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Abstract: Increased knowledge of the immune response of the intestine, a physiologically critical organ involved in absorption, secretion and homeostasis in a non-sterile environment, is needed to better understand the mechanisms involved in the induction of long-lasting immunity and, subsequently, the development of efficacious gastrointestinal immunization approaches. To this end, analysis of isolated gut cells will give an insight into the cell types present and their immune capability. Hence, in this study we first optimised a method for salmonid gut leucocyte isolation and characterised the cells on the basis of their expression of a range of selected cell markers associated with T & B cells and dendritic cells. The GALT leucocytes were then stimulated with a variety of PAMPs, recombinant cytokines and PHA, as a means to help characterise the diversity of the immune repertoire present in such cells. The stimulants tested were designed to examine the nature of the antibacterial, antiviral and T cell type responses in the cells (at the transcript level) using a panel of genes relevant to innate and adaptive immunity. The results showed distinct responses to the stimulants, with a clear delineation seen between the stimulant used (eg viral or bacterial PAMP) and the pathway elicited. The changes in the expression patterns of the immune genes in these cells indicates that the salmonid intestine contains a good repertoire of competent immune cells able to respond to different pathogen types. Such information may aid the development of efficient priming by oral vaccination in salmonids.

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Gene expression analysis of isolated salmonid GALT leucocytes in response to PAMPs and recombinant cytokines

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Abstract

Increased knowledge of the immune response of the intestine, a physiologically critical organ involved in absorption, secretion and homeostasis in a non-sterile environment, is needed to better understand the mechanisms involved in the induction of long-lasting immunity and, subsequently, the development of efficacious gastrointestinal immunization approaches. To this end, analysis of isolated gut cells will give an insight into the cell types present and their immune capability. Hence, in this study we first optimised a method for salmonid gut leucocyte isolation and characterised the cells on the basis of their expression of a range of selected cell markers associated with T & B cells and dendritic cells. The GALT leucocytes were then stimulated with a variety of PAMPs, recombinant cytokines and PHA, as a means to help characterise the diversity of the immune repertoire present in such cells. The stimulants tested were designed to examine the nature of the antibacterial, antiviral and T cell type responses in the cells (at the transcript level) using a panel of genes relevant to innate and adaptive immunity. The results showed distinct responses to the stimulants, with a clear delineation seen between the stimulant used (eg viral or bacterial PAMP) and the pathway elicited. The changes in the expression patterns of the immune genes in these cells indicates that the salmonid intestine contains a good repertoire of competent immune cells able to respond to different pathogen types. Such information may aid the development of efficient priming by oral vaccination in salmonids.

Keywords

GALT leucocytes; Isolation; Salmonids; PAMPs; Recombinant cytokines; Gene expression

Introduction

Despite marked progress in our understanding of the teleost immune system over the last decade, the gut immune response is still not well-understood. From an immune perspective the teleost gut is structurally and morphologically different from the mammalian gut, in that it lacks lymph nodes, Peyer's patches, M cells and IgA secreting lymphocytes, but it does possess gut-associated lymphoid tissue (GALT) that consists of individual and aggregated cells scattered in the lamina propria (lamina propria leucocytes, LPL) and within the epithelia (intraepithelial leucocytes, IEL) [1,2,3]. These cells are diverse and different to those in other immune tissues, such as the spleen and head kidney [2,4], and include lymphocytes, macrophages, eosinophilic & neutrophilic granulocytes, and antigen sampling cells that resemble immature M cells [5,6,7,8,9]. Most studies have used transcript analysis to define the responses occurring in the gut post infection or oral vaccination [10,11,12]. A few have used immunohistochemistry, as with the identification and characterisation of IgT+ B cells that increase in number during parasite infection [13,14]. Further study of these leucocytes and their bioactivities, the molecules they express and secrete, and the way immune responses are established are needed to enable a better understanding of the immune response in the gut [3,15]. Such information will help drive the development of effective oral vaccination strategies, in terms of efficacy, affordability, mass administration and safety.

One approach to study GALT cell immune responsiveness is to isolate the cells and determine their functionality following *in vivo* or *in vitro* stimulation. There are a few reports on isolated IEL and LPL from fish intestine, using methods modified from protocols for isolating such cells from the mammalian GALT [16,17]. For example, in rainbow trout IEL have been isolated and shown to exhibit phagocytosis and reactive oxygen species (ROS) production [18,19], cytotoxicity against a murine thymoma (EL4) cell line [20] and to express a range of T cell markers [21]. TCRβ transcripts in trout IEL were highly diverse and showed marked changes in the TCRβ repertoire after viral infection. Similarly in sea bass lymphocytes isolated from the intestinal mucosa have been shown to have cytotoxic activity against xenogenic and allogeneic targets [22]. Attempts to purify sea bass intestinal T cells with the DTL15 monoclonal antibody showed that such cells had enhanced expression of TCRβ, CD8α and RAG-1 relative to DLT15- cells [23]. In seabream, isolated GALT cells were shown to undergo ROS production, and could be separated into three subpopulations by flow cytometry (FSC/SSC) analysis [24]. Rather few (<10%) B cells are reported in isolated GALT cells [7,13], which in rainbow trout have similar numbers of IgT+ and IgM+ populations (~54% vs ~46%) in cells from unstimulated fish [13]. Following vaccination specific antibody secreting cells can be detected in these isolated cells, with the kinetics varying dependent upon the route of delivery used [25]. Whilst such studies give clues to the function of the cells present, clearly there is still much to be learnt about GALT cell responses in fish.

In the present study we have examined further the methods used to obtain salmonid GALT cells, to obtain high yields of viable cells for experimentation, in a time and cost-effective manner. We first characterised the cells obtained in terms of their expression of T-cell, B-cell and dendritic cell (DC) markers, and then studied their responsiveness to a range of pathogen-associated molecular patterns (PAMPs), cytokines and phytohaemagglutinin, to examine whether it is possible to detect antibacterial, antiviral and T cell responses in such cells. The results revealed clear responses to the different stimulants, with differential effects apparent in the target gene expression profiles dependent upon the stimulus.

Materials and methods

1. Fish

Rainbow trout (*Oncorhynchus mykiss*), average weight 250 g, were used for optimizing the protocol for isolating gut leucocytes. The protocol deemed optimal was then applied to isolate gut leucocytes from Atlantic salmon (*Salmo salar*) parr, average weight 15 g, to investigate their responses post stimulation *in vitro*. Fish were maintained in 1m-diameter fibreglass tanks with recirculating freshwater at 14°C and fed twice a day with a commercial diet (2% body weight). Fish were starved for 48 h prior to use in order to evacuate the gut contents before collecting the gut tissue, and sampled at the same time of day on each occasion used. The experimental procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

2. Gut marker gene analysis

To verify whether particular gut regions were more suitable for GALT cell isolation, a marker gene analysis was undertaken initially. Rainbow trout were killed and the oesophagus, stomach, pyloric caeca, midgut and hindgut were collected, homogenised in TRI reagent (Sigma) and the TRI lysate stored at -80°C until RNA extraction. The genes studied included cell surface markers for T-lymphocytes (CD4-1, CD8α, TCRα, and TCRγ), B-lymphocytes (membrane (m)IgM, secreted (s)IgM, mIgT and sIgT) and dendritic cells (CD83, CD208, CD209 and MHCIIβ), since these are key for adaptive immune responses.

3. Optimizing salmonid gut leucocyte isolation

3.1. Isolation protocols

Initially, the protocol described by Salinas et al. [24] for isolation of gilthead seabream (*Sparus aurata*) gut leucocytes was employed to isolate rainbow trout gut leucocytes and was named protocol-1. Later this protocol was modified and named protocol-2, and was in turn modified and named protocol-3. Protocol-l was carried out as follows: rainbow trout were bled, then the gut from the pyloric caeca to the anus collected from 5 fish and placed into cold PBS. All the connective tissue was removed and any remaining gut contents rinsed off. The guts were opened longitudinally and cut into 1cm long segments, put in 50 ml tubes containing 15-20 ml of a predigestion solution (0.145 mg/ml DTT + 0.37 mg/ml EDTA in Ca^{2+} & Mg^{2+} free HBSS, Sigma) and shaken in an orbital shaker at 50 rpm for 20 min. The supernatants were filtered through 100 µm nylon mesh strainers (Greiner) to get the first suspension (S1) which was kept at 20°C. Tissue fragments were washed with washing media (0.05 mg DNAse I /ml Ca^{2+} & Mg^{2+} free HBSS) containing 5% foetal bovine serum (FBS, Sigma) to remove any remaining DTT and then shaken in 50 ml tubes containing 15-20 ml of digestion solution (0.37 mg collagenase IV /ml washing medium) in an orbital shaker at 50 rpm for 60 min. Supernatants were filtered through 100 µm nylon mesh strainers, and the obtained suspension was added to S1 to get S2, and washed twice in RPMI-1640 culture medium (Sigma) containing 100 units /ml penicillin & 100 µg /ml streptomycin (P/S, Gibco) and 10% FBS. The cells were then counted and adjusted to 10^7 cells /ml. Nylon wool columns (10 ml syringes packed with 0.5 g nylon wool fibre, Dutscher), were loaded with culture medium for 1 h prior to adding 5 ml S2/column. The columns were incubated for 1 h and then washed twice with culture medium to collect the purified cells, which were washed twice with culture medium, counted and adjusted to 10^7 cells /ml. The cell suspension was

then carefully layered over a discontinuous Percoll gradient with two densities (75% and 25%) and centrifuged at 4°C for 30 min at 400 g. Cells in the intermediate density (ID) and high density (HD) bands were collected and washed twice with the culture medium. Protocol-2 was carried out as for protocol-1 but did not include passing S2 through a nylon wool column. Protocol-3 was essentially the same as protocol-2 except that individual fish guts were used as independent samples rather than pooling from 5 fish, and the tissue fragments were shaken in the digestion solution twice for 60 min rather than once. This protocol was also used to isolate GALT leucocytes from Atlantic salmon.

3.2. Leucocyte viability

Isolated GALT leucocytes were distributed into 12-well plates at $2x10^6$ cells /well and incubated at 20°C for 4 h and 24 h, at which times they were counted using a Neubauer chamber and 0.5% trypan blue. The viabilities were determined by comparison to the respective time 0 h controls.

3.3. Marker gene analysis

Isolated GALT leucocytes ($2x10^6$ cells /well) were incubated at 20°C and harvested at 4 h and 24 h by centrifugation at 400 g for 10 min at 4°C. The supernatant was discarded and TRI reagent added, with pipetting up and down several times. The TRI lysate was stored at -80°C until RNA extraction for marker gene analysis (see below). The genes studied were the same as those used for the initial gene expression analysis of the different gut regions.

3.4. Morphological examination

The gut cells isolated by protocol-3 were taken for flow cytometry and transmission electron microscopy analyses to identify the isolated cell types based on their size, granularity and structure.

3.4.1. Flow cytometry (FSC/SSC)

The cells were washed twice by centrifugation at 4°C for 10 min at 400 g in Ca^{2+} & Mg^{2+} free HBSS (Sigma) + 2% FBS. The analysis was then performed with an Accuri C6 Flow Cytometer and software (BD Science).

3.4.2. TEM

The cells were fixed in 2.5% glutaraldehyde in 0.1M Sodium Cacodylate buffer for 2 hours. Cells were postfixed in 1% osmium tetroxide, dehydrated in a graded ethanol series, embedded in Spurr's resin (TAAB, UK), and ultrathin sections cut on a Leica UC6 (Leica Microsystems, Milton Keyes). Sections were stained in a Leica AC20 (Leica Microsystems, Milton Keyes) with 0.5% uranyl acetate followed by lead citrate, and then viewed on a JEOL 1400 plus (JEOL UK) + AMT UltraVUE camera (Deben UK

~~*3.4.*~~*3.5. Stimulation by LPS*

Isolated GALT leucocytes obtained using each protocol, cultured as above (ie $2x10^6$ cells /well, 20^oC), were stimulated with 50 µg /ml lipopolysaccharide from *Pseudomonas aeruginosa* (LPS, Sigma) for 4 h and 24 h, then harvested, homogenised in TRI reagent and

stored at -80°C until RNA extraction. In this case the expression of three key pro-inflammatory genes were studied, namely IL-1β1, IL-6 and TNF-α2.

4. Atlantic salmon GALT leucocyte analysis

Purified Atlantic salmon GALT leucocytes obtained using protocol-3, were suspended in RPMI-1640 containing P/S and 10% FBS, then plated at $2x10^6$ cells /well into 12 well plates and cultured at 20°C. The cells, from individual fish, were then stimulated with 10 µg /ml phytohaemagglutinin from *Phaseolus vulgaris* (PHA, Sigma), 50 µg /ml LPS, 100 µg /ml polyinosinic acid: polycytidylic acid (poly I:C, Sigma), 100 ng /ml recombinant flagellin from *Yersinia ruckeri* (rYRF, [26], 25 ng /ml IL-1β1 [27], 100 ng /ml IL-2B [28], 100 ng /ml IL-6 [29], 200 ng /ml IL-21 [30] and 20 ng /ml IFN-α2/IFN2 [31]. All doses used were previously shown to be optimal for trout and Atlantic salmon cells [32,33,34], and the cytokine paralogues chosen have known bioactivity on leucocytes from other tissues. Untreated cells were included as controls. The GALT cells were incubated for 4 h and 24 h then harvested, homogenised in TRI reagent and stored at -80°C until RNA extraction.

5. RNA extraction, cDNA synthesis and qPCR

Total RNA extraction, cDNA synthesis and real-time PCR (qPCR) analysis of gene expression were as described by Wang et al. [30,35]. Tissue samples (or cells) were homogenised in TRI reagent using a Qiagen Tissue Lyser II and stored at -80°C before further processing. The Tri lysates were thawed at room temperature and total RNA prepared as per the manufacturer's instructions. Using Oligo (dT)28VN (Eurofins), dNTPs (Thermo Scientific) and RevertAid Reverse Transcriptase, RNA was reverse transcribed to cDNA under the following conditions: 42°C for 60 min, 45°C for 30 min, 50°C for 30 min and 90°C for 5 min. The resultant cDNAs were diluted in TE- buffer (pH 8.0) (Sigma) and duplicate real-time qPCR reactions were run in a light Cycler 480 machine (Roche) using SYBR green (Sigma). The amplification conditions were as follow: 1 cycle of 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 62-66 °C for 30 s and extension at 72°C for 30 s, followed by melting curve analysis between 75°C and 95°C. The primer sets used in this study are shown in Table 1, and were designed with at least one primer across an intron.

Elongation factor-1α (EF-1α) was the house-keeping gene used as the endogenous control to normalize gene expression. The transcript levels were calculated using the light Cycler 480 integrated software. Initially leucocyte marker gene relative expression was calculated as arbitrary units by dividing the marker transcript mean concentration by the respective EF-1α transcript mean concentration. In the case of stimulated GALT cells, the $2^{-\Delta\Delta CT}$ method [36] was then used to express the immune gene expression as a fold change relative to the unstimulated control cells. In each experiment 4 independent replicates were analysed (tissues/cells from 4 individual fish or 4 independent pools of cells) and the data expressed as means and standard error of the mean (SEM).

6. Data and statistical analyses

Expression data were analysed statistically using the IBM SPSS Statistics package 24.0 (SPSS Inc., Chicago, Illinois). One-way analysis of variance (ANOVA) followed by the Tukey post hoc test was used to determine the level of significance between protocols in

terms of leucocyte yield and viability. The independent samples T test was used to determine the level of significance between treated and untreated (control) cell samples. $P<0.05$ was deemed significant.

Results

1. Gut marker gene analysis

Gene expression analysis showed detectable expression levels of the lymphocyte (T-cell, B-cell) and dendritic cell markers in the different gut regions (Fig. 1). However, generally the expression levels were lower in the oesophagus and stomach relative to the other gut regions. The expression levels in the pyloric caeca, midgut and hindgut were relatively similar. An exception was the Ig levels that tended to increase posteriorly along the gut from the pyloric caeca to the hind gut. On the basis of these results the oesophagus and stomach were considered unsuitable for GALT cell isolation but there was no clear advantage to using only the pyloric caeca, mid gut or hind gut. Hence the whole gut posterior of the stomach was taken for cell isolation.

2. Optimizing gut leucocyte isolation

The average cell yield per fish obtained by each protocol differed and ranged from $4.2x10^6$ cells /fish, $6.4x10^6$ cells /fish and 12.0 $x10^6$ cells /fish for protocol-1, -2 & -3, respectively. The cell yield using protocol-2 was significantly higher than that of protocol-1, and the yield from protocol-3 was, in turn, significantly higher than that of protocol-2 (Fig. 2a). No significant differences between the protocols were found in the viability of the cells obtained but there was a significant fall in viability from 4 h to 24 h in culture, from ~93% to ~53% (Fig. 2b).

Analysis of the T-cell, B-cell and dendritic cell markers in the cells isolated by the three protocols showed that all of the genes were expressed at 4 h and 24 h post-isolation (Fig. 3). The expression patterns, however, differed to some extent between the protocols and in comparison to the expression patterns in the gut tissue (Fig. 1). MHCIIβ and IgM were relatively highly expressed in cells isolated by all 3 protocols, as was CD83 using protocol-2 and -3 (Fig. 3a-c). Of the T cell markers, CD4-1 was relatively low, whilst IgT was low compared to the IgM transcript levels. The expression patterns did not vary significantly between the sampling times (4 h vs 24 h) using protocol-1 and -2, but there was a decrease of the T cell markers in particular at 24 h using protocol-3, and to a lesser extent with protocol-2. Flow cytometry analysis revealed three distinct populations of the cells isolated by protocol-3. Based on the location of these populations relative to the forward scatter (FSC) and side scatter (SSC) axes, they were tentatively identified as epithelial cells, lymphoid cells and myeloid cells (Fig. 4). In line with this, transmission electron microscopy showed different leucocyte types were present, characterised based on their morphology and structure as lymphocytes, monocytes/macrophages, and granulocytes (Fig. 5).

Stimulating GALT leucocytes isolated by the different protocols with LPS resulted in a significant increase in expression of IL-1β, IL-6 and TNF-α (Fig. 6). Generally the increases occurred at both time points, with the exception of TNF-α at 4 h in cells isolated by protocol-1 (Fig. 6c) and IL-6 at 4 h in cells isolated by protocol-3. The increases were mostly similar at both time points, especially for protocol-3. However, up-regulation of IL-1β was higher at 4 h vs 24 h in protocol-2 isolated cells, whilst the increases in TNF-α were higher at 24 h vs 4 h in cells isolated by protocol-1 and -2.

Overall, the three protocols gave similar cell viabilities and the isolated cells had similar marker gene profiles. Also, cells isolated by all three protocols could respond to LPS stimulation by induction of pro-inflammatory genes. However, the increase in cell yield using protocol-3 allowed the analysis of GALT leucocytes from individual fish, and in addition was high enough to allow relatively small fish to be used, meaning that freshwater stage salmon as well as trout could be studied. Hence protocol-3 (Suppl. Fig. 1) was selected for further experiments to look at salmon GALT leucocyte responsiveness to a wider range of stimulants.

3. Atlantic salmon GALT leucocyte analysis

3.1. Marker gene analysis

Initially the GALT cells isolated from Atlantic salmon parr using protocol-3 were checked for their expression of T-cell, B-cell and dendritic cell marker genes at 4 h and 24 h in culture. As seen in Figure 7, the profiles were similar to the trout cells. CD83 and MHCIIβ were relatively highly expressed, as was TCRα. mIgM was again higher than mIgT, but sIgM/sIgT and CD4-1 /CD8α were more equal in transcript level. No major differences were seen between the 4 h and 24 h samplings.

3.2. Bacterial PAMP and pro-inflammatory stimulation

LPS was a potent stimulator of pro-inflammatory cytokine expression in the GALT leucocytes, with IL-1β, IL-6, IL-8 and TNF-α all increasing, especially at 24 h post-stimulation (Fig. 8). The antimicrobial peptide CATH-2 was also highly induced (Fig. 8). Smaller effects were seen on interferon (type I and II) and IRF3 (Figs. 8&9) gene expression, and with Th17-type cytokine (IL-17A/F and IL-22, Fig. 9) gene expression at 24 h. Curiously a small down-regulation of IL-4/13B was seen at 4 h. Bacterial flagellin (YRF) was also a good stimulator of IL-8 expression and IL-22 expression, and to a lesser extent on IL-1β, IL-6, TNF-α, IFN-γ and CATH-2 transcript levels. Curiously it had no impact on GALT cell IL-1β expression. rIL-1β itself was able to induce IL-8 (4 h and 24 h) and CATH-2 (24 h) but had no impact on the other genes studied. Similarly rIL-6 was only able to impact CATH-2 expression, at 24 h post-stimulation.

3.3. Viral PAMP and interferon stimulation

Poly I:C was a potent inducer of type I and II interferons, and IRF3, as expected (Figs. 8&9), with marked increases seen at 24 h. However, it also induced to a lesser extent IL-2, IL-17A/F and IL-22 expression at 24 h (Fig. 9). Recombinant type I interferon was also a good inducer of IRF3 and IFN-γ expression, at 4 h and 24 h, and had a small effect on itself, IL-2 and IL-22 transcript levels at 24 h post-stimulation.

3.4. PHA and T cell cytokine stimulation

PHA was a strong inducer of cytokines potentially associated with adaptive immunity, including IL-2, IL-4/13B, IL-17A/F, IFN-γ and TNF-α, at 4 h and 24 h post-stimulation (Figs. 8&9). It also had a small impact on IL-1β and IL-22 expression levels at 4 h post-stimulation (Figs. 8&9), and type I and IRF3 expression levels at 24 h post-stimulation (Figs. 8&9). rIL-2 stimulation had relatively few effects, and increased IL-22 expression at 4 h post-

stimulation (Fig. 9), and itself and IRF3 at 24 h post-stimulation (Figs. 8&9). Lastly, rIL-21 similar to PHA induced cytokines of adaptive immunity, including IL-4/13B, IL-22 and IFN-γ at 4h and 24 h post-stimulation (Fig. 9), and IL-17A/F at 24 h (Fig. 9). It also induced a small increase of IL-6, TNF-α, type I interferon and IRF3 at 24 h (Figs. 8&9), but had no effect on IL-1β, IL-2 or IL-8 expression.

Discussion

Fish GALT is structurally and physiologically different from mammalian GALT [15], and the composition of the immune cells present are considered distinct compared to those in other immune tissues such as the head kidney, spleen, skin and gills [37,38]. Such facts underline the necessity for a better basic understanding of the gut immune system in fish. In the present study we used an *in vitro* approach to examine the responses of isolated GALT leucocytes to a variety of PAMPs and cytokines as a means to help characterise the diversity of the immune repertoire present in such cells.

Before isolating GALT leucocytes we examined whether differences existed in different gut regions in terms of the expression of a variety of marker genes typical of T-lymphocytes (CD4-1, CD8α, TCRα and TCRγ), B-lymphocytes (sIgM, mIgM, sIgT and mIgT) and dendritic cells (CD83, CD208, CD209 and MHCIIβ), since this could influence whether to focus on a particular area for cell isolation. That differences may be apparent come from studies in trout showing that IgM+ B-cells are highly recruited to the pyloric caeca after oral stimulation [39], that IgT^+ B-cells are particularly abundant in the midgut/hind gut [40], and that antigen uptake is most prominent in the second segment of the midgut [41,9]. In addition, studies in Atlantic salmon have shown the mid-posterior intestine has the highest expression levels of a similar gene set (to that used here) in post-smolts [42] and in sea bass regional differences in CD8α and MHCIIβ gene expression have been described [22]. In the present study expression levels of the marker genes were relatively low in oesophagus and stomach, especially the latter, in agreement with past immunohistochemical studies of intestinal B cells and sites of antigen uptake [9,40]. However, in the pyloric caeca, midgut and hindgut higher expression levels were seen that were relatively similar. Nevertheless, there was a trend for increasing Ig transcript level from the pyloric caeca to the hindgut, similar to results seen in sea bass with IgM^+ B cells [43]. Given their homology with mammalian cells, the constitutive expression of the selected markers demonstrates that naïve rainbow trout gut, in particular the intestine, has a good repertoire of the immune cell types and subtypes that are required to initiate and establish an adaptive immune response.

On the basis of the marker gene expression results the pyloric caeca, mid gut and hind gut were considered suitable for GALT cell isolation and the whole gut posterior to the stomach was taken for this purpose. Initially we used the method of Salinas et al. [24] for isolation of gilthead seabream (*Sparus aurata*) GALT leucocytes. This approach (designated protocol-1) gave a yield of $4.2x10^6$ cells /fish, which was relatively low, requiring pooling of gut tissue from multiple fish to allow *in vitro* experimentation. Nevertheless, the cells had relatively good viability, expressed the marker genes as seen in the gut regions from which they were derived, and were responsive to LPS in terms of up-regulation of the pro-inflammatory genes IL-1β, IL-6 and TNF-α. However, the marker gene analysis showed there were relatively low levels of IgT and CD4-1 transcripts in the isolated cells, relative to IgM and CD8/TCR expression levels. This may reflect fewer IgT B cells [13,44] and Th cells in the unstimulated gut, consistent with the whole gut tissue analysis (Fig. 1), although it is known that CD4-1 can also be expressed by myeloid cells in fish [45,46]. CD4 levels have also been shown to be relatively low in sea bass intestine, compared to CD8α expression [22]. Since protocol-1

used nylon wool columns that could impact on yield and cell activity/ gene expression, as seen in mice [47,48], this step was excluded in protocol-2. This significantly increased the cell yield, to 6.4x10^6 cells /fish, with no effect on cell viability or LPS responsiveness, but did result in a higher relative expression of some dendritic cell markers. Since the ultimate aim was to get enough cells from a single fish for experimentation *in vitro*, to avoid the potential for reciprocal immune reactions between the cell populations that could give rise to altered gene expression, protocol-2 was modified further by incorporation of a second enzymatic digestion (protocol-3). This again led to a significant increase in cell yield, which went to 12.0 x10^6 cells /fish. The flow cytometry analysis of these cells revealed lymphoid and myeloid populations, and transmission electron microscopy confirmed the presence of lymphocytes, monocytes/macrophages and granulocytes. The viability, expression profiles and responsiveness to LPS of these cells were similar to those from protocol-2. One difference was in the TNF-α responses using protocol-3, where the significant increase at 24 h vs 4 h of stimulation with LPS using protocols-1 and -2 was absent. This may have been an anomaly since the 24 h response was larger when using LPS with the salmon GALT cells (Fig. 8d). However, as stated above protocols-1 and -2 used pooled cells and TNF-α is known to increase during the MLR in trout [49]. Overall, protocol-3 was considered a time- and cost-effective means to isolate GALT leucocytes from salmonids. This method should be suitable for other fish species but the length of enzymatic digestion may need to be optimised further.

Protocol-3 was next used to isolate GALT leucocytes from Atlantic salmon. The cells were once again analysed for marker gene expression, which revealed similar expression profiles to the isolated cells from rainbow trout and little impact of culture time (4 h vs 24 h), and were considered suitable for the stimulation studies. Of the few differences that were seen, such as reduced sIgM expression in the salmon cells, it was unclear whether species differences or fish size/age were the cause. The stimulants tested were designed to examine the nature of the antibacterial, antiviral and T cell type responses in the GALT leucocytes (at the transcript level) using a panel of cytokines relevant to innate and adaptive immunity, the antimicrobial peptide (AMP) cathelicidin-2 (CATH-2) and interferon regulatory factor (IRF) 3. Since multiple paralogues of such genes can exist in salmonid fish [50,51], a particular paralogue was selected where necessary.

Clear responses to bacterial PAMPS (LPS, flagellin) were detected. IL-1β, IL-6, IL-8 and TNF-α were all notably increased, as was CATH-2. These cytokines form part of the cytokine cascade typical of responses to Gram negative bacteria, in fish as in mammals [49], and reveal they are active in GALT leucocytes. They function to attract and activate phagocytes, to clear bacteria, and can induce expression of AMPs such as CATH-2 [29, 49]. Th17/ILC3-type cytokines [52] are also known to be effective inducers of anti-microbial defences, and the PAMPs were shown to induce both IL-17A/F (considered a forerunner of IL-17A and IL-17F, [33] and IL-22 in the isolated GALT cells. rIL-22 has been shown to up-regulate a variety of AMPs (β-defensins and LEAPs) in trout [53], whilst in haddock marked upregulation of IL-22 occurs in gills following challenge of vaccinated fish [54]. CATH-2 is a potent AMP in salmonids, able to kill a range of fish bacterial pathogens as part of the innate immune response [55]. It has been reported to be upregulated by IL-6 and flagellin (YRF) in a trout macrophage (RTS-11) cell line [29,26], in keeping with the present findings. However, the effects of rIL-6 on CATH-2 expression were quite marginal, and indeed both pro-inflammatory cytokines tested (rIL-1β and rIL-6) induced relatively few/small effects.

Curiously the two cathelicidins present in trout are usually not co-expressed upon stimulation [29,56] and it will be interesting to see if this is also the case in GALT leucocytes.

In terms of anti-viral responses, it was shown that poly I:C was a potent inducer of interferons (type I and II) and IRF3 in GALT leucocytes, demonstrating a functional signalling cascade in these cells. Poly I:C is a synthetic double stranded RNA and, as with viruses, is detected by TLR3, which is present in fish in addition to TLR22 that can also bind dsRNA [57]. It serves to induce interferon production, with IRF3 a crucial transcription factor in regulating type I interferon expression [58]. Recombinant type I interferon was also a good inducer of IRF3 and IFN-γ expression, but had a relatively small positive feedback on itself. Neither stimulus impacted on the proinflammatory genes (IL-1β, IL-6, IL-8 and TNF-α) or CATH-2, showing a clear delineation between these signalling pathways within the GALT leucocytes. Lastly, since it has been reported that the majority of teleost GALT leucocytes are T- cells, as reported in carp [36], sea bass [59,60] and rainbow trout [20], we examined the responses induced by the T cell mitogen PHA and two T-cell stimulatory cytokines (IL-2 and IL-21). PHA was a strong inducer of cytokines of adaptive immunity, including IL-2, IL-4/13B, IL-17A/F, IFN-γ and TNF-α. This induction potentially reflects Th cell or ILC responses, and suggests that multiple subpopulations may be present. Interestingly, IL-4/13 exists as two genes in teleost fish (IL-4/13A, IL-4/13B), with IL-4/13A being more highly expressed constitutively but IL-4/13B is more inducible, as seen post vaccination and infection [56,61]. Similar to PHA, rIL-21 was a good inducer of IL-4/13B, IL-17A/F, IL-22 and IFN-γ, and to a small extent TNF-α, however rIL-2 had very limited effects, although found to be an essential regulator of T cell responses in salmonids [28]. Previous studies in trout have shown that rIL-21 is able to markedly upregulate IL-10, IL-22 and IFN-γ in head kidney cells, and can maintain the expression of T and B cell markers at a high level compared with control cultures without IL-21, suggesting it may be a survival factor for such cells [30].

In conclusion, the current study has optimized a protocol for isolation of GALT cells from salmonids, that gives good viability and yield. The cells expressed a wide range of T-cell, B-cell and dendritic cell markers. They were responsive to a panel of PAMPs, cytokines and PHA, and gave distinct expression profiles dependent upon the stimulus used. The gut leucocyte responses appear mostly comparable with known immune responses from other immune sites and reveal the adequacy of gut cells to elicit appropriate humoral and cellular pathways to combat immunological threats. Thus this study creates a benchmark for future examination of salmonid gut mucosal immunity to assay gut cell responses and proliferation to different stimulants and antigens. Further studies are in progress in our laboratory to assess protective immune responses in the salmonid gut, associated with mucosal delivery of antigens, with a view to aid development of efficacious priming of protective responses by oral vaccination in salmonid aquaculture.

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Table & Figures legends

Table 1: qPCR primer sequences and GenBank accession numbers.

Fig. 1. The expression of marker genes for T-lymphocytes (CD4-1, CD8α, TCRα and TCRγ), B-lymphocytes (sIgM, mIgM, sIgT and mIgT) and dendritic cells (CD83, CD208, CD209 and MHCIIβ), in rainbow trout oesophagus, stomach, pyloric caeca, midgut and hindgut. The marker gene expression was determined by qPCR and is presented as arbitrary units (after normalising to EF-1α) relative to the oesophagus transcript level for each gene studied. Bars are means + SEM of four fish.

Fig. 2. (a) The average yield of GALT leucocytes isolated from rainbow trout gut by protocol-1, -2 and -3 and (b) their viability after incubation for 4 h and 24 h. Bars are means ± SEM of four independent samples. Differences amongst the protocols were tested by One-way ANOVA followed by the Tukey post hoc test, with different letters indicating significant differences.

Fig. 3. The expression of marker genes for T-lymphocytes (CD4-1, CD8α, TCRα and TCRγ), B-lymphocytes (sIgM, mIgM, sIgT and mIgT) and dendritic cells (CD83, CD208, CD209 and MHCIIβ), in primary cultures of rainbow trout GALT leucocytes isolated by (a) protocol-1, (b) protocol-2 and (c) protocol-3, and incubated for 4 h and 24 h. The marker gene expression was determined by qPCR, normalised to EF-1α and presented relative to the CD4-1 transcript level at 4 h. Bars are means ± SEM of four independent samples.

Fig. 4. Rainbow trout GALT cell FACS profile. Forward scatter (FSC) and side scatter (SSC) analysis indicates three distinct populations of cells are present. E: presumptive epithelial cells, L: presumptive lymphoid cells, and M: presumptive myeloid cells. The figure shows cells from one representative fish of four analysed.

Fig. 5. Transmission electron micrographs of rainbow trout GALT cells: (a) & (b) Lymphocytes, characterized by a large nuclear:cytoplasmic ratio, (c) Neutrophil, note the lobulated nucleus, (d) Macrophage, note the non-lobulated nucleus, surface ruffles and cytoplasmic granules, (e) Macrophage undergoing phagocytosis, arrow indicates a phagosome containing an ingested cell, and (f) an unknown vacuolated granulocyte.

Fig. 6. Time-dependent induction of (a) IL-1β1, (b) IL-6A and (c) TNF-α2 gene expressions by LPS in primary cultures of rainbow trout GALT leucocytes isolated by protocols-1, -2 and -3. The immune gene expression was determined by qPCR, normalized to EF-1α and

expressed as a mean fold change relative to the control cells. Bars are means ± SEM of four independent samples. Differences between stimulated samples and controls are shown as ***p*<0.05, ****p*<0.01 and *****p*<0.001. Lines above the bars show significant differences between the two time points.

Fig. 7. The expression of marker genes for T-lymphocytes (CD4-1, CD8α, TCRα and TCRγ), B-lymphocytes (sIgM, mIgM, sIgT and mIgT) and dendritic cells (CD83, CD208, CD209 and MHCIIβ), in GALT leucocyte cultures isolated from Atlantic salmon gut and incubated for 4 h and 24 h. The marker gene expression was determined by qPCR, normalised to EF-1α and presented relative to the CD4-1 transcript level at 4 h. Bars are means ± SEM of GALT cells from 4 fish.

Fig. 8. Time-dependent induction of (a) IL-1β1, (b) IL-6A, (c) IL-8, (d) TNF-α2, (e) CATH-2 and (f) IRF3 gene expression by PHA, LPS, poly I:C, YRF, IL-2B, IL-21, IFN-α2, IL-1β1 and IL-6 in GALT leucocyte cultures isolated from Atlantic salmon gut. The immune gene expression was determined by qPCR, normalized to EF-1α and expressed as a mean fold change relative to the control cells. Bars are means ± SEM of GALT cells from 4 fish. Differences between stimulated samples and controls are shown as ***p*<0.05, ****p*<0.01 and *****p*<0.001.

Fig. 9. Time-dependent induction of (a) IFN-α, (b) IFN-γ1, (c) IL-2A, (d) IL-4/13B1, (e) IL-17A/F-1 and (f) IL-22 gene expressions by PHA, LPS, poly I:C, YRF, IL-2B, IL-21, IFN-α2, IL-1β1 and IL-6 in GALT leucocytes cultures isolated from Atlantic salmon gut. The immune gene expression was determined by qPCR, normalized to EF-1α and expressed as a mean fold change relative to the control cells. Bars are means ± SEM of GALT cells from 4 fish. Differences between stimulated samples and controls are shown as ***p*<0.05, ****p*<0.01 and *****p*<0.001.

Supplementary Fig. 1. Zigzag flow diagram of the optimised salmonid gut leucocyte isolation protocol.

Figures and Table

Table 1

Gene	Forward primer (5′ – 3′)	Reverse primer (5′ — 3′)	GenBank Acc. no.
EF-1α	CAAGGATATCCGTCGTGGCA	ACAGCGAAACGACCAAGAGG	AF498320
CD4	GTGTGGAGGTGCTACAGGTTTTTTC	ATCGTCACCCGCTGTCTGTG	AY973028
CD8α	CAAGTCGTGCAAAGTGGGAAA	TCTGTTGTTGGCTATAGGATGT	AF178053
TCRα	CAAACTGGTATTTTGACACAGATGCAA	TTCTTGTGTTGTCTTTGAGGGACTGA	BT073987
TCRγ	CATCCCAAAAGATACTCCAACC	GAGAGAGAGCCCACAGCAATAC	EU072700.1
CD83	GTGAGGTGGTACAAGCTGGGTG	GCTGCCAGGAGACACTTGTAC	AY263797
CD208	ACATGAAAAGCTGTTCCCAACTGC	AGCCCAGCACTCAACCTCCTC	NM_001281412
CD209	CACCACTGACCACAGCGAATTG	GAACATTTCTCATCATTCCACC	FN667662
MHCIIβ	TGTCAGAGTCAGGTGGACCAGGA	GGCTCACCTCAGGTTCCCAGAT	3 alleles
sIgM	TACAAGAGGGAGACCGGAGGAGT	CTTCCTGATTGAATCTGGCTAGTGGT	X65261
mIgM	CCTACAAGAGGGAGACCGATTGTC	GTCTTCATTTCACCTTGATGGCAGT	OMU04616
sIgT	CATCAGCTTCACCAAAGGAAGTGA	TCACTTGTCTTCACATGAGTTACCCGT	AY870268
mIgT	TCGAAGTCCACGGCGAACA	GTGTTCTTCACCGCTTCATCTTGAA	AY870264
IL-1β1	CCTGGAGCATCATGGCGTG	GCTGGAGAGTGCTGTGGAAGAACATATAG	AJ278242
IL-6A	GGGAGAAAATGATCAAGATGCTCGT	GCAGACATGCCTCCTTGTTGG	DQ866150
TNF-α2	CTGTGTGGCGTTCTCTTAATAGCAGCTT	CATTCCGTCCTGCATCGTTGC	AJ401377
IL-8	AGAGACACTGAGATCATTGCCAC	CCCTCTTCATTTGTTGTTGGC	AJ310565
Type I IFN-α	CTGTTTGATGGGAATATGAAATCTGC	CCTGTGCACTGTAGTTCATTTTTCTCAG	AJ580911
IFN-γ1	CAAACTGAAAGTCCACTATAAGATCTCCA	TCCTGAATTTTCCCCTTGACATATTT	AJ616215
IL-2A	TGATGTAGAGGATAGTTGCATTGTTGC	GAAGTGTCCGTTGTGCTGTTCTC	AM422779
IL-4/13B1	GAGATTCATCTACTGCAGAGGATCATGA	GCAGTTGGAAGGGTGAAGCTTATTGTA	HG794522
IRF3	ACTGGTCATGGTCGAGGTGGT	CACAAGTCCATCATCTCCTGCAG	AJ829668
IL-22	GAAGGAACACGGCTGTGCTATTAAAC	GATCTAGGCGTGCACACAGAAGTC	AM748538
IL-17A/F-1	CAAACGTACACTTTTTGATGGTGCTG	GGGACTCATCATAGGTGGTGTTGGT	KJ921977
CATH-2	ACATGGAGGCAGAAGTTCAGAAGA	GAGCCAAACCCAGGACGAGA	AY542963

Fig.1

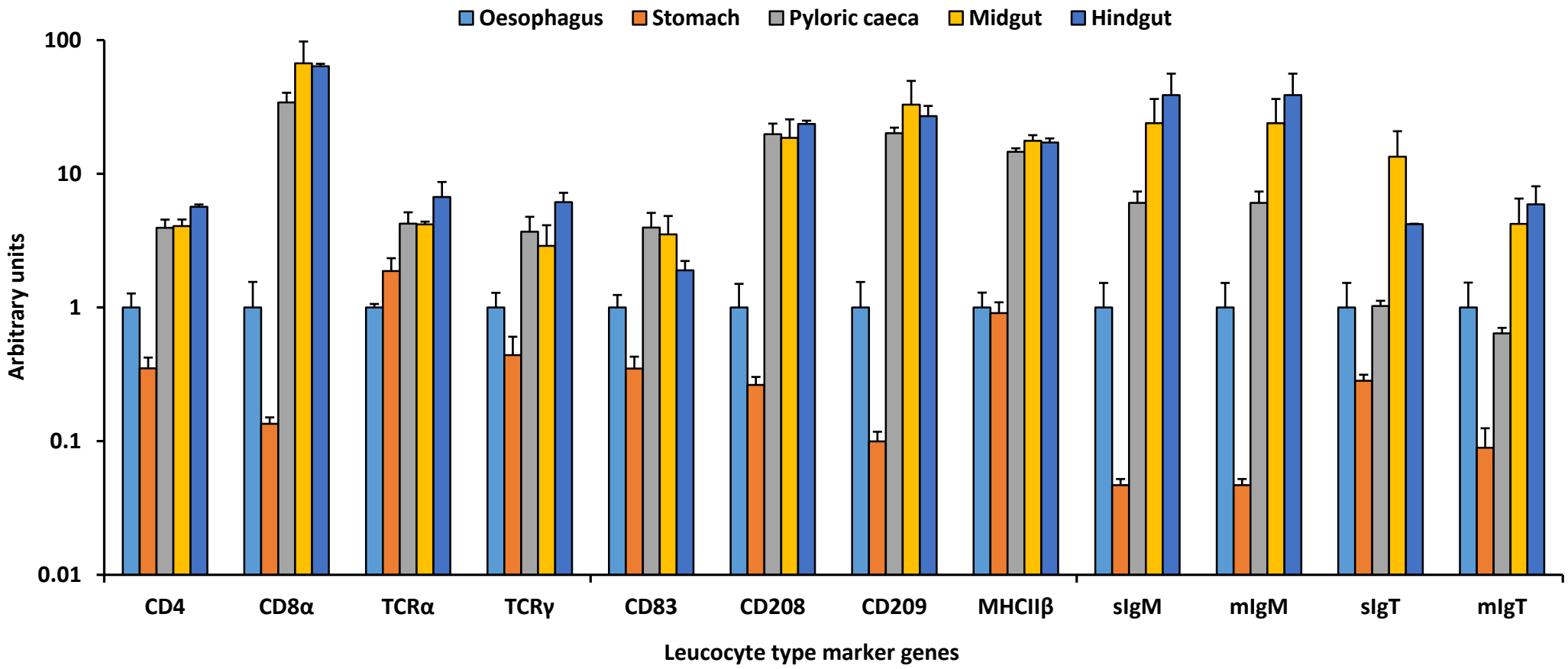
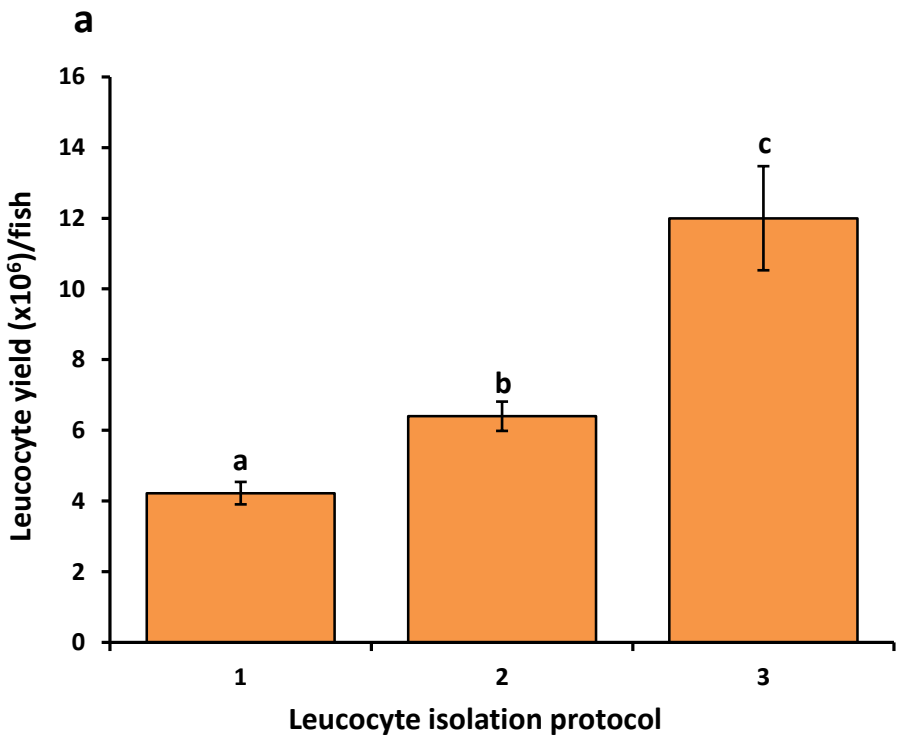

Oesophagus
Stomach
Pyloric caeca
Midgut
Hindgut
100
10
1
0.1
0.01
Arbitrary units
CD4
CD8α
TCRα
TCRγ
CD83
CD208
CD209
MHCIIβ
sIgM
mIgM
sIgT
mIgT
Leucocyte type marker genes

Fig.2a & b


a
Leucocyte yield (x10⁶)/fish
16
14
12
10
8
6
4
2
0
a
b
c
1
2
3
Leucocyte isolation protocol

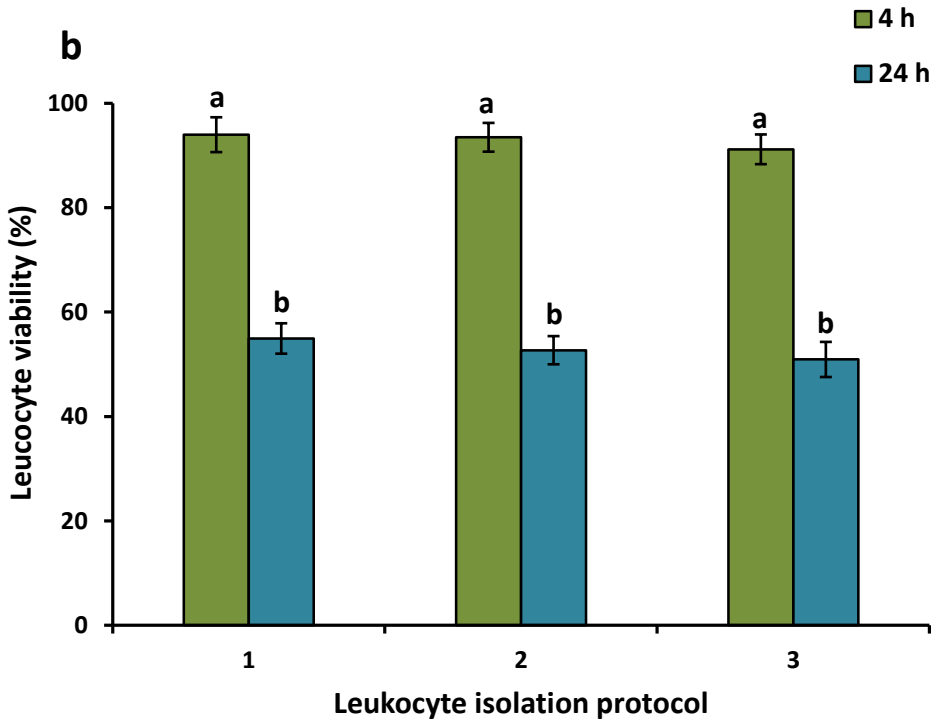

b
4 h
24 h
Leucocyte viability (%)
100
80
60
40
20
0
a
b
a
b
a
b
1
2
3
Leukocyte isolation protocol

Fig.3a

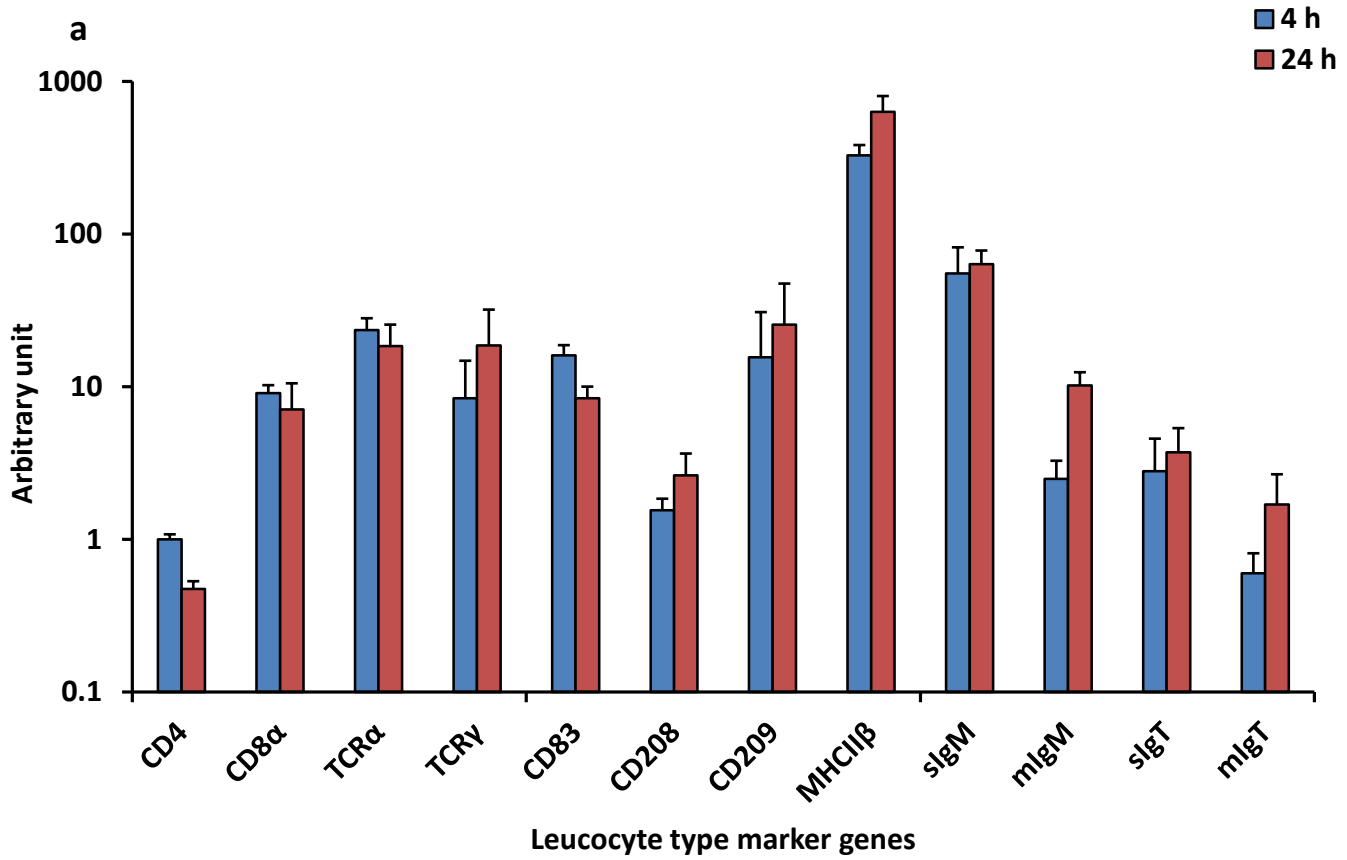

a
4 h
24 h
1000
100
10
1
0.1
Arbitrary unit
CD4
CD8α
TCRα
TCRγ
CD83
CD208
CD209
MHCIIβ
sIgM
mIgM
sIgT
mIgT
Leucocyte type marker genes

Fig.3b

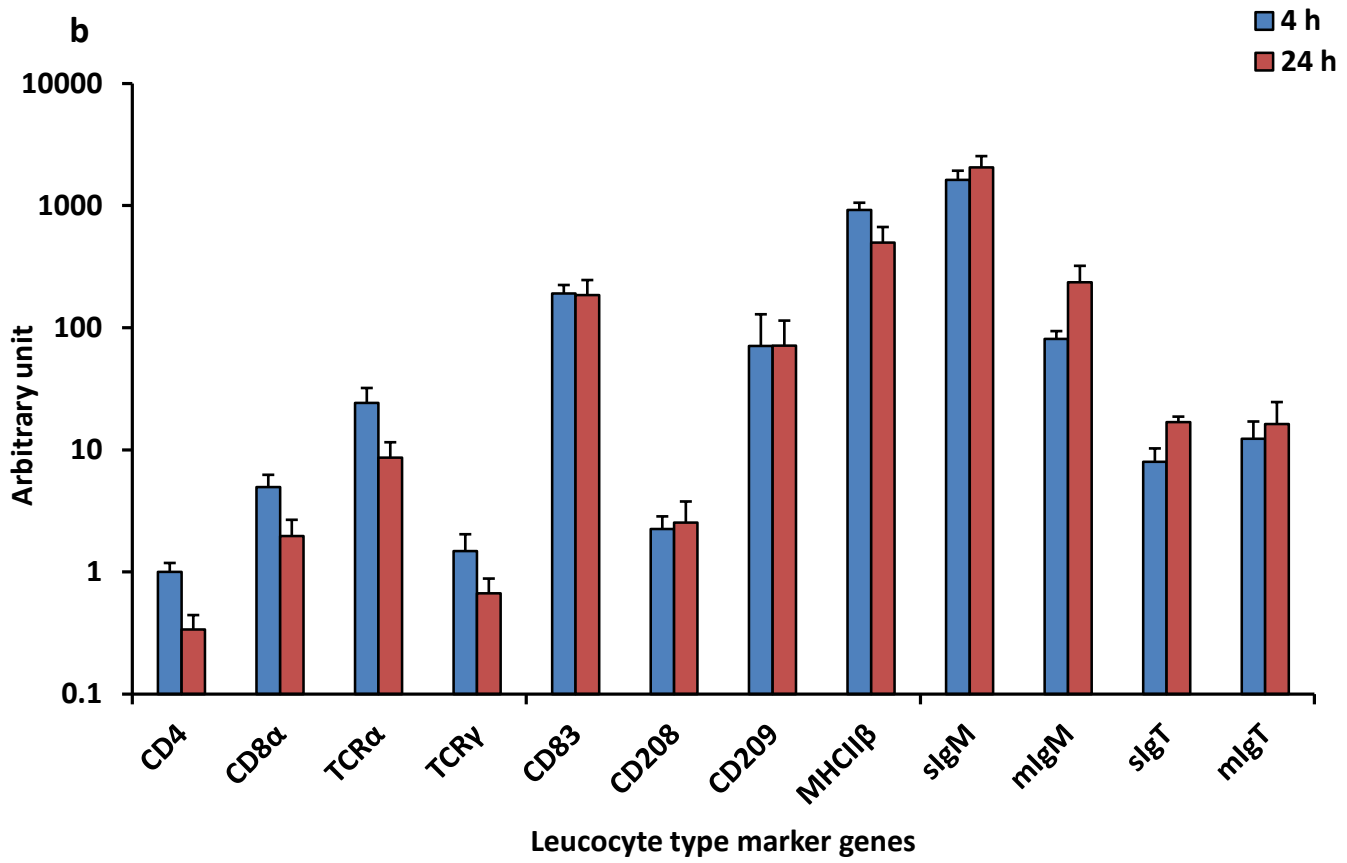

b
4 h
24 h
10000
1000
100
10
1
0.1
Arbitrary unit
CD4
CD8α
TCRα
TCRγ
CD83
CD208
CD209
MHCIIβ
sIgM
mIgM
sIgT
mIgT
Leucocyte type marker genes

Fig.3c

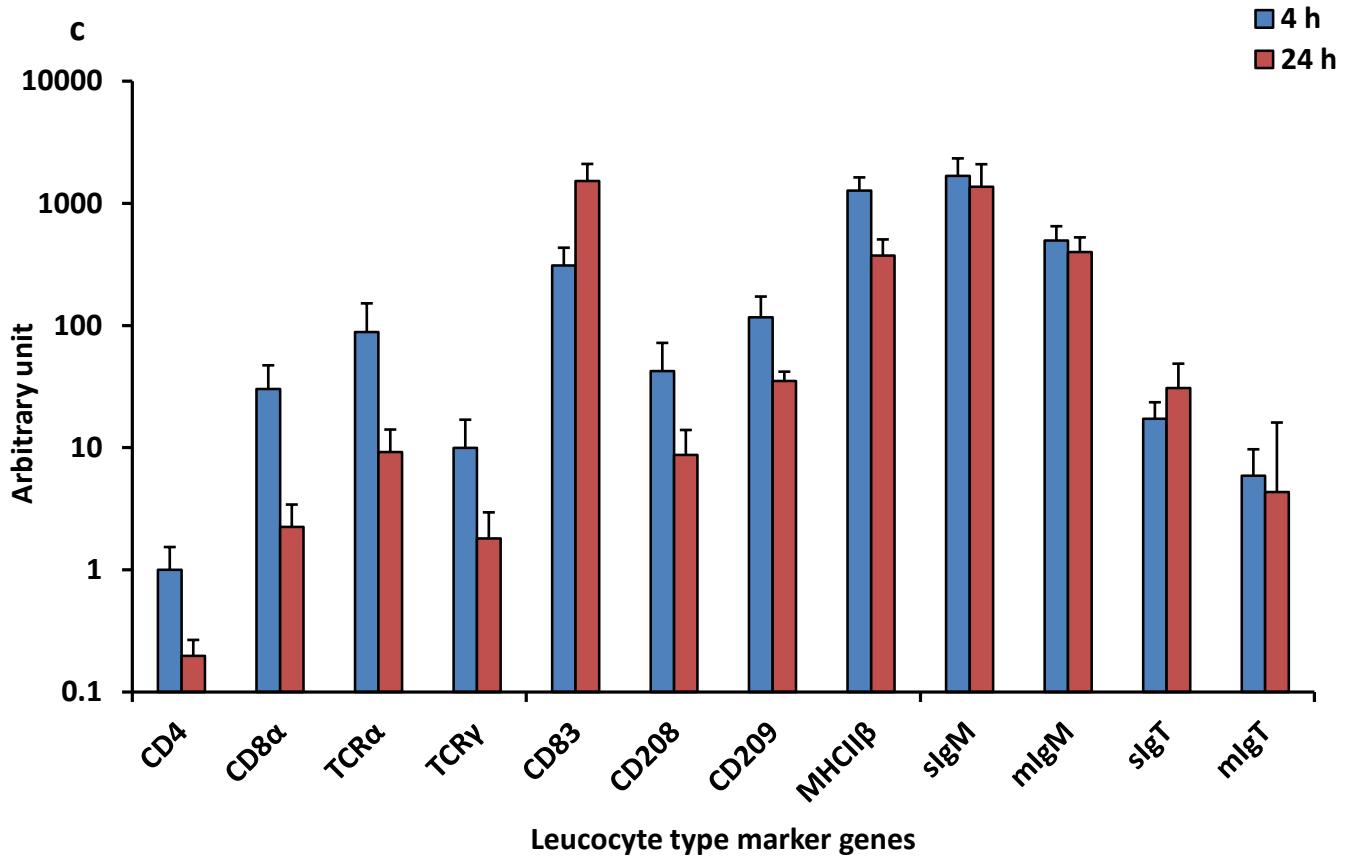

c
4 h
24 h
10000
1000
100
10
1
0.1
Arbitrary unit
CD4
CD8α
TCRα
TCRγ
CD83
CD208
CD209
MHCIIβ
sIgM
mIgM
sIgT
mIgT
Leucocyte type marker genes

Fig.4

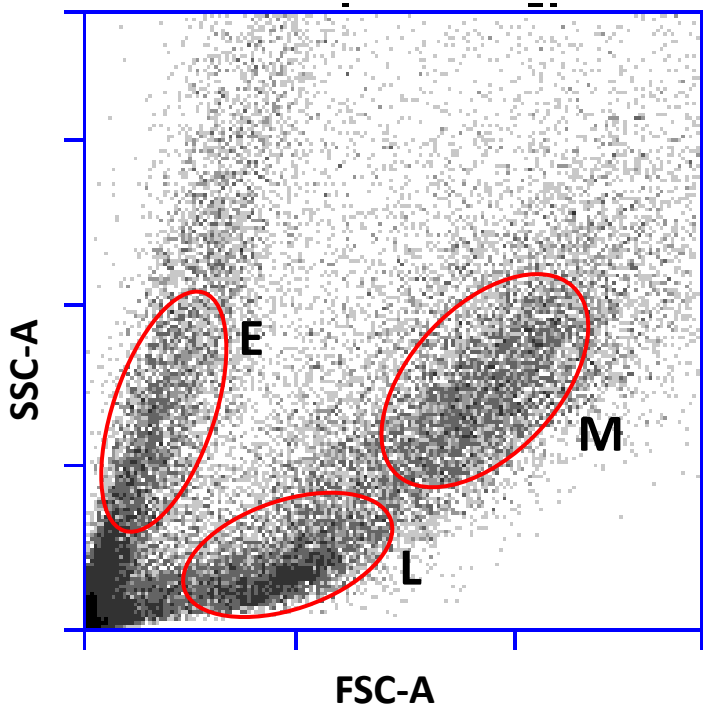


Fig.5a,b,c,d,e &f

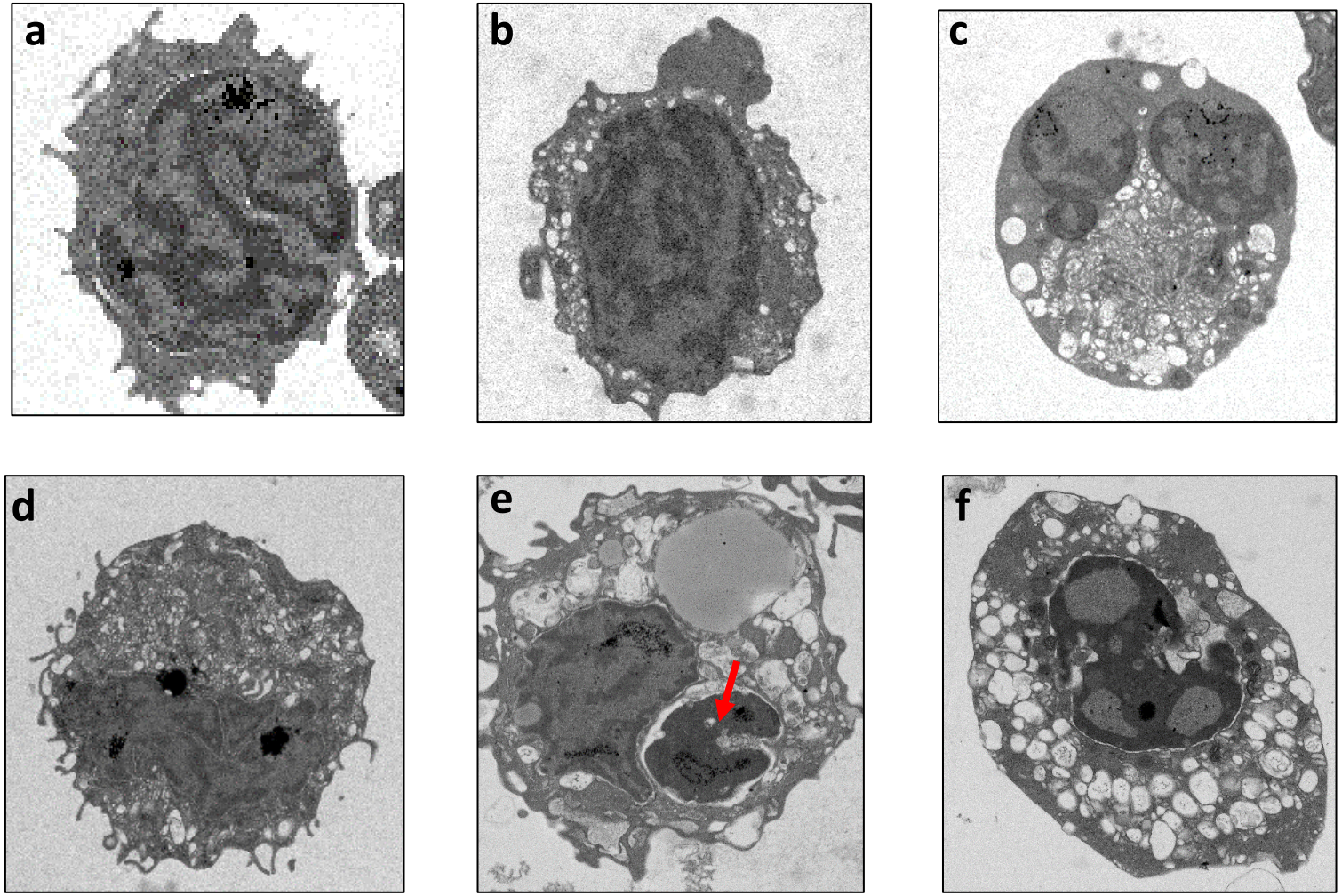


Fig.6a, b & c

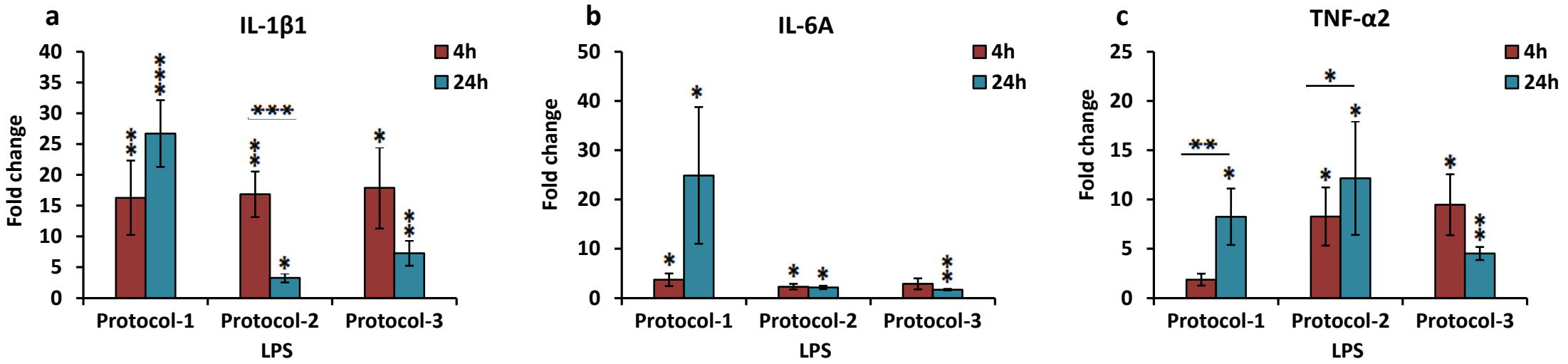

a
IL-1β1
Fold change
40
35
30
25
20
15
10
5
0
4h
24h
Protocol-1
Protocol-2
Protocol-3
LPS
b
IL-6A
Fold change
50
40
30
20
10
0
4h
24h
Protocol-1
Protocol-2
Protocol-3
LPS
c
TNF-α2
Fold change
25
20
15
10
5
0
4h
24h
Protocol-1
Protocol-2
Protocol-3
LPS

Fig.7

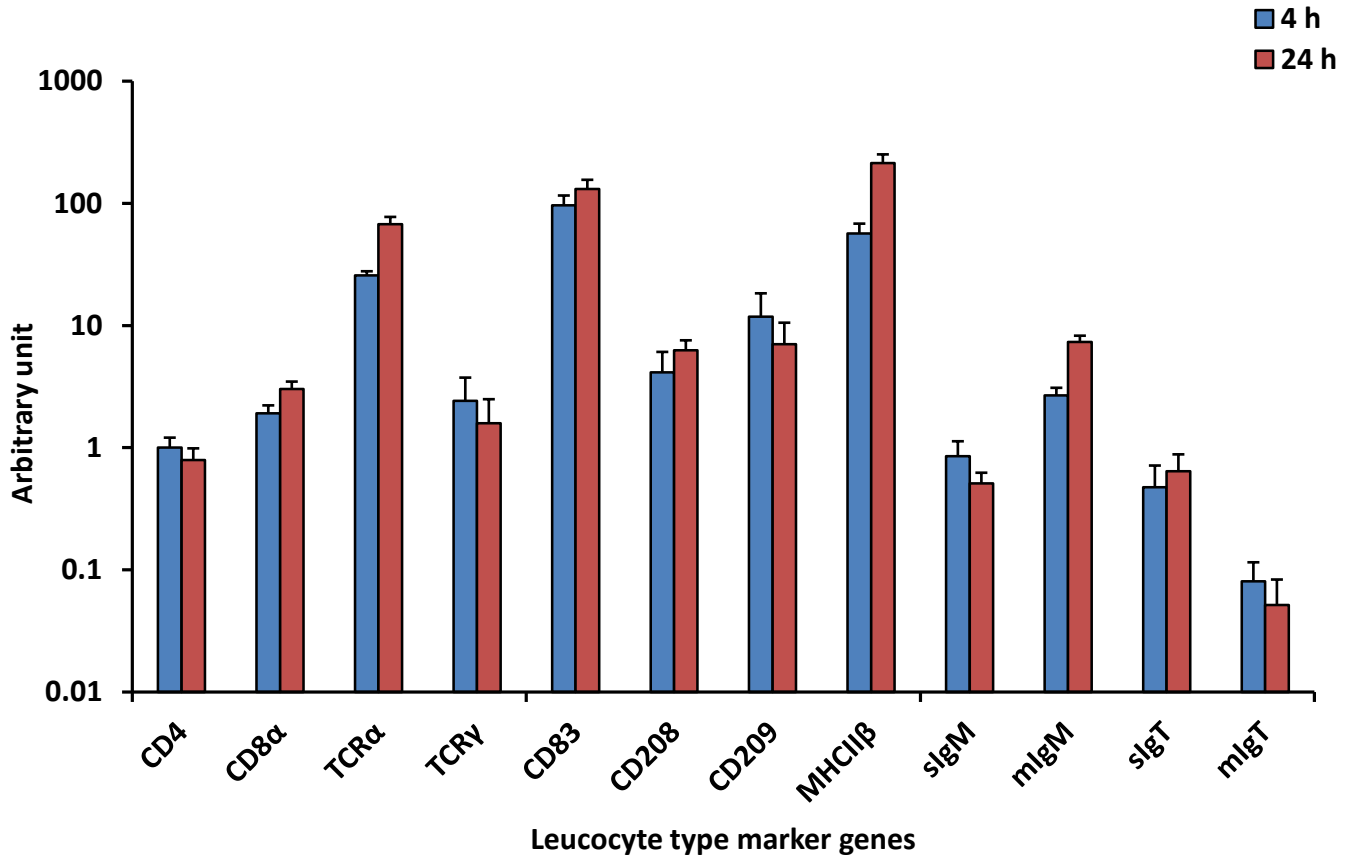

4 h
24 h
1000
100
10
1
0.1
0.01
Arbitrary unit
CD4
CD8α
TCRα
TCRγ
CD83
CD208
CD209
MHCIIβ
sIgM
mIgM
sIgT
mIgT
Leucocyte type marker genes

Fig.8a & b

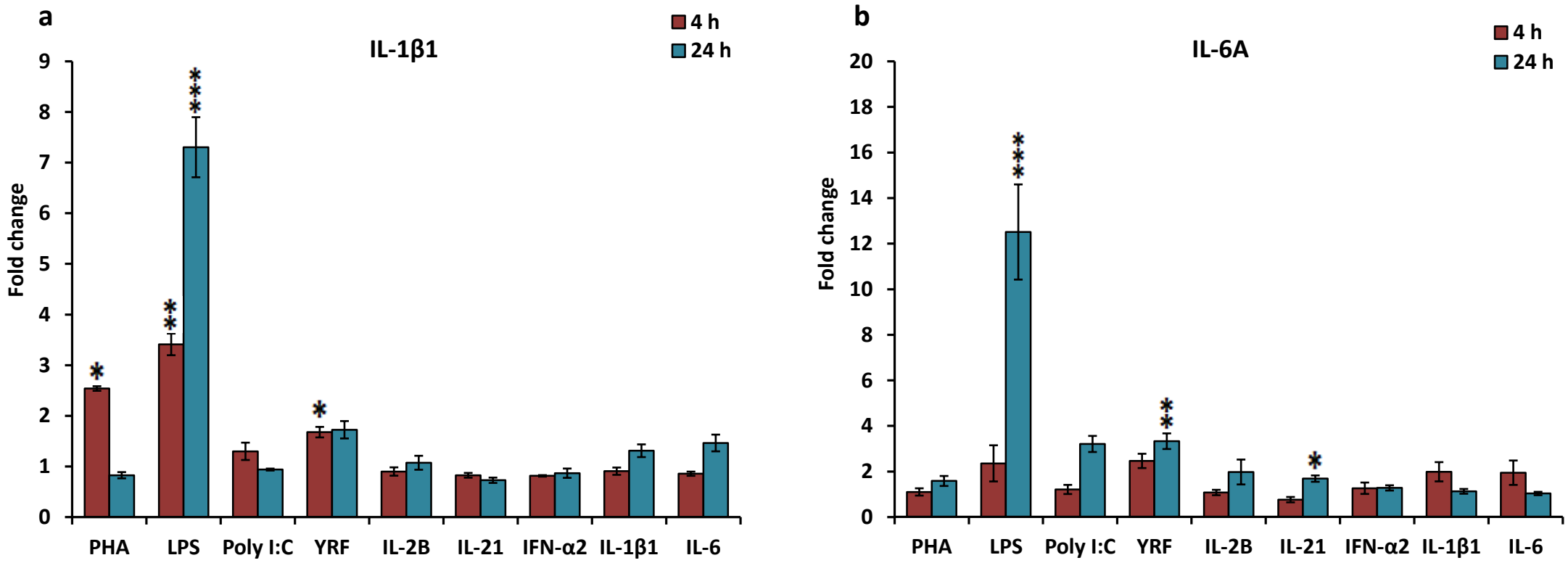
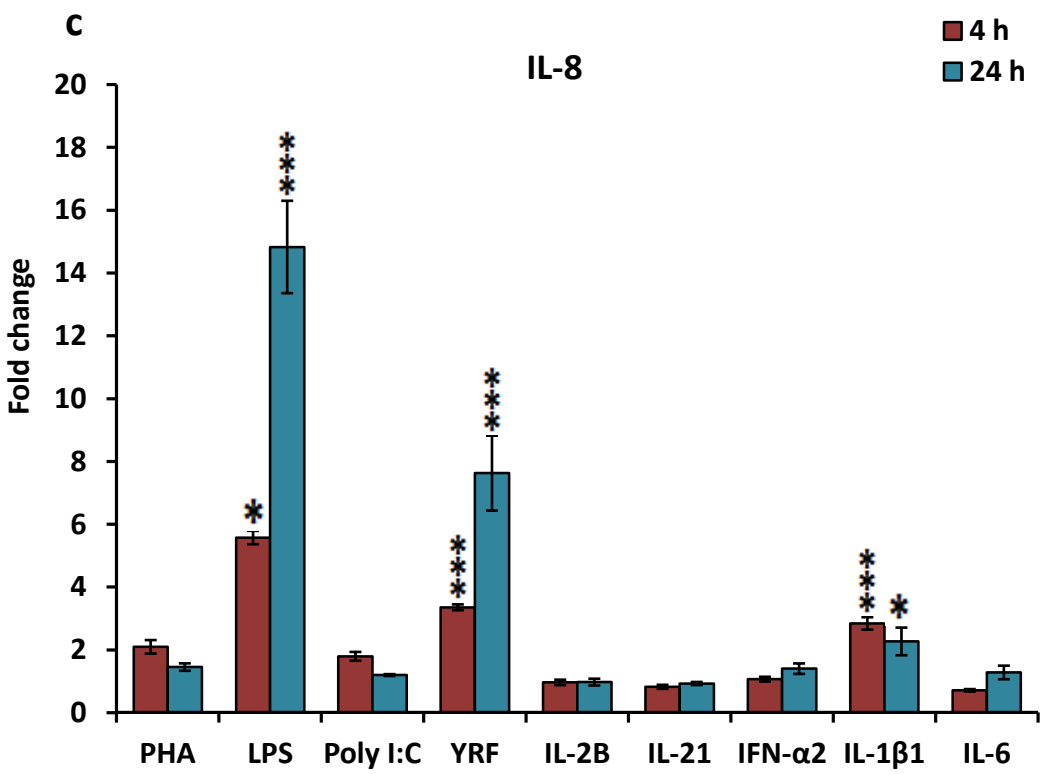

a
IL-1β1
4 h
24 h
Fold change
0
1
2
3
4
5
6
7
8
9
PHA
LPS
Poly I:C
YRF
IL-2B
IL-21
IFN-α2
IL-1β1
IL-6
b
IL-6A
4 h
24 h
Fold change
0
2
4
6
8
10
12
14
16
18
20
PHA
LPS
Poly I:C
YRF
IL-2B
IL-21
IFN-α2
IL-1β1
IL-6

Fig.8c & d


c
IL-8
4 h
24 h
Fold change
0
2
4
6
8
10
12
14
16
18
20
PHA
LPS
Poly I:C
YRF
IL-2B
IL-21
IFN-α2
IL-1β1
IL-6

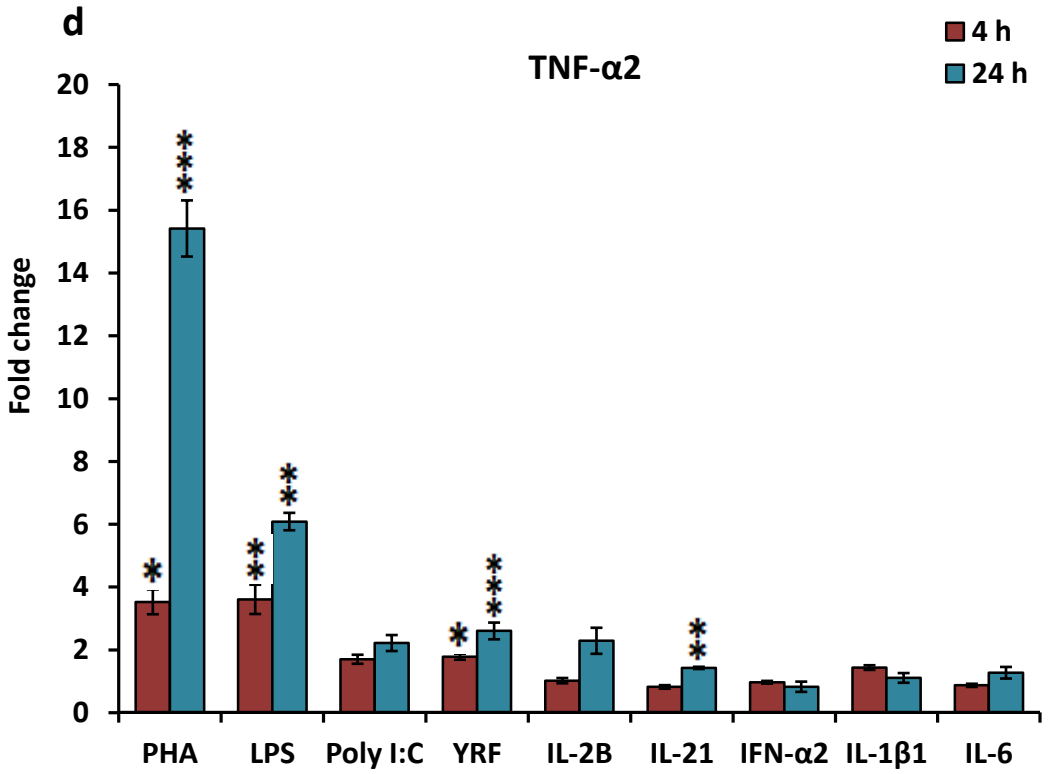

d
TNF-α2
4 h
24 h
Fold change
0
2
4
6
8
10
12
14
16
18
20
PHA
LPS
Poly I:C
YRF
IL-2B
IL-21
IFN-α2
IL-1β1
IL-6

Fig.8e &f

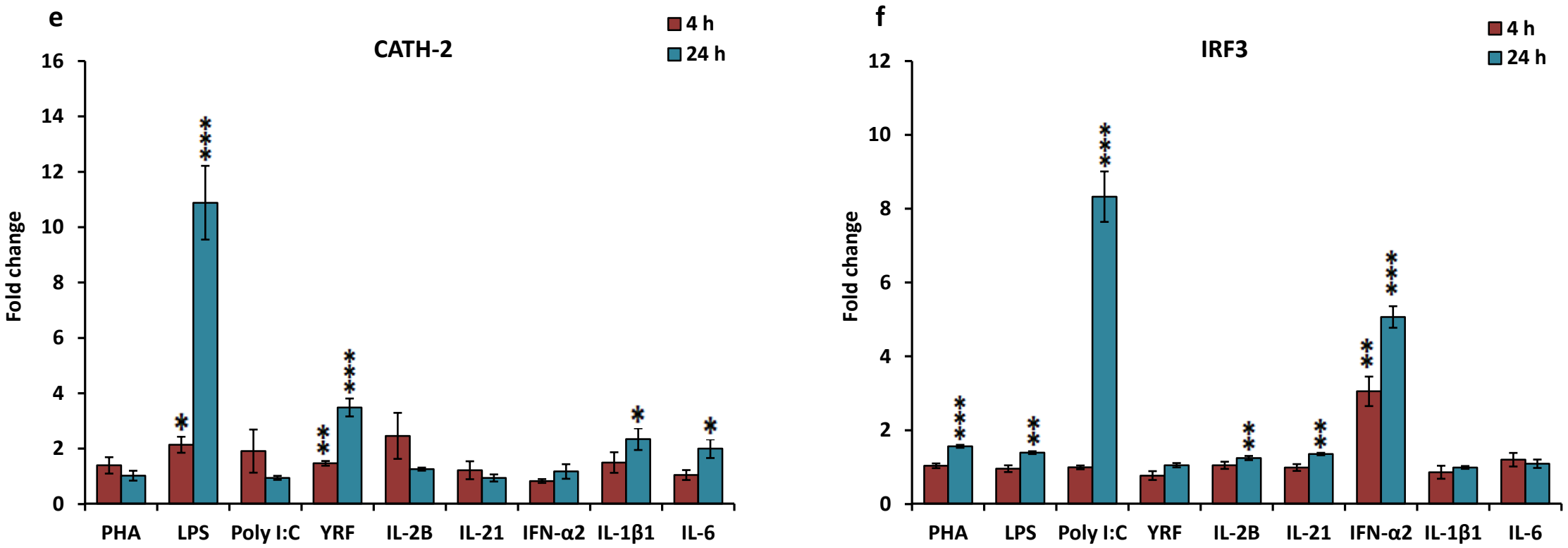

e
CATH-2
4 h
24 h
Fold change
0
2
4
6
8
10
12
14
16
PHA
LPS
Poly I:C
YRF
IL-2B
IL-21
IFN-α2
IL-1β1
IL-6
f
IRF3
4 h
24 h
Fold change
0
2
4
6
8
10
12
PHA
LPS
Poly I:C
YRF
IL-2B
IL-21
IFN-α2
IL-1β1
IL-6

Fig.9a & b

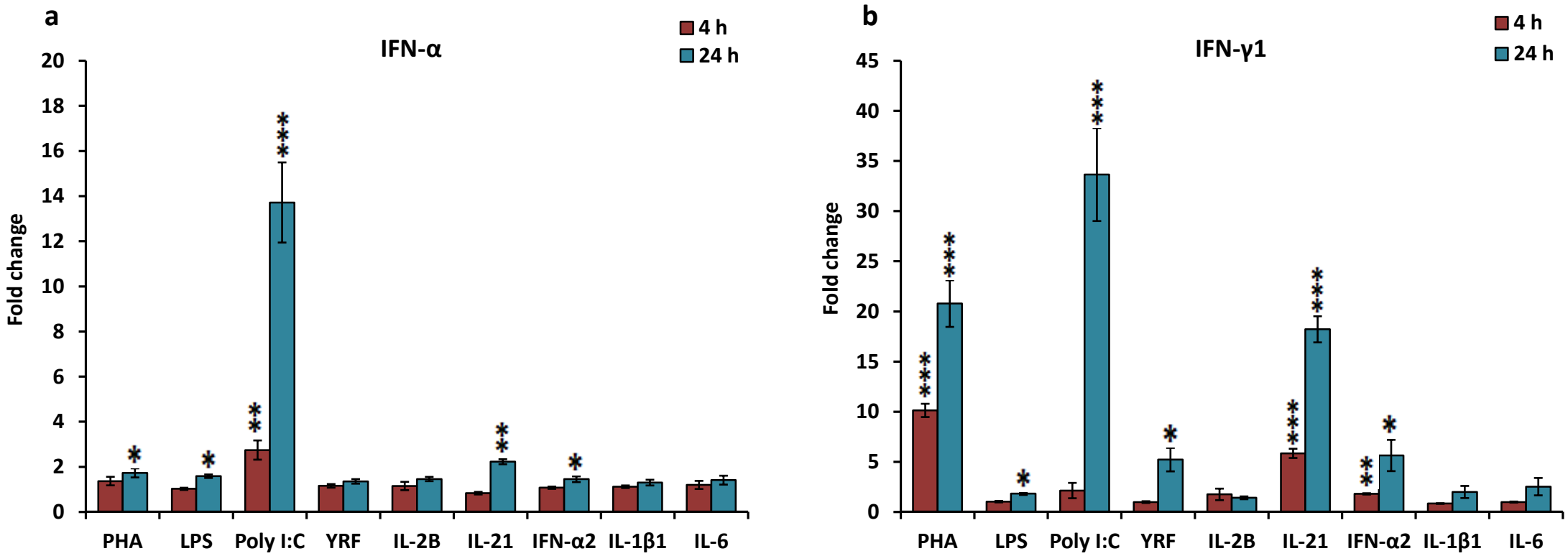

a
IFN-α
4 h
24 h
Fold change
0
2
4
6
8
10
12
14
16
18
20
PHA
LPS
Poly I:C
YRF
IL-2B
IL-21
IFN-α2
IL-1β1
IL-6
b
IFN-γ1
4 h
24 h
Fold change
0
5
10
15
20
25
30
35
40
45
PHA
LPS
Poly I:C
YRF
IL-2B
IL-21
IFN-α2
IL-1β1
IL-6

Fig.9c & d

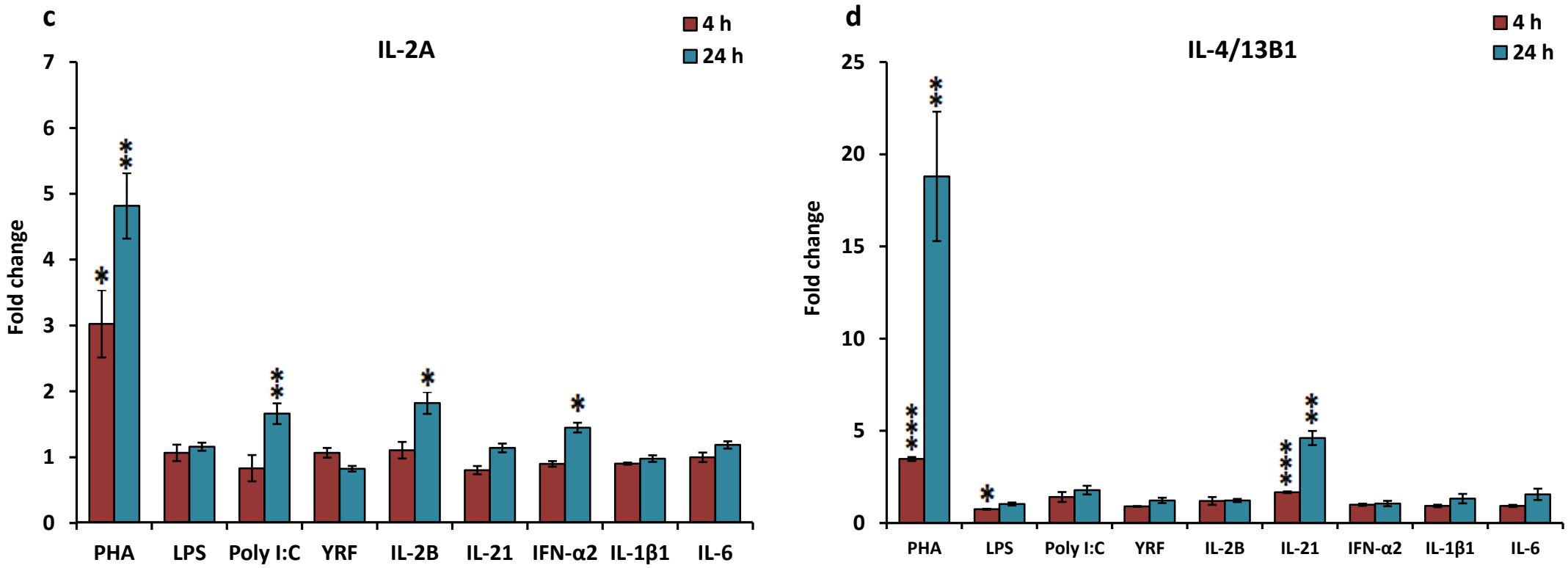
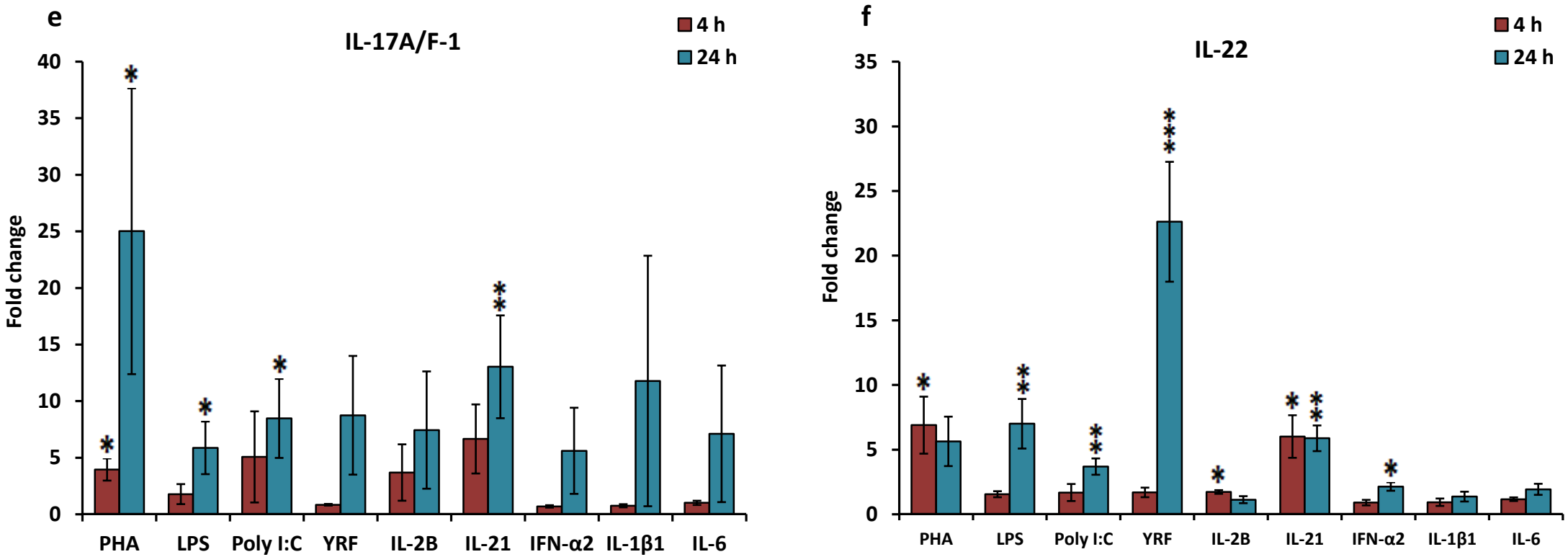
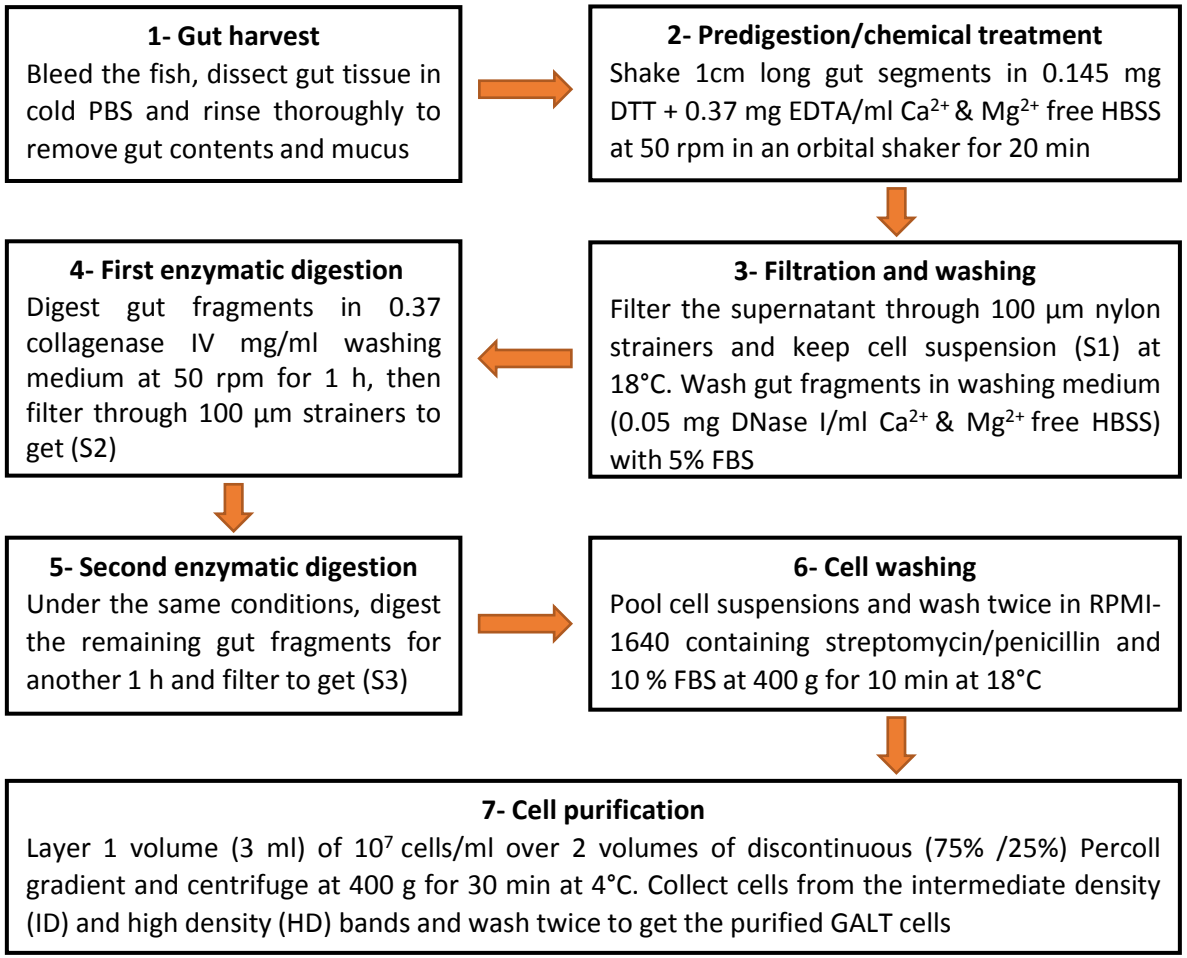

c
IL-2A
4 h
24 h
Fold change
0
1
2
3
4
5
6
7
PHA
LPS
Poly I:C
YRF
IL-2B
IL-21
IFN-α2
IL-1β1
IL-6
d
IL-4/13B1
4 h
24 h
Fold change
0
5
10
15
20
25
PHA
LPS
Poly I:C
YRF
IL-2B
IL-21
IFN-α2
IL-1β1
IL-6

Fig.9e & f


e
IL-17A/F-1
4 h
24 h
Fold change
40
35
30
25
20
15
10
5
0
PHA
LPS
Poly I:C
YRF
IL-2B
IL-21
IFN-α2
IL-1β1
IL-6
f
IL-22
4 h
24 h
Fold change
35
30
25
20
15
10
5
0
PHA
LPS
Poly I:C
YRF
IL-2B
IL-21
IFN-α2
IL-1β1
IL-6

Suppl. Fig. 1


1- Gut harvest
Bleed the fish, dissect gut tissue in cold PBS and rinse thoroughly to remove gut contents and mucus
2- Predigestion/chemical treatment
Shake 1cm long gut segments in 0.145 mg DTT + 0.37 mg EDTA/ml Ca^{2+} & Mg^{2+} free HBSS at 50 rpm in an orbital shaker for 20 min
3- Filtration and washing
Filter the supernatant through 100 µm nylon strainers and keep cell suspension (S1) at 18°C. Wash gut fragments in washing medium (0.05 mg DNase I/ml Ca^{2+} & Mg^{2+} free HBSS) with 5% FBS
4- First enzymatic digestion
Digest gut fragments in 0.37 collagenase IV mg/ml washing medium at 50 rpm for 1 h, then filter through 100 µm strainers to get (S2)
5- Second enzymatic digestion
Under the same conditions, digest the remaining gut fragments for another 1 h and filter to get (S3)
6- Cell washing
Pool cell suspensions and wash twice in RPMI-1640 containing streptomycin/penicillin and 10 % FBS at 400 g for 10 min at 18°C
7- Cell purification
Layer 1 volume (3 ml) of 10^7 cells/ml over 2 volumes of discontinuous (75% /25%) Percoll gradient and centrifuge at 400 g for 30 min at 4°C. Collect cells from the intermediate density (ID) and high density (HD) bands and wash twice to get the purified GALT cells