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Immuno-inhibitory PD-L1 can be induced by a Peptidoglycan/NOD2 mediated pathway in primary monocytic cells and is deficient in Crohn's patients with homozygous NOD2 mutations.

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Abstract Peptidoglycan (PGN) is a ubiquitous bacterial membrane product that, despite its well known pro-inflammatory properties, has also been invoked in immuno-tolerance of the gastrointestinal tract. PGN-induced mucosal IL-10 secretion and downregulation of Toll like receptors are potential mechanisms of action in the gut but there are few data on tolerogenic adaptive immune responses and PGN. Here, using blood-derived mononuclear cells, we showed that PGN induced marked cell surface expression of PD-L1 but not PD-L2 or CD80/CD86, and specifically in the $CD14^+$ monocytic fraction. This was reproduced at the gene level with rapid induction (<4 h) and, unlike for LPS stimulation, was still sustained at 24 h. Using transfected and native muramyl dipeptide (MDP), which is a cleavage product of PGN and a specific NOD2 agonist, in assays with wild type cells or those from patients with Crohn's disease carrying the Leu1007 frameshift mutation of NOD2, we showed that (i) both NOD2 dependent and independent signalling (appearing TLR2 mediated) occurred for PGN upregulation of PD-L1 (ii) upregulation is lost in response to MDP in patients with the homozygous mutation and (iii) PD-L1 upregulation was unaffected in patients with heterozygous mutations as previously reported for cytokine responses to MDP. The uptake of PGN and its cleavage products by the intestinal mucosa is well recognised and further work should consider PD-L1 upregulation as one potential mechanism of the commensal flora-driven intestinal immuno-tolerance. Indeed, recent work

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has shown that loss of PD-L1 signalling in the gut breaks $CD8^+$ T cell tolerance to self antigen and leads to severe autoimmune enteritis.

1. Introduction

Pathogen associated molecular patterns (PAMPs) such as peptidoglycans (PGN) and lipopolysaccharides (LPS) have long been known for their pro-inflammatory properties, typically inducing cytokines from mononuclear cells such as IL-1β, TNF-α, IL-6 and IL-8. Signalling occurs via cell surface toll-like receptors (TLRs) and also via intracellular Nod-like receptors such as NOD1 and NOD2 which are the receptors for distinct peptidoglycan moieties namely *meso*-diaminopimelic acid (*meso*-DAP) [1,2] and *N*-acetyl-muramyldipeptide (MDP), respectively [3,4]. However, more recently, there has been increasing evidence that, at least in the gastrointestinal tract, the presence of PAMPs such as LPS and PGN is required for *downregulation* of inflammation and normal gut health [5,6]. Moreover, polymorphisms of NOD2 that lead to *loss* of function with respect to MDP pro-inflammatory signalling, are associated with an *increased* risk of the inflammatory bowel disease, Crohn's disease. Several factors could explain these apparent paradoxical observations. First it is now clear that, in addition to pro-inflammatory signalling, PAMPs may also stimulate cellular secretion of the regulatory cytokine IL-10 [7,8]. Secondly, cells of the gut are hyporesponsive with respect to pro-inflammatory signalling [9] and, in the absence of additional signalling from invasive bacteria, may be conditioned to respond tolerantly to PAMPs. Indeed it has been proposed that constitutive MDP exposure provides the requisite signal that, through IRF4 upregulation, leads to TLR downregulation on intestinal cells [10].

Less is understood about tolerance through acquired immune signalling in the gut although, recently, Reynoso et al. showed that loss of PD-L1 (B7-H1) signalling in the intestine breaks $CD8^+$ T cell tolerance to self-antigen and leads to severe autoimmune enteritis [11]. The Gram-negative bacterial cell-wall component, LPS, is known to stimulate PD-L1 expression in primary mononuclear cells [12] but this has not yet been demonstrated for the ubiquitous bacterial component (gram positive and gram negative), PGN, although there is limited evidence for such an effect via TLR2 in B cell cancers [13]. Here we demonstrate, in primary monocytic cells, that PGN specifically stimulates PD-L1 expression and, using Crohn's disease derived mutants, that this occurs through both NOD2 dependent and NOD2 independent mechanisms. Thus APC expression of PD-L1 in the gut may provide another mechanism through which luminal PAMPs, such as PGN, promote immuno-tolerance and downregulate mucosal inflammation and autoimmune enteritis.

2. Materials and methods

The study was approved by the research ethics committees of Cambridge (reference 03/296 and 05/Q0108/355).

2.1. Cell preparation and isolation

To investigate PD-L1 cell surface expression and the time course of PD-L1 gene expression following PAMP exposure, 20 ml heparinised venous blood was obtained from healthy volunteers, following informed consent. In some instances, buffy coats were also purchased from the national blood service. Peripheral blood mononuclear cells (PBMC) were then isolated by density gradient centrifugation and, when required, PBMC were further enriched for either monocytes or T cells by negative isolation (Dynal, Invitrogen Ltd., UK).

2.1.1. Stimulation of cells for measurement of surface antigen expression by flow cytometry

Cell suspensions at 1.10^6 cells/ml were incubated at 37 °C in 5% CO_2/95% air for 3 h in 1 ml of tissue culture medium (TCM) (RPMI 1640 (Sigma) supplemented with 10% fetal calf serum (PAA laboratories), 2 mM L-glutamine (Sigma) and penicillin/streptomycin (Sigma; 100 U/ml and 100 μg/ml respectively)) with either 10 ng/ml LPS *Escherichia Coli* (Sigma-Aldrich, UK), 5 μg/ml PGN *Staphylococcus Aureus* (Sigma-Aldrich) or saline (Sigma-Aldrich) before washing and re-suspending in fresh TCM for the remaining period of incubation (21 h). To obtain a dose response curve, 1.10^6 cells/ml were stimulated with increasing doses (ranging from 0.1 to 10 μg/ml) of PGN from *S.Aureus* and incubated for 24 hours. In some experiments, 4 mM calcium chloride was included with the PAMPs described above to induce the formation of calcium phosphate [14] and act as a transfection agent. For the examination of cell surface markers, enriched monocytic cells were stained after stimulation with recommended volumes of the combined antibodies: (i) FITC-PD-L1 (BD 557266), PE-PD-L2 (BD 558066) and PerCP Cy5.5-CD14 (BD 550787) (ii) FITC-CD80 (BD 557266), PE-CD14 (BD 555398) and PE Cy5-CD86 (BD 555659), or (iii) PE PD-L1 (Biolegend 317410) and PerCP Cy5.5 CD14 (BD 550787). Cells were stained for 20 min on ice, in the dark, before washing and fixing with 1% para-formaldehyde and protected from light thereafter. A minimum of 50,000 events per sample were immediately acquired using an EPICS XL-MCL flow cytometer (Beckman Coulter) and EXPO32 ADC software for acquisition and analysis or CYAN ADP flow cytometer and SUMMIT software for acquisition and analysis (Beckman Coulter).

2.1.2. Stimulation of cells for PD-L1 gene expression

PBMC were incubated at 1.10^6 cells/ml for 4, 8 and 24 h in 1 ml of TCM with either 10 ng/ml LPS from *E. Coli* (Sigma-Aldrich, UK), 10 ng/ml ultra pure LPS from *E. coli* (Invivogen), 10 μg/ml PGN from *S. Aureus* (Sigma-Aldrich), 10 μg/ml purified PGN from *S. aureus* (Invivogen, UK), 10 μg/ml soluble purified PGN from *E. coli* (Invivogen, UK), 10 μg/ml synthetic MDP (Sigma-Aldrich), 10 μg/ml synthetic MDP (Alexis, UK) or equivalent volume of saline

(Sigma-Aldrich) as a negative control. Following incubation at 37 °C in 5% CO_2/95% air, cells were centrifuged at 1500 rpm for 10 min and the pellet of cells prepared for RNA extraction.

2.2. Influence of genotype on cellular responses to PAMPs

To investigate whether NOD2 genotype influences PD-L1 gene expression and ensuing cytokine production following PAMP exposure, 20 ml heparinised venous blood was obtained from NOD2-genotype-stratified patients with Crohn's disease during their attendance at gastroenterology out-patient clinics and also from healthy volunteer laboratory staff. A summary of basic demographic data at baseline is given in Table 1.

Monocyte-enriched suspensions at 1.10^6 cells/ml were incubated for 8 h in 1 ml of TCM containing either 10 μg/ml MDP (Sigma-Aldrich), 10 μg/ml PGN (Sigma-Aldrich) or equivalent volume of saline (Sigma-Aldrich) as a negative control. To further facilitate MDP entry into the cytosol, cell suspensions were also incubated with MDP and 4 mM calcium chloride which, in the presence of the high phosphate-containing TCM, induces the formation of calcium phosphate [14], and acts as a transfection agent. Following incubation at 37 °C in 5% CO_2/95% air, cells were centrifuged at 1500 rpm for 10 min. Supernatants were collected and analysed by ELISA for TNF-α and IL-8 according to manufacturers' instructions (R&D systems). Cell pellets were prepared for RNA extraction.

2.3. Real time RT-PCR

Unless otherwise stated, reagents/equipment were supplied by Applied Biosystems, UK. Following experimental treatment, cultured cells were lysed in Total RNA Lysis Solution and stored at −70 °C prior to RNA isolation. Total RNA was extracted using the ABI PRISM 6100 Nucleic Acid PrespStation according to the manufacturer's instructions. This included a DNAse treatment using AbsoluteRNA Wash Solution. Extracted RNA concentration and quality were determined using a NanoDrop ND-1000 Spectrophotometer (Labtech International Ltd., UK). Synthesis of cDNA was performed using TaqMan Reverse Transcription Reagents with random hexamers on a GeneAmp PCR System 9700 Thermal Cycler. Relative mRNA expressions for TNF-α, IL-8 and 18S housekeeping control or PD-L1 were determined on an Applied Biosystems 7000 Sequence Detection System using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich, UK) or TaqMan Gene Expression Assays/Gene Expression Master Mix on an Applied Biosystems 7500 Fast Real-Time PCR System under standard reaction conditions. The following primer pair sequences (forward and reverse, respectively) were used with SYBR Green: TNF-α (5′-CCTGCCCCAATCCCTTTATT-3′, 5′-CCCTAAGCCCCCAATTCTCT-3′ [15], IL8 (5′-ATGACTTCCAAGCTGGCCGTG-3′, 5′-TCTCAGCCCTCTTCAAAAACTTCTC-3′ [16] and 18S (5′-CGGCTACCACATCCAAGGAA-3′, 5′-GGCTGCTGGCACCAGACTT-3′ [17]). Relative mRNA expressions for PD-L1 and 18S housekeeping control were determined using TaqMan Gene Expression Assays as above (primer sequences not available from Applied Biosystems). Results were calculated using the ΔΔCt method and for statistical analysis expressed as log2 gene fold-inductions relative to the untreated control.

Table 1
Summary of basic demographic data for patients and healthy volunteers, at baseline.

Subject	Gender	Genotype
Crohn's disease patient 1	Female	L1007fs/L1007fs
Crohn's disease patient 2	Female	L1007fs/L1007fs
Crohn's disease patient 3	Male	R702W/L1007fs
Crohn's disease patient 4	Male	R702W/L1007fs
Healthy control 1	Male	WT
Healthy control 2	Male	WT
Healthy control 3	Female	WT
Healthy control 4	Male	WT
Healthy control 5	Female	WT
Healthy control 6	Male	WT

2.4. Statistics

Differences in PGN and MDP responses between healthy subjects and patients with Crohn'disease were analysed by unpaired t tests. Differences among means were considered significant at P values < 0.05.

3. Results

Peripheral blood mononuclear cells (PBMC), enriched for monocytic cells from healthy donors and stimulated for 3 h with PGN from *S. Aureus*, increased by 5 fold (i.e. from 18% to 90%) the number of $CD14^+$ gated cells that showed enhanced PD-L1 protein cell surface expression at 24 h as measured by mean fluorescence intensity (Figs. 1A and B). Moreover, the mean fluorescence intensity (MFI) for all $CD14^+$ gated cells increased in a dose responsive fashion upon exposure to PGN from *S. Aureus* (Fig. 1C). Unexpectedly, LPS did not lead to a significant increase in PD-L1 expression (Figs. 1D and E). The PGN effect, however, was specific for PD-L1 amongst the inducible B7 proteins as it was not observed for PD-L2 (Fig. 1B) or CD80 (Supplemental Fig. 1) at 24 h. Expression did not increase significantly for CD86 (Supplemental Fig. 1), although this could have been due to slower expression kinetics of the ligand [18,19]. The PGN effect on PD-L1 was specific to the monocytic cell fraction (i.e. $CD14^+$) and was not observed for $CD14^-$ cells (Fig. 1F). Thus, in further work, monocytic enrichment was not always considered necessary. Previous work with a synthetic viral PAMP, namely Poly I:C, has shown that PD-L1 up-regulation is transcriptionally driven [20]. To determine this for PGN, we undertook real time PCR analyses of the PD-L1 gene at 4, 8 and 24 h following stimulation of PBMC again with crude PGN of *S. aureus*. Expression peaked at 8 h (range of linear induction 12–40 fold) but was also strongly apparent (7–22 fold) at 4 h and was retained at 24 h (Fig. 2A). To confirm that the effect was due to PGN and not some minor contaminant we also used purified PGN from *S. aureus* and showed a similar pattern of PD-L1 upregulation, albeit slightly attenuated in magnitude (6–24 fold) (Fig. 2B). Initial responses to LPS were observed as expected in terms of PD-L1 upregulation, but were not

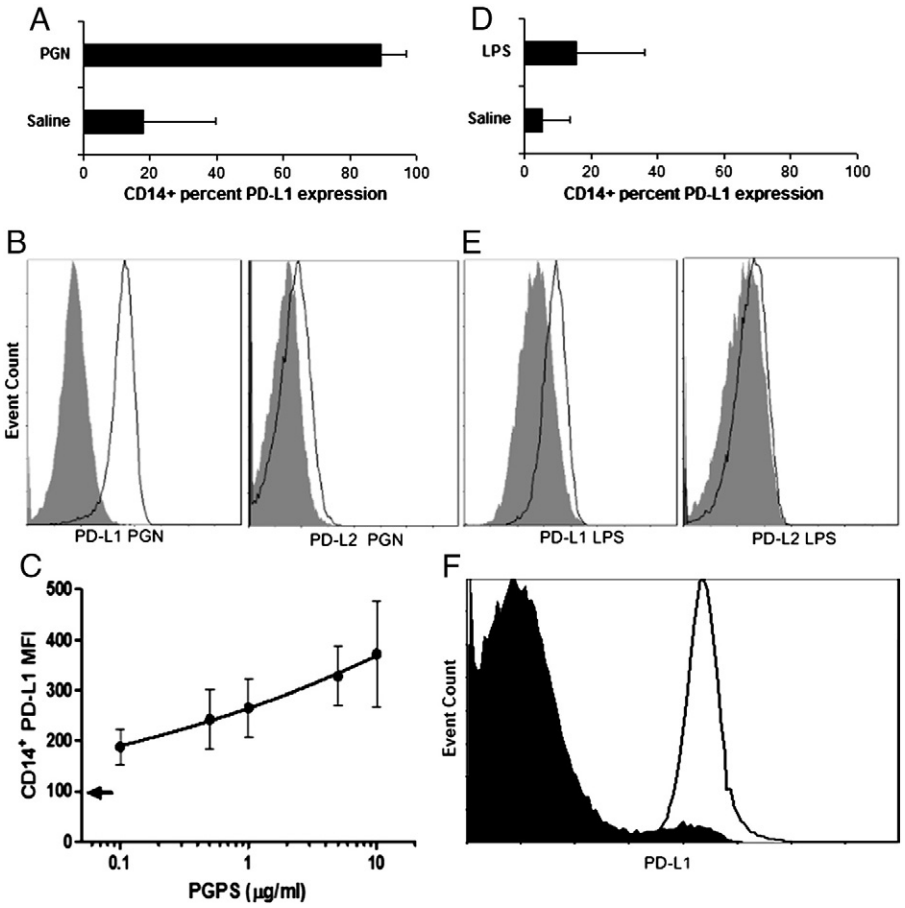

Figure 1 (A). Percentage of $CD14^+$ cells expressing PD-L1, 24 hours after stimulation with PGN from *S. Aureus* (5 μg/ml) (n = 5; p = 0.001 PGN versus saline). (B). Example fluorescence density plots gated on $CD14^+$ cells in the live monocyte gate, showing PD-L1 and PD-L2 expression in response to PGN from *S. Aureus*, grey fill = saline control, no fill = PGN. (C). The effect of increasing doses of PGN from *S. Aureus* on PD-L1 expression measured by mean fluorescence intensity (MFI), n = 6, baseline levels of PD-L1 staining are indicated by the arrow. (D). Percentage of $CD14^+$ cells expressing PD-L1, 24 hours after stimulation with LPS from *E. Coli* (10 ng/ml, n = 5). (E). Fluorescence density plots gated on $CD14^+$ cells in the live monocyte gate, showing PD-L1 and PD-L2 expression in response to LPS from *E. Coli*, grey fill = saline control, no fill = LPS. (F). PD-L1 expression on $CD14^+$ (no fill) and $CD14^-$ (back fill) cells after PGN stimulation.

sustained at 24 h (Fig. 2C) explaining the purported lack of response by flow cytometric analysis (i.e. Figs. 1D and E).

To determine the importance of TLR2 versus NOD2 in the PGN response we used purified soluble PGN which does not engage this receptor significantly [21] but does signal through NOD2. Weak but still significant induction of PD-L1 was observed at 4 h (2–14 fold) and this pattern of stimulation was closely mimicked by the synthetic NOD2 agonist muramyl dipeptide (2–12 fold) (Fig. 2D). MDP has also been suggested to signal through NALP1 and NALP3, at least for inflammasome activation [22,23], so we next isolated cells from patients with Crohn's disease carrying the rare homozygous Leu1007 frameshift mutation in NOD2, where an absolute failure in MDP–NOD2 signalling is observed. To enhance gene expression signal, we used enriched monocytes derived from PBMC and chose an 8 h time point where maximal PGN stimulation and adequate MDP stimulation of PD-L1 mRNA were observed in healthy cells (Figs. 2A and D). In this case MDP was unable to induce PD-L1 gene expression (Fig. 3A; $p < 0.05$ versus healthy subjects) or, as expected, gene expression and secretion of the pro-inflammatory cytokines TNF-α and IL-8 (Figs. 3A and B; $p<0.01$ versus healthy subjects) implying an absolute requirement for NOD2 signalling in MDP regulation of PD-L1 as well as that already known for the pro-inflammatory cytokines. This contrasted with the positive gene expression of PD-L1 and IL-8 as well as the secretion of TNF-α and IL-8 proteins in healthy control cells upon MDP stimulation (Figs. 3A and B). Finally, to confirm that the lack of MDP response in NOD2 Leu1007fs cells was not due to poor cellular uptake of the ligand [4], we transfected MDP using calcium phosphate. As anticipated, this enhanced both message and secretion of IL-8 and TNF-α in healthy control cells (Figs. 3C and 3D versus 3A and 3B), albeit not PD-L1, and showed no effect in NOD2 Leu1007fs cells except for a small variation for cytokine secretion around baseline levels (i.e. versus sham transfection, Figs. 3C and D).

For PGN (*S. aureus*)-stimulation of the above cell types we reasoned the opposite outcomes: namely that PD-L1 and

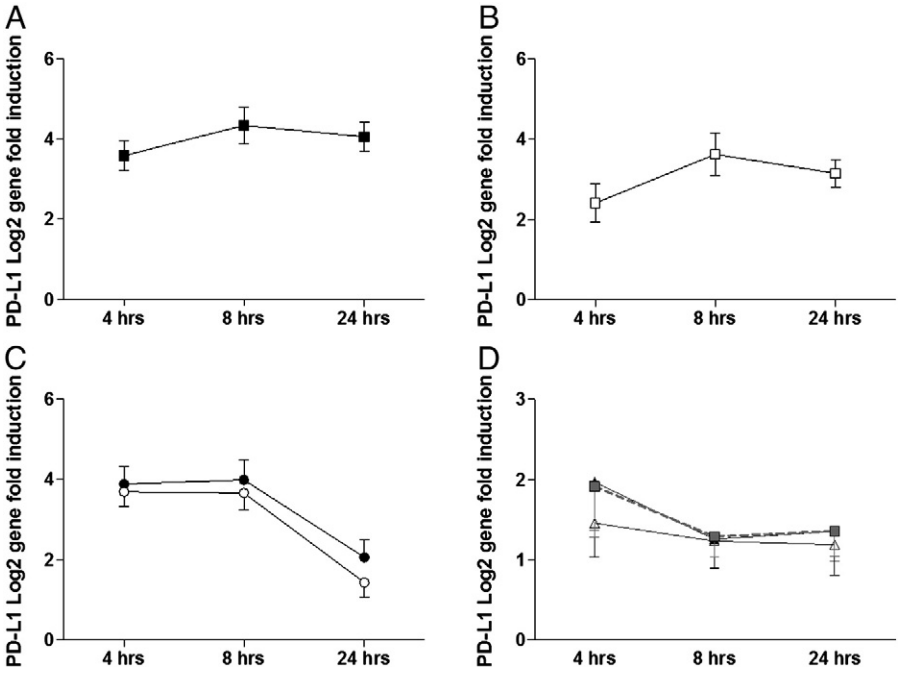

Figure 2 Time course of PD-L1 log2 gene expression in response to PAMPs. PD-L1 log2 gene expression following stimulation of PBMC with (A) PGN from *S. aureus* (10 μg/ml) (B) purified PGN from *S. aureus* (10 μg/ml) (C) regular (black circle) and ultra-pure LPS (open circle) both from *E. coli* (10 ng/ml) and (D) soluble PGN from *E. coli* (grey square; 10 μg/ml) and synthetic MDP from two sources (open triangle from Sigma; closed triangle from Alexis; 10 μg/ml). Data are mean ± SEM (n = 4).

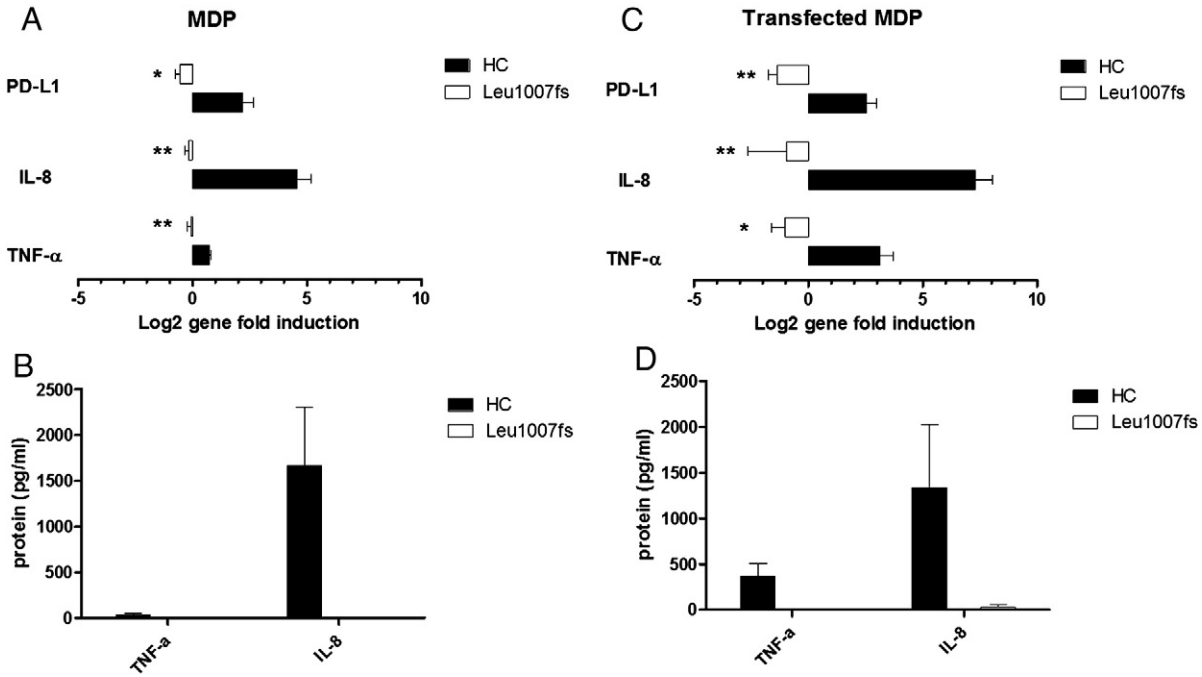

Figure 3 Influence of genotype on cellular responses to MDP. PD-L1, IL-8 and TNF-α log2 gene expression following synthetic MDP stimulation (Sigma, 10 μg/ml) (A) without and (B) with calcium phosphate transfection in enriched monocytes isolated from healthy controls (HC; n = 6) or patients with Crohn's disease homozygous for the NOD2 Leu1007 frameshift mutation (Leu1007fs; n = 2). IL-8 and TNF-α protein secretion (pg/ml) following MDP stimulation (Sigma; 10 μg/ml) (C) without and (D) with calcium phosphate transfection in enriched monocytes isolated from healthy controls (HC, n = 6) or patients with Crohn's disease homozygous for the NOD2 Leu1007 frameshift mutation (Leu1007fs; n = 2).* $p < 0.05$ and ** $p < 0.01$ versus healthy controls.

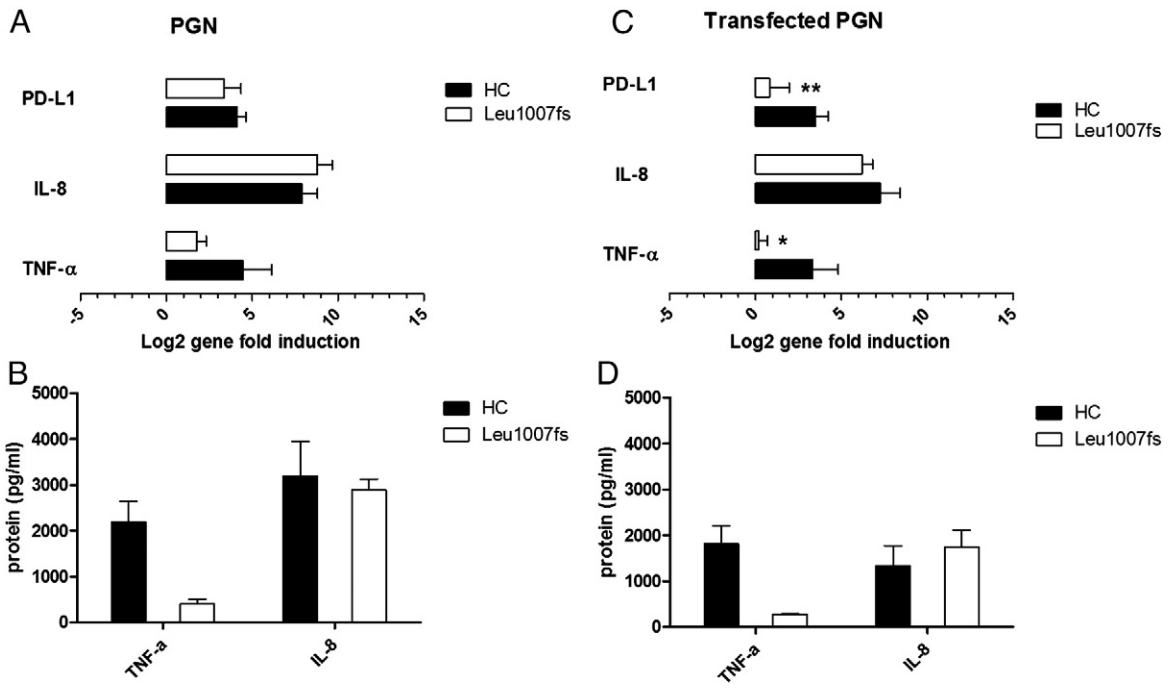

Figure 4 Influence of genotype on cellular responses to PGN. PD-L1, IL-8 and TNF-α log2 gene expression following *S. aureus* PGN stimulation (10 μg/ml) (A) without and (B) with calcium phosphate transfection in enriched monocytes isolated from healthy controls (HC; n=5) or patients with Crohn's disease homozygous for the NOD2 Leu1007 frameshift mutation (Leu1007fs; n=2). IL-8 and TNF-α protein secretion (pg/ml) following *S. aureus* PGN stimulation (10 μg/ml) (C) without and (D) with calcium phosphate transfection in enriched monocytes isolated from healthy controls (HC; n=5) or patients with Crohn's disease homozygous for the NOD2 Leu1007 frameshift mutation (Leu1007fs, n=2). * $p<0.05$ and ** $p<0.01$ versus healthy controls.

cytokine responses would remain intact in response to native PGN in NOD2 Leu1007fs cells due to non-NOD2 signalling, while transfection of PGN would enhance its intracellular delivery such that, upon digestion to its minimal motif, namely MDP, it would favour the (dysfunctional) NOD2 pathway and reduce responsiveness to whole PGN. Indeed, PGN signalling appeared normal in NOD2 Leu1007fs cells and, for both these cells and healthy control cells. PD-L1 and IL-8 responses were lower normal in NOD2 Leu1007fs cells and, in all cases, PD-L1 and IL-8 responses were lower in PGN-transfected cells versus those receiving PGN alone (Fig. 4). Notably, upon transfection of PGN, expression of PD-L1 and TNF-a were especially decreased in NOD2 Leu1007fs cells (Fig. 4C, $p<0.01$ and $p<0.05$ versus healthy subjects, respectively) in NOD2 Leu1007fs cells (Fig. 4C). In all cases, responses to soluble LPS were normal (Supplemental Fig. 2).

Previous work has shown that the absolute failure in MDP–NOD2 signalling is associated only with the homozygous NOD2 Leu1007 frameshift mutation and not single nucleotide mutations or even compound heterozygote mutations involving NOD2 Leu1007fs. We confirmed this here for transfected MDP induction of PD-L1 expression in enriched monocytic cells from patients with Crohn's disease bearing NOD2 mutations other than Leu1007fs (see Table 1 for patient genotype details). After 8 h, PD-L1 expression was upregulated by about 4 fold (linear induction) (Supplemental Fig. 3A). Positive responses were seen for TNF-α and IL-8 both at the message and protein level (Supplemental Figs. 3A and 3B).

4. Discussion

The nature of the cellular response to TLR engagement depends upon the level of exposure to PAMP and the site or "compartment" of exposure (e.g. gut versus lung) [24]. Disruption of such a careful balance and abnormal TLR activation has been linked to a number of inflammatory and autoimmune diseases, including the inflammatory bowel diseases [24,25]. Since (i) under certain circumstances (e.g. in the gut) PGN may invoke tolerogenic responses [5,6] and (ii) PD-1 ligand interactions have been implicated in the maintenance of tolerance and the control of autoimmunity [11,26,27], this study aimed to investigate the expression of PD-1 ligands following PGN/MDP stimulation of mononuclear cells derived from both healthy subjects and from patients with Crohn's disease carrying known NOD2 genetic mutations. The numbers studied were inevitably low due to the very rare occurrence of the NOD2 Leu1007fs mutation in the population but differences were absolute and the data, therefore, compelling.

Our findings indicate that, as with other PAMPs, PGN stimulates the upregulation of PD-L1 in primary monocytic cells. Both NOD2 dependent and NOD2 independent pathways are apparent because a distinct PGN moiety, namely MDP, could not induce PD-L1 in cells carrying a homozygous frameshift mutation in NOD2 whereas whole *S. aureus* PGN signalled normally. The latter effect is likely to occur via TLR2 engagement since *S. aureus* PGN binds this receptor with reasonably high affinity, triggering pro-inflammatory

cytokine secretion [28,29] whereas digested monomeric PGN from *B. anthracis* [30] or purified PGN from *E. coli* have no such effect [21,29] and, similarly in this work, appeared equivalent to MDP in its ability to induce PD-L1. The pathway linking TLR or NOD engagement to PD-L1 upregulation is unclear but the parallels with pro-inflammatory cytokine signalling throughout this work suggest that it may involve NF-κB activation. A second possibility concerns type I interferons: these may be stimulated by cellular exposure to PGN/MDP [31,32] and recent work has indicated that the type I interferons also stimulate PD-L1 upregulation [33].

Irrespective of the mechanisms, an important question is why microbial PAMPs, including the ubiquitous PGN structure, should at all induce a co-stimulatory molecule that is commonly involved in immuno-tolerance in primary phagocytic cells? Reynoso et al. indicate that PD-1:PD-L1 interactions, or CD80:PD-L1 interactions, are vital to check unrestrained proliferation of cytotoxic T cells during neo-antigen presentation within a background of naïve but antigen-specific $CD8^+$ lymphocytes in the small intestine [11]. In their model, inhibition of this process led to severe autoimmune enteritis [11]. It is possible, therefore, that this "PD-L1 safety net" is also operative during bacterial infection, and driven in part by PGN, as a balance would be required to achieve clearance of the pathogen without the development of over-zealous cytotoxic $CD8^+$ T cells that increase the risk of autoimmune responses (towards APCs or even new targets through molecular mimicry). However, increasing evidence points to a down side to such a mechanism, namely that the PD-1/ligand pathway may be exploited by pathogens as a mechanism of immune evasion. Examples are apparent in viral, bacterial, protozoan and parasitic worm infections [27]. In all cases it appears that the PD-1 pathway is utilised by the pathogen to escape host immune responses and establish persistent infection. Parallel to the persistence of infection, PD-1 expression has also been associated with T cell exhaustion and disease severity. Correlations between viral load and PD-1 expression on T cells have been reported in HIV infection, with lower PD-1 expression associated with long-term non-progressors [34,35]. Pertinent to our study, translocation of microbial constituents from the gut into blood in HIV infection has been reported to inhibit T cell expansion and function via up-regulation of PD-1 and IL-10 production by monocytes, thereby restraining HIV specific T cell responses. The systemic modulation of immune responses described was induced by several bacterial TLR ligands [36]. At the level of the gut contemporary understanding is that commensal bacteria have an active role in both shaping and maintaining intestinal immunity, via TLRs and NLRs and that disruption of such signalling risks inflammation and autoimmunity [25]. With this in mind it is even possible that purposeful acquisition occurs, of luminal PGN fragments by mucosal antigen presenting cells, to enable PD-L1 upregulation and tolerogenic signalling to T cells, especially following APC migration to the mesenteric lymph nodes. Certainly, a PGN-based tolerogenic mechanism for the innate immune system has been suggested by Watanabe et al. with downregulation of TLR responses being triggered in mucosal cells following the engagement of NOD2 by luminal PGN fragments [10]. This, or the acquired PD-L1 mechanism proposed here, require the presence of intracellular bacterial fragments in the bowel wall and it is noteworthy that a number of reports have demonstrated this, especially in the Peyer's patch, terminal ileum and even the colon [37–39]. How such fragments are acquired by mucosal immune cells, and whether this leads to the induction of cell surface PD-L1 *in vivo* as we have shown here *in vitro*, remains to be established but are active areas of research. Finally it should be noted that PD-1 interactions are thought to be of considerable importance for hepatic CD4 T cell tolerance [40–41] and interestingly, during preparation of this manuscript, Castellaneta et al. reported that PGN induces PD-L1 expression *in vivo* in hepatic plasmacytoid dendritic cells in a NOD2 dependent fashion [42]. Unlike us they found no effect in *in vitro* cultures, which probably reflects the different cell types investigated, but at least adds evidence to the idea that PGN fragments may induce the functional *in vivo* expression of PD-L1 in splancnic myeloid cells. Additionally, it is noteworthy that other TLR agonists have recently been shown to be important for the generation of tolerogenic APC during dendritic cell differentiation inducing a STAT-3 mediated expression of PD-L1 [43]. Several genetic defects associated with the intracellular identification and processing of bacterial components have been identified in inflammatory bowel disorders, and further work with gut APCs is merited. In summary, *in vitro*, PGN induces PD-L1 upregulation in primary monocytic cells which could further explain how luminal bacteria and bacterial fragments can impact anti-inflammatory and tolerogenic signalling in the gut.

Supplementary materials related to this article can be found online at doi:10.1016/j.clim.2012.01.016.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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