# Deliverable Report

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| **Deliverable No:** | D24.10 | **Delivery Month:** | 48 |
| **Deliverable Title** | Kinetics of antibody and cytokine production established post-pathogen exposure or stimulation with PAMPs | | |
| **WP No:** | 24 | **WP Lead beneficiary:** | P1. HCMR |
| **WP Title:** | Fish health - meagre | | |
| **Task No:** | 24.6 | **Task Lead beneficiary:** | P5. UNIABDN |
| **Task Title:** | Monitor specific immune responses | | |
| **Other beneficiaries:** | P3. IRTA | | |
| **Status:** | Delivered | **Expected month:** | 48 |
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**Objective:** To gain a better understanding of the kinetics and magnitude of antibody (Ig) and cytokine production in meagre established post pathogen exposure or stimulation with pathogen associated molecular patterns (PAMPs).

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## Introduction

The objective of this work was to better understand the kinetics and magnitude of antibody (Ig) and cytokine production in meagre (*Argyrosomus regius*) established post pathogen exposure or stimulation with pathogen associated molecular patterns (PAMPs). This Deliverable has focused on PAMPs as a means to study immune gene modulation rather than pathogen-infection. Tissue samples were collected from fish following

exposure to the PAMPs poly I:C, LPS and β-glucan, with qPCR assays optimized to detect expression of adaptive immune genes (eg IgT/ IgM, TCR, Th cytokines) and key antimicrobial, antiviral and pro-inflammatory genes, based on the results of D24.3. Similarly, primary cell cultures from different tissues (gills, gut, head kidney, spleen) were stimulated *in vitro* with these PAMPS, and the kinetics of immune gene induction followed.

## 2. Materials and Methods

Healthy meagre (20 g) were provided and maintained by P3. IRTA. The fish were injected intraperitoneally with 100 µl of PBS containing either 100 µg of poly I:C, 400 µg of LPS, 100 µg of β-glucan or 100 µl of PBS as a control, with doses used based on past studies in other fish species. The gills, mid-gut, head kidney (HK) and spleen were then sampled after 24 h. Total RNA was extracted from these samples and converted to cDNA, which was then used in qPCR reactions to enable a quantitative comparison of gene expression between the control group and PAMP stimulated groups for each tissue. An *in vitro* experiment was also performed, where the gills, mid-gut, HK and spleen were taken from healthy 20-g meagre and each tissue passed through a 100 µm mesh with L15 medium to create cell suspensions. PBS, poly I:C (500 µg), LPS (250 µg) or beta glucan (250 µg) was then added to the cell suspensions and the resulting impact on immune gene transcript expression was determined by extracting total RNA after 4, 12 or 24 h, producing cDNA and performing qPCR as above.

## 3. Results

*In vivo PAMP stimulation*

The meagre immune system was successfully stimulated through the intraperitoneal injection of PAMPs, each PAMP generating a very different immune profile, as seen in **Figure 1**. Fish stimulated with poly I:C, a viral mimic, typically resulted in the upregulation of antiviral genes such as the type I (IFNc, IFNd and IFNh) and type II (IFNγ) interferons, however IFNh was only upregulated in the gut and IFNγ was not modulated in gills. Poly I:C also upregulated the antimicrobial peptide (AMP) piscidin in the gills, and the pro-inflammatory cytokine IL-1β in gills, gut and spleen. Several of the adaptive immune genes were also upregulated in the spleen, as seen with IL-4/13, IL-17A/F, IL-22, IgM, TCRβ and RAG1, the latter also upregulated in gut and HK. Lastly, the anti-inflammatory cytokine IL-10 was upregulated in gills, as seen with the other PAMPs, likely to reduce the host response to prevent an impact on the other key physiological processes that happen at this site.

Stimulation with LPS resulted in a quite different set of changes. Noticeably it was the only treatment to upregulate the proinflammatory genes TNFα1 and TNFα2, in the HK. It was also a potent stimulant for upregulation of IL-22 in all four tissues, and of IL-1β and IL-17A/F in HK and spleen. It had no effect on the antiviral genes, and only in the HK the AMP defensin was upregulated. It also increased a few of the adaptive immune genes, as seen with IL-4/13, IL-10, IFNγ and RAG1 in HK and IL-10, IFNγ, RAG1 and TCRβ in spleen.

β-glucan stimulation also resulted in distinct set of upregulated genes. The biggest impact was seen on the AMPs, with piscidin, hepcidin and defensin increased in at least one tissue studied, potentially reflecting different cell types present at different sites that express these genes. β-glucan also increased IL-1β in all tissues studied, and IL-4/13 and IFNγ in the HK and spleen. Interestingly it increased IL-22 and IL-17A/F (Th17 associated cytokines) but only in the spleen. However, there was no impact on Ig or TCR transcript expression, or type I IFN gene expression, with the exception of IFNh in the HK.

In few instances down regulation was observed following PAMP stimulation. For example, piscidin and defensin were down regulated in spleen after LPS and poly I:C treatment, TNFα1 and TNFα2 were down regulated in spleen after poly I:C, and IFNc was down regulated in all tissues after β-glucan treatment.

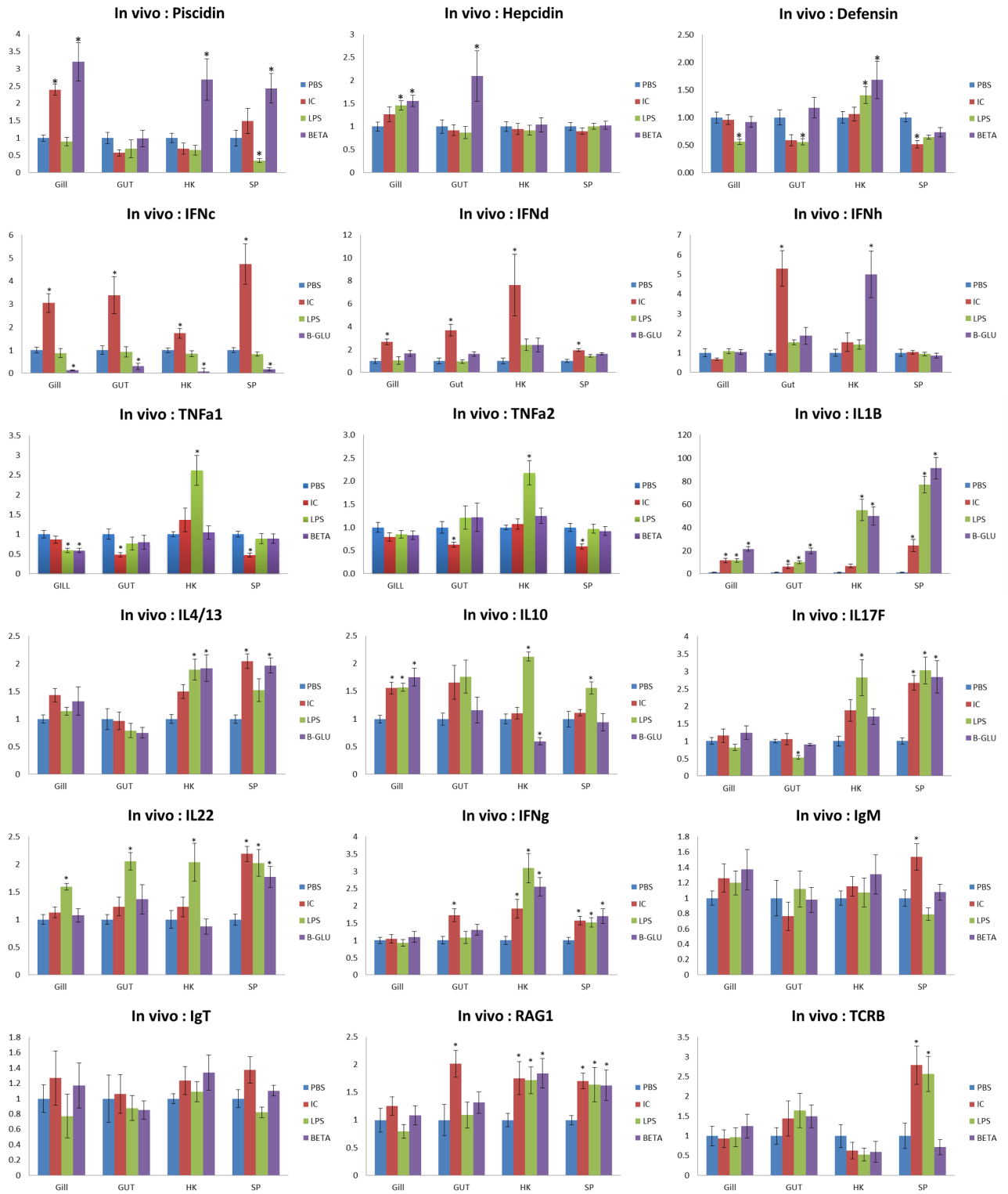


**Figure 1**. Fold change in immune gene expression following *in vivo* PAMP stimulation. The graphs show the modulation of key target immune genes in the gills, mid-gut, head kidney (HK) and spleen (SP) following injection with poly I:C (IC - red), LPS (green) and β-glucan (purple) for 24 h. Target genes were normalized against GAPDH and the data are presented as the mean of 10 fish ± SEM. Groups statistically different from the PBS injected control fish ($P<0.05$) are indicated by an asterisk.

*In vitro PAMP stimulation*

The expression profiles of AMPs, cytokines and Ig related genes following *in vitro* stimulation resemble the *in vivo* stimulation profiles, but with differences in the temporal expression, as seen in **Figures 2-5**. Typically, the expression profiles *in vivo* match the 4 and 12 h post stimulation *in vitro* expression profiles, which is likely the result of a delayed response *in vivo* due to the time taken for the antigen to reach particular tissues.

With gill cell suspensions (**Figure 2**), poly I:C again upregulated the type I IFN genes, although with different kinetics (IFNc and IFNh at 4 h, IFNd at 24 h). It also upregulated hepcidin, defensin, TNFα1 and IL-10 at 4 h, but no other effects were seen. In contrast LPS induced upregulation of piscidin at 4h and 12h, and defensin at 12 h. It also upregulated TNFα1 (but not TNFα2 – Milne et al., 2017) at all time points, and IL-4/13, IL-10, IL-17A/F and IL-22 at 12 h, with IL-17A/F also induced at 4 h post-stimulation. Lastly, β-glucan had relatively little impact but upregulated piscidin and TNFα1 at 4 h, and IL-22 at 4 h and 12 h.

Gut cells (**Figure 3**) also showed a good induction of the type I IFN genes with poly I:C treatment, at 24 h. IL-22 was the only other gene upregulated by poly I:C. LPS was a more potent stimulus, and upregulated a large number of immune genes at 12 and 24 h post-stimulation, as seen with IL-17A/F, IL-22, IgM and IgT at 12 h and hepcidin, defensin, TNFα1, TNFα2, IL-1β, IL-4/13, IL-10, IL-17A/F, IFNγ, IgT and TCRβ at 24 h. β-glucan was more varied, and affected RAG1 at 4 h, defensin, IL-22 and IgM at 12 h, and TNFα2, IL-1β, IL-17A/F, and TCRβ at 24 h.

In the case of HK cells (**Figure 4**), poly I:C was a rapid inducer of type I IFNs (as with gill cells), but had a later effect on piscidin, TNFα2, IL-4/13, IL-22, and IFNγ (24 h). LPS was again a potent inducer of proinflammatory genes such as TNFα1, TNFα2 and IL-1β, but with inhibition of the former by 24 h. Adaptive cytokines were also induced at 4 h, as with IL-17A/F, IL-22 and IFNγ, the latter also upregulated at 12 h. β-glucan induced a rapid increase in piscidin and hepcidin, but with a later effect on defensin. It also induced TNFα2, IL-1β, IL-17A/F and IL-22 at 4 h, with IL-17A/F and IL-22 remaining elevated to 24 h. Lastly, it induced IL-4/13 and IFNγ at 12 h and IgT and RAG1 at 24 h.

**Figure 5** shows the responses of spleen cells. Poly I:C again mainly affected the IFN genes, both type I and II, but with an early (4 h) induction of TNFα2 and IL-1β, and later induction of IL-4/13 (12 h) and IL-17A/F (24 h). LPS induced TNFα2, IL-1β, IL-10 and IFNγ at 4 h post-stimulation, IL-1β, IL-10 and TCRβ at 12 h, but only piscidin and TCRβ by 24 h. Similarly, β-glucan induced an early upregulation of TNFα2 and IL-1β, in addition to IL-4/13, IL-17A/F, IFNγ, IgM and IgT. However, by 24 h only IFNd, IL-17A/F and TCRβ were found upregulated in the splenocytes.

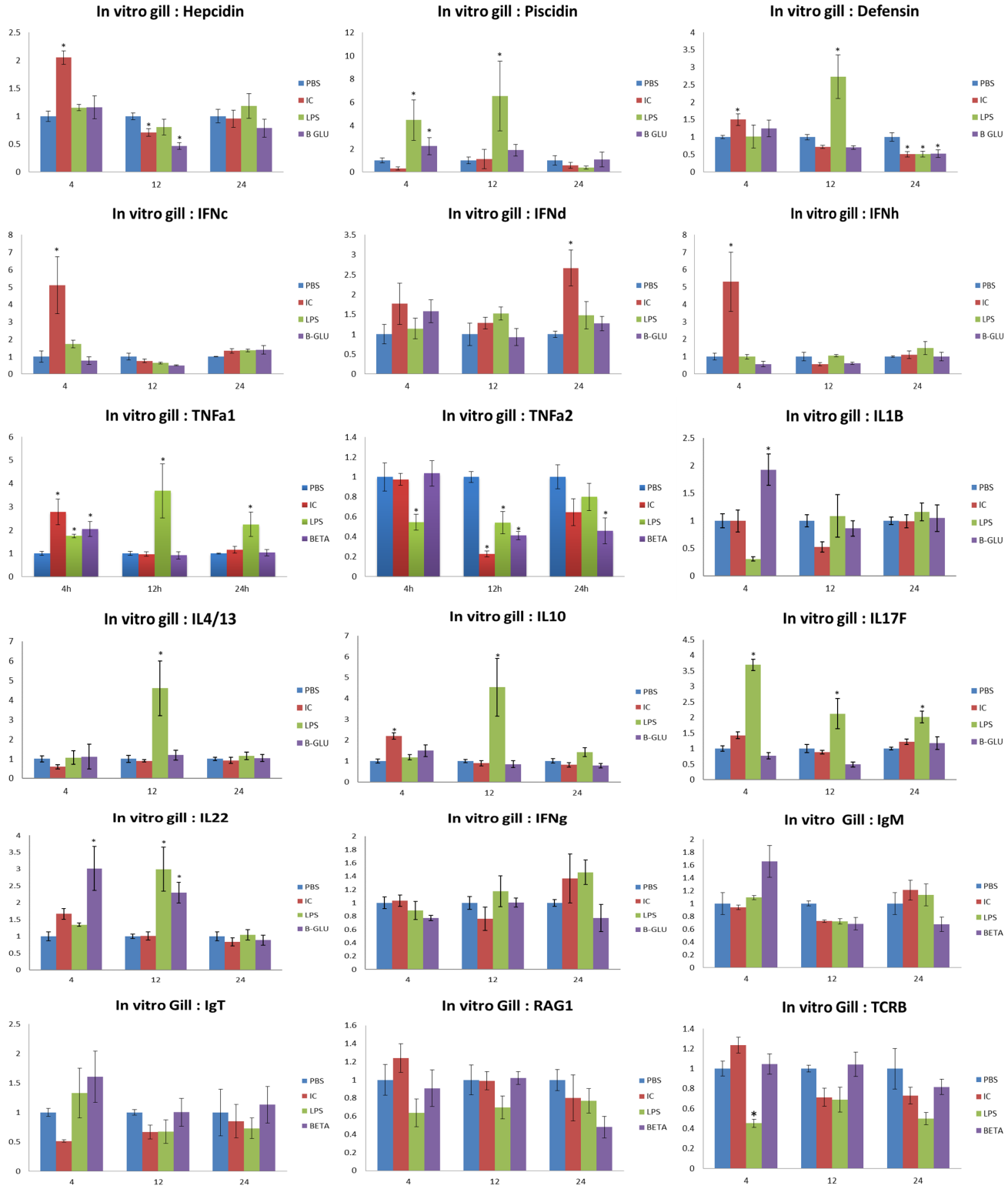


**Figure 2.** Fold change in gene expression following *in vitro* PAMP stimulation of gill cells. The graphs show the fold change in expression of key target immune genes following stimulation with poly I:C (red), LPS (green) and beta glucan (purple) after 4, 12 and 24 h. Expression of the target genes was normalized against GAPDH and data presented as the mean of cell cultures from 10 fish ± SEM. Groups statistically different from the respective PBS treated cells ($P<0.05$) are indicated by an asterisk.

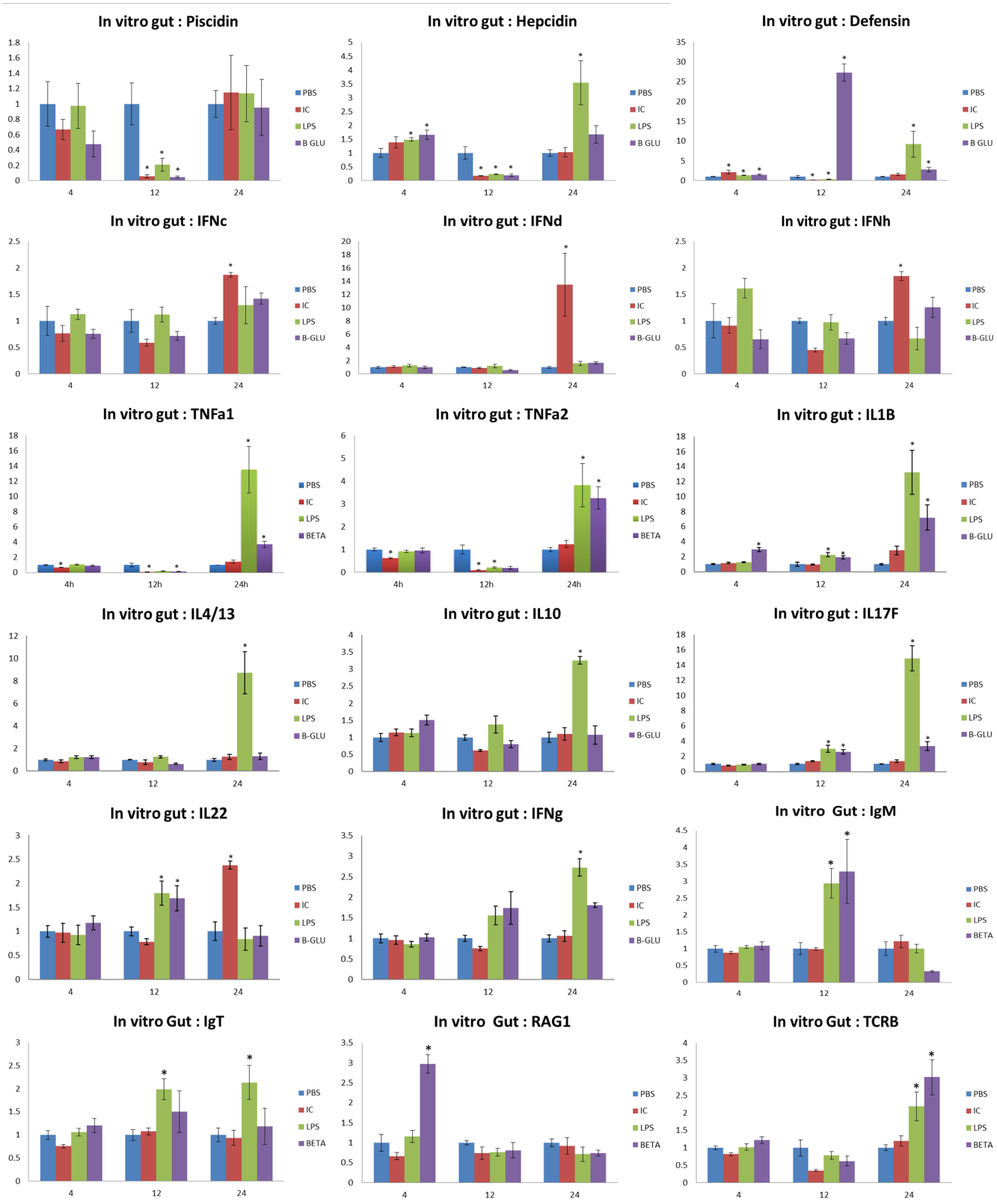


**Figure 3.** Fold change in gene expression following *in vitro* PAMP stimulation of gut cells. The graphs show the fold change in expression of key target immune genes following stimulation with poly I:C (red), LPS (green) and beta glucan (purple) after 4, 12 and 24 h. Expression of the target genes was normalized against GAPDH and data presented as the mean of cell cultures from 10 fish ± SEM. Groups statistically different from the respective PBS treated cells ($P<0.05$) are indicated by an asterisk.

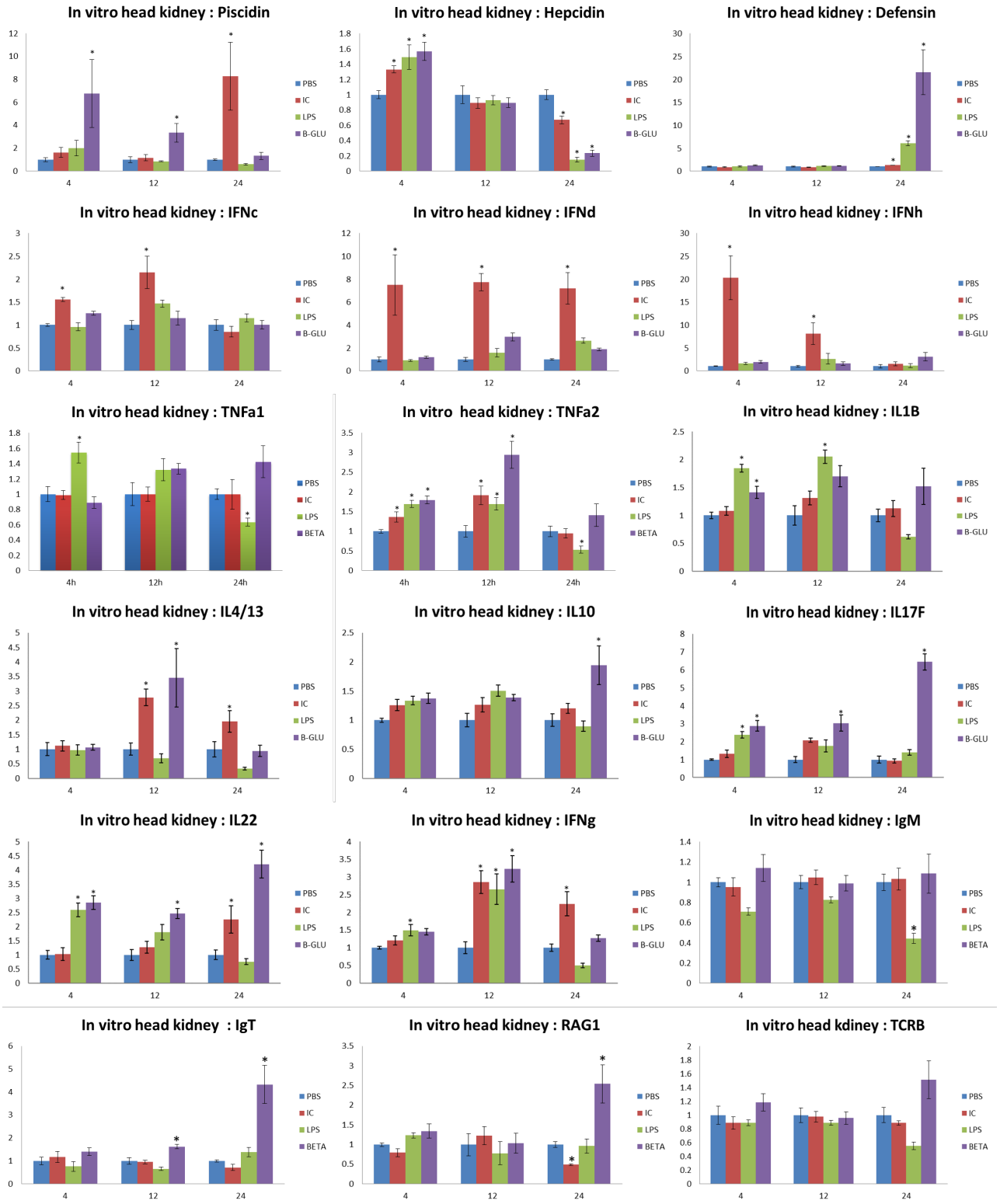


**Figure 4.** Fold change in gene expression following *in vitro* PAMP stimulation of head kidney cells. The graphs show the fold change in expression of key target immune genes following stimulation with poly I:C (red), LPS (green) and beta glucan (purple) after 4, 12 and 24 h. Expression of the target genes was normalized against GAPDH and data presented as the mean of cell cultures from 10 fish ± SEM. Groups statistically different from the respective PBS treated cells ($P<0.05$) are indicated by an asterisk.

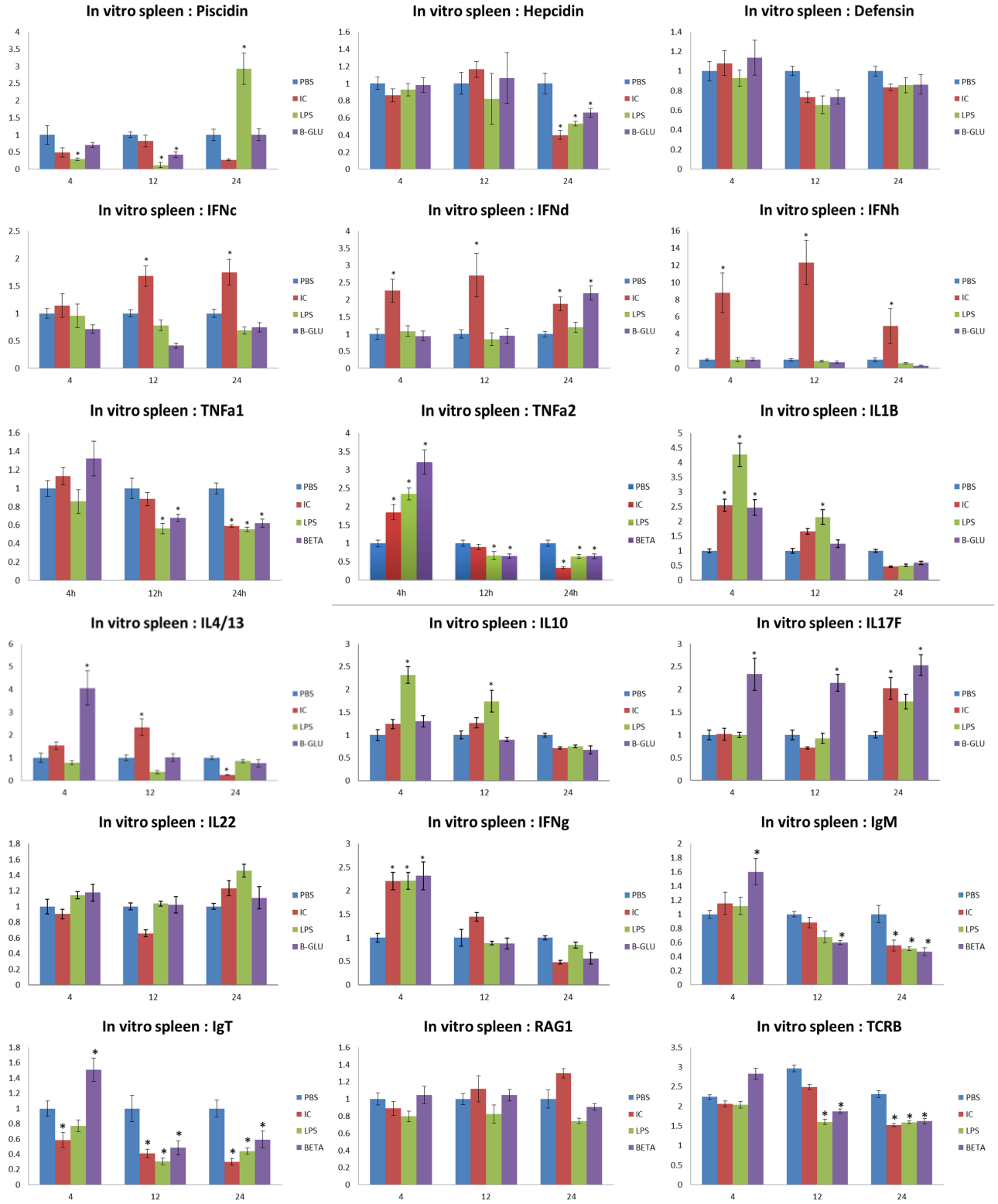


**Figure 5.** Fold change in gene expression following *in vitro* PAMP stimulation of spleen cells. The graphs show the fold change in expression of key target immune genes following stimulation with poly I:C (red), LPS (green) and beta glucan (purple) after 4, 12 and 24 h. Expression of the target genes was normalized against GAPDH and data presented as the mean of cell cultures from 10 fish ± SEM. Groups statistically different from the respective PBS treated cells ($P<0.05$) are indicated by an asterisk.

**4. Discussion**

It is clear that the meagre immune genes examined in this Deliverable are capable of being modulated by a variety of pathogen-derived molecules (PAMPs), with both viral and bacterial mimics studied here. In the *in vivo* stimulus trial clear differences between the different PAMPs were apparent, for example with the viral PAMP poly I:C being a strong inducer of IFN genes, whilst LPS and β-glucan more highly upregulated proinflammatory genes such as TNFα and IL-1β. β-glucan was also the most potent stimulant regarding AMP upregulation. These differential responses make sense in terms of the types on immune responses that must be elicited in response to these pathogen types, and show that meagre immune cells can rapidly induce appropriate innate responses *in vivo* when stimulated.

Some interesting tissue-specific responses were highlighted in these studies, for many of the genes studied. Regarding the AMP response, whilst piscidin had a relatively large tissue distribution, it was not expressed in gut, where only hepcidin was upregulated by the PAMPs studied. The gut was also the only site where IFNh was upregulated by poly I:C. With the adaptive immune genes there was a tendency for the responses to be in the HK and spleen, major sites for lymphocyte activation. This was especially apparent with marker genes for Th1, Th2 and Th17 responses, as with IL-4/13 (Th2), IL-17A/F (Th17), IL-22 (Th17) and IFNγ (Th1) (Wang & Secombes, 2013). However, generally there was little effect on Ig transcript level and the TCR was only affected in spleen (along with RAG expression). The fact that there was also some responsiveness in the gills/gut to IL-22 is in line with other studies in fish (Zhang et al., 2014; Costa et al., 2013) and highlights the importance of this cytokine for mucosal and systemic defense. These differences found likely relate to differences in cellular composition of the tissue studied, with different leucocyte types being present and even endothelial cells being a possible source of some proinflammatory cytokines. One particularly interesting tissue difference was the upregulation of IL-10 in the gills by all three PAMPs. IL-10 is known as a down-regulator of inflammatory responses (Piazzon et al., 2015), and may serve to prevent the gills being compromised in the long term (hours to days) from immune responses at this site that could result in a loss of function and potential death of the fish.

The *in vitro* responses mirrored to a certain degree the *in vivo* responses, although the kinetics were sometimes at odds (typically earlier), possibly reflecting the time it takes the PAMPs to reach some of the tissues after an injection, or that *in vivo* there may be a single pulse exposure to the PAMPs compared to the continuous exposure of the cells during the *in vitro* experiment. Nevertheless, it was again apparent that PAMPs such as poly I:C consistently increased IFN expression with the different cell suspensions, whilst PAMPS such as LPS and β-glucans were more effective at upregulating AMPs, proinflammatory genes and cytokines associated with antibacterial defences (eg IL-17A/F and IL-22). Some differences in kinetics were seen between genes when using particular cell suspensions, and between the tissue sources of the cells. Thus, in gill cells IFNc and IFNh were induced at 4h post stimulation with poly I:C, whilst IFNd was not induced until 24 h. Such subtype specific differences have been noted in other species (Zou et al., 2014), and may allow for more prolonged antiviral defenses to be maintained at this site, or are a means to avoid viral suppressive mechanisms interfering with IFN induction at an important site of pathogen entry. Similarly, in the HK, β-glucan induced piscidin early (4 h and 12 h), but defensin was not induced until 24 h, and in this case may prolong the antibacterial response. With the proinflammatory genes (TNFα, IL-1β), LPS and β-glucan induced increases in gut cells at 24 h, whereas in splenocytes responses (if they occurred) were earlier, and by 24 h were inhibited. Differences between cells from mucosal (gills/gut) and systemic (HK/ spleen) compartments were also apparent with some of the adaptive immune genes, with LPS being a more potent inducer of IL-17A/F in gill cells but β-glucan being more potent in splenocytes. All three PAMPs could induce IFNγ in HK and spleen cells whilst only LPS with gut cells showed induction. Curiously LPS was a good inducer of Igs (M and T) in gut cells, but not in the other cell suspensions.

To conclude, this data shows that the meagre immune response to PAMPs (poly I:C, LPS and β-glucan) is robust and can be tailored to the potential type of pathogen that is encountered. The responses vary by tissue, likely linked to the cell composition present at different immune sites, and the need for immediate or more prolonged responses. These data show the potential to modulate immune responses in meagre in culture, such as by delivery of immunostimulants, to enhance particular immune pathways at a time of disease risk.

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## Deviations

None. However, this Deliverable has focused on PAMPs as a means to study immune gene modulation rather than pathogen-infection. Both were possible models in the DoW.

Co-funded by the Seventh Framework Programme of the European Union