Dengue virus NS1 protein activates immune cells via TLR4 but not TLR2 or TLR6

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Running Title: Dengue virus NS1 protein activates TLR4

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Conflict of Interest Statement: NM, DW, PRY and KJS hold a patent related to TLR4 as a target for dengue virus therapy.

ABSTRACT

The secreted hexameric form of the dengue virus non-structural protein 1 (NS1) has recently been shown to elicit inflammatory cytokine release and disrupt endothelial cell monolayer integrity. This suggests that circulating NS1 contributes to the vascular leak that plays a major role in the pathology of dengue haemorrhagic fever and shock. Pathways activated by NS1 are thus of great interest as potential therapeutic targets. Recent works have separately implicated both Toll-like receptor 4 (TLR4) and the TLR2/6 heterodimer in immune cell activation by NS1. Here we have used mouse gene knockout macrophages and antibodies blocking TLR function in human peripheral blood mononuclear cells to show that recombinant NS1, expressed and purified from eukaryotic cells, induces cytokine production via TLR4 but not TLR2/6. Furthermore, the commercial *E. coli*-derived recombinant NS1 preparation used in other work to implicate TLR2/6 in the response is not correctly folded and appears to be contaminated by several microbial TLR ligands. Thus TLR4 remains a therapeutic target for dengue virus infections, with TLR4 antagonists holding promise for the treatment of dengue disease.

INTRODUCTION

The dengue virus (DENV) NS1 protein functions intracellularly to promote viral replication, but is also secreted from infected mammalian cells as a 300 kDa hexameric glycoprotein.[1]

This hexameric species is found circulating in the blood of infected individuals and its presence is used for the rapid diagnosis of DENV infection.[2] Recent results suggest a critical role for NS1 in the vascular leak that characterises severe infection.[3,4] We demonstrated that NS1 activates both immune and endothelial cells via engagement with TLR4, leading to cytokine secretion and endothelial cell monolayer disruption.[3] Furthermore, we found that TLR2 was not involved in the response to NS1. The best-studied ligand for the TLR4/MD-2 complex is bacterial lipopolysaccharide (LPS),[5,6] although a number of viral proteins including Ebola virus glycoprotein, respiratory syncytial virus F protein, and human immunodeficiency virus Tat protein, have also been shown to be TLR4 agonists.[7-9] In contrast, Chen and colleagues[10] reported that NS1 activates human and murine immune cells via TLR2 and TLR6, which otherwise act as a heterodimer in the recognition of diacylated lipoproteins from some Gram-positive bacteria and *Mycoplasma*.[11,12] To resolve these apparently contradictory reports on innate immune recognition of NS1, we assessed the roles of TLR2, TLR4 and TLR6 in response to the different sources of NS1 used in the two papers.[3,10]

RESULTS AND DISCUSSION
Chen et al.[10] used commercially available recombinant *E. coli*-derived NS1 (Abcam Cat. # 64456) in experiments implicating TLR2/6 in recognition of NS1. The Abcam product is C-terminally His-tagged NS1 from DENV-1, supplied in 1.5 M urea and recommended for use in ELISA and western blot applications. We first compared the oligomeric state of *E. coli*-derived NS1 to DENV-2 NS1 immunoaffinity-purified from the supernatant of stably-transfected S2 cells ("insect NS1"),[3] or from the medium of DENV-2 infected Vero cells cultured without serum ("native NS1"). The secreted form of NS1 has a barrel-like hexameric structure formed by a trimer of stable NS1 dimers. The formation of this oligomeric structure is dependent on both glycosylation and correct disulphide bonding of the 12 cysteines in NS1.[13-17] Immunoaffinity-purified preparations of NS1 used routinely in our laboratory are hexameric, based on both size exclusion chromatography and electron microscopic (EM) analysis (Modhiran et al.[3] Figure S1). Chemical cross-linking confirms the hexameric nature of the particles seen on EM.[15] Insect cell-expressed NS1 hexamers dissociate to dimers upon SDS-PAGE analysis of unboiled samples, and to monomers after boiling[15], regardless of the presence or absence of reducing agent (Figure 1). In contrast, the commercially available *E. coli*-derived NS1 was monomeric under all conditions, with an unknown low molecular weight product also noted. Thus the *E. coli*-derived NS1 is not in its native conformation and is likely denatured given that it is supplied in 1.5 M urea. Such a misfolded protein may be suitable as an antigen substrate for western blot and qualitative ELISA analyses using antibodies recognising linear epitopes, but are less likely to be functional in receptor activation studies, that generally rely on protein structure-based interactions.

Antibodies specific for human TLR2 and TLR6 were used by Chen et al. to demonstrate inhibition of IL-6 production by human PBMC in response to *E. coli*-derived NS1.[10] Using these antibodies, we found no effect of TLR2 or TLR6 blockade on the response to insect NS1 (Figure 2a). As expected for PBMC from four different donors, the absolute level of response to stimuli was variable, but in all cases the activity of insect-derived NS1 was completely prevented by anti-TLR4 antibody as well as the TLR4 antagonist, *Rhodobacter sphaeroides* LPS (LPS-RS)[18] (Figure 2a), in agreement with our previous work.[3] Responses to the control TLR2/6 stimulus Pam$_2$CSK$_4$ and TLR4 stimulus LPS were sensitive to the respective anti-TLR antibodies, as expected. However, none of these TLR-inhibitory agents affected the response to *E. coli* NS1 (Figure 2a), suggesting that it may be contaminated with a variety of TLR ligands acting redundantly. The dominant contaminants from *E. coli* are

likely to be LPS and a number of TLR2/1 agonists including triacylated lipopeptides.[19,20] Despite using the same sources of *E. coli*-derived NS1 and blocking antibodies we were unable to replicate the results of Chen et al.[10] One possible explanation for their results is that the TLR-targeting antibodies bound to cells may lead to cell death if used in combination with serum that has not been adequately heat inactivated to destroy complement function, whilst isotype control antibodies that do not bind cells would have no effect. It is notable that in one experiment shown by Chen et al., anti-TLR2 and TLR6 blocking antibodies inhibited the PBMC response to LPS to the same extent as they inhibited the response to a TLR2/6 ligand.[10] In contrast, a supplementary figure showed LPS response unaffected by the same antibodies, but this data does not appear to have been generated in parallel with the relevant experiments.

A further assessment of TLR-dependence was performed with bone marrow-derived macrophages (BMMs) from *Tlr* knockout mice. Activity of the insect-derived NS1 was lost in $Tlr4^{-/-}$ cells, but was unaffected by knockout of *Tlr1* or *Tlr6* (Figure 2b). Our prior work using $Tlr2^{-/-}$ BMM similarly showed no role for TLR2 in the stimulatory activity of purified insect-derived NS1.[3] Similar to results with PBMC cultures, *E. coli*-derived NS1 at 10 μg/ml was strongly active, with this activity not abolished by any individual *Tlr* knockout. However at 1 μg/ml, its induction of IL-6 was TLR4-dependent. This was distinct from the human cell response, where TLR4 responses did not clearly dominate at this concentration of *E. coli* NS1 (Figure 2a). This may reflect greater sensitivity of the human cells to possible contaminants within the preparation. Overall, these results cannot be reconciled with those of Chen et al.[10] who reported that the response to *E. coli*-derived NS1 was completely lost in $Tlr6^{-/-}$ mouse macrophages.

Any study proposing a new ligand for TLR activation must exclude microbial contaminants.[21] Chen and co-workers[10] used a synthetic 6X His tag peptide (Abcam Cat. # 14943) as a control for NS1, however this preparation is unlikely to provide an adequate experimental control for possible bacterial contaminants present in a recombinant protein purified from *E. coli*. We used a *Limulus* amoebocyte lysate (LAL) assay to estimate the LPS content in 1 μg/ml of the commercially sourced *E. coli*-derived NS1 to be 0.55 EU/ml, whilst 10 μg/ml insect-derived NS1 was below the limit of detection (<0.1 EU/ml). The *E. coli*-derived NS1 preparation was active in induction of IL-6 by PBMC at concentrations as low as 0.64 ng/ml NS1, which was approximately 1000-fold more potent than insect cell-derived NS1 (Figures 3a and 3b). The LPS-binding antibiotic polymyxin B had no effect on the response to insect-cell derived NS1 (Figure 3a), showing a lack of any accessible LPS as being responsible for its activity. In contrast, *E. coli*-derived NS1 activity was reproducibly inhibited by polymyxin B, but only at low concentrations of NS1 (Figure 3b), suggesting the presence of a number of immunostimulatory agents including LPS. LPS and Pam_2CSK_4 treatments were used as positive and negative controls for TLR4 and TLR2/6 activation and polymyxin B sensitivity (Figures 3c and 3d). The broad dose response curve seen with *E. coli*-derived NS1 is also consistent with it containing a number of stimulatory contaminants. In contrast, purified insect cell-derived NS1, ultrapure LPS and Pam_2CSK_4 showed saturation of responses within 1-2 log units (Figures 3a-d).

The molar concentrations required for response to insect NS1 were approximately 10-fold higher than for LPS (Figures 3a and 3c). Whilst NS1 is a somewhat less potent agonist, concentrations of circulating NS1 seen in dengue virus infected patients are higher than levels of circulating LPS in bacterial sepsis. The median LPS reported in sepsis is 300 pg/ml (0.03 nM assuming molecular weight of 10 kDa), ranging up to 5 ng/ml (0.5 nM).[22] NS1 is detected

in serum at ng/ml to µg/ml levels (1 µg/ml is 3.3 nM).[23-25] Consequently, active concentrations of NS1 are within its physiological range. In addition, NS1 may be sequestered *in vivo*, for example by binding to endothelial cells,[26] or masked by antibody, and its functional concentration may be higher than that measurable in serum.

The results presented here clearly show the inadequacy of commercial bacterial-expressed NS1 for studies of innate immune responses, due to protein misfolding and likely immunostimulatory contaminants. Although Chen et al.[10] used a combination of *E. coli*-expressed and baculovirus-expressed NS1, key experiments defining TLR2/6 as the receptor used the bacterial protein; all data in the paper other than Figures 3 A-D, G and H used only *E. coli* NS1 (Justin Chu, personal communication). The only data presented with baculovirus-expressed NS1 suggesting a role for TLR2/6 utilised TLR2/6-transfected HEK293 cells. This showed a detectable but low response to all ligands tested, including LPS that should not activate this pathway, and consequently results are not clear. Although we have found no TLR2/6 response to purified insect-derived NS1, this does not exclude a role for TLR2/6 activation in other aspects of the response to DENV infection. In fact, Chen et al. demonstrated that relative to wild-type mice, newborn *Tlr6*^{-/-} mice had improved survival upon infection with high doses of DENV,[10] suggesting a function for TLR6 in dengue pathology in this model. However a link between TLR6 and NS1 cannot currently be drawn; the observation of toxicity of NS1 similarly injected into newborn mice[10] is compromised by our demonstration that the NS1 used was a denatured protein likely to contain several inflammatory microbial contaminants. The authors also demonstrated that TLR2 and TLR6 are induced by DENV infection.[10] This shows that they are transcriptional targets of inflammatory signalling pathways induced by DENV infection, but provides no evidence for a role of TLR2/6 in recognition of the virus.

Although we provide further evidence for TLR4 recognition of purified NS1, the work presented here alone is not sufficient to exclude LPS as the source of TLR4-stimulating activity in our NS1 preparation. To this end we have previously shown complete loss of activity following NS1 immuno-depletion, as well as the stimulatory activity of NS1 secreted from mammalian cells and used without purification.[3] The NS1 hexamer is a barrel-like structure carrying a lipid cargo in its core[14,15] and whether protein or lipid is required for TLR4 stimulation remains to be established. It is still conceivable that trace amounts of LPS within tissue culture reagents could be bound and concentrated on NS1 in a manner inaccessible to polymyxin B and the LAL assay, with NS1 becoming an efficient delivery agent for LPS to the TLR4 receptor complex. Importantly, any such function would be biologically relevant, since there are detectable amounts of LPS in circulation, particularly in the portal vein draining the gut.[27] Indeed it is worth noting that circulating LPS levels become further elevated in DENV infection, possibly via gut leakiness, and may play a role in pathology.[28] However, in support of an LPS-independent role for NS1, is its increased activity relative to LPS in induction of human compared with mouse cytokine mRNAs, and the greater effectiveness of NS1 than LPS in the disruption of endothelial monolayers.[3] Irrespective of the underlying mechanism, the TLR4 antagonist LPS-RS prevented vascular leak in DENV-infected mice.[3] This finding emphasises the importance of TLR4 responses in this disease, and the potential for therapeutic reduction of pathology with TLR4 antagonists.

METHODS
Materials
Ultrapure LPS from *E. coli* 0111:B4 strain, ultrapure LPS-RS, Pam$_2$CSK$_4$ and polymyxin B were obtained from InvivoGen (San Diego, CA, USA). The anti-TLR2 and anti-TLR6

antibodies were purchased from InvivoGen (MAb-hTLR2 and Anti-hTLR6-IgG). The anti-TLR4 antibody was prepared in-house by transient expression of heavy and light chains encoding mouse HTA125 antibody[29] in CHO cells.[3] Mouse IgG1 and IgG2a isotype control antibodies were purified from ascites fluid using a protein G column. *E. coli*-expressed DENV1 NS1 was purchased from Abcam (Melbourne, Australia. Cat. # 64456).

Cell culture

BMMs were obtained by differentiation of mouse bone marrow progenitors of *Tlr1⁻/⁻*, *Tlr4⁻/⁻*, *Tlr6⁻/⁻* and C57BL/6 mice in the presence of 10^4 U/ml CSF1 for 7 days.[30] Breeding and use of mice was approved by the University of Queensland and James Cook University Animal Ethics Committees. Blood was obtained from healthy donors to the Red Cross Blood Service, under approval from the University of Queensland Medical Research Ethics Committee, and PBMCs were isolated by Ficoll gradient.[31]

Generation and purification of DENV NS1

The recombinant DENV2 NS1 protein was expressed in stably transfected *Drosophila* S2 cells as previous described.[3] Native DENV2 NS1 was prepared from medium of Vero cells infected by DENV2 strain ET00.300 (GenBank: AY422469.1) at multiplicity of 1 and maintained in serum free opti-MEM I medium (Invitrogen) for 5 days. The recombinant and native NS1 proteins were affinity purified from culture medium using immobilised 2A5.1 anti-NS1 monoclonal antibody.[3] The purified proteins were concentrated and the buffer exchanged using an Amicon 100 kDa cutoff filter (Merck Millipore, Darmstadt, Germany) and quantified by BCA assay (Pierce, ThermoFisher Scientific, Waltham, MA, USA). A LAL Chromogenic Endotoxin Quantitation Kit (Pierce, ThermoFisher Scientific) was used to quantify endotoxin contamination from both insect-derived and *E. coli*-derived NS1. Purified proteins were assessed on SDS-PAGE gels stained with Coomassie blue.

Detection of cytokine production by ELISA

Mouse BMMs were seeded at 80,000 cells/well and duplicate treatment wells were incubated with Pam_2CSK_4, ultrapure LPS, *E. coli*-derived NS1 or insect-derived NS1 in a final volume of 100 µl. Mouse cell experiments were performed three times. Human PBMCs from four separate donors seeded at 50,000 cells/well were left untreated or pre-incubated with LPS-RS, anti-TLR2, anti-TLR4, anti-TLR6 antibodies or isotype control antibodies for 1 hour and subsequently stimulated with designated stimuli in a final volume of 100 µl. Experiments with human cells were performed twice with cells from two donors in each experiment. For both human and mouse cells, medium was harvested after 24 hours and IL-6 quantified by ELISA (R&D Systems, Minneapolis, MN, USA), with each sample assayed in duplicate to ensure reliability. No samples were excluded from analysis.

CONFLICT OF INTEREST
NM, DW, PRY and KJS hold a patent related to TLR4 as a target for dengue virus therapy.

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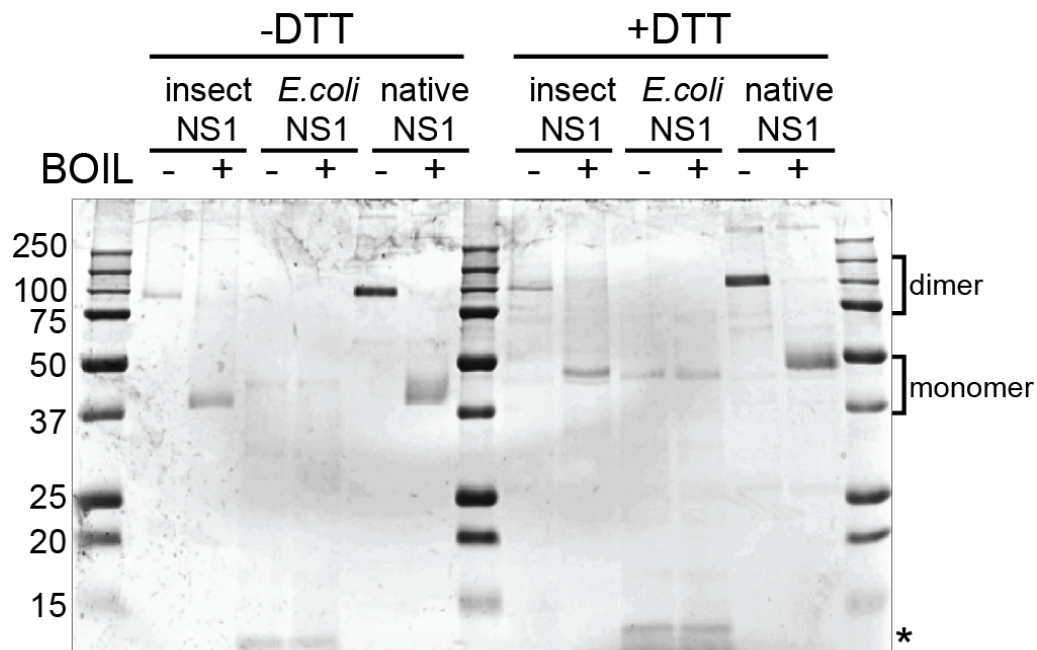
Figure 1 *E. coli*-expressed NS1 does not form dimers. A 15% SDS-PAGE gel stained with Coomassie blue shows immunoaffinity-purified S2 cell-expressed DENV2 NS1 (insect NS1), *E. coli*-expressed DENV1 NS1 (Abcam) (*E. coli* NS1) and immunoaffinity-purified native NS1 from DENV2-infected Vero cells (native NS1), with or without sample boiling, and in the presence or absence of the reducing agent dithiothreitol (DTT). The asterisk indicates an unknown band in the *E. coli* NS1 sample.
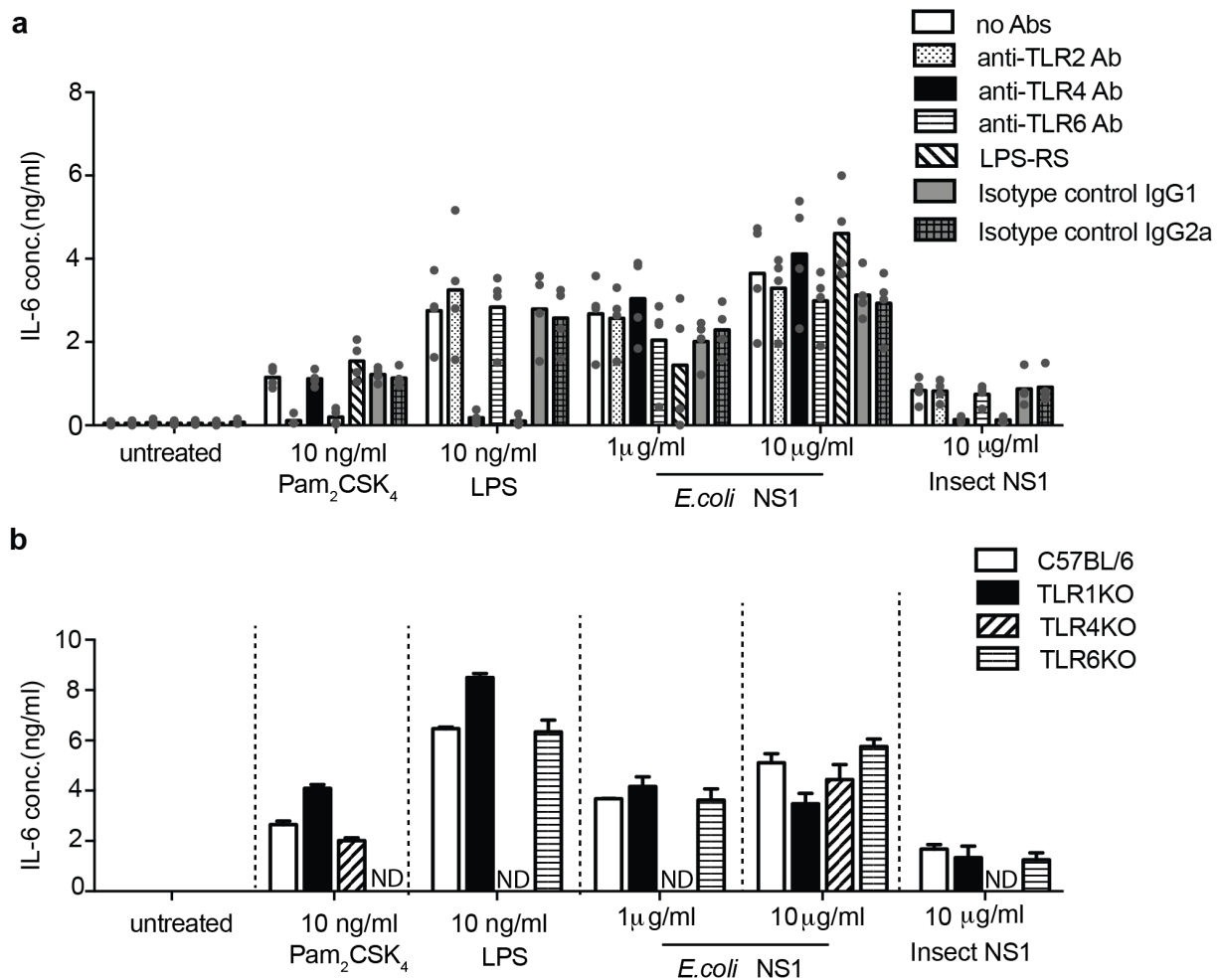
Figure 2 Purified NS1 activates mouse and human immune cell responses via TLR4 and not TLR2/6. (a) Purified insect cell-derived NS1 activation of PBMCs is blocked by anti-TLR4 antibodies and TLR4 antagonist LPS-RS, but impure Abcam *E. coli*-derived NS1 activity was not prevented by anti-TLR2, TLR4 or TLR6 antibodies, nor by LPS-RS. Human PBMCs were pre-incubated with anti-TLR2, anti-TLR4, anti-TLR6 or isotype control antibodies (all at 1 μg/ml), or ultrapure LPS-RS (10 μg/ml) for 1 hour and subsequently stimulated with TLR2/6 ligand Pam$_2$CSK$_4$, TLR4 ligand *E. coli* ultrapure LPS, *E. coli*-derived NS1, or immunoaffinity-purified S2 insect cell-derived NS1. Medium was harvested after 24 hours and IL-6 quantified by ELISA. Data shown is mean and individual data points for four independent donors (n=4). (b) Purified NS1 activates mouse macrophages via TLR4. BMM were prepared from *Tlr1$^{-/-}$*, *Tlr4$^{-/-}$*, *Tlr6$^{-/-}$* and control C57BL/6 bone marrow. BMMs were treated for 24 hours with Pam$_2$CSK$_4$, *E. coli* ultrapure LPS, *E. coli*-derived NS1, and insect-derived NS1. IL-6 production was measured by ELISA. Results shown are mean ± range of duplicate cell treatments within one experiment, and similar effects of knockouts were observed in two further experiments. ND = not detected.
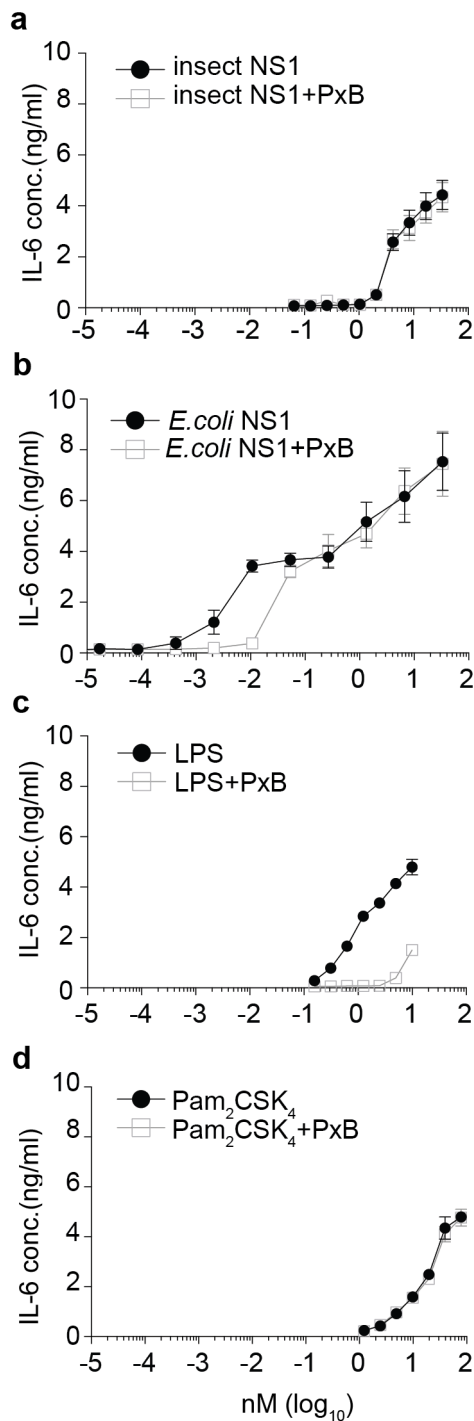
Figure 3 LPS-binding antibiotic polymyxin B (PxB) blocks the activity of low concentrations of *E. coli*-derived NS1, but has no effect on purified insect cell-derived NS1. NS1, LPS or Pam_2CSK_4 were pre-incubated for 30 minutes with or without polymyxin B in a volume of 25 µl, then added to PBMCs in 50 µl and incubated for 24 hours. The final concentration of polymyxin B was 2.5 µg/ml, with varying concentrations of (a) insect-derived NS1 (2-fold dilutions from 10 µg/ml or 33.3 nM), (b) *E. coli*-derived NS1 (5-fold dilutions from 10 µg/ml or 33.3 nM) (c) *E. coli* ultrapure LPS (2-fold dilutions from 100 ng/ml or 10 nM assuming average molecular weight of 10 kDa), and (d) Pam_2CSK_4 (2-fold dilutions from 100 ng/ml or 78.6 nM using molecular weight of 1271.85 Da). IL-6 production was quantified by ELISA. Results shown are the mean ± SEM for cells from four donors (n=4).