Characterization of IL-22BP expression during Crohn's disease shows lack of evidence for IL-22BP production by T cells

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Abstract

Background & aims: IL-22 binding protein (IL-22BP) is a soluble and specific inhibitor of IL-22, a cytokine with known protective actions on intestinal epithelial cells during inflammation. Although eosinophils and mononuclear phagocytes (MNP) were shown to be the producers of IL-22BP in the intestine *lamina propria* (LP), it has recently been proposed that $CD4^+$ T cells represent an additional source of IL-22BP in the gut during Crohn's disease (CD). In this study we sought to confirm these findings and to assess IL-22BP secretion levels.

Methods: We investigated IL-22BP cellular sources in CD gut samples using qPCR and ELISA on FACS-sorted LP and mesenteric lymph nodes (MLN) cell subsets. We also evaluated IL-22BP levels in *ex-vivo* culture of intestinal biopsies.

Results: MNP (HLA DR^+ $CD11c^+$ cells) and eosinophils isolated from LP CD tissues expressed *IL22RA2*, while the expression measured in the $CD4^+$ and $CD8^+$ T cells fractions was undetectable or 100-1000 times lower than in MNP. In MLN, *IL22RA2* expression in T cells was either undetectable of 1000-fold lower than in MNPs without differences between naïve and non-naive subsets. *IL22RA2* expression was undetectable after *ex vivo* activation of both $CD4^+$ and $CD8^+$ T cells from LP or MLNs and IL-22BP was not detected in their supernatant, either activated or not. LP eosinophils appeared capable of secreting IL-22BP but not DCs likely due to *in vitro* activation as confirmed with monocyte-derived DCs. We also identified higher production of IL-22BP from ileum as compared to colon biopsies and demonstrated that IL-22BP levels correlated with those of eotaxins but not with TNF and IL-6. Finally, high *ex vivo* production of IL-22BP in mucosa biopsies from CD patients was only observed in smokers.

Conclusion: We confirmed that eosinophils and MNPs are the major sources of IL-22BP in the gut during CD and found no clear evidence for IL-22BP expression by T cells. This work also provides new insights on factors regulating intestinal IL-22BP levels.

Introduction

Inflammatory bowel disease (IBD) is a group a chronic inflammatory conditions of the gastrointestinal tract, the main clinical entities of which are Crohn's disease (CD) and ulcerative colitis (UC)[1]. IBD progressively leads to bowel damage and disability that significantly alter the quality of life[2]. IBD prevalence has increased steadily worldwide[3]. IBD is thought to result from complex interactions between environmental[4], genetic[5], microbial[6] and immune factors responsible[7] for sustained inflammatory responses damaging the gut epithelium and leading to disease-associated intestinal remodeling[8]. Interleukin-(IL) 22, a member of the IL-10 family of cytokines, is strongly induced during IBD flares[9], as a result of increased production by populations of lymphocytes including $CD4^+$ T cells and group 3 innate lymphoid cells (ILC3)[9–11]. IL-22 receptor (IL-22R) expression is mostly limited to epithelial cells[12] and protective actions of IL-22 on the gut epithelium have been largely suggested in different rodent models of gut inflammation[13–17]. IL-22 contributes to increase epithelial barrier properties by inducing the secretion of antimicrobial peptides[15,18] and mucins[13] as well as intestinal epithelial cell (IEC) survival and proliferation to support epithelial regeneration and healing[19,20]. When unregulated, however, IL-22 actions on IEC can also promote tumor cell proliferation[21–24]. IL-22 binding protein (IL-22BP) is a soluble, specific and potent IL-22 inhibitor [25] which controls the level of IL-22-induced epithelial response *in vivo*[26–30]. We[31] and others[32] previously showed that IL-22BP expression is moderately increased during IBD but strongly down-regulated during infectious colitis. We also reported increased IL-22-dependent protection against DSS-induced colitis in IL-22BP-deficent rats[31]. Murine IL-22BP was shown by others to protect against T cell-dependent colitis[32] and to prevent lasting pro-proliferative actions of IL-22 that can support tumorigenesis[26]. *Il22ra2* expression is detected in subsets of dendritic cells (DCs)[26,32–35] and macrophages[35–38] in the gut *lamina propria* (LP) and gut-associated lymphoid structures in rodents. We confirmed the high expression of *IL22RA2* in human gut MNP and identified

74 eosinophils as another source of IL-22BP in the LP of healthy and IBD tissues[31], as later
75 confirmed by Pelczar et al[32]. Interestingly, the latter further revealed unsuspected expression of
76 IL-22BP by $CD4^+$ T cells in human and mouse gut tissues[32,39] and suggested T cells also
77 contributed to increased IL-22BP levels observed in the inflamed IBD intestine. This is of
78 particular importance in IBD given the major pathophysiological implications linked to the
79 increased production of IL-22BP observed in inflamed tissues[31,32]. In this study, we sought to
80 thoroughly revisit the cellular sources of IL-22BP in the LP and in mesenteric lymph nodes
81 (MLNs) of CD patients. While we verified the expression of IL-22BP in eosinophils and
82 mononuclear phagocytes (MNP), we could not obtain evidence for significant *IL22RA2*
83 expression in T cells. We also provided new insights on factors regulating intestinal IL-22BP
84 levels in healthy and CD tissues.

85 # Materials and Methods

86 **Human samples**

87 For identification IL-22BP sources, specimens were obtained from surgical pieces from CD
88 patients undergoing resection due to relapse (**Supp Table 1**). For *ex vivo* culture, colonic and
89 ileum biopsies of mucosal areas were also obtained from 43 active CD and 6 active UC patients
90 undergoing endoscopy (**Supp Table 2**). For RT-qPCR analysis, colonic and ileum biopsies of
91 mucosal areas were also obtained from 24 active CD and 10 active UC patients undergoing
92 endoscopy (**Supp Table 3)**. Control biopsies (n=16) from patients undergoing resection for
93 CRC were also included. Normal colonic tissue samples were taken 10 cm downstream to the
94 tumor. The tissue fragments were processed according to the French guidelines for research on
95 human tissues. Informed patient consent was obtained according to the French bioethics law.
96 Ethical approval was obtained from the local ethics committee (DC-2008-402).

97

98 **Purification of human cells**

99 Immune cells were isolated from fresh human intestine and MLN. First, in human intestine
100 samples the mucosa was isolated. To remove epithelial cells, the tissue was cut in small pieces
101 and further incubated for 15 min at 37°C under slow rotation (100 rpm) twice time with
102 dissociation solution (HBSS without Ca^{2+} Mg^{2+} - HEPES 10mM – EDTA 5mM). The
103 remaining tissue was washed in PBS, further minced with a scalpel and incubated for 40 min at
104 37°C in HBSS (with Ca^{2+} and Mg^{2+}) with Collagenase IV (0,5 mg/mL-Sigma ref C5138) and
105 DNase I (0,1 mg/mL-Sigma) under 100 rpm rotation and vortexing every 20 min. Cells
106 suspension was then filtered through 70µm pore size cell strainers (BD Biosciences) and
107 processed for cell sorting. After removal adipose tissue, human MLN were cut in small pieces
108 and incubated for 30 min at 37°C on a shaking incubator in Collagenase D solution (2mg/mL-

109 Sigma ref 1108882001) containing 0,1 mg/mL DNase I. Then, cell suspension was then filtered
110 through 70µm pore size cell strainers (BD Biosciences) and processed for cell sorting.
111
112 **Cells sorting from human LP and MLN**
113 Human intestine LP and MLN cells were stained with antibodies specific for CD45 (clone
114 2D1/APCCy7), CD3 (clone UCHT1/FITC), CD4 (clone SK3/PECy7), CD19 (clone HIB19
115 /PE), CD11c (clone B-LY6/APC), HLA-DR (clone G46-6/V500) (BD Biosciences) and
116 SIGLEC-8 (clone 7C9/PE) (BioLegend, San Diego, CA). The addition of the CD14 mAb (clone
117 M5E2/PECy7) then allowed a precise separation between the DCs and macrophages. Cells
118 isolated from MLN were also stained with the following CD45RA (clone HI100/APC) and
119 CD62L (clone Dreg56/PE) mAbs to isolate naïve and non-naïve (memory/effector) T cells.
120 Dead cells were excluded by gating on 4', 6-diamidino-2-phenylindole (DAPI)-negative cells.
121 Cell sorting was performed on a BD FACS Aria Cell sorter (BD Biosciences).
122
123 **Real-time quantitative PCR**
124 After sorting, cells were suspended in TRIzol reagent (Thermo Fisher Scientific) and frozen at
125 80°C. Total RNA was prepared using an RNeasy mini kit (Qiagen,Valencia, CA) according to
126 manufacturer's instructions. Reverse transcription was performed using Murine Moloney
127 Leukemia Virus Reverse Transcriptase (Thermo Fisher Scientific) following manufacturer's
128 instructions. Gene expressions were assessed with the TaqMan Fast Advanced Master Mix
129 reagent (Applied Biosystems, Foster City, Calif). Primers and probes were purchased from
130 Applied Biosystems (see **Supp Table 4** for complete list). Real-time PCR was performed using
131 the StepOne Plus System (Applied Biosystems). Then, relative expression was normalized to
132 hypoxanthine-guanine phosphoribosyltransferase and calculated using 2^{-DDCt}. Results were
133 expressed in arbitrary unit (a.u.).

134 **Monocyte-derived dendritic cells**

135 Dendritic cells were generated from monocytes isolated from healthy volunteers. Monocytes
136 were isolated either by elutriation of PBMCs (Clinical Development and Transfer Platform,
137 Nantes, France) or by magnetic labelling (untouched cells, Human monocyte Isolation kit II).
138 To obtain monocytes-derived dendritic cells (moDC), $2.5x10^6$ monocytes were incubated in a
139 6-well plate in 5 mL of complete medium (RPMI 1640 medium containing 10% Fetal Calf
140 serum (FCS), 1% L-glutamin, 1% antibiotics, 1mM Sodium Pyruvate, 1mM HEPES, 1% non-
141 essential amino acids) supplemented with recombinant human IL-4 (200U/mL) and
142 recombinant human GM-CSF (100U/mL) for 6 days at 37°C with 5% CO_2. When indicated,
143 cells were treated with retinoic acid (RA) (100nM, Sigma Aldrich), LPS (1 µg/mL, Sigma
144 Aldrich) and TNFα (50 ng/mL, Miltenyi). After 6 days, moDC and supernatants were collected
145 and frozen at −80°C until use.

146

147 ***Ex- vivo* culture of gut biopsies**

148 Biopsies from healthy and CD patients were cultured *ex vivo* during 6 hours at 37°C – 5% Co2
149 under slow rotations. Biopsy were placed (1 biopsy per well) in 4-well Petri dishes in 500µL
150 serum-free medium (Media 1640, Gibco) supplemented with BSA (0.01%), 200µg/mL
151 Penicillin/Streptomycin (Gibco ref 15140-122) and 0.25µg/mL Fungizone (Gibco ref 15290-
152 026). After 6 hours, supernatants were collected and frozen -80°C until use.

153

154 **ELISA assay for 22BP**

155 Measurement of IL-22BP in the supernatant of stimulated biopsy was performed with the
156 Human IL-22BP ELISA DuoSet kit (R&D System, DY-1087-05) according to manufacturer's
157 instruction. Briefly, a 96-well microplate was coated with a rabbit monoclonal anti-IL-22BP
158 [4µg/mL] and incubated at room temperature (RT) overnight. The next day, after blocking 100-

159 µL standard dilutions and pure samples were added to each well and incubated 2 hours at RT.
160 Then, 100µL/well of goat anti-IL-22BP [70ng/mL] were added and 2 hours incubation at RT
161 was performed. Finally, ELISA was revealed, and plates were read at 450 nm with TECAN
162 Spark instrument. According to the supplier's instructions (R&D Systems), the detection limit
163 was 94 pg/mL.
164
165 **Multiplex assay**
166 Cytokines (IL-6 and TNFα) and chemokines (MIP-1β, CXCL1, CCL11 (eotaxin-1), CCL24
167 (eotaxin-2) and CCL26 (eotaxin-3)) were measured in 6 hours culture supernatants of biopsies
168 from normal (n=13) and CD mucosa (n=29 colons) with a multiplex assay from Biotechne
169 (Rennes, France) according to the supplier's instructions. Analysis was carried out using a
170 Luminex MAGPIX® instrument.
171
172 **Statistical analysis**
173 Statistical analysis was performed with GraphPad Prism Software (GraphPad Software, San
174 Diego, CA). Means comparisons of unpaired samples were performed using the Mann–Whitney
175 U-test or the Kruskal–Wallis test with Dunn's post-test. The Wilcoxon signed-rank test was
176 used for paired samples. P-values <0.05 were considered statistically significant.

177 # Results

178 **High levels of IL-22BP expression is limited to MNP and eosinophils in human**

179 We previously showed that human MNP (DCs and macrophages) and eosinophils purified from
180 uninflamed colons of controls expressed significant levels of *IL22RA2* mRNA, whereas almost
181 no expression was detected in epithelial cells, lymphocytes and neutrophils[31]. To revisit the
182 cellular distribution of IL-22BP mRNA expression in CD patients, we FACS-sorted
183 populations of HLA-DR$^+$ CD11c$^+$ MNP, eosinophils, CD4$^+$ and CD8$^+$ T cells and B cells from
184 uninflamed and inflamed LP tissues (n= 5 CD patients with only 2 of them having matched
185 areas, **supp Table 1**) (**Supp Figure 1**) and analyzed them by RT-qPCR for *IL22RA2* expression
186 (**Figure** 1A). HLA-DR$^+$ CD11c$^+$ MNP presented the highest levels of *IL22RA2* levels followed
187 by eosinophils, in which average expression was about 3-fold lower, though with more
188 variability, as 2 out of the 5 samples analyzed had low-to-no IL-22BP mRNA detected.
189 Compared to MNP and eosinophils, IL-22BP expression in CD4$^+$ T cells was 100-1000-fold
190 lower and two samples had undetectable levels (**Figure** 1A). In addition, IL-22BP expression
191 in CD4$^+$ T cells was similar to that detected in CD8$^+$ T cells and B cells (**Figure** 1A).

192 Pelczar et al. reported *Il22ra2* expression in mouse MLN CD4$^+$ T cells which was especially
193 high in memory cells[32]. We FACS-sorted naïve and non-naïve populations of CD4$^+$ and CD8$^+$
194 T cells, MNP and B cells from MLN collected on CD surgical resections (n=5). In order to
195 dissect standard naïve vs effector/memory T cells populations in lymph node, we stained for
196 CD45RA and CD62L. The common presence of both CR45RA and CD62L is routinely and
197 used here to identify naïve T cells[40] (**Supp Figure 2**). The absence of both markers is
198 characteristic of effector memory T cells while the singular expression of CDR45RA and
199 CD62L is associated to T_{EMRA} cells and central memory T cells, respectively[40]. Cells not
200 positively stained for CD45RA and CD62L were sorted together and will later be called non-
201 naïve T cells. As shown in **Figure 1**A, significant expression of *IL22RA2* was consistently

202 detected in MNP whereas very low levels were observed in lymphocytes, which did not differ
203 between naïve and non-naïve subsets of $CD4^+$ and $CD8^+$ T cells. Again, similar expression
204 existed between T and B lymphocytes (**Figure** 1A). To assess whether IL-22BP expression
205 could be induced in T cells upon TCR-mediated activation, we FACS-sorted T cells from CD
206 intestine and MLN and stimulated them with PMA plus Ionomycine (PMA+Iono). T cell
207 activation was verified by the induction of *IL2RA* (encoding for CD25) expression (**Figure 1**B).
208 However, upon stimulation, neither $CD4^+$ and $CD8^+$ T cells upregulated *IL22RA2* expression
209 in intestine, nor naïve and non-naïve fractions T cells in MLN (**Figure 1**C).
210 It is possible that *IL22RA2* mRNA expression is transitorily induced upon TCR-dependent
211 stimulation and was not captured in our single time point analysis. We thus quantified the levels
212 of secreted IL-22BP protein in cell supernatants by an ELISA assay that we first validated using
213 monocyte-derived DCs (moDC), which express *IL22RA2*[33,41]. MoDC were differentiated with
214 or without retinoic acid (RA) (moDC-RA), which we[33] and others[41,42] identified as a major
215 inducer of IL-22BP expression in moDC, a finding we confirmed here at the protein level in
216 **Figure 2**A. As previously demonstrated[33,41], LPS or TNF-induced maturation of moDCs
217 dramatically decreased *IL22RA2* mRNA expression, independently of RA preconditioning
218 (**Figure 2**A). Secreted IL-22BP was only detected in supernatants of moDC-RA and higher
219 levels were observed in unstimulated vs. LPS-stimulated cells (**Figure 2**B). Secreted IL-22BP
220 reflected IL22RA2 expression except for moDC-RA and LPS-stimulated cells, as confirmed by
221 the significant correlation existing between IL-22BP levels quantified by RT-PCR and ELISA
222 (**Figure 2**C).
223 We then specifically isolated and cultured overnight HLA DR^+, $CD11c^+$, $CD14^-$ cells,
224 corresponding to DCs, eosinophils and T cells from three CD LP tissues (**supp Figure 3**). When
225 applying this ELISA assay to the quantification of IL-22BP in culture supernatants, only one
226 eosinophil supernatant returned detectable levels (**Table 1**). We did not detect DC-derived IL-

227 22BP likely because of rapid *ex vivo* associated maturation induced by tissue processing, cell
228 sorting and cell culture, a phenomenon well-described for tissue DC[33,43]. Concordant with RT-
229 qPCR data in **Figure 1C**, IL-22BP was not detected in supernatants of both resting and
230 stimulated LP T cells, whereas the production of IFNγ was strongly induced upon stimulation
231 (**Table 2**). Similarly, in MLN, IL-22BP was not detected in supernatants of both resting and
232 stimulated T cells, naïve and non-naïve fractions (data not shown). While the sensitivity of the
233 ELISA assay remains low, these data, together with the mRNA expression ones, do not support
234 human gut T cells as significant sources for IL-22BP.
235
236 **IL-22BP levels correlate with eosinophil attracting eotaxins in human gut tissues.**
237 We previously reported variable levels of *IL22RA2* mRNA expression in IBD biopsies accross
238 patients[31] and we sought here to assess protein levels by measuring IL-22BP in the supernatant
239 of biopsies from non-IBD intestinal tissues (healthy) and CD lesions (both uninflamed and
240 inflamed biopsies) after 6h culture (**Figure 3**A) (**Supp Table 3&4**). High amounts of IL-22BP
241 (ranging from 94 to 812 pg/ml in normal tissues and up to 8795 pg/mL in CD lesions) were
242 detected in some samples again with wide inter-sample variability. Compared to colonic
243 biopsies, levels of IL-22BP were significantly higher in ileum biopsies from both healthy and
244 IBD tissues (**Figures 3**A), ~~as confirmed by RT-qPCR~~ (**Figure 3**B). IL-22BP levels were not
245 significantly different between non-IBD and CD tissues in both ileum and colon, as well as
246 between CD and UC biopsies (**Figures 3**A). Interestingly, we observed small but significant
247 correlations between levels of IL-22BP and eotaxins (**Figure 3**C), chemokines involved in
248 eosinophil recruitment in the gut LP[44], but not between IL-22BP and proinflammatory
249 cytokines (TNF, IL-6) / chemokines (MIP-1β and CXCL1) (**Figure 3**D). Because eotaxin levels
250 reflect eosinophil abundance in LP mucosa[45], these results suggest that higher production of IL-
251 22BP observed in some biopsies could reflect increased infiltration of eosinophils. We finally

252 investigated whether clinical parameters could contribute to IL-22BP heterogeneity observed
253 in CD tissues. We observe no correlation between secreted IL-22BP levels and disease activity
254 at the time of collection, nor at 3 years post-endoscopy with disease progression or response to
255 anti-TNF. Unexpectedly, we found that all high IL-22BP producers, in both colon and ileum
256 CD tissues, were active smokers (**Figure 3**E).

257 ## Discussion

258 Data presented in this study confirm the production of IL-22BP by MNP and eosinophils in CD
259 gut tissues, but do not support a production by T cells in LP nor in MLN as recently reported
260 by Huber's group[32,39]. The reason for the discrepancies regarding expression of *IL22RA2* in
261 human remain to be elucidated. *IL22RA2* expression was either undetected or detected at
262 extremely low levels in T cells than MNP from CD mucosa, similar to what we observed
263 previously in normal colonic mucosa[31]. *IL22RA2* expression levels in T cells were in fact
264 equivalent to those in B cells which are not currently identify as a cellular source of IL-22BP.
265 In Pelczar's study, results for *IL22RA2* expression in IBD mucosa were reported on two
266 separate graphs, making thus the comparison between $CD4^+$ T cells and MNP impossible[32].
267 They also assessed *Il22ra2* expression in $CD4^+$ T cell subsets from mice LN and reported
268 increased expression in memory as compared to naïve fractions. IL-22BP expression by murine
269 T cells, however, was not confirmed by several other groups[34,36,46,47] or by the analysis of the
270 ImmGen database (www.immgen.org)[48]. In our study, we could not confirm these data in
271 human MLN in which *IL22RA2* expression levels in both naïve and non-naïve $CD4^+$, as well
272 as $CD8^+$ T cells, were either undetectable or 1000 times less than DCs. Although we could not
273 exclude that a rare subset of LP T cells expresses *IL22RA2* during IBD, mining of our recently
274 published single cell analysis data of CD inflammatory lesions[49] as well as other public data
275 revealed a lack of *IL22RA2* mRNA expression by T cells.

276 Several assays have been used to measure IL-22BP protein production. We previously used
277 tissue immunofluorescence to demonstrate that LP eosinophils were by far the most abundant
278 IL-22BP expressing cells in normal and CD mucosa with few DCs expressing IL-22BP and
279 none of IL-22BP-expressing cells expressing CD3[31]. Huber group used intracellular flow
280 cytometry to demonstrate IL-22BP expression by DCs, eosinophils and $CD4^+$ T cells from
281 LP[32,39], the levels of which were reported to be enhanced during IBD only in $CD4^+$ T cells[32].

282 Although not mentioned by authors, DCs actually also appear to upregulate intracellular IL-
283 22BP in their data. Strikingly, virtually all DCs appear to express IL-22BP in their report which
284 is contradictory with the previously described restricted expression of *IL22RA2* by a specific
285 subset of DCs (DC2)[31,50]. Here, we used an ELISA assay for IL-22BP but found no evidence
286 for production by T cells from LP or MLN in line with RT-qPCR results yet the sensitivity of
287 this immunoassays was rather low.
288 IL-22BP levels in culture supernatants from normal and CD biopsies are consistent with the
289 fact that eosinophils are the major source of IL-22BP in the gut. Indeed, higher levels of IL-
290 22BP in the ileum compared to the colon is consistent with the known intestinal distribution of
291 eosinophils[44,51–53] and IL-22BP levels ae weakly but significantly correlated with eotaxins ones
292 [44]. We found no correlation between TNF and IL-22BP as shown by Pelczar et al at the mRNA
293 level[32], suggesting that IL-22BP levels are not modulated by the state of inflammation.
294 Surprisingly, we did not detect higher secreted IL-22BP in IBD biopsies as compared to normal
295 tissue, but rather a large interindividual variability. Intriguingly, all CD biopsies with high
296 levels of IL-22BP were harvested from active smokers patients both in ileal and colonic
297 mucosa. Although the same observation was made in our small cohort of UC patients, these
298 findings remain to be confirmed in larger number of samples. Although the exact mechanism
299 remains unknown, it provides an additional argument for a damaging effect of smoking during
300 CD.

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306 ## References

307 1. Kaplan, G. G. The global burden of IBD: from 2015 to 2025. *Nat Rev Gastroenterol*
308 *Hepatol* **12**, 720–727 (2015).
309 2. Baumgart, D. C. & Sandborn, W. J. Inflammatory bowel disease: clinical aspects and
310 established and evolving therapies. *Lancet* **369**, 1641–1657 (2007).
311 3. Ananthakrishnan, A. N. Epidemiology and risk factors for IBD. *Nat Rev Gastroenterol*
312 *Hepatol* **12**, 205–217 (2015).
313 4. Ananthakrishnan, A. N. *et al.* Environmental triggers in IBD: a review of progress and
314 evidence. *Nat Rev Gastroenterol Hepatol* **15**, 39–49 (2018).
315 5. Jostins, L. *et al.* Host-microbe interactions have shaped the genetic architecture of
316 inflammatory bowel disease. *Nature* **491**, 119–124 (2012).
317 6. Turpin, W., Goethel, A., Bedrani, L. & Croitoru Mdcm, K. Determinants of IBD
318 Heritability: Genes, Bugs, and More. *Inflamm. Bowel Dis.* **24**, 1133–1148 (2018).
319 7. Friedrich, M., Pohin, M. & Powrie, F. Cytokine Networks in the Pathophysiology of
320 Inflammatory Bowel Disease. *Immunity* **50**, 992–1006 (2019).
321 8. de Souza, H. S. P. & Fiocchi, C. Immunopathogenesis of IBD: current state of the art. *Nat*
322 *Rev Gastroenterol Hepatol* **13**, 13–27 (2016).
323 9. Andoh, A. *et al.* Interleukin-22, a member of the IL-10 subfamily, induces inflammatory
324 responses in colonic subepithelial myofibroblasts. *Gastroenterology* **129**, 969–984 (2005).
325 10. Brand, S. *et al.* IL-22 is increased in active Crohn's disease and promotes proinflammatory
326 gene expression and intestinal epithelial cell migration. *Am. J. Physiol. Gastrointest. Liver*
327 *Physiol.* **290**, G827-838 (2006).
328 11. Geremia, A. *et al.* IL-23-responsive innate lymphoid cells are increased in inflammatory
329 bowel disease. *J. Exp. Med.* **208**, 1127–1133 (2011).

330 12. Sabat, R., Ouyang, W. & Wolk, K. Therapeutic opportunities of the IL-22-IL-22R1 system.
331 *Nat Rev Drug Discov* **13**, 21–38 (2014).
332 13. Sugimoto, K. *et al.* IL-22 ameliorates intestinal inflammation in a mouse model of
333 ulcerative colitis. *J. Clin. Invest.* **118**, 534–544 (2008).
334 14. Zenewicz, L. A. *et al.* Innate and adaptive interleukin-22 protects mice from inflammatory
335 bowel disease. *Immunity* **29**, 947–957 (2008).
336 15. Zheng, Y. *et al.* Interleukin-22 mediates early host defense against attaching and effacing
337 bacterial pathogens. *Nat. Med.* **14**, 282–289 (2008).
338 16. Monteleone, G. *et al.* Phase I clinical trial of Smad7 knockdown using antisense
339 oligonucleotide in patients with active Crohn's disease. *Mol. Ther.* **20**, 870–876 (2012).
340 17. Couturier-Maillard, A. *et al.* Interleukin-22-deficiency and microbiota contribute to the
341 exacerbation of Toxoplasma gondii-induced intestinal inflammation. *Mucosal Immunol* **11**,
342 1181–1190 (2018).
343 18. Coorens, M. *et al.* Innate lymphoid cell type 3-derived interleukin-22 boosts lipocalin-2
344 production in intestinal epithelial cells via synergy between STAT3 and NF-κB. *J. Biol.*
345 *Chem.* **294**, 6027–6041 (2019).
346 19. Lindemans, C. A. *et al.* Interleukin-22 promotes intestinal-stem-cell-mediated epithelial
347 regeneration. *Nature* **528**, 560–564 (2015).
348 20. Gronke, K. & Diefenbach, A. Regenerative biology: Innate immunity repairs gut lining.
349 *Nature* **528**, 488–489 (2015).
350 21. Hernandez, P., Gronke, K. & Diefenbach, A. A catch-22: Interleukin-22 and cancer. *Eur.*
351 *J. Immunol.* **48**, 15–31 (2018).
352 22. Jiang, R. *et al.* IL-22 is related to development of human colon cancer by activation of
353 STAT3. *BMC Cancer* **13**, 59 (2013).

354 23. Kirchberger, S. *et al.* Innate lymphoid cells sustain colon cancer through production of
355 interleukin-22 in a mouse model. *J. Exp. Med.* **210**, 917–931 (2013).
356 24. Kryczek, I. *et al.* IL-22(+)CD4(+) T cells promote colorectal cancer stemness via STAT3
357 transcription factor activation and induction of the methyltransferase DOT1L. *Immunity* **40**,
358 772–784 (2014).
359 25. Dumoutier, L., Lejeune, D., Colau, D. & Renauld, J. C. Cloning and characterization of IL-
360 22 binding protein, a natural antagonist of IL-10-related T cell-derived inducible factor/IL-
361 22. *J. Immunol.* **166**, 7090–7095 (2001).
362 26. Huber, S. *et al.* IL-22BP is regulated by the inflammasome and modulates tumorigenesis in
363 the intestine. *Nature* **491**, 259–263 (2012).
364 27. Broquet, A. *et al.* Interleukin-22 level is negatively correlated with neutrophil recruitment
365 in the lungs in a Pseudomonas aeruginosa pneumonia model. *Sci Rep* **7**, 11010 (2017).
366 28. Martin, J. C. *et al.* Limited Presence of IL-22 Binding Protein, a Natural IL-22 Inhibitor,
367 Strengthens Psoriatic Skin Inflammation. *J. Immunol.* **198**, 3671–3678 (2017).
368 29. Kleinschmidt, D. *et al.* A Protective Function of IL-22BP in Ischemia Reperfusion and
369 Acetaminophen-Induced Liver Injury. *J. Immunol.* **199**, 4078–4090 (2017).
370 30. Perriard, G. *et al.* Interleukin-22 is increased in multiple sclerosis patients and targets
371 astrocytes. *J Neuroinflammation* **12**, 119 (2015).
372 31. Martin, J. C. *et al.* IL-22BP is produced by eosinophils in human gut and blocks IL-22
373 protective actions during colitis. *Mucosal Immunol* **9**, 539–549 (2016).
374 32. Pelczar, P. *et al.* A pathogenic role for T cell-derived IL-22BP in inflammatory bowel
375 disease. *Science* **354**, 358–362 (2016).
376 33. Martin, J. C. J. *et al.* Interleukin-22 binding protein (IL-22BP) is constitutively expressed
377 by a subset of conventional dendritic cells and is strongly induced by retinoic acid. *Mucosal*
378 *Immunol* **7**, 101–113 (2014).

379 34. Laaksonen, H. *et al.* The multiple sclerosis risk gene IL22RA2 contributes to a more severe
380 murine autoimmune neuroinflammation. *Genes Immun.* **15**, 457–465 (2014).
381 35. Jinnohara, T. *et al.* IL-22BP dictates characteristics of Peyer's patch follicle-associated
382 epithelium for antigen uptake. *J. Exp. Med.* **214**, 1607–1618 (2017).
383 36. Savage, A. K., Liang, H.-E. & Locksley, R. M. The Development of Steady-State
384 Activation Hubs between Adult LTi ILC3s and Primed Macrophages in Small Intestine. *J.*
385 *Immunol.* **199**, 1912–1922 (2017).
386 37. Leonardi, I. *et al.* CX3CR1+ mononuclear phagocytes control immunity to intestinal fungi.
387 *Science* **359**, 232–236 (2018).
388 38. Baratin, M. *et al.* T Cell Zone Resident Macrophages Silently Dispose of Apoptotic Cells
389 in the Lymph Node. *Immunity* **47**, 349-362.e5 (2017).
390 39. Kempski, J. *et al.* IL22BP Mediates the Antitumor Effects of Lymphotoxin Against
391 Colorectal Tumors in Mice and Humans. *Gastroenterology* **159**, 1417-1430.e3 (2020).
392 40. Geißler, K. *et al.* Functional characterization of T-cells from palatine tonsils in patients with
393 chronic tonsillitis. *PLoS One* **12**, e0183214 (2017).
394 41. Lim, C., Hong, M. & Savan, R. Human IL-22 binding protein isoforms act as a rheostat for
395 IL-22 signaling. *Sci. Signal.* **9**, ra95–ra95 (2016).
396 42. Gómez-Fernández, P. *et al.* Long Interleukin-22 Binding Protein Isoform-1 Is an
397 Intracellular Activator of the Unfolded Protein Response. *Front Immunol* **9**, 2934 (2018).
398 43. Schlecht, G., Mouriès, J., Poitrasson-Rivière, M., Leclerc, C. & Dadaglio, G. Purification
399 of splenic dendritic cells induces maturation and capacity to stimulate Th1 response in vivo.
400 *International Immunology* **18**, 445–452 (2006).
401 44. Loktionov, A. Eosinophils in the gastrointestinal tract and their role in the pathogenesis of
402 major colorectal disorders. *World J Gastroenterol* **25**, 3503–3526 (2019).

403 45. Adar, T. *et al.* The Importance of Intestinal Eotaxin-1 in Inflammatory Bowel Disease: New
404 Insights and Possible Therapeutic Implications. *Dig Dis Sci* **61**, 1915–1924 (2016).
405 46. Fukaya, T. *et al.* Pivotal Role of IL-22 Binding Protein in the Epithelial Autoregulation of
406 Interleukin-22 Signaling in the Control of Skin Inflammation. *Front Immunol* **9**, 1418
407 (2018).
408 47. Guendel, F. *et al.* Group 3 Innate Lymphoid Cells Program a Distinct Subset of IL-22BP-
409 Producing Dendritic Cells Demarcating Solitary Intestinal Lymphoid Tissues. *Immunity* **53**,
410 1015-1032.e8 (2020).
411 48. Shay, T. & Kang, J. Immunological Genome Project and systems immunology. *Trends*
412 *Immunol* **34**, 602–609 (2013).
413 49. Martin, J. C. *et al.* Single-Cell Analysis of Crohn's Disease Lesions Identifies a Pathogenic
414 Cellular Module Associated with Resistance to Anti-TNF Therapy. *Cell* **178**, 1493-
415 1508.e20 (2019).
416 50. Watchmaker, P. B. *et al.* Comparative transcriptional and functional profiling defines
417 conserved programs of intestinal DC differentiation in humans and mice. *Nat. Immunol.* **15**,
418 98–108 (2014).
419 51. Lee, E. H., Yang, H. R. & Lee, H. S. Quantitative Analysis of Distribution of the
420 Gastrointestinal Tract Eosinophils in Childhood Functional Abdominal Pain Disorders. *J*
421 *Neurogastroenterol Motil* **24**, 614–627 (2018).
422 52. Matsushita, T. *et al.* The number and distribution of eosinophils in the adult human
423 gastrointestinal tract: a study and comparison of racial and environmental factors. *Am J*
424 *Surg Pathol* **39**, 521–527 (2015).
425 53. DeBrosse, C. W., Case, J. W., Putnam, P. E., Collins, M. H. & Rothenberg, M. E. Quantity
426 and distribution of eosinophils in the gastrointestinal tract of children. *Pediatr Dev Pathol*
427 **9**, 210–218 (2006).
428

429 ## Legends to figures:

430 **Figure 1: IL-22BP is expressed by human MNP and eosinophils but not T cells.**

431 (**A**) *IL22RA2* expression was analysed by RT-qPCR on FACS-sorted MNP, eosinophils and T
432 cells from LP and MLN CD tissues (n=5). For gut results, red circles correspond to inflamed
433 mucosa and the black one to uninflamed mucosa. Concerning MLN graph, naïve lymphocytes
434 refer to $CD45RA^+$ $CD62L^+$ cells while non-naïve to all the others. Each point represents the
435 ratio of *IL22RA2* to HPRT (hypoxanthine guanine phosphoribosyl transferase) expression, as
436 determined by the $2^{\wedge -ddct}$ method of relative quantification. (**B**)&(**C**) Fresh isolated T cells from
437 LP and MLN CD tissues have been stimulated with media culture or PMA (50ng/m) +
438 Ionomycine (250ng/mL). After 20 hours, *IL22RA2* and *IL2Rα* gene expression were evaluated
439 by RT-qPCR in resting and stimulated T cells. Each symbol represents one sample. Means
440 comparisons of unpaired samples were performed using the Kruskal–Wallis test. P-value < 0,05
441 were considered statistically significant. CD: Crohn's disease; DCs: dendritic cells, Mθ:
442 macrophages; MLN: mesenteric lymph node, MNP: mononuclear phagocytes.

443

444 **Figure 2: IL-22BP level assessed using ELISA in monocytes-derived DCs.**

445 Human monocytes from peripheral blood of healthy donors were differentiated into DCs
446 (moDCs) in complete medium with GM-CSF and IL-4 for 6 days. When indicated, ligands were
447 added of culture. **(A)** On day 6, *IL22RA2* expression was analysed by quantitative RT–qPCR.
448 Bars represent mean±s.e.m. ratio of *IL22RA2* gene to *HPRT* expression as determined by the
449 $2^{\wedge -ddCt}$ method of relative quantification from n=11 independent experiments. **(B)** At day 6,
450 supernatants were collected and frozen -80°C until measurement of IL-22BP level with ELISA.
451 **(C)** Correlation between *IL22RA2* expression and IL-22BP protein levels. Means comparisons
452 of paired samples were performed using the Wilcoxon test. Correlation were calculated using
453 the Spearman rank correlation test. P-value < 0,05 were considered statistically significant. ***

454 p<0,001; ** p<0,01; * p<0,05; moDCs: monocyte-derived dendritic cell; RA: retinoic acid;
455 LPS: Lipopolysaccharide; TNFα: Tumor necrosis factor alpha.
456
457 **Figure 3: In human gut tissue, IL-22BP levels are stronger in the ileum and correlate with**
458 **those of eotaxins.**
459 (**A**) ELISA assay of IL-22BP in 6 hours culture supernatants of biopsies from normal mucosa
460 (He) (n=4 ileum and n=12 colons), CD mucosa (n=11 ileum and n=32 colons) and UC mucosa
461 (n=6 colons) biopsies. (**B**) *IL22RA2* gene expression evaluated by RT-qPCR in fresh normal
462 gut tissue (He) (n=7 ileum and n=15 colons), CD mucosa (n=14 ileum and n=10 colons) and
463 UC mucosa (n= 10 colons). Correlation between secreted IL-22BP and eotaxins (**C**) or IL-6,
464 TNFα, CXL1 and MIP-1β (**D**) measured in 6 hours culture supernatants of biopsies from normal
465 (n=13) and CD tissues (n=29). **(E)** ELISA assay of IL-22BP in 6 hours culture supernatant of
466 biopsies from CD gut mucosa (n=11 ileum and n=32 colons) according to smoking status. Each
467 symbol represents one sample. Means comparisons of unpaired samples were performed using
468 the Mann Whitney test. Correlations were calculated using the Spearman rank correlation test.
469 P-value < 0,05 were considered statistically significant. *** p<0,001; * p<0,05 n.s: non-
470 significant. He: normal tissue; CD- Crohn's disease; UC: ulcerative colitis.

471 **Table 1 : IL-22BP protein assay in culture supernatant of fresh sorted cells from CD LP**

472 Data are representative of three independent experiments on three different donors. IL-22BP
473 dosages are expressed in pg/mL and values below the detection limit, according to the supplier's
474 instructions, are reported as <94pg/mL. n.d: not done.

CD-LP	DCs	Eosinophils	T cells
Inflamed Ileum#1	n.d	321	<94
Inflamed Ileum#2	<94	<94	<94
Inflamed Ileum#3	<94	<94	<94

475

476 **Table 2: IL-22NP and IFNγ protein assays in culture supernatant of fresh sorted T cells**
477 **from CD LP.**

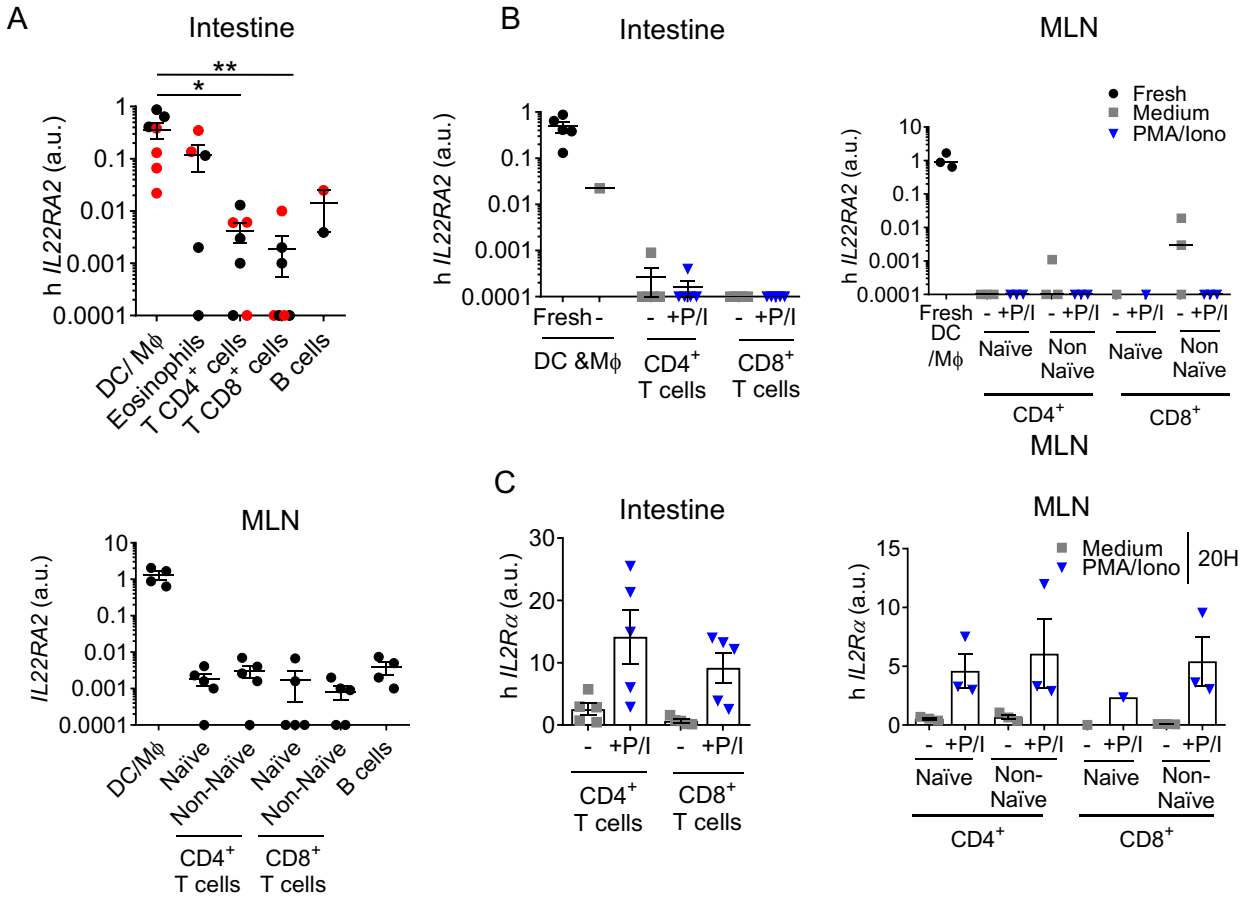
478 Data are representative of five independent experiments on three different donors. IL-22BP and
479 IFNγ dosages are expressed in pg/mL and values below the detection limit, according to the
480 supplier's instructions, are reported as < 94 pg/mL and < 17 pg/mL respectively. n.d: not done.

CD LP	**CD4$^+$ T cells**			
	medium		**PMA+Ionomycine**	
	IFNγ (pg/mL)	IL-22BP (pg/mL)	IFNγ (pg/mL)	IL-22BP (pg/mL)
Uninflamed colon-CD#1	<17	<94	8946	<94
Inflamed colon-CD#1	<17	<94	2060	<94
Uninflamed ileum-CD#2	<17	<94	36275	<94
Inflamed ileum-CD#2	<17	<94	6138	<94
Uninflamed colon-CD#3	<17	<94	23440	<94

481

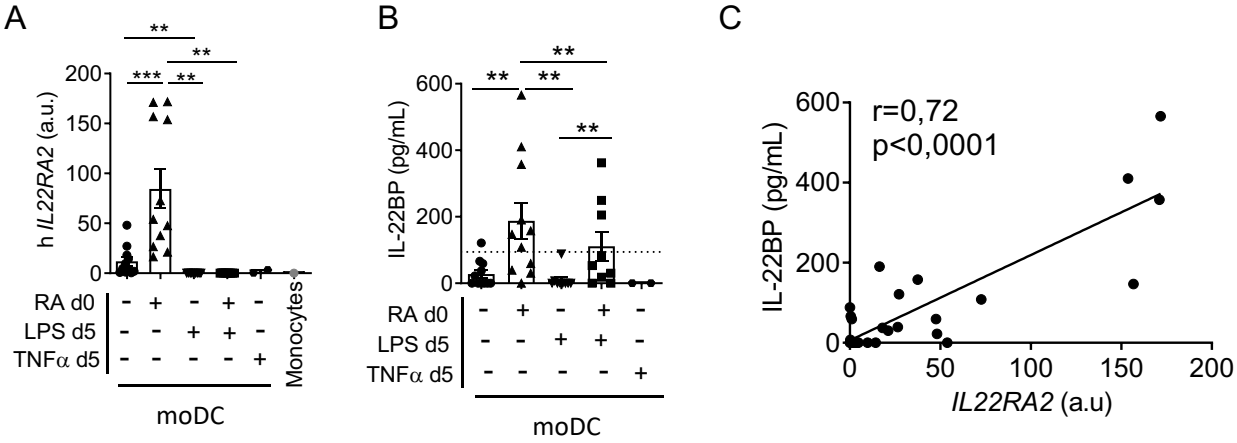
482 **Figures**

483 **FIGURE 1**



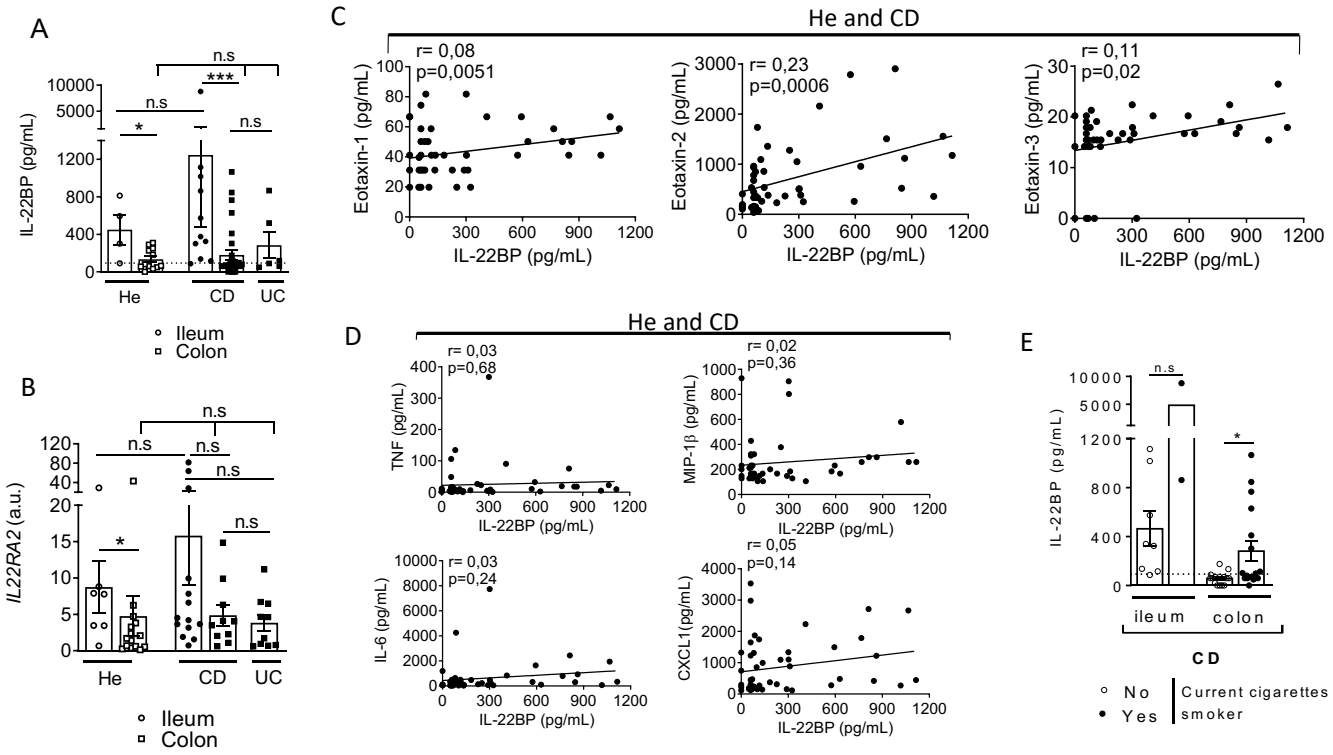
484

485 **FIGURE 2**



486

FIGURE 3


A
IL-22BP (pg/mL)
n.s
***
n.s
*
n.s
He
CD
UC
Ileum
Colon
B
IL22RA2 (a.u.)
n.s
n.s
n.s
n.s
n.s
*
He
CD
UC
Ileum
Colon
C
He and CD
r= 0,08
p=0,0051
Eotaxin-1 (pg/mL)
IL-22BP (pg/mL)
r= 0,23
p=0,0006
Eotaxin-2 (pg/mL)
IL-22BP (pg/mL)
r= 0,11
p=0,02
Eotaxin-3 (pg/mL)
IL-22BP (pg/mL)
D
He and CD
r= 0,03
p=0,68
TNF (pg/mL)
IL-22BP (pg/mL)
r= 0,02
p=0,36
MIP-1β (pg/mL)
IL-22BP (pg/mL)
r= 0,03
p=0,24
IL-6 (pg/mL)
IL-22BP (pg/mL)
r= 0,05
p=0,14
CXCL1(pg/mL)
IL-22BP (pg/mL)
E
IL-22BP (pg/mL)
n.s
*
ileum
colon
CD
No
Yes
Current cigarettes smoker