

# ***Toxoplasma gondii* profilin does not stimulate an innate immune response through bovine or human TLR5**

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## **Abstract**

*Toxoplasma gondii* is responsible for one of the most prevalent infections in people. *T. gondii* profilin (TgPr) is a protein integral to parasite movement and cellular invasion. Murine TLRs has been described to bind TgPr. Furthermore, more recently, human TLR5 has been described to recognise recombinant TgPr, as well as bacterial flagellin. In addition to infections in humans, *T. gondii* infects farm animals, but little information is available about its innate recognition. We aimed to investigate whether, similarly to their human orthologue, bovine and porcine TLR5 could also be stimulated by TgPr by using a combination of reporter cell lines expressing full length TLR5 from each species as well as primary cells. Although human and bovine TLR5-transfected cells responded to flagellin, no response was detected upon stimulation with profilin. Furthermore, TgPr failed to elicit IL-6 secretion in human peripheral blood mononuclear cells and CD14<sup>+</sup> monocytes. In contrast, exposure of RAW cells, known to express TLR11, to TgPr slightly increased the IL-6 response. Our data cast doubts on the possibility that profilin is a specific ligand for human TLR5 and bovine TLR5. This leaves the immunogenic properties of this potential target antigen (Ag) uncharacterised outside of the murine system.

## **Keywords**

*Toxoplasma gondii*, profilin, Toll-like receptor 5, bovine

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## **Introduction**

*Toxoplasma gondii* is an obligate intracellular protozoan and arguably the most successful known parasite.<sup>1</sup> It is capable of infecting all mammals and birds, with members of the *Felidae* family serving as its definitive host. *T. gondii* is also widely recorded as the most prevalent infection in humans, with 30–50% of the global population estimated to be infected.<sup>2</sup> Such broad species tropism is undoubtedly a key factor contributing to this wide distribution, as it only increases the likelihood of a human encountering *T. gondii*. Transmission of *T. gondii* occurs through the ingestion of tissue cysts from the raw or undercooked meat of an infected intermediate host, such as pigs or cattle, or environmentally resistant oocysts shed into water, soil or vegetation by previously uninfected felines. Despite the widespread seroprevalence, *T. gondii* rarely presents clinically. Instead, it remains largely asymptomatic in most infected hosts, with symptoms of mild flu observed

occasionally.<sup>3</sup> This ability to maintain a healthy, subclinically infected host is a hallmark of an effective parasite, allowing the host to remain active and prolonging the period during which they may communicate the infection further.

In addition to the threat towards humans, toxoplasmosis is also of concern in livestock as a major cause of production loss.<sup>4,5</sup> In temperate regions around the world, *T. gondii* is often a leading cause of reproductive

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failure in sheep and goats, with complications ranging from weak offspring to mummified or aborted foetuses.<sup>6</sup> Neonatal pigs can also succumb to toxoplasmosis, though ingestion of *T. gondii* is much more frequent than vertical transmission.<sup>7</sup> Acquired infections during adulthood also result in tissue cyst formation; as seen in humans and small ruminants.<sup>8</sup> As pork is the most widely consumed red meat by humans,<sup>9</sup> the impact that porcine toxoplasmosis can have on global health and economy is evident. In contrast to small ruminants, cattle appear to be largely resistant to *T. gondii* infections and rarely present with tissue cysts or aborted pregnancies.<sup>10</sup> Beyond pathology-based findings, these species-specific differences in *T. gondii* infections remain relatively undescribed and further understanding of the molecular interactions involved would greatly benefit the field.

The ability to invade host tissues is one common among parasites of the Apicomplexa phylum and would not be possible without the arrangement of organelles at their apical end, to which they owe their name. Instead of long, stable and filamentous actin polymers, Apicomplexa such as *T. gondii* have been found to form transient, short and unstable actin polymers. Essential to the recruitment and rapid assembly of monomeric actin in *T. gondii* is the protein profilin, which is present in all eukaryotic cells and, more specifically, at the apical end of tachyzoites.<sup>11</sup>

In addition to its role in regulating the actin cytoskeleton, profilin is also passively secreted in a soluble form by tachyzoites through an unknown mechanism and serving an unknown purpose.<sup>12</sup> It is likely because of this that profilin has also been identified as a potent ligand for TLR11 and 12 on murine dendritic cells (DCs),<sup>13</sup> with the response characterised by the production of IL-12 and the subsequent production of IFN- $\gamma$  by activated NK and CD4<sup>+</sup> Helper T-cells (Th1).<sup>14</sup> Knockout studies revealed that this response is dependent on the adapter protein MyD88 and its activation of the NF- $\kappa$ B protein complex, a hallmark of TLR signalling.<sup>15</sup> It is this same response that contains the acute stage of infection by live parasites and forces *T. gondii* into latency, conferring resistance and survival in mice.<sup>16</sup> Understanding the murine mechanisms of innate recognition and resistance to *T. gondii* has been invaluable in furthering the somewhat limited knowledge of the human response to this parasite. Given the distinct association between reactivated *T. gondii* infections and AIDS patients,<sup>17</sup> it became evident that the role of IL-12-dependent Th1 cell-mediated immunity in controlling the infection was conserved throughout evolution. However, genes encoding functional TLR11 and 12 do not exist in other mammalian species,<sup>18</sup> leaving the TLR responsible for eliciting this response unidentified.

In 2014, human TLR5 (huTLR5), a receptor already known to bind bacterial flagellin,<sup>19</sup> was reported to specifically bind profilin, and this interaction seemed to mediate the same production of cytokines as in response to live tachyzoites.<sup>20</sup> In this study, dose-dependent differences in the production of IL-6, IL-8 and IL-12 in both TLR5-transfected human embryonic kidney (HEK) 293 cells as well as *ex vivo* isolated human monocytes were reported, when cells were stimulated with recombinant *T. gondii* profilin (TgPr).<sup>20</sup> Responses to profilin were compared with that induced by flagellin and LPS, and the interaction of TgPr with TLR5 was blocked using either an Ab specific for huTLR5 or by using small interfering RNA.<sup>20</sup> These findings were the first documenting the recognition of soluble *T. gondii* proteins by cells of the human innate immune system, whereas previous studies had concluded that only the recognition of live tachyzoites was possible,<sup>21</sup> and that soluble Ag detection was exhibited only in mice expressing either TLR11 or TLR12.

TLR5 has also been described as the innate sentinel for bacterial flagellin in the bovine immune system.<sup>22</sup> Given the sequence similarities between TLR5 of other mammals,<sup>23</sup> it could be postulated that porcine (po) and bovine (bo) TLR5 may also function as the PRR for TgPr in a manner similar to that reported for huTLR5, and it was hypothesised that boTLR5 may also act as a receptor for profilin. The aim of this study was therefore to assess the binding of recombinant TgPr to bo and poTLR5.

## Materials and methods

### SEAP assay

To detect specific TLR5 stimulation, we used a HEK 293T cell line, stable expressing secreted embryonic alkaline phosphatase (SEAP) reporter (293T/SEAP) and transiently transfected with species-specific full length TLR5 genes. The generation and culture of the 293T/SEAP cell line is described elsewhere.<sup>24</sup> The sequences of hu, bo or poTLR5 have been described before.<sup>22,23</sup> TLR5 of all three species inserted into pUNO, ultrapure *Salmonella* Typhimurium flagellin (FLA-ST) and the alkaline phosphatase substrate QUANTI-Blue were purchased from Invivogen (San Diego, USA). Due to the discontinued production of recombinant purified TgPr by Invivogen, the TgPr used in the present study, as well as phorbol 12-myristate 13-acetate (PMA) and FCS, was obtained from Sigma Aldrich (St. Louis, USA). TurboFect transfection reagent, DMEM and PBS were purchased from ThermoFisher Scientific (Waltham, USA). Tissue culture plates were manufactured by Greiner Bio-One (Kremsmünster, Austria).

The transfection protocol was optimised for the pUNO vector (data not shown) and reactions were set up similarly to as described recently.<sup>24</sup> Briefly, 4 ml 293T/SEAP cell suspension per well was seeded into four wells of a six-well tissue culture plate at  $10^5$  cells  $\text{ml}^{-1}$  density. After allowing cells to attach overnight (18–21 h), the cells were transfected using a mixture of 2  $\mu\text{g}$  vector DNA (either hu, bo or poTLR5-pUNO, or mock) and 8  $\mu\text{l}$  TurboFect reagent in a total of 400  $\mu\text{l}$  DMEM (ThermoFisher Scientific). The mixtures were incubated at 20–25°C for 20 min before dropwise addition to the reporter cells. After a 24 h incubation, the cells were lifted using sterile PBS (ThermoFisher Scientific) and seeded into 96-well flat bottom tissue culture plates in stimulation media (DMEM with 2% FCS) at a density of  $4 \times 10^4$  per well. Specific TLR5 stimulants and controls were set up in duplicates: negative control (media only), FLA-ST at concentration of 10 and 100  $\text{ng ml}^{-1}$ , recombinant TgPr at 0.01, 0.1 and 1  $\mu\text{g ml}^{-1}$  and 200  $\text{ng ml}^{-1}$  PMA. The remainder of the transfected cells were cultured in selection media containing 10  $\mu\text{g ml}^{-1}$  blasticidin.

Supernatants were collected after 48 h, and 10  $\mu\text{l}$  of each sample was added to 200  $\mu\text{l}$  QUANTI-Blue reagent and incubated at 37°C for at least 24 h. Optical densities were recorded at 635 nm with a Tecan Infinite M200 Pro plate reader. Remaining supernatants were frozen until analysis for IL-8 by ELISA (Quantikine, R&D Systems, Minneapolis, USA). Supernatant samples were assayed undiluted and according to the manufacturer's instructions. Optical density values were measured and IL-8 concentrations were calculated using four parameter logistic regression in Prism version 7 (GraphPad Software, San Diego, USA).

### Assays using primary human PBMC and monocytes

Based on the results obtained in the SEAP assays, we proceeded to assay TgPr on primary innate immune cell cultures. Whole blood samples were collected from healthy volunteers after ethical approval was obtained and PBMCs were isolated using density gradient centrifugation as described.<sup>25</sup> Monocytes were isolated from PBMC by Magnetic-Activated Cell Sorting using anti-human CD14 conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) as described.<sup>25</sup>

One hundred microlitres of PBMC or CD14<sup>+</sup> monocytes were seeded at  $10^6$  cells  $\text{ml}^{-1}$  into the wells of a U-bottom 96-well plate (Greiner Bio-One). Stimulation conditions and controls were applied in triplicate, and consisted of either media only, FLA-ST (0.01, 0.1 and 1  $\mu\text{g ml}^{-1}$ ), recombinant FliC (VacciGrade

recombinant *S. Typhimurium* flagellin, Invivogen; 0.01, 0.1 and 1  $\mu\text{g ml}^{-1}$ ), TgPr (0.01, 0.1 and 1  $\mu\text{g ml}^{-1}$ ) or 100  $\text{ng ml}^{-1}$  LPS (*Escherichia coli* K-12; Invivogen). Plates were incubated for 24 h at 37°C, 5%  $\text{CO}_2$ ; thereafter, supernatants were collected and frozen for IL-6 detection by ELISA after 1:10 dilution (Quantikine, R&D Systems). All reagents were tested for their LPS contamination using the EndoSafe PTS device (Charles River Laboratories, Edinburgh, UK) using 0.01–1 EU  $\text{ml}^{-1}$  LPS cartridges.

### Stimulation of murine RAW 264.7 cells with TgPr and LPS

To confirm the biological activity of the TgPr used in our study, the murine monocytic RAW 264.7 cell line, which has been shown to express TLR11 and 12,<sup>26,27</sup> was used. Cells were seeded at  $10^5$  cells per well and incubated with TgPr (0.01, 0.1 and 1  $\mu\text{g ml}^{-1}$ ) and LPS (0.1  $\mu\text{g ml}^{-1}$ , Invivogen) for 24 h. Thereafter, supernatants were harvested and assayed for IL-6 by ELISA (IL-6 Mouse ELISA Kit, ThermoFisher Scientific, Loughborough, UK), according to the manufacturer's instructions.

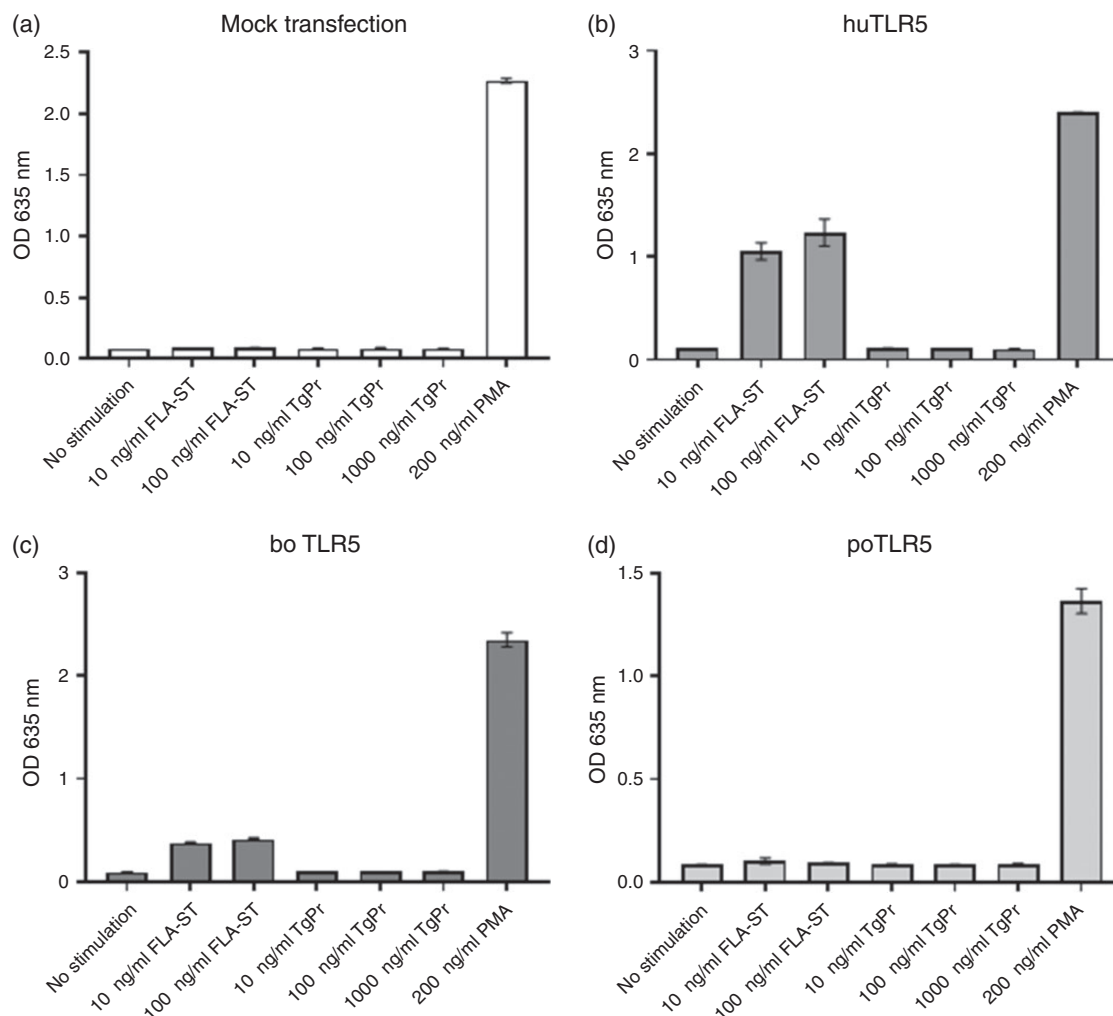
### SEAP assay using HEK293 expressing murine TLR11

To further confirm the biological activity of the TgPr preparation used in the RAW cells, a SEAP assay was performed using a commercially available HEK293 cell line stably expressing both a SEAP reporter and murine TLR11 intracellularly (Mouse TLR11 NF- $\kappa$ B/SEAP SEAPorter cell line; Novus Biologicals), as has been described before.<sup>28</sup> To do so, cells were seeded into 96-well flat bottom tissue culture plates at a density of  $4 \times 10^4$  cells per well. Ligands and controls were set up in duplicate: negative control (media only), recombinant *S. Typhimurium* flagellin (FliC VacciGrade, Invivogen) at concentration of 50 and 500  $\text{ng ml}^{-1}$ , rTgPr at 0.05, 0.5 and 5  $\mu\text{g ml}^{-1}$ , and PMA at 100  $\text{ng ml}^{-1}$ . After 48 h, 20  $\mu\text{l}$  of supernatant was collected and added to 180  $\mu\text{l}$  QUANTI-Blue. This was incubated at 37°C for 4 h before the optical densities were recorded.

## Results

### *T. gondii* profilin does not stimulate a human or bovine TLR5 dependent response

The reporter cell line 293T/SEAP was transfected with either hu, bo, poTLR5 or mock, followed by culturing with a range of ligands and controls (Figure 1). Stimulation with PMA, a non-specific activator of NF- $\kappa$ B, resulted in consistently high SEAP secretion, as



**Figure 1.** Profilin fails to stimulate a TLR5-dependent SEAP response. HEK 293T cells harbouring NF- $\kappa$ B-inducible SEAP reporter genes were transfected with empty plasmid (a) or either human (b), bovine (c) or porcine (d) TLR5 genes. Transfectants were stimulated with *S. Typhimurium*-derived flagellin; 10 ng ml<sup>-1</sup> or 100 ng ml<sup>-1</sup>, *T. gondii* profilin (TgPr; 0.01, 0.1 or 1  $\mu$ g ml<sup>-1</sup>), PMA or media only. SEAP activity was measured by the colour change resulting from alkaline phosphatase substrate conversion at 635 nm wavelength. Values are expressed as mean  $\pm$  SD, and are one of three repeats with each condition run in triplicate.

SEAP: secreted embryonic alkaline phosphatase; HEK: human embryonic kidney; FLA-ST: *S. Typhimurium*-derived flagellin; TgPr: *T. gondii* profilin; huTLR5: human TLR5; boTLR5: bovine TLR5; poTLR5: porcine TLR5.

measured by QUANTI-Blue conversion detected using absorption at 635 nm. In supernatants from cells cultured without a ligand (no stimulation), only a very low background absorption could be measured, indicating that there was no measurable endogenous alkaline phosphatase activity interfering with the assay, and this is confirmed by the results of mock-transfected cells stimulated with all ligands (Figure 1a). Cells transfected with hu (Figure 1b) and boTLR5 (Figure 1c) responded to specific stimulation with FLA-ST, confirming the overexpression of functional TLR5 as well as the species-specific differences seen before.<sup>22</sup> However, neither transfection resulted in

a response to TgPr added in a range of concentrations. In contrast, no response, apart from the response to PMA, was seen to any ligand in cells transfected with poTLR5 (Figure 1d). In an attempt to enrich successfully transfected cells, transfectants were maintained in selection media and the stimulations repeated 1 wk (Supplemental material online Figure 1a–d) and 2 wk (Supplemental Figure 1 e–h) after transfection, with consistent results.

To confirm SEAP assay results, IL-8 levels were measured from the assay supernatants obtained in the initial stimulation assay (Supplemental Figure 2). These results matched those of the SEAP assay.



To try to confirm the activity of TgPr in a SEAP assay, a commercially available HEK293 cell line stably expressing both a NF- $\kappa$ B driven SEAP reporter and murine TLR11 was used. Whereas the technical control (PMA) showed a clear response, neither TgPr nor FliC induced any NF- $\kappa$ B SEAP response in these cells (Supplemental Figure 3).

### *T. gondii* profilin does not induce an IL-6 response in primary human cells

After being unable to detect a TLR5-specific response to TgPr with regard to NF- $\kappa$ B stimulation or IL-8 release, we abandoned our original aim of species comparison and further assessed whether profilin stimulated primary human cells. For this, we isolated PBMCs as well as CD14<sup>+</sup> monocytes. In addition to ultrapure flagellin and TgPr, we also used monomeric FliC flagellin, which is less prone to endotoxin contamination. LPS was used as a positive control, and IL-6 production was assessed, similarly to as described in Salazar Gonzales et al.,<sup>20</sup> and the resulting IL-6 values are shown in Figure 2. While we consistently detected high levels of IL-6 in the supernatant of cells stimulated with either flagellin, FliC or LPS in a dose-dependent

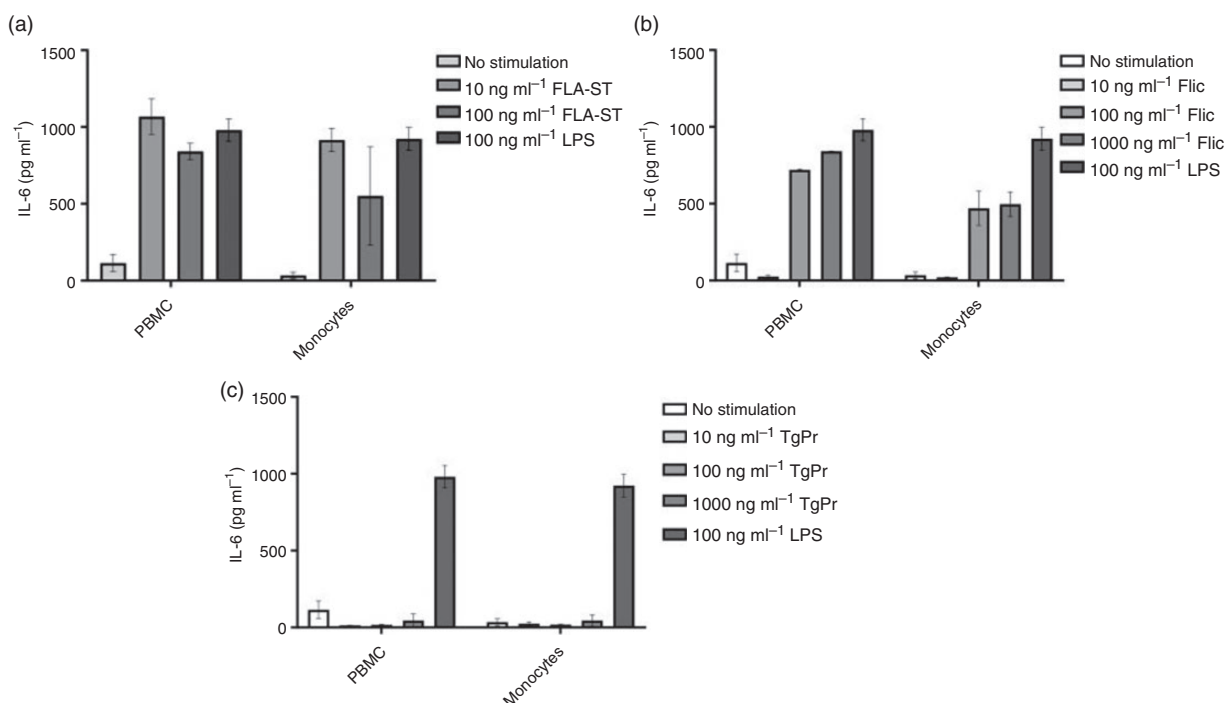
manner, no IL-6 was measured in supernatants from cells stimulated with TgPr at various concentrations.

### TgPr stimulates IL-6 release in RAW 264.7 cells

To assess whether the absence of any measurable response to TgPr (Sigma) was due to its lack of biological activity, we assessed IL-6 production in a murine macrophage-like RAW 264.7 cell line, which has been described to express both TLR11 and TLR12<sup>26,27</sup> and was originally used to clone TLR11.<sup>29</sup> As shown in Figure 3, RAW 264.7 cells responded with increased IL-6 production in a concentration-dependent manner, but this response was substantially lower compared with the response induced by LPS.

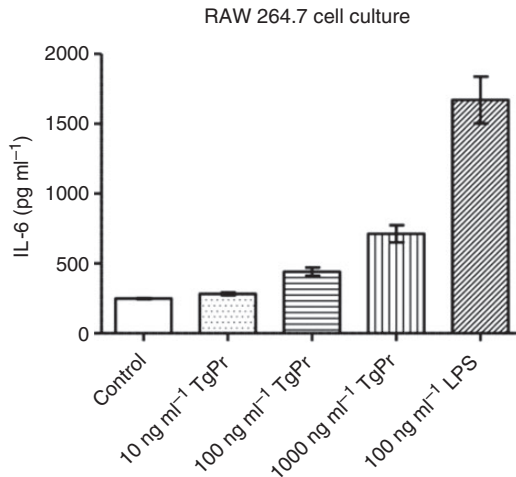
## Discussion

The present study aimed to elucidate the functionality of bo and poTLR5 in the recognition and response to the protein TgPr, an indispensable protein for movement and cellular invasion for *T. gondii*. The motivation for this was threefold: first, the species-specific differences noted on the progression and outcome of infection by *T. gondii* in different mammalian species;



**Figure 2.** Profilin does not stimulate IL-6 release in primary human cells. PBMC isolated from whole blood as well as isolated CD14<sup>+</sup> monocytes were incubated with flagellin (a), FliC (b) and *T. gondii* profilin (c) in the concentrations shown. Cells left untreated were used as negative, LPS as positive control for TLR activation. Data are a representative set of data from two biological repeats, with samples run in triplicate. IL-6 secretion into supernatants, analysed in duplicates, was measured by ELISA in a 1:10 dilution, and values are expressed as mean  $\pm$  SD.

FLA-ST: *S. Typhimurium*-derived flagellin; TgPr: *T. gondii* profilin.



**Figure 3.** Profilin stimulates IL-6 release in murine monocytic RAW 264.7 cells. Cells were cultured in the presence of *T. gondii* profilin (0.01, 0.1 or 1  $\mu\text{g ml}^{-1}$ ). Cells left untreated were used as negative, LPS as positive control for TLR stimulation. IL-6 release into supernatants was measured by ELISA; values are expressed as mean  $\pm$  SD. Data are mean of two repeats, with each condition run in duplicate.  
TgPr: *T. gondii* profilin.

second, the recently described role of huTLR5 in the innate recognition of profilin and protection against this parasite;<sup>20</sup> last, the evolutionarily conserved action of hu and boTLR5 in the response to bacterial flagellin,<sup>22</sup> another protein integral to motility.

Whereas the robustness of the SEAP-based TLR stimulation assay was recently described,<sup>24</sup> and the already described differences with regard to flagellin as well as FliC between hu and boTLR5<sup>22</sup> were confirmed in the present study, we were unable to obtain a TLR5-dependent response to TgPr in either system. Indeed, a dose-dependent TLR5-driven NF- $\kappa$ B-SEAP response was obtained using different concentrations of flagellin, suggesting that receptor-ligand interactions were responsible for such activity. This was coherent with previous studies investigating human<sup>30</sup> and bovine<sup>31</sup> TLR5-dependent recognition of flagellin in transfected cells, in addition to those demonstrating the SEAP reporter assay for other TLRs and their respective ligands.<sup>24</sup> The result related to TLR5-driven NF- $\kappa$ B-SEAP response was further confirmed by assessing the corresponding supernatants for IL-8 by ELISA. Similar to the SEAP assay, huTLR5-transfected cells increased IL-8 production in a concentration-dependent manner to flagellin, whereas cells transfected with boTLR5 responded to a lesser extent, consistent with previous findings.<sup>22</sup> Interestingly, using either the reporter assay or ELISA, SEAP activity and IL-8 production were negligible using the commercially available poTLR5 construct following culture with flagellin. These results are again consistent

with earlier data using an own poTLR5 construct derived from a Large White breed pig.<sup>22</sup> This was not examined any further as part of this investigation. However, similar data regarding poTLR5 expression in HEK cells were published recently,<sup>32</sup> indicating that poTLR5 is non-responsive in HEK cells, despite the fact that the main adaptor, MyD88, shares a 94% homology between human and porcine MyD88 (Yamakawa and Werling, unpublished data).

Contrary to expectation, TgPr did not elicit any detectable NF- $\kappa$ B activity in huTLR5 transfected cells across all three SEAP assays. As this was mirrored in the IL-8 concentration of supernatant taken on the first stimulation, we concluded that TgPr did not provoke any effect measured in this study on the transfected cells, opposing previously published findings.<sup>20</sup> The functional confirmation of huTLR5 indicates that TgPr does not simply bind TLR5 in the manner it is expressed in the cells of this study. The different starting receptor profiles of SEAP reporter cells and those used in the original study<sup>20</sup> with regard to huTLR5, together with the differences presented in TLR5-transfected cell activity towards profilin, might suggest the possibility of other participating co-stimulatory molecules being already present, undetected, in the cells used.

Further investigations of this possibility required primary human cells expressing a greater repertoire of cell surface molecules. To do so, PBMC and CD14<sup>+</sup> monocytes were isolated for their characteristically high TLR5 expression.<sup>33</sup> Both cell populations presented a largely homologous TLR5-specific activity to flagellin, FliC and LPS. All ligands tested increased IL-6 production compared with that of unstimulated cells, giving functional confirmation of TLR5 and TLR4 (and any required signalling molecules); however, as seen with TLR5-transfected HEK cells, TgPr did not elicit any significant IL-6 increase.

All these data suggested that either the profilin used in the present study was not bioactive, or that hu and boTLR5 may not be receptors for TgPr. As we had failed to obtain TgPr from the same source as the original study,<sup>20</sup> due to the product being discontinued, we assessed the response of the TgPr used in the present study using the murine monocytic RAW 264.7 cell line. Here, we were indeed able to show a TgPr dose-dependent induction of IL-6.

To further assess whether TgPr used in this study is bioactive, we purchased a HEK cell line stable transfected with a NF- $\kappa$ B driven SEAP and TLR11. TLR11 has been shown to be an intracellularly expressed TLR, residing in the ER, and binding to the profilin protein of *Toxoplasma* after the invasion of the parasite. However, to be able to respond to this, the 12 membrane-spanning endoplasmic reticulum-resident

protein UNC93B1 needs to be present, to regulate TLR11-dependent activation.<sup>34</sup> Indeed, deficiency in functional UNC93B1 protein completely abolished TLR11-dependent IL-12 production by murine DCs.<sup>34</sup> Furthermore, there seems to be some contradictory data presented whether profilin binds to TLR11, TLR12 or indeed needs a hetero-dimer complex formed by both TLRs to be recognised correctly. This comes from observations that, similarly to TLR11, TLR12 is an endolysosomal innate immune receptor that colocalises and interacts with UNC93B1. More interestingly, both receptors seem to signal mainly through the transcription factor IFN regulatory factor 8 (IRF8), rather than NF- $\kappa$ B, to elicit an IL-12 response in DCs.<sup>35</sup> Thus, the lack of a profilin response in our system could be explained by a) a lack of the HEK cells performing the necessary endocytosis, b) the lack of functional TLR12 expression on HEK cells, which seems to be necessary for appropriate recognition of profilin,<sup>35</sup> c) the fact that the reporter system is NF- $\kappa$ B rather than IRF8 driven and, d) a lack of bioactivity of the TgPr used. Against the latter, the argument is the positive response seen in RAW cells.

Our data indicate that TgPr may not be a ligand for TLR5, and that the response described earlier<sup>20</sup> may have instead been due to impurities in the profilin preparation. Our data would indeed be in accordance with the previously established understanding of the interactions between *T. gondii* and the human host, in which phagocytosis of live parasites is required to generate a pro-inflammatory response involving IL-6, IL-8 and IL-12.<sup>21</sup> This is a mechanism distinct from that of murine hosts, which have been shown to instead respond to soluble parasite Ag in a TLR-dependent manner.<sup>12,13</sup> Viewed from an evolutionary perspective, it would be plausible for mice alone to possess defence mechanisms to this parasite that differ from other potential hosts; given their primary prey status and coevolution with cats and *T. gondii*. Recognition of profilin and other soluble parasite Ags may confer protection and survival in the mouse, thus strengthening the chance of ingestion by the feline definitive host and the continuation of the *T. gondii* lifecycle.

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