

1 ***TLR4, IL10RA, and NOD2* mutation in paediatric Crohn's disease patients: An association**
2 **with *Mycobacterium avium paratuberculosis* and TLR4 and IL10RA expression**

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29 **ABSTRACT**

30 **BACKGROUNDS:**

31 *Mycobacterium avium* subspecies *paratuberculosis* (MAP) has been implicated in the
32 pathogenesis of Crohn's disease (CD). The role of CD susceptibility genes in association with
33 these microbes is not known.

34 **METHODS:**

35 Sixty-two early onset paediatric CD patients and 46 controls with known MAP status were
36 analysed for an association with 34 single nucleotide polymorphisms (SNPs) from 18 CD
37 susceptibility genes. Functional studies on peripheral blood mononuclear cells (PBMCs) were
38 conducted on 17 CD patients with known CD mutations to assess *IL-6*, *IL-10*, and *TNF-α*
39 expression upon stimulation with MAP precipitated protein derivative (PPD) and
40 lipopolysaccharide (LPS). In addition, surface expression of *IL10R* and *TLR4* on resting B cells,
41 NK cells, T cells and monocytes were assessed.

42 **RESULTS:**

43 A mutation in *TLR4* (rs4986790) and *IL10RA* (rs22291130) were significantly associated with
44 MAP positive CD patients compared to MAP negative CD patients (27.6% versus 6.1%, $p =$
45 0.021, and 62.1% versus 33.3%, $p = 0.024$, respectively). PPD and LPS significantly increased
46 IL-6, IL-10, and TNF- α production in PBMCs. IL-10 and TNF- α production was significantly
47 lower in a subgroup of CD patients (5/12) with a known *NOD2* mutation. Receptor for IL-10 was
48 significantly higher expressed on NK cells (CD56^{low}) and on NK T cells harboring a *NOD2*
49 mutations compared to wildtype cells ($p=0.031$ and 0.005, respectively). *TLR4* was significantly
50 higher expressed on NK cells (CD56^{high}) harboring a *NOD2* mutations compared to wildtype
51 cells ($p = 0.038$).

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53 **KEYWORDS:**

54 Crohn's disease, paediatric, *Mycobacterium avium* subspecies *paratuberculosis*, mutation,
55 cytokine

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60 INTRODUCTION

61 Crohn's disease (CD) is a multifactorial disease in which a T-cell mediated mucosal
62 inflammation is caused by a combination of environmental triggers in genetically susceptible
63 individuals [1]. The most researched microbe is *Mycobacterium avium* subspecies
64 *paratuberculosis* (MAP). MAP is the cause of Johne's disease in ruminants, a disease clinically
65 very similar to CD in humans [2-4]. However, its role in human CD remains controversial [5-7]
66 possibly due to discrepancies in detection methodology and the influence of antibiotic therapy
67 on the gut microflora [8]. Many of the prevalence studies have been conducted in adult
68 populations whose gut microbiota might have been altered by long term drug treatment. Despite
69 these confounding factors, numerous studies and meta-analysis confirmed that CD patients
70 have an increased risk of being positive for MAP [9-11]. We have previously reported the
71 detection of MAP in a cohort of paediatric onset CD patients with no prior drug therapy. MAP
72 was detected by specific PCR and culture more often in children with CD compared to control
73 children [12, 13]. MAP may be implicated in the pathogenesis of CD, perhaps in conjunction
74 with susceptibility genes. An association with *Nod2* (nucleotide oligomerisation domain 2)
75 deficiency and reduced inflammatory response was reported in *Nod2* *-/-* mice infected with
76 pulmonary mycobacteria [14]. Studies with human peripheral blood mononuclear cells (PBMCs)
77 have demonstrated that individuals homozygous for the minor allele of *NOD2* rs5743293
78 (3020inC) had an up to 70% defective cytokine response (*TNF- α* , *IL-1 β* , and *IL-10*) after
79 stimulation with *M. paratuberculosis* compare to PBMCs with the wildtype gene.

80

81 The recognition of *Mycobacterium tuberculosis* by pattern recognition receptors (PRR) is
82 essential for the activation of both the adaptive and innate immune response. An important
83 component is the activation of the NFkappaB signaling pathway, which results in the
84 transcription of proinflammatory cytokine genes and thus innate immune responses [15]. The
85 activation of NFkappaB pathway takes place via recognition of bacterial components from cell

86 surface receptors such as Toll like receptor (TLR) receptors and *NOD2* signaling [16, 17].
87 Conflicting data are available for an association between *TLR4* mutations and CD in humans.
88 However, a meta-analysis of studies of the most common *TLR4* mutation rs4986790
89 (Asp299Gly) revealed that 10 of 12 studies had increased 299Gly carrier frequency in CD
90 patients [18]. *In vitro* cell culture studies and *in vivo* mouse experiments have shown that *NOD2*
91 and *TLR* are two of the non-redundant mechanisms for the detection of *Mycobacterium* [14, 19,
92 20]. Ferwerda and colleagues showed that *M. tuberculosis* and MAP stimulated Chinese
93 hamster ovary (CHO) cell lines transfected with human *TLR2* or *TLR4* [19, 20]. The same group
94 has also shown up to 50% decrease in cytokine production in *TLR2* and *TLR4* knock-out mice
95 models after stimulation with Mycobacteria.

96

97 The function of interleukin 10 (IL-10) as a pleiotropic cytokine produced by T-cells, B-cells, and
98 macrophages, is well defined as an immune and anti-inflammatory regulator in both innate and
99 cell-mediated immunity [21]. IL10 receptor alpha (IL10RA) plays an essential role in IL10
100 mediated immune regulation [22]. The observation that *IL10* *-/-* mice developed spontaneous
101 enterocolitis [23] and the implication of *IL10RA* in ileitis mouse models [24] have suggested that
102 human *IL10RA* mutation(s) play a role in the regulation of the inflammatory response in the
103 human gut. At least 11 single nucleotide polymorphisms (SNPs) are known on the *IL10RA* gene
104 of which many are silent mutations [25]. Gasche and colleagues identified two novel *IL10RA*
105 variants in humans (rs2229113 and rs3135932) and showed that carriers have a reduced
106 sensitivity to IL10. As well, there is reduced ability of IL10 to inhibit lipopolysaccharide induced
107 TNF-alpha production in human monocytes, indicating that this variant may be a loss-of function
108 allele [26].

109 Animal studies and *in vitro* cell culture studies have provided some insights into the role of CD
110 susceptibility genes and microbial infection. The role of human CD susceptibility genes
111 associated with microbial infection is largely unknown. In our study we investigated the

112 association between 34 SNPs and MAP infection in a paediatric CD cohort, and explored
113 association of *TLR4* and *IL10RA* polymorphisms and cytokine expression with MAP infection in
114 early onset paediatric CD patients.

115

116 **MATERIAL AND METHODS**

117 **Patient cohort**

118 A total of 116 children (62 with CD and 54 with non-IBD) were previously analysed for the
119 presence of MAP in mucosal gut biopsies, peripheral blood mononuclear cells (PBMCs) and by
120 culture of gut biopsies [12]. A subsequent genetic study genotyped 72 CD patients and 98 non-
121 IBD control patients for 34 CD susceptibility SNPs present in 18 genes [27]. Thus MAP status
122 and genotype data were available for 62 paediatric onset CD patients and 46 non-IBD controls
123 (Supplementary Table 1). Logistic regression analysis was performed on this group to test for
124 an association between MAP infection and presence of susceptibility genotypes (Supplementary
125 table 1). All statistical analysis was performed using STATA 11. All statistical cytokine analysis
126 was performed using GraphPad prism (version 5.0). Ethics approval for the study was obtained
127 from the Human Ethics Committee of the Royal Children's Hospital. Informed consent was
128 obtained from each individual parent or guardian.

129

130 **Functional studies**

131 Functional cytokine studies and cell surface receptor staining were performed on PBMCs
132 isolated from 17 CD patients with an average age of 12.8 years (8-17.2) (Table 1).

133

134 ***In vitro* PBMC stimulation with MAP precipitated protein derivative (PPD) and 135 lipopolysaccharide (LPS).**

136 PBMCs were isolated from whole blood as previously described [12]. Frozen PBMC were
137 thawed and re-suspended in RPMI-1640 supplemented with 5% FCS and

138 penicillin/streptomycin. Half a million PBMCs were stimulated with PPD (1:200 dilution of 0.5
139 mg/ml: final concentration) or LPS (100 ng/ml) for 20 hrs at 37°C, 5% CO₂, 95% humidity. The
140 culture supernatants were collected and stored at -70°C for later cytokine ELISA. PPD was
141 prepared from MAP cultures at the CSIRO Australian Animal Health Laboratory as described by
142 Wynne *et al* [28].

143

144 **Cytokine ELISA**

145 The culture supernatants were assayed for TNF, *IL-6* and *IL-10* using a commercial ELISA (BD
146 Biosciences) according to the manufacturer's specifications. Assay's limit of detection was 8
147 pg/ml.

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149 **Flow Cytometry of *TLR4* and *IL10R* expression**

150 TLR4 and IL10 expression was performed on PBMC using flow cytometry. PBMC were stained
151 with the following anti-human monoclonal antibodies: anti-CD14 APC Cy7, anti-CD3 (Pacific
152 Blue), anti-CD56 APC, anti-CD19 PE Cy7 (BD Biosciences), anti-TLR4 PE (eBioscience) and
153 anti-IL10R (CDw210) PE (BD Pharmingen). CD14-positive monocytes (n = 10,000) were
154 acquired on a FACS Canto (BD Bioscience), and FlowJo software (Tree Star) was used for
155 analysis and the geometric mean fluorescence of each sample was normalized to its own
156 isotype-matched control.

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163 **RESULTS**

164 **MAP - genotype interaction**

165 The potential association of MAP and SNP genotypes were analysed for 62 CD patients and 46
166 non-IBD controls (Supplementary Table 1). The proportion of wildtype alleles of each of the 34
167 SNPs were stratified with MAP status (positive and negative) in the CD group. Linkage between
168 *TLR4* mutation (rs4986790) and *IL10RA* mutation (rs2229113) and the presence of MAP
169 infection was detected in a significant subgroup of patients. Both wildtype alleles of SNPs
170 rs2229113 and rs4986790 were significantly more often detected in MAP negative patients
171 compared to MAP positive patients (66.7% versus 37.9%, OR = 3.27, 95% CI 1.15-9.28, p =
172 0.024, and 93.9% versus 72.4%, OR = 5.90, 95% CI = 1.14-30.61, p = 0.021, respectively)
173 (Figure 1a). The very conservative Bonferroni correction would have generated no significant
174 association. In the non-IBD control group no statistically significant associations were detected
175 between MAP positive and MAP negative individuals (Supplementary Table 1).

176
177 Manual interaction analysis of minor allelic SNPs rs2229113 (*IL10RA*) and rs4986790 (*TLR4*)
178 revealed that both minor allelic SNPs were detected in combination more often in MAP positive
179 patients compared to the same SNP variant at the individual levels (82.8% versus 62.1% and
180 27.6%, respectively). The difference between MAP positive and MAP negative CD patients
181 carrying a combination of minor allelic *IL10RA* SNP rs2229113 and *TLR4* SNP rs4986760 was
182 highly significant (p <0.001, OR = 7.39, 95% CI = 2.25-24.27) (Figure 1b). Both SNP variants
183 remained significantly associated with MAP infection after adjustment by logistic regression
184 (Figure 1.b).

185
186 Logistic regression analysis was performed to analyse SNP-SNP interaction and MAP infection
187 between *IL10RA* rs2229113 variant and *TLR4* rs4986790 variant within and with all other SNPs
188 included in the previous genotype study. Logistic regression analysis of *TLR4* SNP rs4986790

189 with all other SNPs revealed that all interactions were not significant. Logistic regression
190 analysis was significant for one MAP-SNP-SNP interaction involving *IL10RA* SNPs rs2229113
191 and *IL23R* SNPs rs7517847 ($p = 0.042$, OR = 9.55 95% CI = 1.08-84.21). Due to the large
192 confidence interval and not passing Bonferroni correction ($p = 0.0014$) this combination was not
193 considered significant.

194
195 In our previous study we cultured MAP from biopsy tissue from CD patients [12]. All four
196 patients from whom MAP was cultured contained a mutation in one of the MAP associated
197 genes, either *IL10RA* or *TLR4* (Table 2). In contrast, only one of the six MAP culture negative
198 patients had a MAP associated mutation however, the same patient was MAP positive in the
199 biopsy sample.

200

201 **Expression of IL-6, IL-10 and TNF**

202 PBMCs from 17 CD patients harboring a range of mutations including *NOD2* variants, *TLR4*
203 variants, and *IL10RA* variants were stimulated with PPD, LPS, and combined PPD+LPS.
204 Cytokine production for IL-6, IL-10, and TNF- α , regardless of mutation status, were 3.7 fold, 3.1
205 fold, and 2.3 fold higher upon stimulation with PPD compared to un-stimulated PBMCs (Figure
206 2). The same cytokines were 5.1 fold, 7.2 fold and 8.4 fold higher upon LPS stimulation
207 compared to un-stimulated PBMCs (Figure 2). IL-10 and TNF- α expression was significantly
208 higher upon stimulation with LPS compared with PPD (Figure 2). No significant changes in *IL-6*,
209 *IL-10* and TNF- α production were detected between PBMCs harboring a mutation in *NOD2* and
210 *TLR4* and/or *IL10RA* compared to PBMCs with the wildtype form of the genes (Supplementary
211 Figure 1a to 1f).

212

213 Adjustment for MAP infection revealed no significant changes in IL-6, IL-10 and TNF- α
214 production between PBMCs from MAP positive CD patients compared with MAP negative CD

215 patients (Supplementary Figure 1g to 1i). We further analysed each patient individually for the
216 effect of polymorphisms in 34 SNP variants by comparing IL-6, IL-10, and TNF- α production
217 upon stimulation with PPD, LPS and PPD+LPS. The 34 SNP variants were located on 19 genes
218 including, *NOD2* (n=4 SNPs), *NOD1* (n=1 SNP), *TLR4* (n=1 SNP), *IL10RA* (n=2 SNPs), *PSMG1*
219 (n=1 SNP), *TNFRSF6B* (n=2 SNPs), *IBD5* (n=2 SNPs), *SCL22A4* (4/5) (n=2 SNPs), *IL23R* (n=3
220 SNPs), *ATG16L1* (n=1 SNP), 10q21.1 (n=1 SNP), 3q21 (n=1 SNP), *NKX2-3* (n=1 SNP), *ABCB1*
221 (n=1 SNP), *IRGM* (n=1 SNP), *NELL1* (n=1 SNP), *MYO9B* (n=3 SNPs), *DLG5* (n=6 SNPs).
222 Primary adjustment was conducted with *NOD2* mutations versus *NOD2* wildtype alleles (Figure
223 3). In a subgroup of CD patients (5/12) with at least one mutation in the main *NOD2* allele
224 cytokine expression for *IL-10* and *TNF- α* were significantly lower compared to the remaining
225 seven *NOD2* positive patients ($p < 0.01$) (Figure 3). Similar results were obtained between the
226 same subgroup for IL-6 expression but the differences did not reach statistical significance
227 ($p = 0.07$) (Figure 3). Analysis of the *NOD2* gene variants revealed a distinct subgroup with
228 significant lower cytokine expression, where the subgroup had a significant reduction of IL-6 and
229 TNF production when compared with the *NOD2* wildtype patients (Figure 3). This was not
230 observed with any of the other genes (data not shown). None of 34 individual SNP variants
231 could be specifically associated with the low cytokine expressing subgroup (CD patients 1 to 5,
232 data not shown). Also, the MAP status of the low cytokine expressing subgroup (patients 1 to 5
233 Figure 3) was not significantly different from the seven high cytokine expressing subgroup
234 (patients' 6-12 Figure 3) or to MAP status of the *NOD2* wildtype group (Figure 3).

235

236 ***IL10* receptor and *TLR4* receptor expression**

237 Basal *IL10R* and *TLR4* protein expression was assessed on the surface of B cells, NK cells
238 (CD56 high), NK cell (CD56 low), NK T cells, T cells, and monocytes. Both receptors were
239 chosen because their two mutated forms were identified significantly more often in MAP positive
240 CD patients compare to MAP negative CD patients. Adjustment for *NOD2* mutation revealed a

241 significantly higher level of *IL10R* expression in NK cell (CD56 low) ($p = 0.031$) and in NK T cells
242 ($p = 0.005$) and a significantly higher level of TLR4 expression in NK cell (CD56 high) ($p =$
243 0.038) harboring at least one main *NOD2* mutation (Figure 4). No significant differences in
244 IL10R and TLR4 expression was observed between patients with or without *TLR4* and/or
245 *IL10RA* mutations and between MAP positive and MAP negative patients (data not shown).

246

247 **DISCUSSION**

248 In this study, we explored whether the presence of MAP was associated with any of 34 SNP
249 variants present on 19 CD susceptibility genes in a paediatric CD cohort. The expression levels
250 of three common cytokines were then analysed in the context of MAP and mutation status in
251 these paediatric CD patients. This study demonstrated an association between the presence of
252 MAP and mutations in *TLR4* (rs4986790 Asp299Gly) and *IL10RA* (rs2229113 Gly330Arg) in
253 paediatric CD. A synergistic interaction between the MAP associated genetic variants was
254 observed, with an increase in odds ratio from 3.27 to 6.05, and 5.9 to 13.14 if *IL10RA* mutation
255 was adjusted for *TLR4*, and *TLR4* mutation was adjusted for *IL10RA*, respectively.

256

257 The importance of *TLR4* and *IL10* dependency was reported by Ronni *et al* [29] previously, who
258 revealed that functional TLR4 is required in macrophages for activation of anti-inflammatory IL-
259 10 promoters. TLR4 also plays a central role in bacterial LPS signal transduction by activation of
260 multiple pathways including the anti-inflammatory *IL-10* genes [29]. The *IL10RA* Gly330Arg
261 polymorphism was associated with reduced sensitivity of *IL-10* and consequently reduced
262 inhibition of TNF- α production [26]. In our previous study the *IL10RA* Gly330Arg polymorphism
263 was not associated with CD [27], however, in other studies non-synonymous polymorphisms in
264 *IL10RA* were detected more frequently in adult and paediatric CD adult patients compared to
265 controls [26, 27]. Thus *IL10RA* mutations alone are not necessarily implicated in CD but may be
266 associated with specific microbial infections.

267 Mutations in *TLR1*, *TLR2* and *TLR4* genes can alter the ability to recognize pathogen
268 associated molecular patterns (PAMPs) [30]. The important role of *TLR4* Asp299Gly
269 polymorphism in human disease was reported in pulmonary tuberculosis patients [31], [32], and
270 osteomyelitis patients [33]. Similarly this study identified an association between *TLR4* mutation
271 (Asp299Gly) and MAP infection. This association maybe specific for humans, since in previous
272 studies neither of the human *TLR4* mutations (Asp299Gly and Thr399Ile) were associated with
273 MAP infection in sheep and cattle [30, 34]. While, TRL1 and, TLR 1 and TLR 2 have been also
274 reported to be significantly associated with MAP infection in cattle and sheep [34]. The
275 interaction of TLR and MAP infection are further supported by studies that reported changes in
276 cytokine expression in TLR knockout mice models with and without MAP infection ,[19, 20].

277

278 Our observed lack of an association between *NOD2* mutations and MAP infection in paediatric
279 CD is in concordance with Bernstein *et al* (2004) who did not identify any association between
280 *NOD2* variants and serological response to MAP in IBD cases [35]. Conversely, an association
281 has been reported between bovine *NOD2* variants and MAP infection in an animal study [36], as
282 well as *in vivo* mouse studies which implicated *NOD2* mutation in defective MAP recognition
283 [37, 38]. A link between *NOD2* mutations and *Mycobacterium* species infection is further
284 supported by the association of *NOD2* mutations, genetically and functionally, with leprosy,
285 caused by *M. leprae* [39].

286

287 In the second part of this study, we evaluated IL-6, IL-10 and TNF- α expression levels in
288 PBMCs. Each stimulant (PPD or LPS) significantly increased the production of cytokines
289 regardless of CD associated mutation or MAP status, and in no instance was a statistically
290 significant synergistic or antagonistic effect of the activation observed. The only significant
291 difference observed (demonstrated by the p value) was the difference to the unstimulated value
292 and the difference of LPS to PPD. The last was true for both TNF and IL-10 production.

293 In contrast, Gasche *et al* (2003) previously reported that *IL10RA* mutant monocytes showed a
294 significantly lower TNF- α inhibition (78%) if challenged by LPS compare to wildtype monocyte
295 cells (96%) [26]. However in the same study, IL10RA receptor staining on monocytes revealed
296 no significant differences between wildtype and mutant cells suggesting that mutant SNP
297 rs2229113 of the *IL10RA* gene stabilizes receptor expression or increase receptor expression
298 [26]. A functional involvement of any of the 34 SNP variants including mutations in *IL10RA* and
299 *TLR4* genes could not be established in this study.

300

301 Previous studies have demonstrated that individuals homozygous for the minor allele of *NOD2*
302 rs5743293 (3020inC) had up to an 80% defective cytokine response in PBMCs [19, 20]. In our
303 study, no significant change in IL-6, IL-10 and TNF- α expression between PBMCs with *NOD2*
304 and without *NOD2* mutations was identified. Although, a subgroup of five *NOD2* positive
305 patients did exhibit a significant reduction in IL-6 and TNF expression. The five *NOD2* positive
306 patients did not contain any unique mutations (including 3020inC) when compared to the
307 remaining *NOD2* positive and *NOD2* negative patient groups, in this study. This suggests that
308 another genetic defect may be present in the five patients which impacts on cytokine
309 expression.

310

311 This study showed a significantly higher level of IL10R expression in NK cells (CD56 low) and
312 IL10R and TLR4 expression in NK T cells harboring a *NOD2* mutation. The importance of an
313 increased IL10 and/or TL4 receptor expression on NK cells with a *NOD2* mutation in paediatric
314 CD is unknown. The significantly higher level of TLR4 expression in NK cells (CD56high)
315 harboring a *NOD2* mutation raises questions regarding the *NOD2/TLR4* interaction. Studies
316 have shown that NK cells express NOD2 in response to muramyl dipeptide, a peptidoglycan
317 constituent of both Gram positive and Gram negative bacteria [40]. It was also shown that in
318 mice over-expression of *Nod2* could lead to increased negative regulation of the TLR2 response

319 and protection from Trinitrobenzene sulfonic acid (TNBS) induced colitis. However, no reduction
320 in TLR responses were observed with cells isolated from *Nod2* deficient mice [41]. Kullberg *et al*
321 proposed the absence of *NOD2/TLR4* cross-tolerance as the central mechanism for the
322 implication of *NOD2* mutation in CD [42]. In animal models *Nod2* is a key regulator of intestinal
323 microbiota, as microflora in the terminal ileum are dysregulated in *Nod2*-deficient mice, and
324 *Nod2*-deficient ileal intestinal epithelia are unable to kill bacteria efficiently [43]. It is possible that
325 *NOD2* mutations in CD may increase disease susceptibility by altering interactions between ileal
326 microbiota and mucosal immunity, however the exact role of *NOD2* in recognition of bacteria,
327 including MAP, remains to be elucidated.

328

329 **CONCLUSION**

330 Our study provided evidence that genetic variance may confer susceptibility to specific microbial
331 pathogens implicated in CD. This is the first study, which implicates mutations in *TLR4* and
332 *IL10RA* with MAP infection in paediatric CD patients. No association between *NOD2* mutations
333 and MAP infection in human CD was identified in this study. However, a significantly higher
334 expression of IL10R and TLR4 receptors in NK cell harboring *NOD2* mutations in CD individuals
335 was identified, highlighting the complexity of the role of *NOD2* in CD pathogenesis. This study
336 suggests that CD results where a set of specific mutations predisposes an individual to disease,
337 and subsequent infection with one or more specific microbes triggers disease onset.

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345 **ACKNOWLEDGMENTS**

346 We thank the children and their families for their participation in this study

347

348 **FUNDING**

349 This work was supported by research grants from the Murdoch Childrens Research Institute,

350 The CASS Foundation, and The Lynne Quayle Charitable Trust, Equity Trustees LTD. Dr C

351 Kirkwood is supported by an NHMRC CDA Fellowship (609347). This work was supported by

352 the Victorian Government Operational Infrastructure Support Program.

353

354 **CONFLICT OF INTEREST**

355 None to declare

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494 **FIGURE LEGENDS**

495 **Fig 1**

496 **a)** Associated toll like receptor 4 (*TLR4*) and interleukin 10 receptor A (*IL10RA*) mutations with
497 *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection in paediatric Crohn's
498 disease (CD) patients. The proportion of positive CD patients harboring either a *TLR4* mutation
499 (SNP variant rs4986790) or/and *IL10RA* mutation (SNP variant rs229133) were determined and
500 compared with MAP negative CD patients. Statistical analysis was conducted using Fisher
501 exact tests in GraphPad Prism (version 5.0d).

502 **b)** Logistic regression was used to determine the odds ratio and 95% confidence interval for the
503 association of *IL10RA* and *TLR4* mutations in CD patients and adjusted for either mutation.
504 Logistic regression was done in Stata (version 11.0).

505

506 **Fig 2**

507 Production of cytokines by peripheral blood mononuclear cells (PBMCs) collected from
508 paediatric CD patients. Interleukin 6 (IL6), interleukin 10 (IL10), and tumor necrosis factor alpha
509 (TNF- α) were measured by ELISA upon stimulation with MAP precipitated protein derivative
510 (PPD), lipopolysaccharide (LPS), and combined PPD+LPS. Statistical analysis was conducted
511 using unpaired student t-test in GraphPad Prism (version 5.0d). Significant p values are shown.

512

513 **Fig 3**

514 Cytokine production by PBMCs collected from paediatric CD patients adjusted for main *NOD2*
515 mutations, MAP infection, *TLR4* mutation, and *IL10RA* mutation. The fold change for IL-6
516 expression (figure 3.a), IL-10 expression (figure 3.b), and TNF- α expression (figure 3.c) for each
517 of the individual 17 CD patients are shown. PBMCs were stimulated with PPD, LPS, or
518 combined PPD+LPS and the level of cytokine expression was measure by ELISA. Statistical
519 analysis was conducted using unpaired student t-test in GraphPad Prism (version 5.0d). Note, a

520 group of CD patients (5/12) with a *NOD2* mutation (CD Pt 1-5) showed a significant lower IL10
521 and TNF- α production and a near significant lower IL6 production compared to the remaining 7
522 CD patients with a *NOD2* mutation.

523 PBMCs = peripheral blood mononuclear cells, CD = Crohn's disease patients, *NOD2* =
524 Nucleotide-binding oligomerisation domain-containing protein 2, = *Mycobacterium avium*
525 subspecies *paratuberculosis*, TLR = toll like receptor, IL10RA = interleukin receptor 10A, PPD =
526 precipitated protein derivative from, LPS = lipopolysaccharide

527

528 **Fig 4**

529 **a)** The cell surface expression of IL10 receptors on B cells, NK cells (CD56high), NK cells
530 (CD56 low), NK T cells, T cells, and Monocytes were measured by flow cytometry. The IL10R
531 expression fold change compare to background expression was adjusted for least one of the
532 main *NOD2* mutations (variant rs2066845, variant rs2066844, and variant rs5743293). A
533 significant higher expression of *IL10R* was observed on NK cell (CD56low) and on NK T cells
534 from CD patients harboring a *NOD2* mutation.

535 **b)** The expression of TLR4 on B cells, NK cells (CD56high), NK cells (CD56 low), NK T cells, T
536 cells, and monocytes, were measured by flow cytometry. The TLR4 receptor expression fold
537 change compare to background expression was adjusted for least one of the main *NOD2*
538 mutations (variant rs2066845, variant rs2066844, and variant rs5743293). A significant higher
539 expression of TLR4 was observed on NK cell (CD56 high) and on NK T cells from CD patients
540 harboring a *NOD2* mutation.

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542 **Supplementary Fig 1**

543 Cytokine production by PBMCs in paediatric CD patients was adjusted for *NOD2* mutations (a,
544 b, c), for *TLR4* and/or *IL10RA* mutations (d, e, f), and for MAP infection (g, h, i). IL-6, IL-10, and
545 TNF- α was measured by ELISA following stimulation with MAP precipitated protein derivative

546 (PPD), lipopolysaccharide (LPS), and a combined PPD+LPS. No significant differences were
547 observed. PBMCs = peripheral blood mononuclear cells, CD = Crohn's disease patients, NOD2
548 = Nucleotide-binding oligomerisation domain-containing protein 2, = *Mycobacterium avium*
549 subspecies *paratuberculosis*, IL-6 = interleukin 6 cytokine, IL-10 = interleukin 10 cytokine, TNF-
550 α = tumor necrosis factor alpha, IL10RA = interleukin receptor 10A, TLR4 = toll like receptor 4.

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Table 1: CD patient characteristic included in the functional cytokine study

Patient	Phenotype	Sex	Age	MAP status	NOD2 rs2066844	NOD2 rs2066845	NOD2 rs5743293	IL10RA rs2229113	TLR4 rs4986790
1	A1/L3/B1	f	13.9	Neg	0	1	0	1	0
2	A1/L3+4/B	m	14.9	Pos	0	2	0	0	0
3	A1/L3+4/B	m	12.1	Neg	1	0	0	1	0
4	A1/L3/B1	f	13.9	Pos	0	0	0	0	0
5	A1/L3+4/B	m	8.0	Pos	1	0	0	2	0
6	A1/L3/B1P	m	9.6	Pos	0	0	0	2	0
7	A1/L3/B1	m	16.6	Pos	0	1	0	1	0
8	A1/L2/B1	m	13.2	Neg	0	0	0	0	0
9	A1/L3/B1+	m	12.3	Pos	1	0	0	0	0
10	A1/L2+4/B	m	10.2	Pos	0	0	0	1	1
11	A1/L3/B1P	m	15.5	Pos	0	1	0	2	0
12	A1/L3/B2P	m	11.7	Pos	0	2	0	1	0
13	A1/L3+4/B	m	15.0	Pos	0	0	0	0	1
14	A1/L2/B1	f	10.6	Pos	0	0	0	1	1
15	A1/L3+4/B	m	11.5	Neg	0	0	0	1	0
16	A1/L3/B1	f	17.2	Pos	0	0	0	0	1
17	A1/L3+L4/	m	11.3	Neg	0	0	0	0	0

571 L1 ± L4 = ileal disease with or without upper gastrointestinal tract (GI) involvement

572 L2 ± L4 = colonic disease with or without GI involvement

573 L3 ± L4 = ileal/colonic disease with or without GI involvement

574 L4 = upper GI disease

575 B1 ± P = inflammatory appearance with or without perinial (P) disease

576 B2 ± P = stricturing disease with or without P disease

577 B3 ± P = penetrating disease with or without P disease

578 F = female

579 M = male

580 IL10RA = interleukin receptor 10A

581 TLR4 = toll like receptor 4

582 MAP = *Mycobacterium avium subspecies paratuberculosis*

583 0 = wildtype form of the SNP

584 1 = heterozygote form of the SNP

585 2 = minor homozygote form of the SNP

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600 **Table 2:** MAP associated genotypes in MAP positive culture patients

Patient	Phenotype	sex	age	IL10RA rs2229113	TLR4 rs4986790	MAP culture outcome	Overall MAP status
1	A1/L3+4/B1P	f	11.3	0	AG	positive	positive
2	A1/L2+4/B1	m	12.3	0	AA	positive	positive
3	A1/L3/B1	m	8.2	0	AG	positive	positive
4	A1/L3/B1P	m	6.5	GA	0	positive	positive
5	A1/L3/B1	m	14.6	0	AG	negative	positive
6	A1/L3/B1+P	m	12.3	0	0	negative	positive
7	A1/L3/B1P	m	9.3	0	0	negative	positive
8	A1/L3/B1	m	13.6	0	0	negative	negative
9	A1/L2/B1	m	13.2	0	0	negative	negative
10	A1/L3/B1	f	11.1	0	0	negative	negative

601 L1 ± L4 = ileal disease with or without upper gastrointestinal tract (GI) involvement

602 L2 ± L4 = colonic disease with or without GI involvement

603 L3 ± L4 = ileal/colonic disease with or without GI involvement

604 L4 = upper GI disease

605 B1 ± P = inflammatory appearance with or without perinial (P) disease

606 B2 ± P = stricturing disease with or without P disease

607 B3 ± P = penetrating disease with or without P disease

608 F = female

609 M = male

610 IL10RA = interleukin receptor 10A

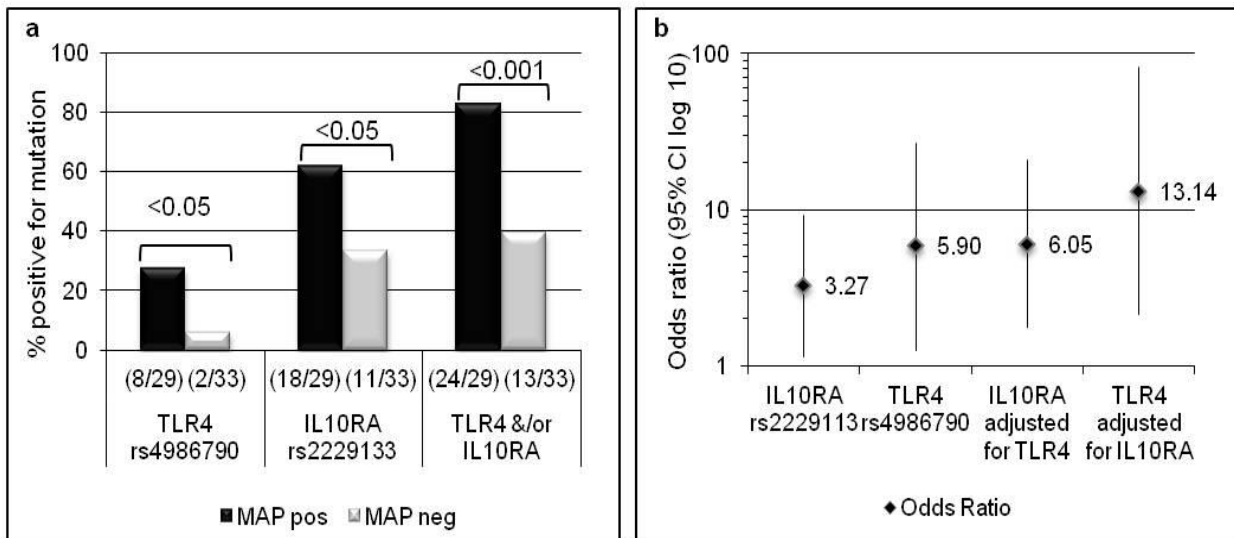
611 TLR4 = toll like receptor 4

612 MAP = *Mycobacterium avium subspecies paratuberculosis*

613 0 = wildtype form of the SNP

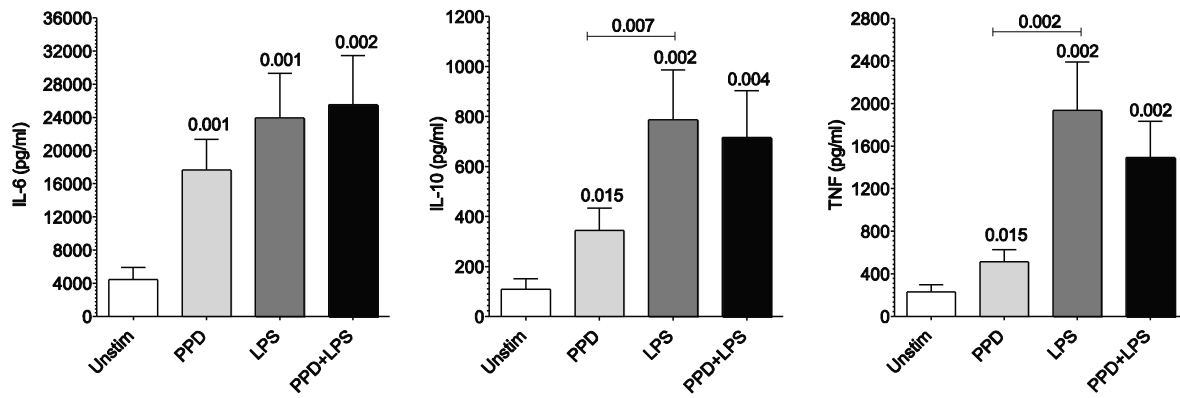
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Figure 1: Stratification of TLR4 and IL10RA mutations with MAP infection in CD patients



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Fig 2 Cytokine production by PBMCs in paediatric Crohn's disease patients



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Fig 3 Single patient analysis for *NOD2* mutation, MAP infection and *TLR4* and *IL10RA* mutation

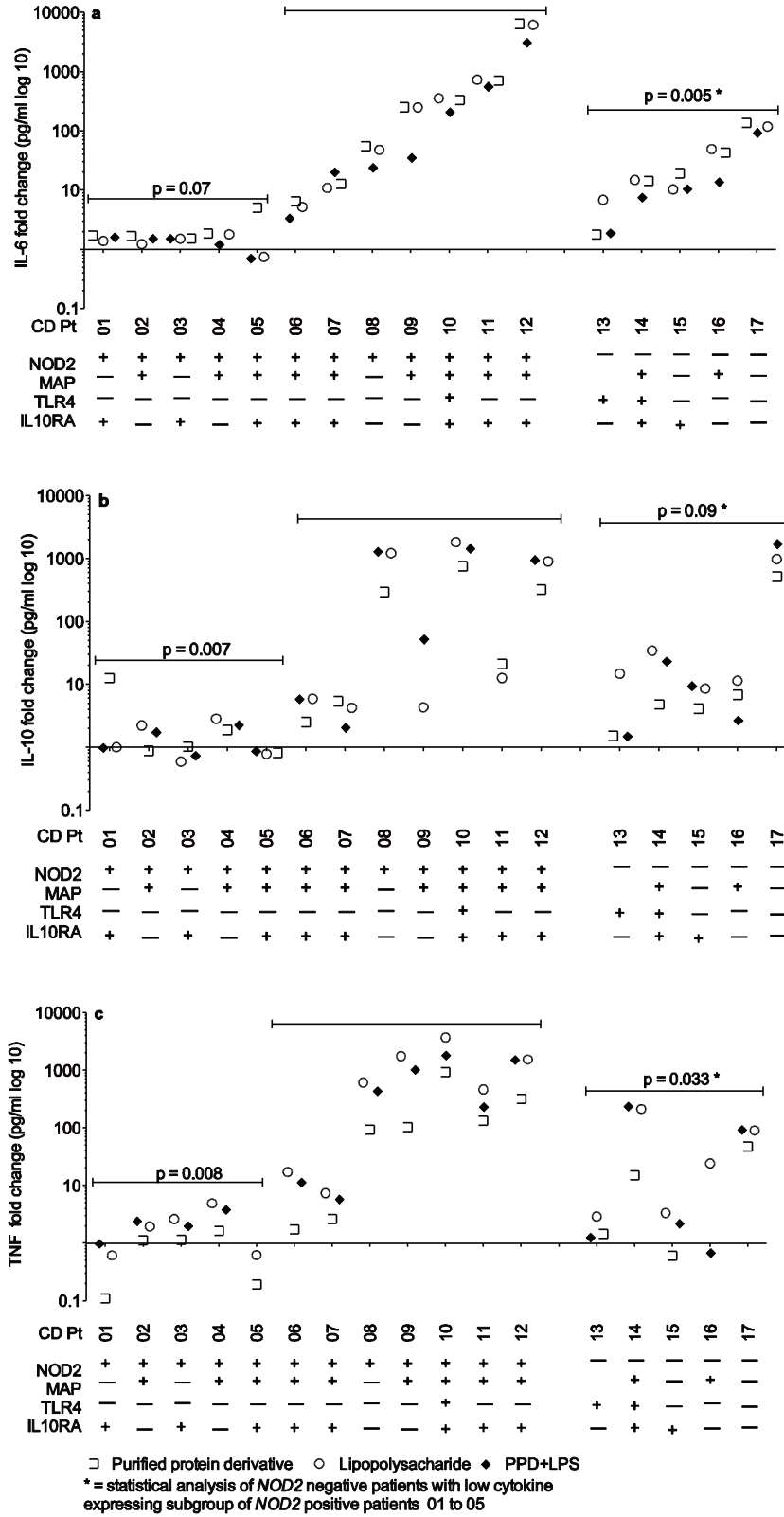


Fig 4.a IL10R expression adjusted for *NOD2* mutations

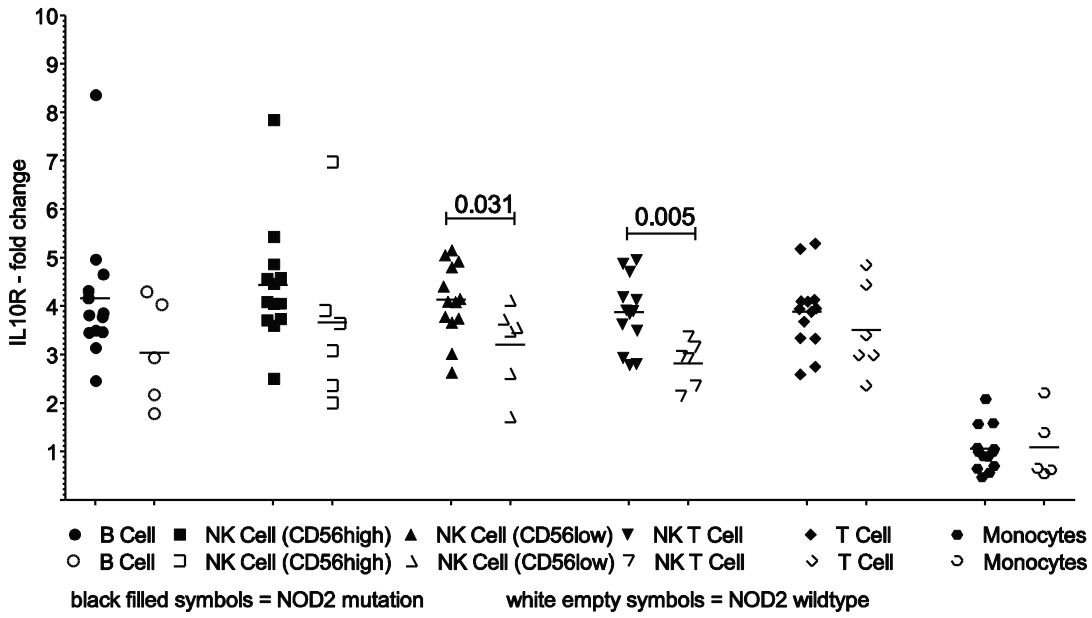


Fig 4.b TL4R expression adjusted for *NOD2* mutations

