rYRF at 1-24 h except for IL-4/13B2 at 4 h when a small induction (3-fold) was seen (Figs. S2-S3).

The expression of APPs and AMPs: APPs, eg serum amyloid A protein (SAA) and serum amyloid protein P (SAP)1 and SAP2, and AMPs, eg CATH1, CATH2 and hepcidin, are evolutionarily conserved effector molecules of the innate immune system that have important roles in the resolution of infection and activation of the adaptive immune response (Douglas, et al., 2003; Chang et al., 2006; Mickels et al., 2015; Choi et al., 2015). Thus their expression was examined in response to flagellin stimulation. SAA expression was increased from 1 h, reached the highest induction level (521-fold) at 4 h and maintained this high level to 24 h. SAP1 expression was also induced from 1 h to 12 h and peaked at 4 h (10-fold) but SAP2 expression was refractory (Fig. 4A-C). The expression of all the three AMPs was induced from 1 h to 24 h and reached the highest levels at 4 h (CATH2, 70-fold; hepcidin, 328-fold) or at 8 h (CATH1, 41-fold) (Fig. 4D-F).

3.4 Time dependent modulation of gene expression by rYRF in HK cells

rYRF is a potent stimulant of pro-inflammatory cytokines, APPs and AMPs in the macrophage RTS-11 cell line. Human T cells also express TLR5 and increase TCR-induced adaptive cytokine

289 expression when co-stimulated or pretreated with flagellin (Tremblay et al., 2014). It is not possible to
290 isolate pure T cells in salmonids because of lack of tools, eg antibodies to T cells. To investigate the
291 potential of flagellin modulation of T cell cytokine expression, HK cells containing macrophages, T
292 cells and B cells etc. were stimulated with flagellin for 1 h to 48 h, and the expression of adaptive
293 cytokines, B cell related molecules, and genes upregulated in RTS-11 cells was quantified (Table 2).
294 To give an indication of expression level in HK cells, Δcp that is the cp (the crossing point at which
295 the fluorescence crosses the threshold) of the target gene minus that of EF-1α, were also provided
296 (Table 2). A higher cp value indicates a lower expression level. The expression of TLR5M and
297 TLR5S was detectable but refractory to rYRF stimulation. The expression of the pro-inflammatory
298 cytokines, including IL-β1, IL-1β2, nIL-1Fm, TNF-α2, TNF-α3, IL-6, IL-8, IL-11 and IL-34, was
299 induced in HK cells by rYRF albeit to a lower fold change compared to that in RTS-11 cells. The
300 exceptions were TNF-α1, IL-17C2 and M17, that showed no significant up-regulation. Up-regulation
301 of the expression of AMPs, APPs and anti-inflammatory cytokines was also seen in HK cells.
302 However, the expression of the subunits of the IL-12 cytokine family was not modulated in HK cells
303 except for that of p40B2 where a 2-fold increase at 2 h was seen after rYRF stimulation. Interestingly,
304 the expression of all the adaptive cytokines IFN-γ, IL-2 (Diaz-Rosales et al., 2009), IL-4/13 (Wang et
305 al., 2015a), IL-17 paralogues (Wang et al., 2015a), IL-21 and IL-22, that are transcribed in T cells,
306 and molecules related to B cells (IgM, IgD and IgT, secreted or membrane bound, and pIgR) was
307 refractory to rYRF (Table 2).

308 **3.5 Modulation of the flagellin-mediated response by rIFN-γ in RTS-11 cells**

309 Although no up-regulation of expression of adaptive cytokines such as IFN-γ was found in RTS-11
310 cells and HK cells by flagellin, such cytokines may be present *in vivo*, eg during a Th1-type immune
311 response, and could potentially modulate the immune response to PAMPs including flagellin. Thus,
312 RTS-11 cells was pre-treated with rIFN-γ overnight and then stimulated with rYRF for 4 h, and the
313 expression of the flagellin-responsive pro-inflammatory cytokines, AMPs, APPs and the IL-12
314 cytokine family members was examined. Treatment with rIFN-γ alone had no effect on the expression
315 of IL-1β2, nIL-1Fm, IL-8, IL-17C2, M17, IL-34, TNF-α2, TNF-α3, SAA, SAP1, CATH1, CATH2,
316 hepcidin, EBI3, p28B, p35A1, p35A2 and p19, but inhibited the expression of IL-1β1, IL-6, IL-
317 11,TNF-α1 and p40B1 and increased p40B2 expression (6-fold) (Figs. 6-7). rIFN-γ pre-treatment
318 down-regulated flagellin-induced expression of the pro-inflammatory cytokines IL-1β1 (3-fold), IL-
319 1β2 (6-fold), IL-17C2 (2-fold), IL-11 (14-fold), M17 (2-fold) and TNF-α1(6-fold), and up-regulated
320 flagellin-induced expression of TNF-α2 (3-fold) and TNF-α3 (5-fold) (Fig. 6). In regard to the APP
321 and AMP genes, rIFN-γ pre-treatment had no effects on the expression of SAA and CATH1, but
322 down-regulated flagellin-induced expression of SAP1 (2-fold), CATH2 (11-fold) and hepcidin (12-
323 fold) (Fig. 7A-E). Although no effect on the expression of the α-chains of IL-12 family cytokines was

seen when rIFN-γ was used alone, rIFN-γ pre-treatment greatly enhanced flagellin induced expression, with a 312-, 9- and 52-fold increase seen for p28B, p35A1 and p35A2, respectively, compared with rYRF treatment alone but no effects on p19 expression were found (Fig. 7G-J). The expression of p40B1 and p40B2 was comparable in unstimulated RTS-11 cells. Pre-treatment with rIFN-γ increased p40B2 expression (8-fold) but decreased p40B1 expression (4-fold) after rYRF stimulation (Fig. 7K-L). Both rYRF and rIFN-γ had no effect on the expression of the other β-chains EBI3 (Fig. 7F) and p40C (data not shown) alone or in combination.

4. Discussion

In this study we show that recombinant *Y. ruckeri* flagellin (rYRF) is a potent stimulant of rainbow trout macrophages and HK cells, and is able to up-regulate a large number of pro-inflammatory cytokines, APPs, AMPs and members of the IL-12 cytokine family but not cytokines typical of adaptive immunity. Flagellin modulated gene expression has been investigated previously in fish but with only a limited number of pro-inflammatory genes studied (Chettri et al., 2011; Gonzalez-Stegmaier et al., 2015). With the recent success in characterising many salmonid cytokine genes, most of which have multiple paralogues, it was clear that the effects of flagellin on the fish immune system needed to be revisited. Moreover, a flagellin from the pathogenic bacterium *Y. ruckeri* has been shown to induce a non-specific protection against a variety of bacterial pathogens *in vivo* in rainbow trout (Scott et al., 2013) but the mechanism(s) of this flagellin-mediated protection is unknown. The results from the present study go some way to address these issues.

The responses to flagellin typically peaked within the first 4h post-stimulation and decreased thereafter, with the notable exception of nIL-1Fm, which is an IL-1 receptor antagonist (Yao et al., 2015) and is therefore expected to quench IL-1β action. The responses of some genes were particularly sensitive to flagellin stimulation, in terms of low dose induction (eg TNFα3, IL-8, SAA) and fold of induction seen (eg IL-6, IL-8). Previous work by Gonzalez-Stegmaier et al. (2015) suggested that rainbow trout were less responsive to flagellin stimulation, in comparison to the responses seen in seabream. We now demonstrate that this is not the case, with fold increases for genes such as IL-1β, IL-8 and TNFα being >100-fold at optimal flagellin concentrations. Also following immunisation with flagellin as an adjuvant, IL-12 (p40) was reported previously as being largely down-regulated *in vivo* in Atlantic salmon (*Salmo salar*) (Hynes et al., 2011). In the present study both chains that form IL-12, p35 and p40, were shown to be up-regulated following stimulation *in vitro*, and so again this conclusion must be treated with caution, especially in light of the multiple paralogues present which will be discussed further below.

Many immune genes in teleost fish are known to have multiple paralogues, especially in species that have undergone additional whole genome duplication events, as seen in the salmonids. For example, there are three genes for IL-1β and TNFα in salmonids (Husain et al., 2012; Hong et al., 2013) and three each of the p35 and p40 genes, that potentially could make 9 heterodimeric IL-12 isoforms with different functions (Wang and Husain, 2014; Wang et al., 2014). Thus, it is necessary to determine whether all of the genes present react in a similar manner. In this study it is clear that major differences can occur. The biggest differences were seen when one of the paralogues was responsive and the other not, as with IL-10A vs IL-10B, IL-17C1 vs IL-17C2, p40B vs p40C, and SAP1 vs

SAP2. More subtle differences were also seen in sensitivity or level of increase seen, as with the IL-1β, TNFα and p35A paralogues. These differences likely reflect differences in the promoters, with some of the paralogues becoming more or less responsive to particular signalling pathways, perhaps in particular cell types, or genes that are being pseudogenised. Little is known about the differential expression of the SAP paralogues, but previous studies on trout IL-10 have shown that IL-10A is often more highly expressed in response to different stimulants, in contrast to the present study. It is highly expressed in the spleen following bacterial (*Y. ruckeri*) infection, with IL-10B induced in the gills (Harun et al., 2011b). In the case of the IL-17C paralogues, we have shown that IL-17C2 is generally more highly induced (eg by bacteria or oomycetes) although IL-17C1 can be induced significantly in both situations (Wang et al., 2010; de Bruijn et al., 2012). Lastly, in the case of p40 in trout, p40C is generally less inducible than p40B (B1 and B2), although a small induction by rIL-1β and TNFα has been found (Wang & Husain, 2014) and in response to infection (Wang et al., 2014). Interestingly p40B expression was not induced by viral or parasite infection, at least in the HK. Two isoforms of rainbow trout rIL-12 have been made that differ in the p40 chain (ie p40B or p40C). These proteins can induce IFN-γ expression in HK cells but only the isoform containing p40C was able to also induce IL-10 (Wang et al., 2014), suggesting subtle differences in bioactivity dependent upon the p40 chain used. Differential responsiveness to flagellin was also demonstrated in the kinetics of induction by the TNF-α paralogues. In agreement with our previous study that showed TNF-α3 is an early responsive gene to crude LPS stimulation (Hong et al., 2013), TNF-α3 expression peaked at 1 h post flagellin stimulation whilst TNF-α1 and TNF-α2 expression peaked at 4 h. rTNF-α3 can induce the expression of other inflammatory cytokines including paralogues of IL-1β and TNF-α, IL-6 and IL-8 (Hong et al., 2013). Thus the early induction suggests that TNF-α3 is a key cytokine in the cascade of cytokine expression induced by PAMPs.

The induction of APPs and AMPs by rYRF is particularly interesting in the context of the non-specific protection seen after flagellin administration *in vivo* (Scott et al., 2013). Clearly these molecules may contribute to induction of an antimicrobial state. Cathelicidins are a group of AMPs that share a highly conserved preproregion containing the cathelin-like domain at the N terminus but carry a substantially heterogeneous C-terminal domain that encodes the mature antimicrobial peptide. Two cathelicidin genes, CATH1 and CATH2, are present in salmonids that exhibit potent antimicrobial activity (Chang et al., 2006). In many situations one or other of these genes is preferentially induced in trout (Costa et a., 2011; Hong et al., 2013; Wang et al., 2015b), but here both are up-regulated in a comparable way by flagellin. Hepcidin is the master regulator of iron homeostasis in vertebrates and contributes to host defence by withholding iron from invading pathogens (Michels et al., 2015). SAA is a highly conserved APP exhibiting significant immunological activity by, for example, inducing the synthesis of several cytokines, being chemotactic for neutrophils and mast cells, and by activating the inflammasome cascade (Villarroel et

al., 2008; Eklund et al., 2012). SAP is a well-known APP and an important component of the innate immune system in vertebrates. It binds to extracellular antigens (eg pathogens, dead cells, or cellular debris) and supports their rapid clearance by phagocytosis (Choi et al., 2015). Whilst flagellin from *Y. ruckeri* promotes a strong induction of a variety of APPs and AMPs that may contribute to non-specific protection, clearly fish still succumb to infection with *Y. ruckeri*. The highly pathogenic serovar 1 strains that are motile (biotype 1, BT1) apparently repress flagellin expression during infection (Synder & Welch, 2015), potentially to evade the host immune response. In addition, the non-motile biotypes (BT2) that have emerged independently on several occasions over the last decade, have a phenotype that has lost motility by loss of flagellar secretion (Welch et al., 2011). The emergence of BT2 strains has been associated with loss of protection in fish vaccinated with BT1, suggesting that flagellar secretion or the flagellum have been important past targets for the immune system post vaccination with BT1.

To gain further insights into the cascade of inflammatory gene expression and pathways involved in RTS-11 cells, the flagellin-responsive genes with over 10-fold induction were used for cluster analysis and this revealed three major clusters (C1-3, Fig. 8). C1 contains TNF-α3 and IL-1β2, and represents the early responsive genes where induction peaked at 1 h after stimulation. This cluster likely contains the key players in the flagellin initiated proinflammatory pathway that serve to amplify the inflammatory response by induction of genes in C2 and C3. C2 contains most of the inflammatory cytokines and SAP1, and their expression was delayed, transient and peaked at 4 h. These inflammatory cytokines, eg IL-1β, TNF-α and IL-6, are known to induce the expression of AMPs and nIL-1Fm in rainbow trout (Wang et al., 2009; Costa et al., 2011; Hong et al., 2013). Thus the C2 genes may represent the major amplifiers of the flagellin-mediated inflammatory response and induce the expression of effector AMPs and negative regulators (eg nIL-1Fm) of the inflammatory response seen in C3. The C3 gene expression was delayed, peaked later (eg nIL-1Fm and CATH1) or lasted longer (eg Hepcidin, CATH2 and SAA) (Fig. 8), essential attributes for defense against microbes (eg AMPs) or for resolution of the inflammatory response (eg nIL-1Fm).

The rYRF induced expression of cytokines, APPs and AMPs in the macrophage RTS-11 cell line was also seen in HK cells, but with some notable exceptions (eg TNF-α1, IL-17C2, M17 and IL-12 family subunits) and with generally lower fold increases compared to RTS-11 cells. Although no increase in IL-12 family members was seen in HK cells, no decreases were seen unlike the situation in spleen following flagellin administration in vivo (Hynes et al., 2011). This difference in response to rYRF between HK and RTS-11 cells may be a consequence of the low level of TLR5M expression in HK cells, with a Δcp of 16.7 compared to a Δcp of 12.1 in RTS-11 cells. Indeed, HK cells are a mixed population of leukocytes, which include macrophages, neutrophils, T cells and B cells, amongst others. Despite this, the expression of the adaptive cytokines (eg IFN-γ, IL-2, IL-4/13 and IL-17

paralogues, IL-21 and IL-22) that are known to be transcribed in T cells, and molecules related to B cells (eg IgM, IgD, IgT and pIgR) was unaffected in HK cells following rYRF stimulation. This may also be due to the low level of TLR5M expression in HK cells, or the need for secondary signals for fish T cell and/or B cell stimulation.

Whilst no induction of adaptive cytokines was seen following flagellin stimulation, it is likely that *in vivo* during infection such cytokines will be released and may impact on the responses seen. In this study we chose to look at the interaction of prior exposure to a type 1 cytokine namely IFN-γ. Cells that had been pre-treated with rIFN-γ overnight were then stimulated with the rYRF for 4 h, and the expression of the flagellin-responsive genes studied. One of the most noticeable effects was on the IL-12 cytokine family members, where IFN-γ treatment synergised with flagellin to enhance p35 expression (both paralogues), with the potential to enhance IL-12 production, and p28 expression with the potential to enhance IL-27 expression. The role of IL-12 in driving Th1 type immune responses is well documented. IL-27 was also thought to drive such responses but it is now recognised to have more diverse activities and to modify $CD4^+$ cell, $CD8^+$ cell and Treg cell responses (Yoshida & Hunter, 2015). Interestingly, p40B2 was also up-regulated and may be the preferred partner for IL-12 production in this model. p19 was not modulated by the combined IFN-γ/flagellin exposure, hinting that IL-23 was likely unaffected. Since the expression of EBI3 that makes IL-27 and IL-35, and p40C that contributes to specific isoforms of IL-12 and IL-23, was unaffected, the presence of IFN-γ in a type 1 environment may promote the production of specific IL-12 isoforms (eg p35A/p40B2) in response to flagellin, that may have a distinct function relative to other isoforms (eg p35A/p40C) (Wang et al., 2014).

In contrast to synergising with flagellin to promote the expression of p28, p35 and p40B2 of the IL-12 family, rIFN-γ pre-exposure down-regulated the expression of many inflammatory mediators, including IL-1β, IL-11, IL-17C2, M17, TNFα1, SAP1, CATH2 and hepcidin. This may represent a regulatory mechanism to limit the inflammation induced by cytokines of adaptive immunity (Wang and Secombes, 2013). Interestingly, rIFN-γ pre-exposure differentially regulated flagellin-mediated upregulation of TNF-α paralogues, with TNF-α1 down-regulated, and TNFα2 and TNFα3 up-regulated. It is known that rainbow trout TNF-α2 and TNF-α3 behave similarly compared to TNF-α1, in terms of expression and modulation, and may be the major isoforms expressed in immune cells, eg T cells and macrophages (Hong et al., 2013). TNF is a central player within a complicated network of cytokines in mammals, and regulates not only pro-inflammatory responses but also processes as diverse as cellular communication, cell differentiation and cell death (Brenner et al., 2015). TNF-α can be expressed by multiple cell types, including macrophages, Th1 cells, Th2 cells and Th17 cells and acts to potentiate the ongoing immune response by increasing the transcription of critical Th1 or Th2 cytokines (Wang and Secombes, 2013). The ability of flagellin to induce the expression of pro-

inflammatory cytokines, and in synergy with a type 1 cytokine (IFN-γ) to up-regulate the expression of specific isoforms of IL-12 and TNF-α isoforms, suggests that flagellin has the potential to be an immune stimulant or adjuvant in future novel vaccines for fish aquaculture as suggested by others (Hynes et al., 2011; Scott et al., 2013; Gonzalez-Stegmaier et al., 2015).

5. Conclusions

This study provides new insights into the role of flagellin in rainbow trout immune responses to flagellated microorganisms. It is clear that rYRF is a potent stimulant able to up-regulate pro-inflammatory genes, APPs, AMPs and IL-12 cytokine family members. This and the synergy seen with rIFN-γ to enhance further expression of specific IL-12 and TNF-α isoforms may suggest that flagellin could be a useful immune stimulant or adjuvant for use in fish aquaculture. This study also highlights the need to study all of the paralogues of immune genes present in fish to gain a full understanding of the effects of PAMPs or other stimulants, and the potential immune responses elicited.

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673

674 **Figure legend**

675

676 **Fig. 1 SDS-PAGE analysis of rYRF expressed and purified from *E. coli* BL21 Star (DE3).** The
677 cell lysate from un-induced BL21 cells (lane 1), BL21 cells induced with 1 mM IPTG for 4 h (lane 2);
678 and purified rYRF (lane 3) was run on an SDS-PAGE gel and stained with SeeBlue (Invitrogen).
679 Protein marker, SeeBlue Plus2 (Invitrogen).

680 **Fig. 2 Dose-dependent induction of the expression of cytokines, AMPs and APPs by rYRF.** RTS-
681 11 cells were cultured overnight and then stimulated with serial 10-fold dilutions of rYRF (0.01, 0.1,
682 1, 10, 100 and 1,000 ng/ml) for 4h. Un-stimulated RTS-11 cells were used as control. The gene
683 expression of IL-1β1 (A), IL-1β2 (B), nIL-1Fm (C), IL-6 (D), TNF-α1 (E), TNF-α2 (F), TNF-α3 (G),
684 IL-8 (H), IL-12 p35A1 (H), IL-23 p19 (J), SAA (K) and hepcidin (L) was determined by real-time
685 PCR, and expressed as a fold change relative to the control samples. The means ± SEM of four
686 independent samples are shown. Differences between stimulated samples and controls were tested by
687 One way-ANOVA followed by the Bonferroni post hoc test. The p values are shown as *p<0.05,
688 **p<0.01, and ***p<0.001.

689 **Fig. 3 Time-dependent induction of the expression of cytokines by rYRF.** RTS-11 cells were
690 cultured overnight and then stimulated with 100 ng/ml of rYRF for 1 h, 2 h, 4h, 8 h, 12 h and 24 h. A
691 mock stimulation (Control) was conducted by incubation with the same amount of storage buffer.
692 The gene expression of IL-1β1 (A), IL-1β2 (B), IL-1β3 (B), nIL-1Fm (D), TNF-α1 (E), TNF-α2 (F),
693 TNF-α3 (G), IL-6 (H), IL-8 (I), IL-17C1 (J), IL-17C2 (K) and IL-34 (L) was determined by real-time
694 PCR, and expressed as a fold change relative to the time-matched control samples. The means + SEM
695 of four independent samples are shown. Differences between stimulated samples and time matched
696 controls were tested by One way-ANOVA followed by the Bonferroni post hoc test. The p values are
697 shown as **p<0.01, and ***p<0.001.

698 **Fig. 4 Time-dependent induction of the expression of subunits of the IL-12 cytokine family by**
699 **rYRF.** RTS-11 cells were cultured overnight and then stimulated with 100 ng/ml of rYRF for 1 h, 2 h,
700 4h, 8 h, 12 h and 24 h. A mock stimulation (Control) was conducted by incubation with the same
701 amount of storage buffer. The gene expression of p19 (A), p35A1 (B), p35A2 (C), p28B (D), EBI3
702 (E), p40B1, (F), p40B2 (G) and p40C (H) was determined by real-time PCR, and expressed as a fold
703 change relative to the time-matched control samples. The means + SEM of four independent samples
704 are shown. Differences between stimulated samples and time matched controls were tested by One
705 way-ANOVA followed by the Bonferroni post hoc test. The p values are shown as *p<0.05,
706 **p<0.01, and ***p<0.001.

707

Fig. 5 Time-dependent induction of the expression of APPs and AMPs by rYRF. RTS-11 cells were cultured overnight and then stimulated with 100 ng/ml of rYRF for 1 h, 2 h, 4h, 8 h, 12 h and 24 h. A mock stimulation (Control) was conducted by incubation with the same amount of storage buffer. The gene expression of SAA (A), SAP1 (B), SAP2 (C), CATH1 (D), CATH2 (E), and Hepcidin (F) was determined by real-time PCR, and expressed as a fold change relative to the time-matched control samples. The means + SEM of four independent samples are shown. Differences between stimulated samples and time matched controls were tested by One way-ANOVA followed by the Bonferroni post hoc test. The p values are shown as ***p<0.001.

Fig. 6 Modulation of flagellin-mediated cytokine induction by rIFN-γ. RTS-11 cells were cultured overnight with/without 20 ng/ml of rIFN-γ (IFNγ) and then stimulated with/without 100 ng/ml of rYRF (YRF) for 4h. The gene expression of IL-1β1 (A), IL-1β2 (B), nIL-1Fm (C), IL-6 (D), IL-8 (E), IL-17C2 (F), IL-11 (G), M17 (H), IL-34 (I), TNF-α1 (J), TNF-α2 (K) and TNF-α3 (L) was determined by real-time PCR, and expressed as a fold change relative to control samples. The means + SEM of four independent samples are shown. The expression levels between different groups are statistically different ($p<0.05$) where letters over the bars are different, as determined by one way-ANOVA.

Fig. 7 Modulation of flagellin-mediated induction of APPs, AMPs and subunits of the IL-12 cytokine family by rIFN-γ. RTS-11 cells were cultured overnight with/without 20 ng/ml of rIFN-γ (IFNγ) and then stimulated with/without 100 ng/ml of rYRF (YRF) for 4h. The gene expression of SAA (A), SAP1 (B), CATH1 (C), CATH2 (D), Hepcidin (E), EBI3 (F), p28B (G), p35A1 (H), p35A2 (I), p19 (J), p40B1 (K) and p40B2 (L) was determined by real-time PCR, and expressed as a fold change relative to control samples. The means + SEM of four independent samples are shown. The expression levels between different groups are statistically different ($p<0.05$) where letters over the bars are different, as determined by one way-ANOVA.

Fig. 8. Agglomerative hierarchical clustering analysis of the induction of flagellin-mediated expression in RTS-11 cells. The induction of gene expression (A) was normalised to the highest induction level (100) during the time course. The Dendrogram (B) was produced using XLSTAT software based on relative induction. Only the genes with over 10-fold induction are shown. The expression profiles (C) shown are the average induction of all genes in each cluster shown in (B).

Table 1 Primers used for expression analysis by real-time PCR

Gene	Forward (5' to 3')	Reverse (5' to 3')	Acc. No.
House-keeping gene			
EF-1α	CAAGGATATCCGTCGTGGCA	ACAGCGAAACGACCAAGAGG	AF498320
Acute phase proteins and antimicrobial peptides			
Serum amyloid A (SAA)	GGTGAAGCTGCTCAAGGTGCTAAAG	GCCATTACTGATGACTGTTGCTGC	AM422447
Serum amyloid P (SAP)1	GCTGTTATGGTGACCTTCAAGATCTCTC	GCGTTTGTACAACAACAAATCATTGTC	X99385
SAP2	GGTTGTTATGCTGAACATCAAGATCTCTC	CCACCCTTTGATTGCATACACAGATT	EZ763346
Cathelicidin (CATH)1	ACCAGCTCCAAGTCAAGACTTTGAA	TGTCCGAATCTTCTGCTGCAA	AY594646
CATH2	ACATGGAGGCAGAAGTTCAGAAGA	GAGCCAAACCCAGGACGAGA	AY542963
Hepcidin	GCTGTTCCTTTCTCCGAGGTGC	GTGACAGCAGTTGCAGCACCA	CA369786
Cytokines			
IL-1β1	CCTGGAGCATCATGGCGTG	GCTGGAGAGTGCTGTGGAAGAACATATAG	AJ278242
IL-1β2	GAGCGCAGTGGAAGTGTTGG	AGACAGGTTCAAATGCACTTTATGGT	AJ245925
IL-1β3	CTG AAG GCC GTC ACA ATC CA	CTGGTCCTTACAGCGCTCCAA	AM181685
nIL-1Fm	CCCATTCCTCGTGACACCAG	CTGGACGACCTGGAGAGTGACT	AJ555869
IL-2	TGATGTAGAGGATAGTTGCATTGTTGC	GAAGTGTCCGTTGTGCTGTTCTC	AM422779
IL-4/13A	ACCACCACAAAGTGCAAGGAGTTCT	CACCTGGTCTTGGCTCTTCACAAC	FN820501
IL-4/13B1	GAGATTCATCTACTGCAGAGGATCATGA	GCAGTTGGAAGGGTGAAGCTTATTGTA	HG794522
IL-4/13B2	GAGACTCATCTATTGCGTATGATCATCG	TGCAGTTGGTTGGATGAAACTTATTGTA	HG794523
IL-6	GGGAGAAAATGATCAAGATGCTCGT	GCAGACATGCCTCCTTGTTGG	DQ866150
IL-8	AGAGACACTGAGATCATTGCCAC	CCCTCTTCATTTGTTGTTGGC	AJ310565
IL-10A	GGATTCTACACCACTTGAAGAGCCC	GTCGTTGTTGTTCTGTGTTCTGTTGT	AB118099
IL-10B	GGGATTCTAGACCACATCAAGAGTCC	GATGGGAGATTTAAAGTTGTGTGTTCC	FR691804
IL-11	CTCTCGCTGCTATTGGCCCA	TCTCGAATGCATGTTCCTTCAATAGAT	AJ535687
M17	GTGGACCTCTTAAAAACATACAAGCTCAG	GGATGGTGGCTGTAAGTCTGTCTG	FM866399
CNTF	GCACTTATCTTCTGGAGCTATATAGGGAGA	AACTCCATCAACCTCCTCATTGC	FM866401
IL-12 p35A1	GGAACACCACATTCAGTGAGAGTGC	CGTCTGCAACTTGTGAGGAAGGAT	HE798148
IL-12 p35A2	GGAACACCACATTCAGTGAGAGTGA	CAACCTGTGAGGAAGACACCCA	HG917950
IL-12 p35B1	TGCCAAACGCCAAGCTTTATTTTG	GCTGTTGAGTGCTTTTGGTCTTTGG	HG917951
IL-12 p40B1	CCCTTCTACATCCGAGAAATAGTGAAAC	GTTGGTTTCACTTATAAACACCTTTTCCTT	HE798149
IL-12 p40B2	CCGTTCTACATACGAGAAATAGTGGAGA	TCAGAGTCACAGCTTTCCCTGG	HG917952
IL-12 p40C	TTAAAGACAACGGAAAGGAGGAGC	CCTCCCGTAACCACATTTTTCC	AJ548830
IL-23 p19	ACCTAAGAGCAGATTCAATGCCTTG	TCTTCCCAGCTCTTCACTTCCTG	KP410548
IL-27 p28A	GCAGCTGCTCAGGAGATATAAGGAGG	TCTCTCAGGTATGCTGGGTTTTGG	HG794528
IL-27 p28B	GCAGCTGCTCATGAGATATAAAGAGGA	GCTGCTCTCTGTTCCACCTTATCCAC	HG794529
EBI3	ACATCGCCACCTACAGTATGAAAGG	GGGTCCGGCTTCACAATGT	AJ620467
IL-15	TGGAATTGCTTCATAATATTGAGCTGCC	TGGTAGTTATCTGTGACCGACATGTCCTC	AJ628345
IL-17A1A	CAAACGTACACTTTTTGATGGTGCTG	GGGACTCATCATAGGTGGTGTTGGT	KJ921977
IL-17A2A	CACCCTGGACCTGGAAAAGCAC	GGCCACAGACAGGAAGGAGG	AJ580842
IL-17C1	CTGGCGGTACAGCATCGATA	GAGTTATATCCATAATCTTCGTATTCGGC	FM955455
IL-17C2	CTGGCGGTACAGCATCGATA	CAGAGTTATATGCATGATGTTGGGC	FM955456
IL-18	GAGCAATGCAAAGCAGATGATTG	CATGTTTTGAGCAGCCAATGTAGTC	AJ556990
IL-21	AAAGTTATCAAAAACCTCAACAACCGAA	CCAGTCTACTGATGGCCTTTTGAAG	FM883702
IL-22	GAAGGAACACGGCTGTGCTATTAAAC	GATCTAGGCGTGCACACAGAAGTC	AM748538
IL-34	AGGCAGAAGACGTAACATGAAACACA	TCCACCCTCGCCCTCAGCTT	FN820429
IFN-γ1	CAAACTGAAAGTCCACTATAAGATCTCCA	TCCTGAATTTTCCCCTTGACATATTT	AJ616215
IFN-γ2	CAAACTGAAAGTCCACTATAAGATCTCCA	GGTCCAGCCTCTCCCTCAC	FM864345
TNF-α1	TGTGTGGGGTCCTCTTAATAGCAGGTC	CCTCAATTTCATCCTGCATCGTTGA	AJ277604
TNF-α2	CTGTGTGGCGTTCTCTTAATAGCAGCTT	CATTCCGTCCTGCATCGTTGC	AJ401377
TNF-α3	GCTGCACTCTTCTTTACCAAGAAACAAG	CCACTGAGGACTTGTAATCACCATAGGT	HE798544
TGF-β1A	CTCACATTTTACTGATGTCACTTCCTGT	GGACAACTGCTCCACCTTGTG	OMY7836
TGF-β1B	CATGTCCATCCCCCAGAACT	GGACAACTGTTCCACCTTGTGTT	FN822750
TLR5			
TLR5M	GCGCATCACTTCAGGGGGAT	GCATTTCACCACTTGCAGGTAGA	AB062504
TLR5S	GCGCTCATAACTTCAGGGGGAT	GCATTTCACCACCTGCAGGTATT	AB091105
B cell related molecules			
IgM, secreted	TACAAGAGGGAGACCGGAGGAGT	CTTCCTGATTGAATCTGGCTAGTGGT	X65261
IgM, membrane	CCTACAAGAGGGAGACCGATTGTC	GTCTTCATTTCACCTTGATGGCAGT	OMU04616
IgD, secreted	TGAACATATCCAAACCAGGTGTCTG	GTCCTGAAGTCATCATTTTGTCTTGA	JQ003979
IgD, membrane	TGAACATATCCAAACCAGAGCTCC	GTCCTGAAGTCATCATTTTGTCTTGA	AY870260
IgT, secreted	CATCAGCTTCACCAAAGGAAGTGA	TCACTTGTCTTCACATGAGTTACCCGT	AY870268
IgT, membrane	TCGAAGTCCACGGCGAACA	GTGTTCTTCACCGCTTCATCTTGAA	AY870264
pIgR	GAGCAGACCACCAAAGGCCACTAT	TCTTGTCCTGTGGGTTTTGTTGTATTG	FJ940682

Table 2

Table 2 Fold change of transcript expression after stimulation of HK cells with rYRF. The gene expression levels were determined by real-time PCR, and expressed as a fold change relative to the time-matched control samples. The means of cell samples from four fish are shown. Numbers in bold indicate significant ($p<0.05$, paired sample T tests) up-regulation.

Gene	ΔCP[a]	1 h	2 h	4 h	8 h	12 h	24 h	48 h
Pro-inflammatory cytokines								
IL-1β1	6.4	**3.45**	**4.79**	**6.05**	**5.32**	**3.59**	**6.74**	**6.67**
IL-1β2	14.1	**12.32**	**10.62**	**16.51**	**11.71**	**7.03**	**9.71**	3.03
nIL-1Fm	10.6	1.14	**2.71**	**2.81**	**2.08**	1.58	1.75	**3.10**
TNF-α1	11.3	1.24	1.67	1.23	0.78	0.99	1.02	2.85
TNF-α2	15.8	**3.05**	**2.82**	**1.96**	**2.27**	**2.26**	**2.21**	**4.39**
TNF-α3	13.8	**9.12**	1.67	2.17	**3.45**	**1.89**	**3.54**	**2.60**
IL-6	14.7	**10.37**	**4.37**	**8.51**	**3.65**	**2.69**	3.51	2.08
IL-8	9.0	**9.94**	**9.78**	**7.75**	**6.30**	**5.04**	**7.43**	**4.81**
IL-11	15.6	2.18	**2.90**	2.44	1.37	1.44	2.48	2.32
IL-17C2	18.2	1.59	1.34	0.66	0.98	1.23	1.09	1.50
IL-34	9.5	1.21	**2.57**	**3.77**	1.79	1.47	1.10	0.97
Anti-inflammatory cytokines								
IL-10A	14.6	1.26	1.43	**2.52**	**2.47**	**3.85**	**3.87**	1.58
IL-10B	15.4	1.16	1.34	**3.53**	2.40	2.04	2.97	2.28
TGF-β1A	11.3	1.07	1.13	1.16	1.96	1.17	1.71	1.37
TGF-β1B	11.6	1.45	1.52	1.28	2.28	1.03	1.17	1.08
Adaptive cytokines								
IFN-γ1	14.8	1.16	1.40	1.23	1.26	1.42	1.10	0.84
IFN-γ2	15.9	1.27	1.25	2.37	2.33	2.46	2.30	1.35
IL-2	15.8	1.03	1.48	1.50	1.17	1.19	1.04	0.86
IL-4/13A	13.2	0.95	1.29	0.84	1.29	0.86	1.54	1.03
IL-4/13B1	15.1	1.16	1.40	1.20	1.13	0.81	1.00	0.69
IL-4/13B2	16.9	0.70	1.13	1.44	1.74	0.82	1.38	1.21
IL-17A1A	18.9	1.26	0.57	0.87	0.51	0.92	0.89	0.92
IL-17A2A	21.8	0.63	1.33	0.10	0.45	0.81	1.68	0.47
IL-21	16.6	1.10	1.59	1.50	1.13	0.69	1.09	2.06
IL-22	15.4	2.69	2.10	2.31	1.00	1.04	1.05	0.50
Subunits of the IL-12 family								
p35A1	16.4	1.15	1.75	1.18	1.06	0.77	1.62	1.19
p35A2	17.2	1.04	2.59	1.36	0.71	1.25	1.47	0.93
p35B1	20.0	1.14	1.89	0.77	1.20	1.10	0.77	0.45
EBI3	19.0	0.99	1.86	0.92	1.30	0.84	1.45	1.47
p40B1	11.8	1.03	1.59	1.98	1.90	1.41	1.77	1.31
p40B2	11.7	1.21	**1.92**	1.51	1.17	1.08	0.91	1.03
p40C	13.4	1.17	1.29	1.29	1.22	0.90	1.24	0.70
Other genes								
IL-15	8.1	0.96	1.42	0.84	1.11	1.03	1.29	1.01
IL-18	7.7	1.02	1.42	1.67	1.30	1.35	1.28	1.10
M17	11.7	1.37	1.28	2.58	3.45	3.71	1.72	1.12
CNTF	18.9	1.01	1.09	0.97	0.66	0.82	1.08	0.74
TLR5m	16.7	0.99	1.06	1.13	1.04	0.81	0.79	0.36
TLR5s	18.4	1.76	1.94	0.67	3.82	2.35	5.72	5.87
AMPs and APPs								
SAA	9.1	**4.14**	**5.82**	**6.46**	**5.31**	**4.22**	**6.53**	**23.77**
SAP1	17.4	0.88	1.11	2.65	4.04	**4.06**	2.33	0.80
SAP2	14.1	0.95	1.09	1.13	1.07	1.06	1.24	1.17
CATH1	9.0	**1.92**	**3.38**	**5.18**	**7.54**	**6.74**	**8.21**	**8.76**
CATH2	12.2	**5.63**	**6.67**	**7.90**	**7.20**	**3.19**	**1.76**	**2.18**
Hepcidin	13.4	1.55	**2.65**	**5.24**	**2.31**	**2.96**	**6.59**	**7.39**
B cell related								
IgM, secreted	0.9	1.01	1.27	1.34	1.14	1.06	1.19	1.39
IgM, membrane	3.8	0.89	1.51	1.24	1.15	1.05	1.11	1.09
IgD, secreted	14.0	0.60	1.20	0.88	1.29	0.92	0.92	0.94
IgD, membrane	7.1	1.08	1.53	1.15	0.77	1.16	0.95	1.12
IgT, secreted	8.7	0.84	1.22	1.65	0.97	0.99	0.95	0.96
IgT, membrane	10.1	0.98	1.19	1.30	1.13	1.05	1.01	1.29
pIgR	10.4	0.84	1.45	1.08	0.91	0.93	1.08	1.23

Note:
[a]Δcp is the average cp value (the crossing point at which the fluorescence crosses the threshold) of the target gene minuses that of the house-keeping gene EF-1α in the control samples at 4 h. The average cp of EF-1α is 12.9. A higher cp value indicates a lower expression level.

Figure 1

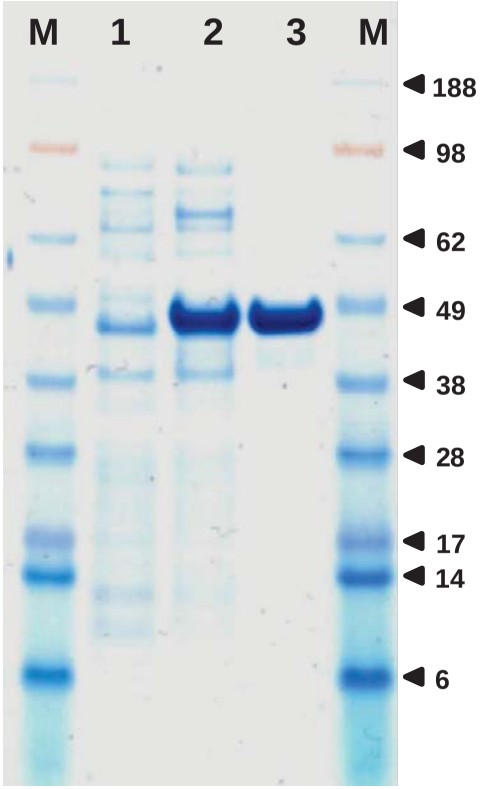
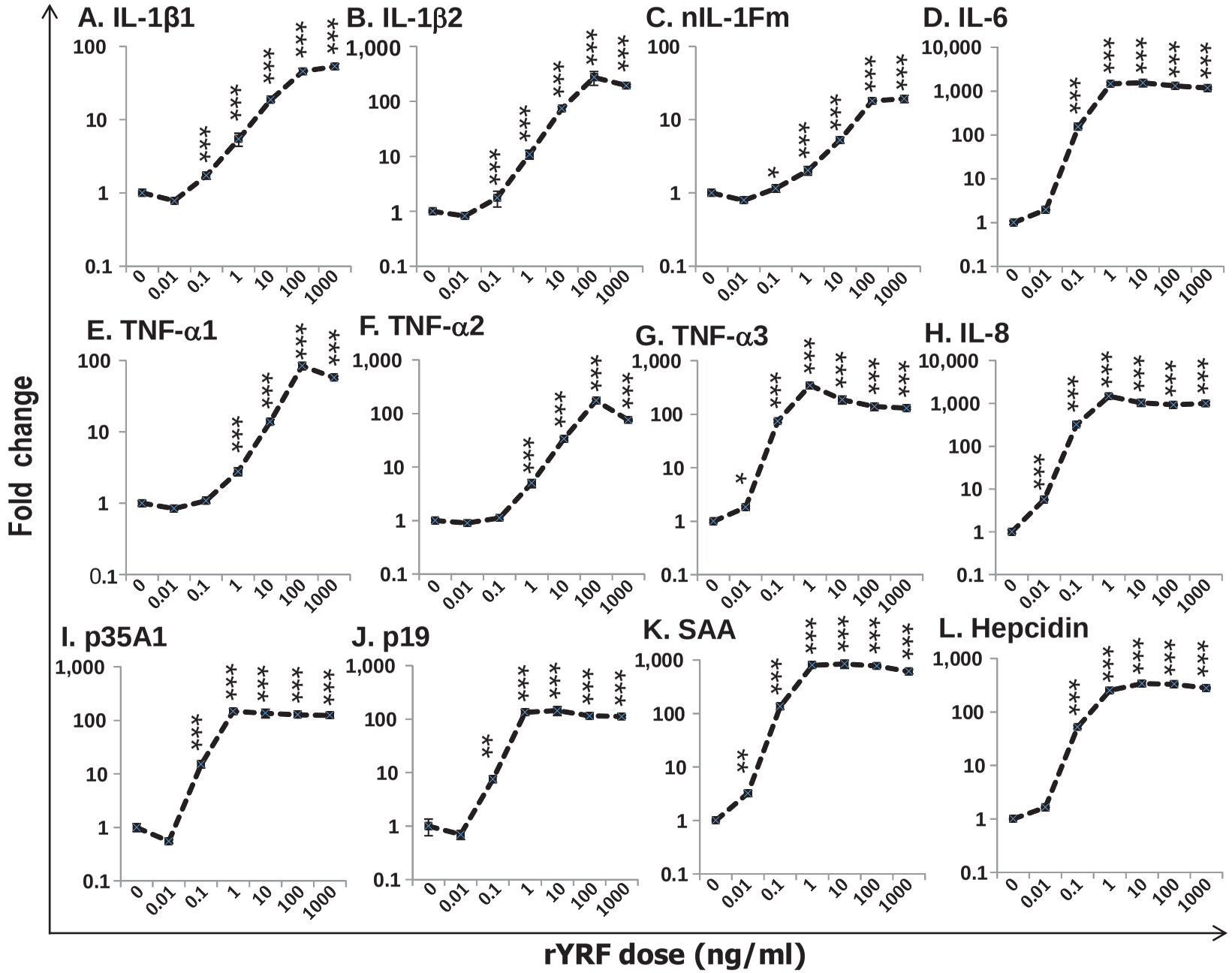


Figure 2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B. IL-1β2
C. nIL-1Fm
D. IL-6
E. TNF-α1
F. TNF-α2
G. TNF-α3
H. IL-8
I. p35A1
J. p19
K. SAA
L. Hepcidin
Fold change
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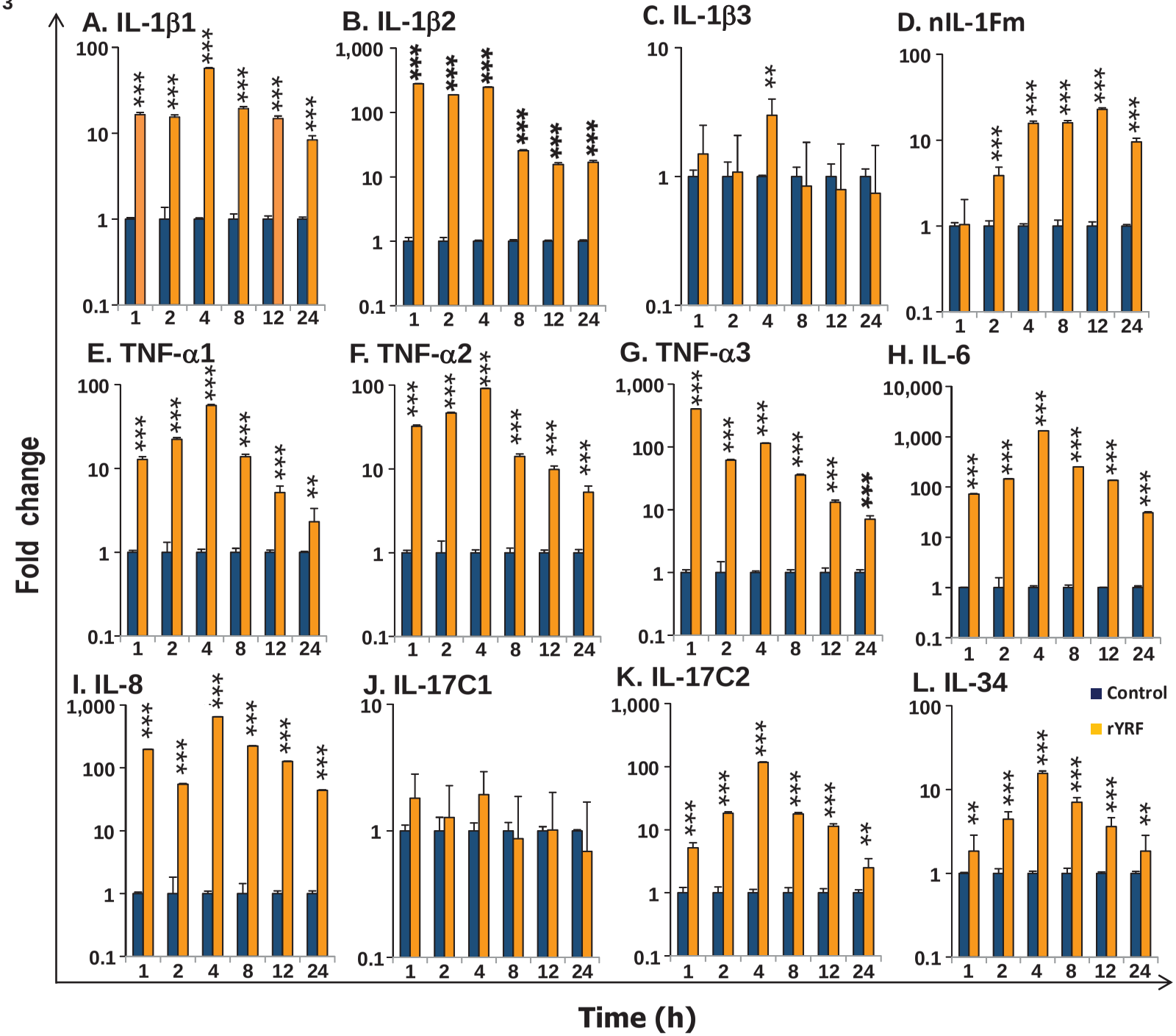
0.01
0.1
1
10
100
1000

Figure 3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C. IL-1β3
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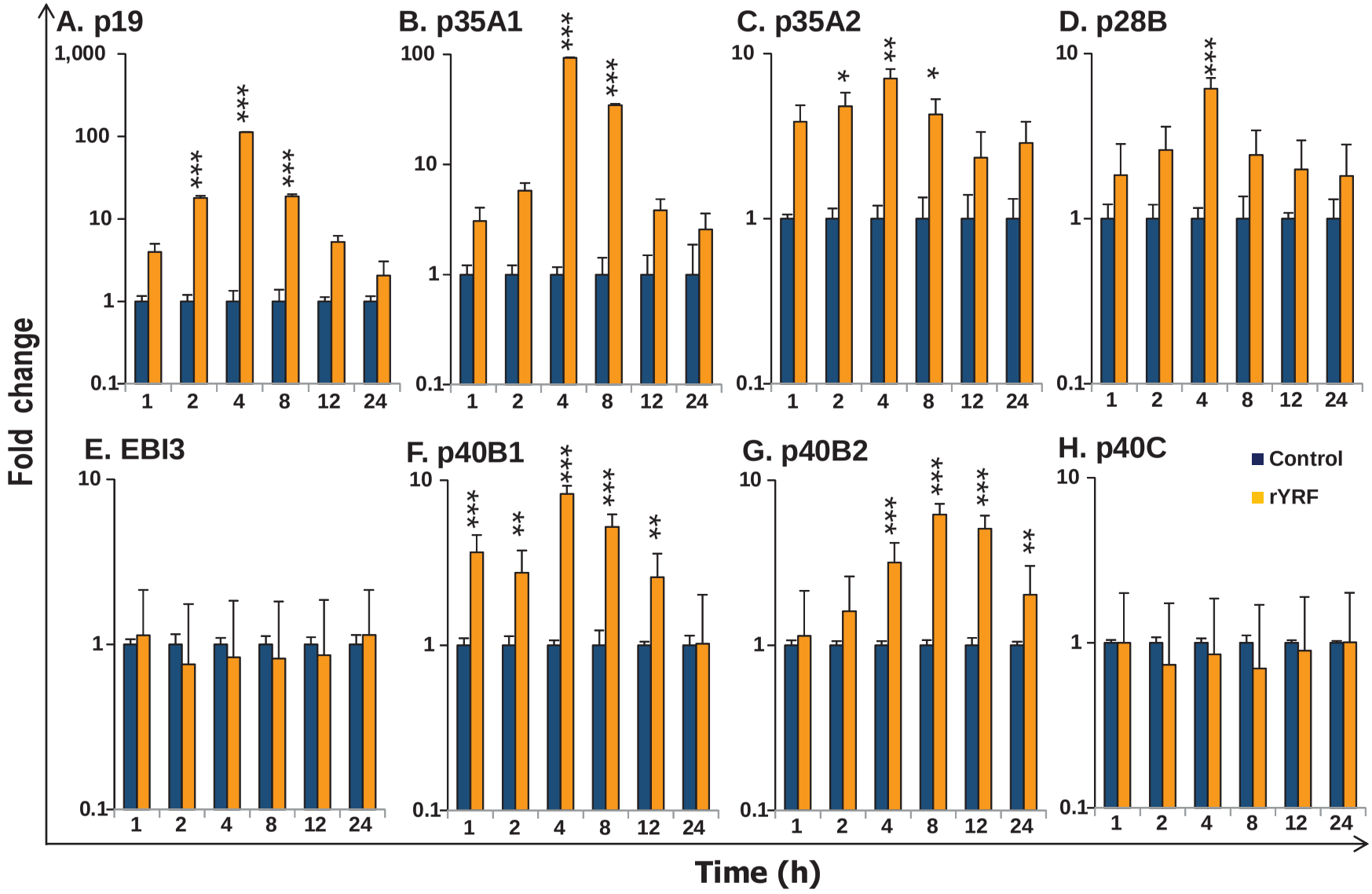
E. TNF-α1
F. TNF-α2
G. TNF-α3
H. IL-6
I. IL-8
J. IL-17C1
K. IL-17C2
L. IL-34
Control
rYRF
Fold change
Time (h)

Figure 4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C. p35A2
D. p28B
E. EBI3
F. p40B1
G. p40B2
H. p40C
Control
rYRF
Fold change
Time (h)
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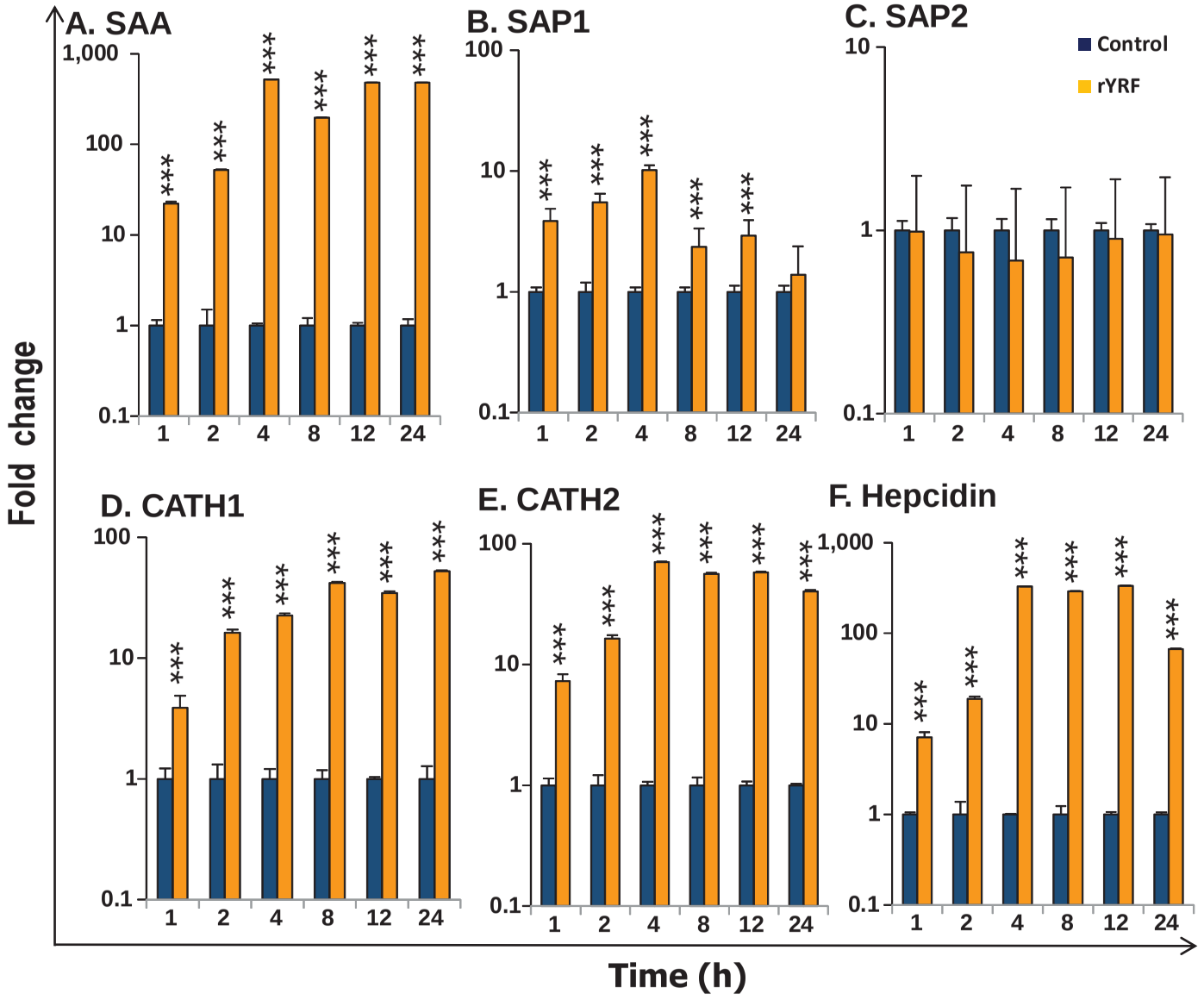
100
10
1
0.1
1
2
4
8
12
24
*
**
***
****

Figure 5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D. CATH1
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F. Hepcidin
Control
rYRF
Fold change
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100
10
1
0.1
1
2
4
8
12
24
***

Figure 6

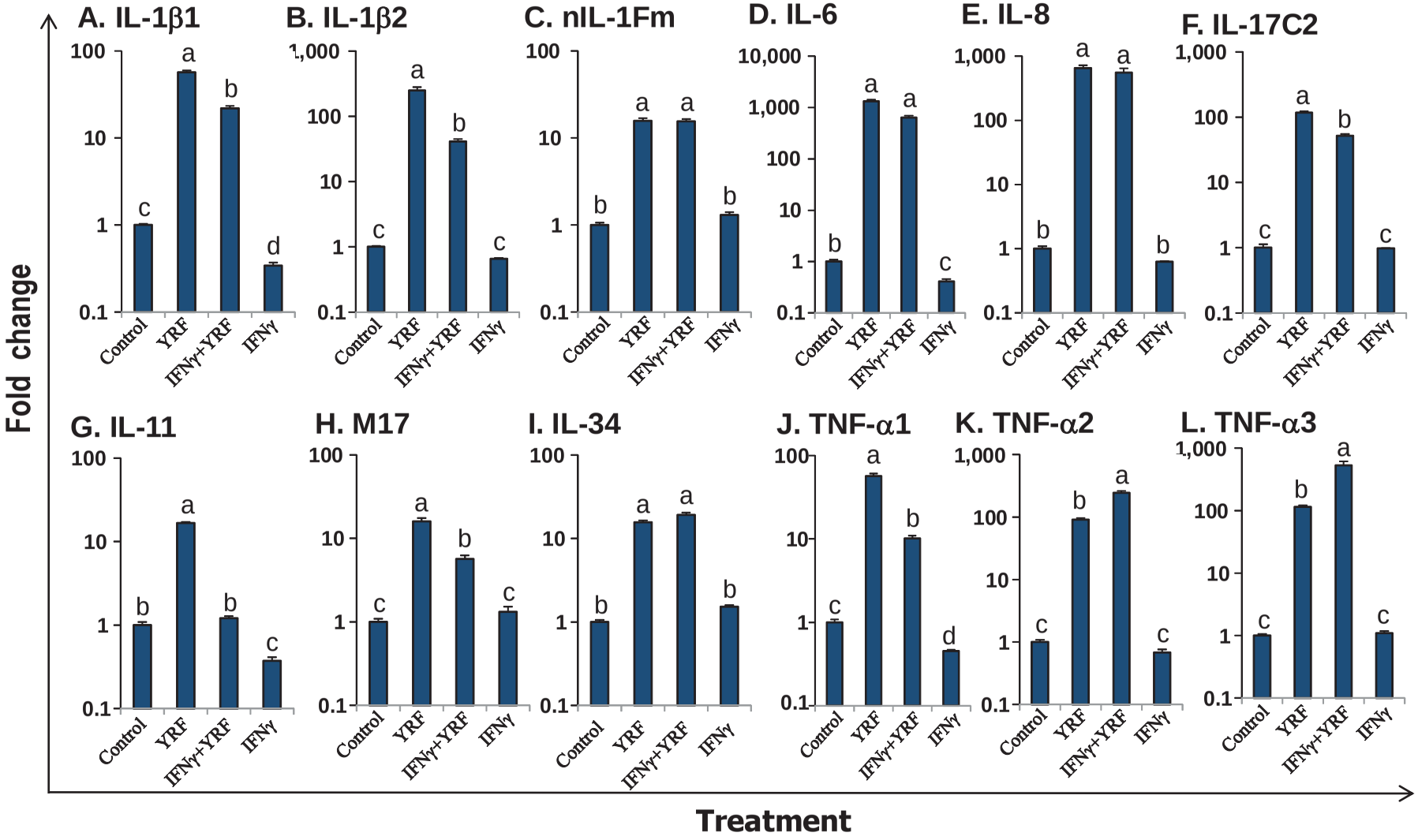

A. IL-1β1
B. IL-1β2
C. nIL-1Fm
D. IL-6
E. IL-8
F. IL-17C2
G. IL-11
H. M17
I. IL-34
J. TNF-α1
K. TNF-α2
L. TNF-α3
Fold change
Treatment
Control
YRF
IFNγ+YRF
IFNγ

Figure 7

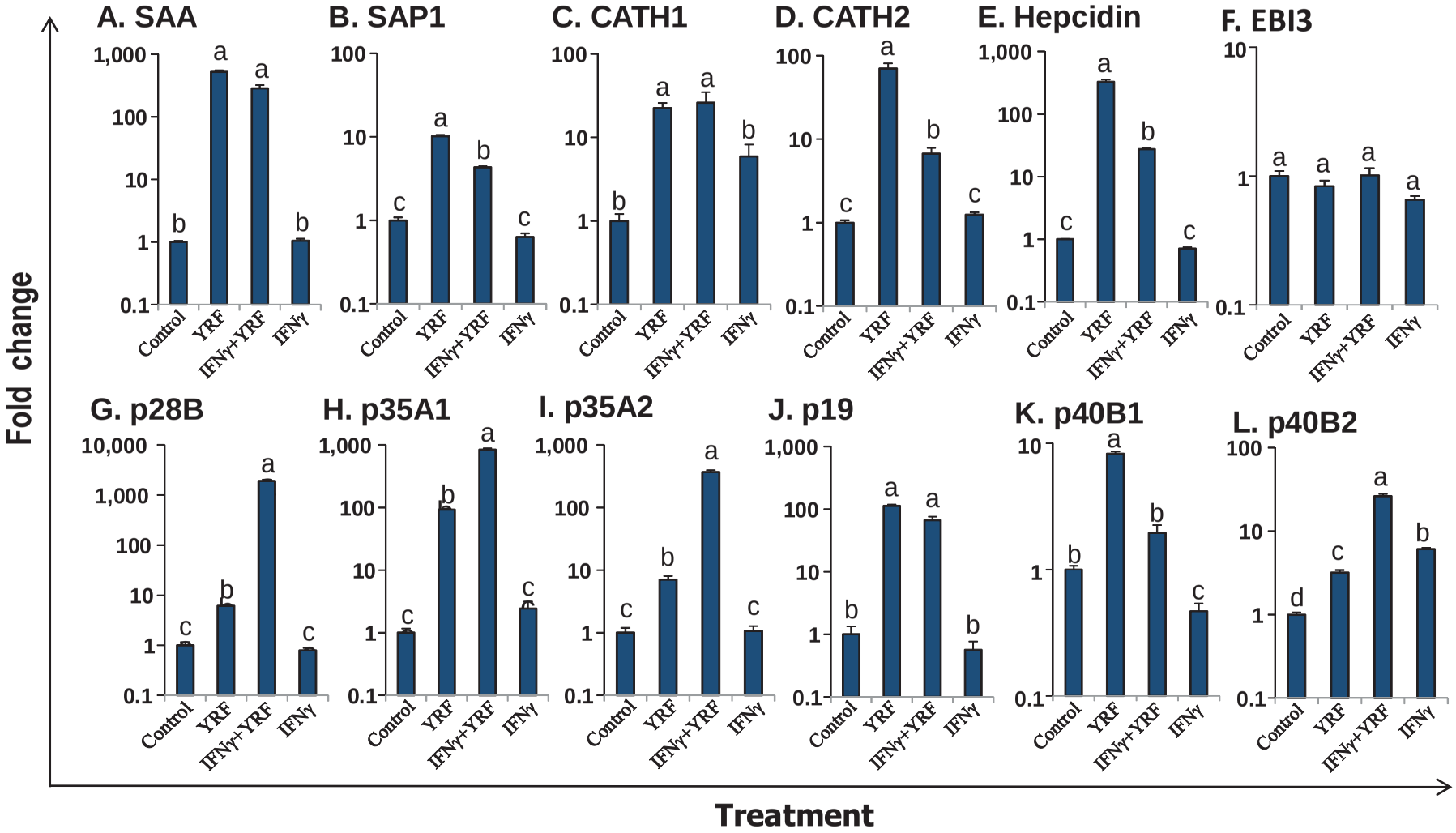
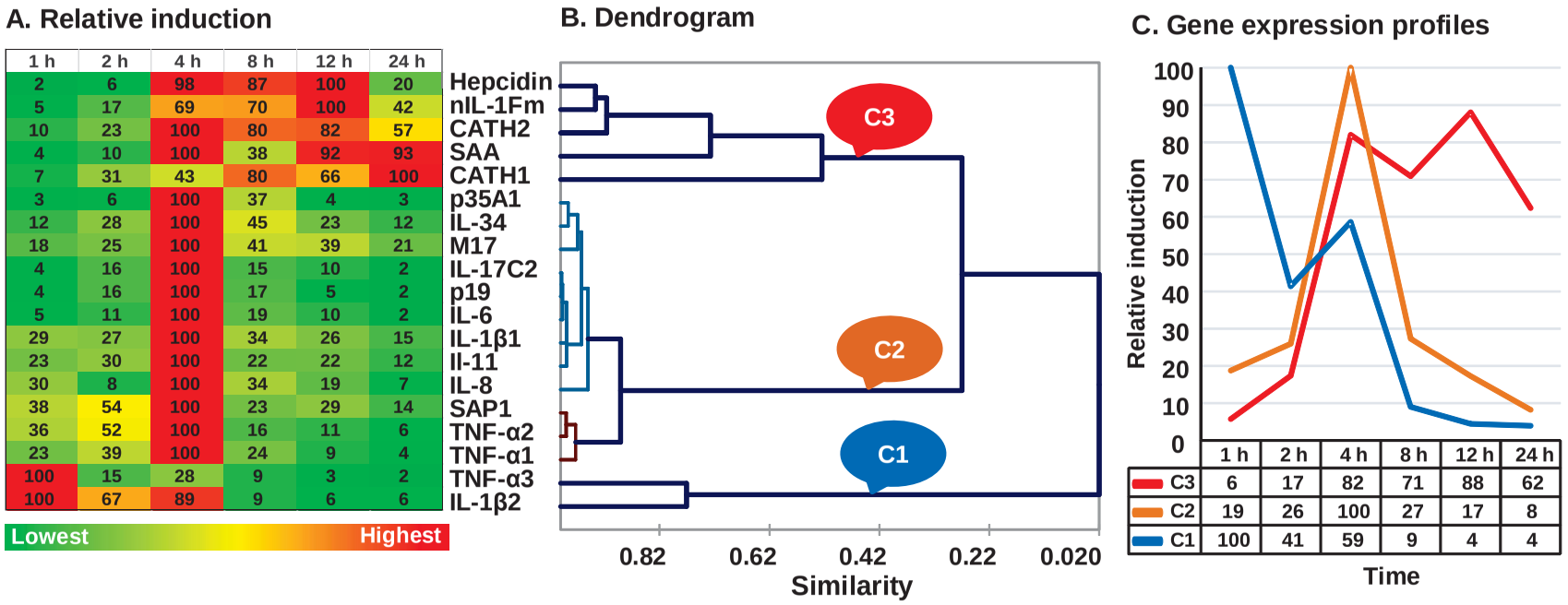

A. SAA
B. SAP1
C. CATH1
D. CATH2
E. Hepcidin
F. EBI3
G. p28B
H. p35A1
I. p35A2
J. p19
K. p40B1
L. p40B2
Fold change
Treatment
Control
YRF
IFNγ+YRF
IFNγ

Figure 8


A. Relative induction
1 h 2 h 4 h 8 h 12 h 24 h
2 6 98 87 100 20 Hepcidin
5 17 69 70 100 42 nIL-1Fm
10 23 100 80 82 57 CATH2
4 10 100 38 92 93 SAA
7 31 43 80 66 100 CATH1
3 6 100 37 4 3 p35A1
12 28 100 45 23 12 IL-34
18 25 100 41 39 21 M17
4 16 100 15 10 2 IL-17C2
4 16 100 17 5 2 p19
5 11 100 19 10 2 IL-6
29 27 100 34 26 15 IL-1β1
23 30 100 22 22 12 Il-11
30 8 100 34 19 7 IL-8
38 54 100 23 29 14 SAP1
36 52 100 16 11 6 TNF-α2
23 39 100 24 9 4 TNF-α1
100 15 28 9 3 2 TNF-α3
100 67 89 9 6 6 IL-1β2
Lowest
Highest
B. Dendrogram
C3
C2
C1
0.82
0.62
0.42
0.22
0.020
Similarity
C. Gene expression profiles
Relative induction
100
90
80
70
60
50
40
30
20
10
0
1 h 2 h 4 h 8 h 12 h 24 h
C3 6 17 82 71 88 62
C2 19 26 100 27 17 8
C1 100 41 59 9 4 4
Time