

# Immunomodulatory properties of Tamm-Horsfall glycoprotein (THP) and uromodulin

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## Summary

**Background:** Tamm-Horsfall glycoprotein (THP) and uromodulin are the most abundant glycoproteins in non-pregnant women's/men's and pregnant women's urine, respectively. However, the bioactivities of these glycoproteins are still unclear.

**Objective:** To evaluate the immunomodulatory properties of THP and uromodulin on human peripheral blood mononuclear cells (PBMC)

**Methods:** THP and uromodulin isolated with diatomaceous earth filtration were subjected to several bioassays, such as MTS viability assay, immunophenotyping and cytokine analysis.

**Results:** MTS viability assay and immunophenotyping analysis showed that uromodulin has greater inhibitory activities in suppressing PBMC viability and the percentage of CD4<sup>+</sup> T helper cells and CD8<sup>+</sup> cytotoxic T cells, compared to that of THP. In cytokine analysis, THP tended to induce pro-inflammatory cytokines such as IL-1 $\beta$ , TNF and Th1 cytokine IFN- $\gamma$ ; while uromodulin only induced IL-1 $\beta$  and suppressed both Th1 cytokine IFN- $\gamma$  and Th2 cytokine IL-10.

**Conclusion:** These results demonstrate that uromodulin has greater immunosuppressive activities and lower inductive property in relation to activation of immune cells, which provides a more tolerant environment for the developing fetus. (*Asian Pac J Allergy Immunol* 2015;33:26-32)

**Keywords:** cytokine, immunophenotyping, immunosuppressive, MTS assay, Tamm-Horsfall glycoprotein, uromodulin

## Introduction

Cytokines are soluble mediators between both immune cells and non-immune cells that regulate a wide range of biological processes such as in immunity, inflammation, suppression, organ development, cell differentiation, survival and tissue repair.<sup>1,2</sup> As cytokines are produced in all immune responses, the activation or suppression of immune response can be determined through the concentration of secreted cytokines. In the case of microbial infections, monocytes and macrophages are activated with increased secretion of IL-1, IL-6 and TNF.<sup>3-5</sup> Production of these pro-inflammatory cytokines serves to recruit and activate T cells and other cells to mount adaptive immune responses. On the other hand, T regulatory cells (Treg) and anti-inflammatory cytokines such as IL-10 and TGF- $\beta$  are produced to control the magnitude of the inflammatory response and to prevent an overwhelming immune response.<sup>6-9</sup>

Tamm-Horsfall glycoprotein (THP) is the most abundant glycoproteins in non-pregnant women's and men's urine.<sup>10,11</sup> This glycoprotein is thought to protect the kidney from urinary tract infection,<sup>12</sup> renal papillary calcification and ureteral stone formation.<sup>13</sup> It also serves as a regulatory factor of innate and adaptive immunity,<sup>14</sup> or as a chemoattractant, activating neutrophils and increases the phagocytosis activity.<sup>15,16</sup> On the other hand, uromodulin is isolated from pregnant women's urine and has a similar protein backbone to that of Tamm-Horsfall glycoprotein but is differentially glycosylated.<sup>17-19</sup> Uromodulin has been reported to be a 13-fold more active inhibitor in suppressing the antigen-specific T cell proliferation *in vitro* compared to its counterpart, THP.<sup>20</sup> However, the effects of THP and uromodulin on cytokine secretion of immune cells are largely unidentified. In this study, we examined the effects of these glycoproteins on PBMC viability, the

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percentage of immune cell subsets, pro- and anti-inflammatory cytokines secretion in healthy human.

## Methods

### Sample collections

Institutional ethics approval and individual consent was obtained. First morning urine specimens were collected weekly from a healthy non-pregnant woman and a healthy pregnant woman over a period of 8 weeks. The urine volume was measured and it was neutralized with 1 M HCl or 1 M NaOH to pH 7.0.

### Isolation and purification of THP and uromodulin

THP and uromodulin were isolated from non-pregnant and pregnant women's urine, respectively, using the diatomaceous earth filtration method described by Serafini-Cessi *et al.*<sup>21</sup> Briefly, diatomaceous earth suspended in deionized water was poured into Büchner funnel, lined with no.1 Whatman filter paper. Layer of diatomaceous earth were washed with deionized water and a 0.02 M sodium phosphate buffer containing 0.14 M NaCl (PBS). Urine was poured carefully into the funnel and filtered through the diatomaceous earth layer. At the end of filtration, the diatomaceous earth layer was washed with PBS exhaustively. Then, the layer was scrapped off from the filter paper and suspended in deionized water, with occasional stirring for 30 minutes. The suspension was centrifuged at 20 000 x g for 20 minutes and pellet was discarded. The supernatant was brought to the salt concentration of PBS and left for 20 minutes at room temperature. Then, the mixture was filtered once again through a new diatomaceous earth layer. The layer was washed exhaustively with PBS and suspended in deionized water to extract the protein. Finally, the supernatant from centrifugation was collected, dialyzed against water at 4°C for 48 hours and freeze-dried. Protein content of THP and uromodulin was determined by a Bradford assay. Crude proteins were purified with Sephadex G-100 chromatography using 50 mM ammonium bicarbonate (AmBic), pH 8.5 as the eluting buffer.

### Isolation of peripheral blood mononuclear cells (PBMC)

Blood samples were obtained from healthy individuals selected randomly from postgraduate students in the Universiti Putra Malaysia with their consent. Ethics approval was obtained from Medical Research Ethics Committee, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia.

PBMC were isolated from heparinized whole blood samples using density gradient centrifugation. Briefly, diluted blood was gently layered over Ficoll-Paque™ PLUS (specific density 1.077 g/L, GE Healthcare, Sweden) and centrifuged at 2,500 rpm for 20 minutes at 10°C. PBMC were aspirated from the buffy coat at the interface, washed twice and re-suspended in complete RPMI 1640 medium, containing 10% FBS, 1% Penicillin-Streptomycin, 0.5% Amphotericin and 0.1% Gentamicin (all of the antibiotics used were obtained from Gibco, Invitrogen, USA). Cells were maintained in an incubator at 37°C supplied with 95% humidified air and 5% CO<sub>2</sub>.

### MTS assay

Cell viability was determined using the 72 hours-MTS assay (Promega, USA), according to the manufacturer's protocol. PBMC at 200,000 cells/well were induced with 1 µg/ml PHA-L (phytohaemagglutinin-L) and treated with different concentrations of THP and uromodulin for 72 hours at 37 °C, 5% CO<sub>2</sub>. The absorbance for the formazan product was measured at 490 nm. The percentage of cell viability was calculated based on the equation:

$$\text{Percentage of cell viability} = \frac{A_t - A_b}{A_c - A_b} \times 100\%$$

$A_t$  = Absorbance for PBMC induced with PHA + extracts (treatment)

$A_b$  = Absorbance for culture medium (RPMI 1640) alone (background)

$A_c$  = Absorbance for PBMC induced with PHA only (positive control)

### Immunophenotyping of PBMC surface markers

PBMCs at  $1 \times 10^6$  cells/well were induced with 1 µg/ml PHA-L (phytohaemagglutinin-L) and treated with or without 250 µg/ml of THP/ uromodulin for 72 hours at 37 °C in 5% CO<sub>2</sub>. Treated PBMCs were then analyzed for phenotyping. A volume of 100 µl PBMCs containing  $1 \times 10^6$  cells was mixed and incubated with appropriate quantities of monoclonal antibodies anti-human CD4 labeled PerCP (clone SK3), anti-human CD8 labeled FITC (clone SK1) and anti-human CD14 labeled APC (clone MφP9). All antibodies were from BD Biosciences. After 30 minutes incubation in the dark, the samples were fixed with 2% paraformaldehyde and analysed with BD FACSDiva software on a LSRFortessa flow

cytometer (BD Bioscience). The cell analysis and gates were restricted to lymphocytes or monocytes, while 10000 gated events were recorded for each sample. The results were expressed as a percentage of expression compared to the control.

#### **Quantification of PBMC cytokines using a CBA (Cytometric Bead Array)**

PBMCs at 200,000 cells/well were induced with 1  $\mu\text{g/ml}$  PHA-L (phytohaemagglutinin-L) and treated with or without 375  $\mu\text{g/ml}$  of THP/uromodulin for 72 hours at 37  $^{\circ}\text{C}$  in 5%  $\text{CO}_2$ . Cytokine production in treated PBMC was analyzed in the supernatants of cells cultured using Cytometric Bead Arrays (BD Biosciences). IL-1 $\beta$ , IL-10, TNF and IFN- $\gamma$  were detected using the human inflammation CBA Kit (Cat. No. 551811; BD Biosciences) and T helper (Th) 1/Th2 Cytokine Kit II (Cat. No. 551809; BD Biosciences). Tests were performed according to the manufacturer's instructions. Briefly, a volume of 50  $\mu\text{l}$  of mixed capture beads, 50  $\mu\text{l}$  of supernatants/ standards and 50  $\mu\text{l}$  of PE detection reagent were mixed well and incubated in the dark for 3 hours at room temperature. A standard curve was generated for each set of experiments. Acquisition of samples and standards was performed with the BD FACSDiva software on a LSRFortessa flow cytometer (BD Biosciences). Data analysis was performed using a BD FCAP Array<sup>TM</sup>. The results were expressed as percentage of expression compared to the control. The lower limit of detection for each cytokine was 20  $\text{pg/ml}$ .

#### **Statistical analysis**

The data in this study were represented as mean  $\pm$  S.D. The comparison between two groups was performed by one-way ANOVA (SPSS). A  $p$  value  $< 0.05$  was considered as statistically significant.

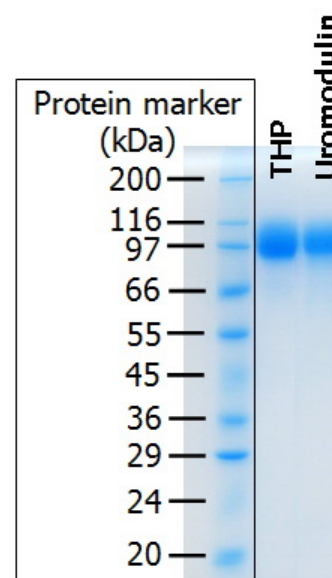
## **Results**

#### **Isolation of THP and uromodulin**

Crude THP and uromodulin isolated by diatomaceous earth filtration were further purified on Sephadex G-100 size exclusion column equilibrated in 50 mM AmBic buffer. SDS-PAGE indicated that THP and uromodulin have molecular sizes of approximately 97 kDa (Figure 1).

#### **MTS viability assay**

The higher cytotoxic activity of uromodulin on PBMC was observed in a 72-hours MTS assay, indicating the higher immunosuppressive activity in uromodulin. As shown in Figure 2, 250  $\mu\text{g/ml}$  of

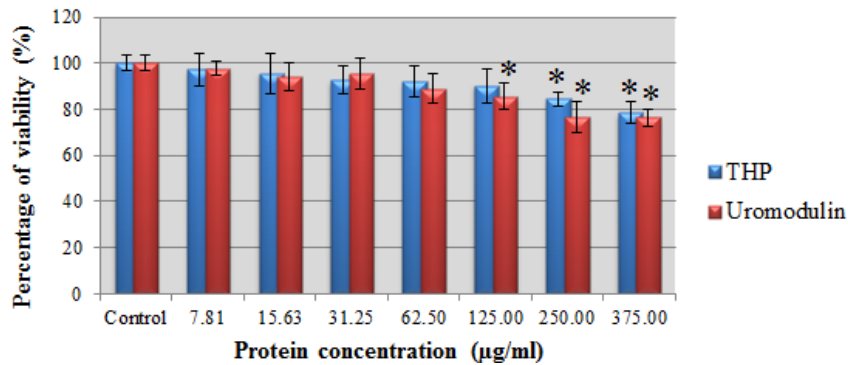


**Figure 1.** 10% SDS-PAGE analysis of THP (Lane 2), uromodulin (Lane 3) and protein marker (Lane 1, SigmaMarker<sup>TM</sup> S8445). THP and uromodulin both have a molecular weight of approximately 97kDa.

THP reduced PBMC viability to  $84.59 \pm 2.94\%$ , while the same concentration of uromodulin significantly decreased the PBMC viability to  $76.75 \pm 6.95\%$ . A concentration of 375  $\mu\text{g/ml}$  of THP and uromodulin significantly decreased PBMC viability to  $78.65 \pm 4.87\%$  and  $76.40 \pm 3.80\%$  respectively. It is worth noting that, a concentration of 125  $\mu\text{g/ml}$  of uromodulin statistically suppressed the PBMC viability to  $85.64 \pm 5.92\%$  but not THP.

#### **Immunophenotyping assay**

T cells subsets, such as T-helper ( $\text{CD4}^+$ ), T-cytotoxic ( $\text{CD8}^+$ ) and monocytes ( $\text{CD14}^+$ ), were phenotyped by flow cytometric analysis using monoclonal antibodies. Figure 3 shows that 250 $\mu\text{g/ml}$  of both THP and uromodulin significantly suppressed the  $\text{CD4}^+$  T helper cells to  $20.92 \pm 3.90\%$  and  $20.73 \pm 4.51\%$ , respectively, compared to the control groups (cells +PHA) at  $29.54 \pm 2.81\%$ . Similarly, the percentage of  $\text{CD8}^+$  for THP- and uromodulin-treated group was  $15.01 \pm 2.86\%$  and  $14.17 \pm 3.52\%$ , respectively, comparing the untreated activated control group at  $20.07 \pm 2.09\%$ . The percentage of  $\text{CD14}^+$  monocytes was significantly reduced from  $5.94 \pm 2.05\%$  (cells +PHA) to  $1.95 \pm 0.46\%$  and  $3.38 \pm 1.17\%$  after treatment with THP and uromodulin, respectively. Nonetheless, uromodulin was found to have a stronger immunosuppressive effect as compared to THP.



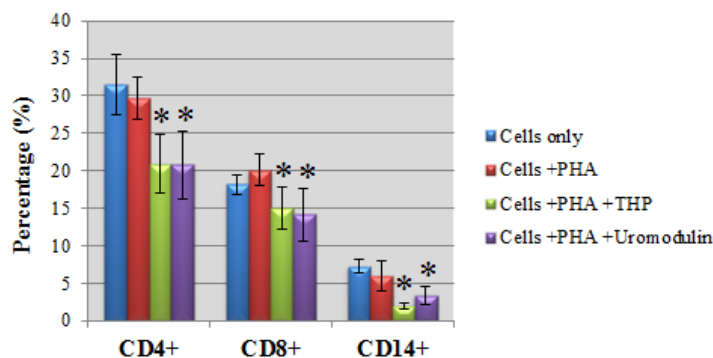
**Figure 2.** PHA-activated PBMCs viability with the stimulation by THP and uromodulin for 72-hours. The control group consisted of PHA-activated PBMCs. Uromodulin concentrations in the range from 125µg/ml to 325µg/ml significantly suppressed PBMC viability compared to untreated controls. (\*denotes  $p < 0.05$ , one-way ANOVA). Results are expressed as mean  $\pm$  S.D. of triplicate wells in a 96-well plate and are representative of six independent experiments.

**Effects of THP and uromodulin on cytokine production**

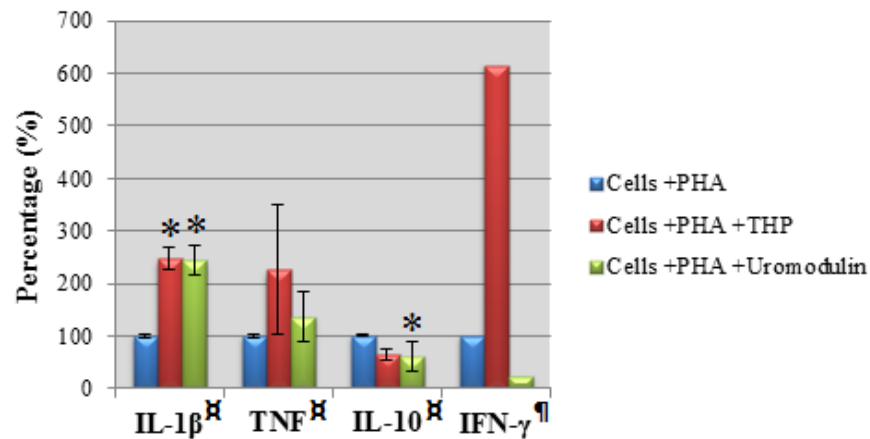
The cytokine profile of PBMCs treated with 375 µg/ml of THP and uromodulin was studied (Figure 4). Upon treatment with these glycoproteins, the IL-1 $\beta$  secretion was significantly increased to  $247.17 \pm 21.02\%$  and  $243.61 \pm 27.23\%$ , respectively. IL-10 was reduced by both THP and uromodulin to  $65.18 \pm 11.53\%$  and  $60.67 \pm 27.98\%$ , respectively, with the reduction by uromodulin being significant. Although not significant, THP tended to increase the TNF (to  $226.40 \pm 123.74\%$ ) and IFN- $\gamma$  production (to  $615.53\%$ ), while uromodulin either maintained (TNF at  $136.39 \pm 47.90\%$ ) or suppressed (IFN- $\gamma$  at  $23.83\%$ ) these cytokines production.

**Discussion**

Until recently the exact role of uromodulin in promoting toleration of the fetus during pregnancy was still unclear, though various immunosuppressive activities have been reported, such as inhibition of lymphocyte proliferation and its role as an IL-1 inhibitor.<sup>20,22</sup> Our MTS assay showed that THP and uromodulin significantly suppress PHA-activated PBMCs viability at 250 µg/ml and 125 µg/ml, respectively. The suppression of PBMC cell viability could be the result of an increase in cell death and/or a decrease in cell proliferation. Further investigation showed that THP and uromodulin suppress the CD4, CD8 and CD14 expression of PBMCs, indicating that T helper, cytotoxic T cells



**Figure 3.** CD4, CD8 and CD14 expression in PBMCs after treatment with 250 µg/ml of THP and uromodulin. Both THP and uromodulin significantly suppressed CD4<sup>+</sup> T helper cells, CD8<sup>+</sup> and CD14<sup>+</sup> monocytes compared to the control groups (PHA activated PBMC). Results are expressed in mean  $\pm$  S.D. of six independent experiments. \* denotes  $p < 0.05$  (one-way ANOVA).



**Figure 4.** Serum cytokine profile of PBMCs after stimulation with 375  $\mu$ g/ml of THP and uromodulin. (\*denotes  $p < 0.05$  one-way ANOVA). The control group consisted of untreated PHA-activated PBMC. Results are expressed in mean  $\pm$  S.D. of three ( $\square$ ) or one ( $\blacksquare$ ) independent experiment(s).

and monocytes were suppressed by both glycoproteins. Uromodulin had higher inhibitory activity in suppressing CD4<sup>+</sup> and CD8<sup>+</sup> cells while THP had greater effect in suppressing CD14<sup>+</sup> monocytes. The reduced percentage of CD4<sup>+</sup>, CD8<sup>+</sup> and CD14<sup>+</sup> cells could partially due to the decreased T cells and monocytes viability in PBMC, as shown in the MTS assay (Figure 2). THP and uromodulin may also suppress the CD4, CD8 and CD14 percentage by reducing the number of the cells, decreasing the cell proliferation rate or increasing the cell apoptosis.

IL-1 $\beta$  is one of the cytokines mediating autoinflammatory responses and it is mainly produced by activated monocytes and tissue macrophages.<sup>5,23</sup> The present study showed that IL-1 $\beta$  secretion is increased in PBMCs treated with THP and uromodulin, a finding in agreement with earlier data from Su and Yeh.<sup>24</sup> We suggest that the binding and activation of THP/uromodulin on monocytes is through the binding of monocyte CD14 as these glycoproteins were shown to interact with soluble CD14 in serum.<sup>24</sup> On the other hand, a study by Darisipudi et al.<sup>25</sup> reported the release of IL-1 $\beta$  in response to THP/ uromodulin was initiated when monocytes phagocytosed the glycoproteins, followed by activation of the NLRP3 inflammasome, its linker protein ASC and caspase-1. Tumour necrosis factor (TNF), mainly produced by activated macrophages is a pro-inflammatory and pleiotropic cytokine, in that it mediates a wide variety of biologic activities.<sup>4,26</sup> THP, but not uromodulin, increased TNF secretion, though the

increment was not statistically significant. Recently, the induction of TNF secretion by THP has been shown to be Toll-like receptor (TLR) 4-dependent, involving the activation of NF- $\kappa$ B in immature dendritic cells (DCs).<sup>15</sup> By inducing production of pro-inflammatory cytokines, such as IL-1 $\beta$  and TNF, THP might be able to protect the kidney through its immunostimulatory properties by activation of the host innate immune system, such as monocytes.<sup>27</sup> On the other hand, uromodulin tended to be more immunosuppressive than THP, in that it induced IL-1 $\beta$  only and withheld TNF secretion. This could be one of uromodulin's fetomaternal defences in tolerating the fetus as a semi-allograft (by maintaining TNF production) while protecting the kidney from diseases (by inducing IL-1 $\beta$  production).

IL-10 is a cytokine with anti-inflammatory properties and always associated with Th2 cells.<sup>28</sup> Both glycoproteins suppressed IL-10 secretion although the decrement by THP was not statistically significant. The suppression of IL-10 by THP could be partially due to the increment of IFN- $\gamma$ , because IL-10 and IFN- $\gamma$  are antagonistic to each other.<sup>29,30</sup> On the other hand, suppression of IL-10 by uromodulin might be due to its direct effect on the IL-10 production because uromodulin has the ability to delay Th2 differentiation, and it has a greater inhibitory effect in decreasing the cell viability of CD4<sup>+</sup> T and CD8<sup>+</sup> cells in PBMCs, in that these cells are able to secrete IL-10. IFN- $\gamma$  is secreted by many cell types such as T cells, NK cells and

monocytes. It is always associated with Th1 cells because it skews the immune response towards a Th1 phenotype.<sup>31,32</sup> It has been observed that the effect of THP and uromodulin on IFN- $\gamma$  secretion is different, in that THP induced IFN- $\gamma$  secretion, while uromodulin suppressed it. This observation suggests that THP might skew the CD4<sup>+</sup> T cells differentiated into Th1 phenotype during inflammation, while uromodulin suppressed this process. The fact that uromodulin was able to suppress both Th1 (IFN- $\gamma$ ) and Th2 (IL-10) cytokines, indicating that uromodulin has higher immunosuppressive activity with minimum immune response. This observation may well have importance implications during pregnancy.

During pregnancy it is scientifically mysterious that the fetus, which carries both maternal and paternal genes, is not rejected by the mother as an allograft. Therefore, the maternal body system must have undergone some modifications so that the maternal allogeneic response is suppressed and the fetal semi-allograft is tolerated in the maternal body.<sup>33</sup> These modifications of the maternal body during pregnancy include functional and structural adaptations in the urinary system to accommodate the increment of renal blood flow, nutrient and waste exchange from the fetus.<sup>34,35</sup> Hence, in this study, it is suggested that uromodulin is secreted abundantly from the kidney during pregnancy and might undergo alteration such that it has higher immunosuppressive activity and a minimal immune response, compared to that of THP. Pregnancy-associated glycosylation changes in uromodulin are most likely to play an important role in its advanced immunosuppressive activities.<sup>18,19</sup>

In conclusion, this study proved that uromodulin has higher suppressive and lower inductive properties in relation to activation of immune cells (PHA-activated PBMCs) which provides a safer and more tolerant environment for the fetus to develop.

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