Human Immunology 80 (2019) 878–882

Contents lists available at ScienceDirect

Human Immunology

journal homepage: www.elsevier.com/locate/humimm

Effect of MUC1 length polymorphisms on the NLRP3 inflammasome response of human macrophages

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A B S T R A C T

Mucin 1 is a cell-membrane associated mucin, expressed on epithelial and immune cells that helps protect against pathogenic infections. In humans, MUC1 is highly polymorphic, predominantly due to the presence of a variable number tandem repeat (VNTR) region in the extracellular domain that results in MUC1 molecules of typically either short or long length. A genetic link is known between these *MUC1* polymorphisms and inflammation-driven diseases, although the mechanism is not fully understood. We previously showed that MUC1 on murine macrophages specifically restricts activation of the NLRP3 inflammasome, thereby repressing inflammation. This study evaluated the effect of *MUC1* VNTR polymorphisms on activity of the NLRP3 inflammasome in human macrophages, finding that long *MUC1* alleles correlated with increased IL-1β production following NLRP3 inflammasome activation. This indicates that the length of MUC1 can influence IL-1β production, thus providing the first evidence of an immune-modulatory role of *MUC1* VNTR polymorphisms in human macrophages.

1. Introduction

Mucins are heavily glycosylated proteins that are at the forefront of defence against pathogens and external stimuli [1]. One such mucin, expressed at virtually all mucosal surfaces is MUC1. The human Mucin 1 (*MUC1*) gene, located on the long arm of chromosome 1, at 1q21, consists of 7 exons and 6 introns that encode a cell-membrane associated glycoprotein [2]. MUC1 is expressed on epithelial and immune cells, where it acts both as a barrier to pathogens and to limit inflammation [3–6]. The MUC1 molecule is made up of three domains: a heavily O-glycosylated extracellular domain, a transmembrane domain, and a cytoplasmic tail [7]. The extracellular domain contains a variable number tandem repeat (VNTR) region which provides a high degree of polymorphism in the length of MUC1. This VNTR region contains 60 base-pair repeats that can occur between 20 and 120 times, leading to MUC1 proteins with different lengths in the extracellular domain [8]. These *MUC1* polymorphisms are associated with susceptibility to *Helicobacter pylori*-induced inflammation and gastric cancer, as well as lung adenocarcinoma [9–11].

One mechanism by which MUC1 is known to restrict inflammation (and resultant pathology) is by suppressing production of the pro-inflammatory cytokine IL-1β [12,13]. The production of IL-1β is a two-step process. Cells are first primed, for example via activation of a Toll-like receptor (TLR) signalling pathway which results in the production of pro-IL-1β [14]. Next, activation of a multiprotein complex called an inflammasome activates the protease caspase-1, which cleaves pro-IL-1β to yield active IL-1β cytokine [14]. While there exist different types of inflammasome, we recently showed that MUC1 on mouse immune cells specifically down-regulates activation of the NLRP3 inflammasome to suppress IL-1β production [13]. Given this finding and the association of VNTR polymorphisms with human inflammation and cancer, we asked if *MUC1* VNTR polymorphisms could influence the IL-1β response of human immune cells. To do this we evaluated the effect of *MUC1* VNTR length polymorphisms on the expression and activity of the NLRP3 inflammasome in human macrophages.

2. Experimental methods

Experiments were performed under guidelines laid down by the Human Research Ethics Committee of the Royal Children's Hospital, Melbourne, Australia.

MUC1 polymorphisms in the VNTR region were identified by using Southern blotting to analyse the length of the MUC1 *Hin*fI fragment sizes, using DNA extracted from peripheral blood mononuclear cells

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https://doi.org/10.1016/j.humimm.2019.06.001
Received 3 February 2019; Received in revised form 20 May 2019; Accepted 6 June 2019
Available online 15 June 2019

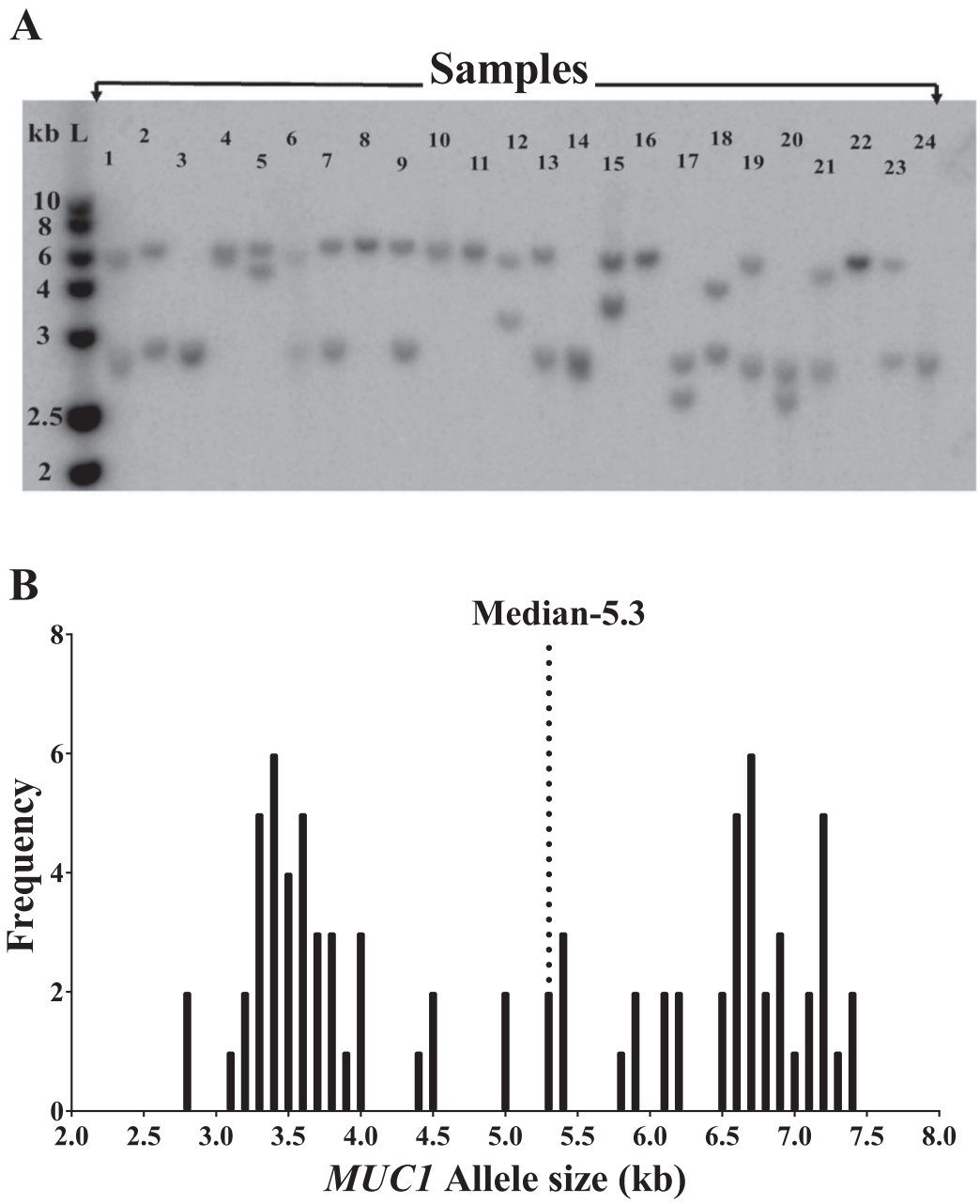

Fig. 1. Identification of *MUC1* VNTR polymorphisms in human DNA samples. Southern blotting was performed to identify *MUC1* allele size from *Hinf*1 digested DNA samples. A) A representative Southern blot membrane. By example, lane 2 was considered short/long, lane 3 short/short and lane 4 long/long. B) Frequency distribution of *MUC1* VNTR alleles (in kilobase pairs). The dotted line shows the median allele size (5.3) which was used as a cut off for separating short and long *MUC1* alleles. L-Ladder; kb-kilobase.

(PBMC), collected from healthy children recruited to the ChiLdhood Arthritis Risk factor Identification sTudY [15]. Only samples from male children were available for this analysis, as the female samples in this collection of controls were required for research into juvenile idiopathic arthritis, which has a significant female bias.

Monocyte-derived macrophages (MDM) were made by differentiating peripheral blood monocytes into macrophages (as previously described [16]). The NLRP3 inflammasome was activated by priming MDM with 100 ng/mL lipopolysaccharide (LPS), then stimulating with 3 μm nigericin (Sigma-Aldrich, St Louis, Missouri, USA) also as previously described [13]. The expression of inflammasome markers *Nlrp3*, *Nlrp1*, *Nlrc4* and *AIM2* in control and stimulated MDM were determined by quantitative Polymerase Chain Reaction (qPCR) as described [17].

IL-1β secretion was measured by Enzyme Linked ImmunoSorbent Assay (ELISA) using anti-IL1β capture (0.2 μg/well) and detection (0.05 μg/well) antibodies (R&D Systems, Minneapolis, Minnesota, USA).

3. Results

Southern blotting was performed on PBMC DNA from 51 male children (aged 12–17); a representative blot is shown in Fig. 1A. The allele size distribution of MUC1 was bimodal, ranging from 2.5 to 8 kb, with two peaks at 3.5 kb and 6.5 kb (Fig. 1B). Using the median allele size of all samples analysed (5.3 kb) as the cut off, alleles < 5.3 kb were classified as short while those > 5.3 kb where considered as long. Individuals were then classified as having either short/short alleles, long/long alleles or one short and one long allele. The bimodal MUC1 allele distribution observed in the current study, as well as the cut off used, are consistent with those of previously published studies [10,18]. Of the

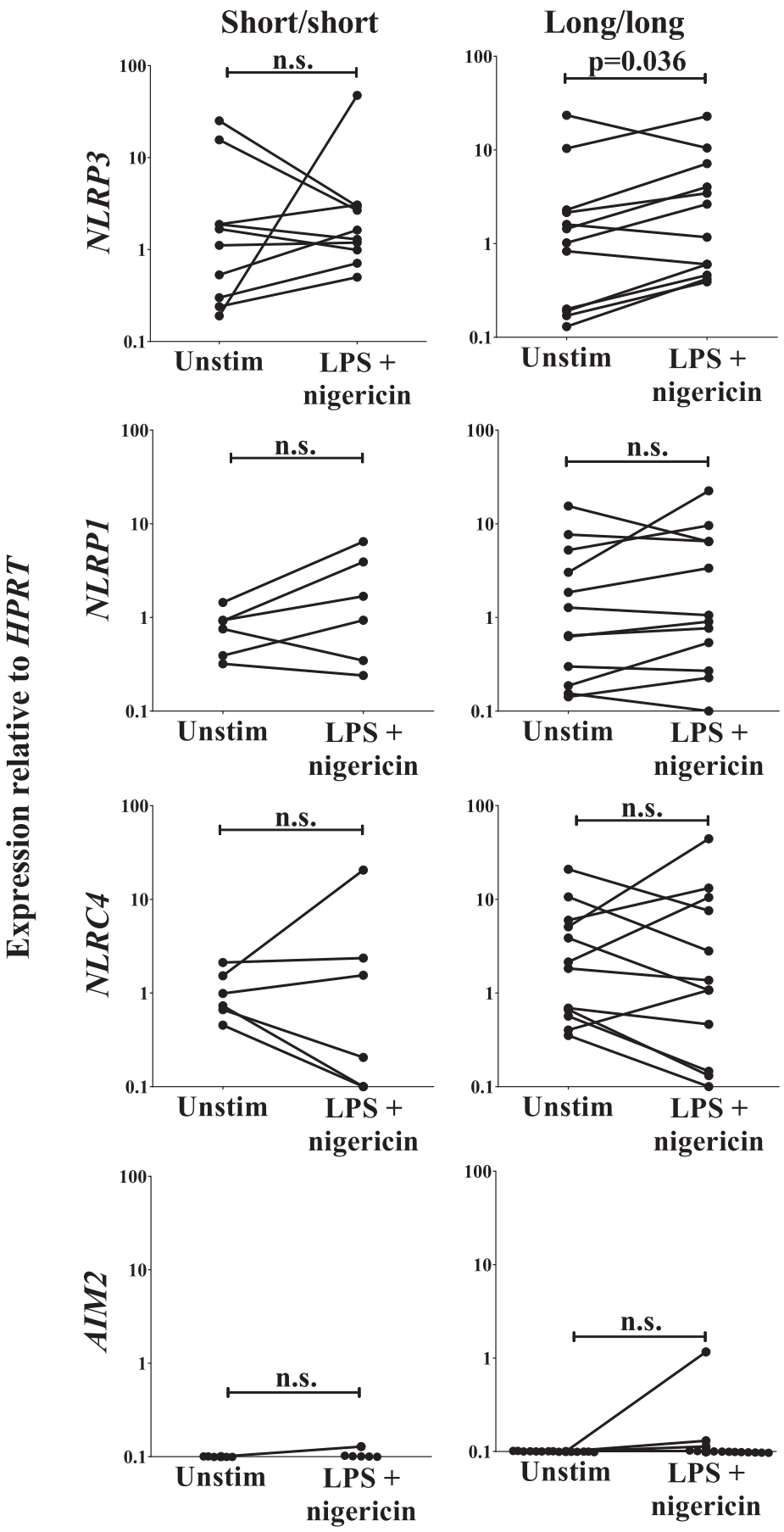

Fig. 2. *MUC1* VNTR genotype affects macrophage expression of *NLRP3*. Human monocyte-derived macrophages (MDM), from individuals in whom *MUC1* allele size had been classified into either short/short or long/long by Southern blotting, were primed with LPS then stimulated with the NLRP3 inflammasome activating ligand nigericin. The expression levels of the major markers for different inflammasome complexes were then quantified by qPCR, specifically: *NLRP3*, *NLRP1*, *NLRC4* and *AIM2*. Data show changes in expression following NLRP3 activation for each individual and were log transformed for analysis. Activation of MDM with LPS followed by nigericin upregulated mRNA levels of *NLRP3* but not of the other inflammasome markers, and only in macrophages from individuals with the long/long *MUC1* allele (Mann Whitney *U* test).

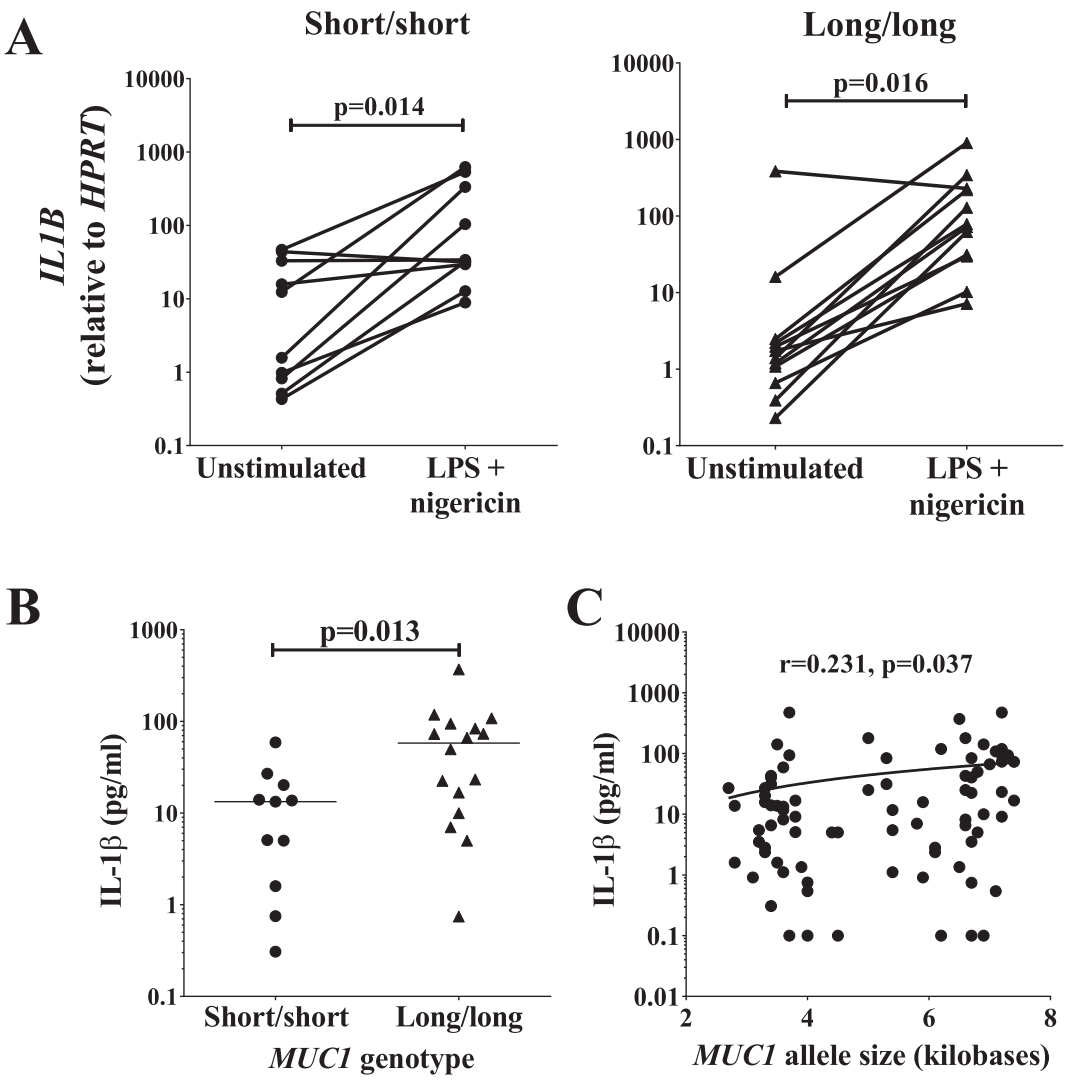

Fig. 3. *MUC1* VNTR genotype and effect on macrophage expression of *IL1B* and IL-1β secretion. (A) *IL1B* expression in human monocyte derived macrophages described in Fig. 2 was analysed by qPCR. NLRP3 inflammasome activation upregulated expression of *IL1B* mRNA in macrophages from both short/short and long/long *MUC1* VNTR genotypes (non-parametric Mann Whitney *U* test). Data show changes in expression following NLRP3 activation for each individual and were log transformed for analysis. (B) IL-1β secretion was measured by ELISA. No basal secretion of IL-1β was detected by any unstimulated cells. Macrophages from individuals with a L/L *MUC1* genotype secreted significantly more IL-1β as compared to cells from S/S individuals in response to NLRP3 inflammasome activation (Mann Whitney *U* test). Other group comparisons were not significantly different. Horizontal bars show the group medians. (C) Correlation plot of *MUC1* allele size with IL-1β secretion. *MUC1* allele sizes of individuals are plotted against their respective levels of IL-1β secretion. *MUC1* allele size positively correlated with levels of IL-1β secretion following NLRP3 inflammasome activation in the human macrophages (r and p calculated by Spearman correlation).

51 samples analysed, 47% were characterised as short/long, 31% were long/long and 22% short/short.

PBMC from these individuals were converted to MDM then the NLRP3 inflammasome activated by priming via TLR4 with LPS, then activation of the NLRP3 inflammasome complex with nigericin. Upon activation of the NLRP3 inflammasome, the levels of *NLRP3* mRNA in MDM from individuals with short/short or long/long MUC1 alleles were compared by qPCR. A significant association with increased NLRP3 expression following LPS priming and nigericin stimulation was observed in cells with long/long but not short/short alleles (Fig. 2), suggesting the length of MUC1 influenced NLRP3 transcription following activation of the NLRP3 inflammasome. In contrast, no significant increase in expression of markers for other inflammasome complexes, specifically *NLRP1*, *NLRC4* or *AIM2*, was detected (Fig. 2). Blood cells from the same individuals were also used to analyse the effect of *MUC1* VNTR on NLRP3 inflammasome activation. No difference was observed in the levels of *MUC1* mRNA in MDM with either short/short or long/long MUC1 alleles, either basally or after NLRP3 inflammasome activation (data not shown). *IL1B* levels were equally upregulated in cells with both the short/short and long/long *MUC1* genotypes (Fig. 3A), indicating *MUC1* VNTR length did not directly affect the expression of *IL1B*.

A main function of the NLRP3 inflammasome is cleavage of pro-IL-1β, which allows subsequent secretion of the active form of this pro-inflammatory cytokine (IL-1β). The release of this important cytokine by stimulated cells into culture supernatant was therefore measured. While MDM from all individuals produced IL-1β in response to activation of the NLRP3 inflammasome, those with a long/long *MUC1* genotype produced significantly more cytokine compared to cells from individuals with the short/short allele (Fig. 3B). A correlation analysis confirmed the presence of a positive and significant correlation between *MUC1* allele size and IL-1β production (Fig. 3C), indicating that a long MUC1 length is associated with a higher secretion of IL-1β.

4. Discussion

The MUC1 molecule has a cytoplasmic tail capable of cell signalling and an extremely large extracellular component; indeed we have shown previously that MUC1 can restrict the binding of *H. pylori* to epithelial cells by stearic hindrance [19]. This latest study shows that polymorphisms in the *MUC1* VNTR allele influences NLRP3 inflammasome activation in human macrophages; that the VNTR region affects the length of the extracellular portion of the MUC1 molecule points to this polymorphism most likely exerting its affect via interactions on the cell surface, rather than by modifying cell signalling. For example, changes in MUC1 VNTR length could potentially impact on the ability of LPS to interact with cell surface TLR4 during the priming event.

However the finding that it is the cells with a long/long *MUC1* allele that have the highest IL-1β secretion following activation of the NLRP3 inflammasome is perhaps surprising, as with stearic hindrance it might be expected that a longer mucin would result in lower not greater activation by TLRs.

One possible explanation is that short MUC1 molecules are more efficient at restricting the access of LPS than their longer counterpart. While counterintuitive, a shorter MUC1 molecule might be able to get into closer proximity to TLR4 and thereby be more efficient at inhibiting LPS activation of this receptor. Alternatively, MUC1 could act as an intermediary, binding LPS and guiding its interaction with TLR4. Such a scenario would potentially be facilitated by a longer MUC1, explaining an increased response in cells from individuals with such molecules. Arguing against this model is the fact we observed no difference in *IL1B* levels between cells with short/short and long/long alleles which would have also been expected in such a scenario. We also have data (not shown) indicating that the LPS-induced production of IL-8 is no different between cells with short/short and long/long alleles. Finally, a previous study has shown that the immuno-modulatory effects on MUC1 on TLR signalling require the cytoplasmic tail but are independent of the MUC1 extracellular domain [20]. Hence it seems unlikely that the effect of VNTR polymorphisms on the NLRP3 inflammasome is mediated via effects on TLR-signalling.

Activation of the NLRP3 inflammasome is a two-step process and the finding that the long/long MUC1 allele is associated with increased expression of *NLRP3* but not *IL1B* suggests the long MUC1 molecule exerts its influence at the level of the formation and activity of the inflammasome complex itself that results in the generation of active caspase-1, rather than with the production of the pro-IL-1β substrate.

In summary, this is the first study to show an immuno-modulatory role of *MUC1* VNTR polymorphisms (and the extracellular domain length) in human macrophages. This study provides evidence of an interesting and unexpected positive association between the length of the *MUC1* VNTR, and thus an increased extracellular length of this mucin molecule, and production of IL-1β after activation of the NLRP3 inflammasome in human macrophages. The data suggest the possibility that the long MUC1 VNTR in some way facilitates formation of the NLRP3 inflammasome, though how this is achieved is currently unknown.

As dysregulated IL-1β production (due to NLRP3 inflammasome activation) is associated with a range of inflammatory diseases, including type-2 diabetes and atherosclerosis [21,22] this observation has implications for the genetic susceptibility of individuals to a range of disease states. Future studies to analyse *MUC1* polymorphisms in individuals with these inflammatory diseases will extend the findings of this study and help in understanding the potential role of MUC1 VNTR length on macrophages in controlling the severity of these diseases.

Acknowledgements

We thank Professor Anne-Louise Ponsonby for her contributions to the recruitment of the healthy child participants as part of the Paediatric AutoImmune Disorder (PAID) research platform. This work was supported by the Victorian Government's Operational Infrastructure Support Program, and National Health and Medical Research Council project grant GNT1046254. PD was supported by a Victoria India Doctoral Scholarship. JAE was supported by an Australian Research Council Future Fellowship.

Conflict of interest

The authors declare no conflict of interest.

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