IL-22BP production is heterogeneously distributed in Crohn's disease

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Abstract

Crohn's disease (CD), a form of inflammatory bowel disease (IBD), is characterized by impaired epithelial barrier functions and dysregulated mucosal immune responses. IL-22 binding protein (IL-22BP) is a soluble inhibitor regulating IL-22 bioactivity, a cytokine proposed to play protective roles during CD. We and others have shown that IL-22BP is produced in IBD inflamed tissues, hence suggesting a role in CD. In this work, we extended the characterization of IL-22BP production and distribution in CD tissues by applying enzyme-linked immunosorbent assays to supernatants obtained from the culture of endoscopic biopsies of patients, and reverse transcription-quantitative polymerase chain reaction on sorted immune cell subsets. We reveal that IL-22BP levels are higher in inflamed ileums than colons. We observe that in a cell-intrinsic fashion, populations of mononuclear phagocytes and eosinophils express IL-22BP at the highest levels in comparison to other sources of T cells. We suggest the enrichment of intestinal eosinophils could explain higher IL-22BP levels in the ileum. In inflamed colon, we reveal the presence of increased IL-22/IL22BP ratios compared to controls, and a strong correlation between IL-22BP and CCL24. We identify monocyte-derived dendritic cells (moDC) as a cellular subtype co-expressing both cytokines and validate our finding using *in vitro* culture systems. We also show that retinoic acid induces the secretion of both IL-22BP and CCL24 by moDC. Finally, we report on higher IL-22BP levels in active smokers. In conclusion, our work provides new information relevant to therapeutic strategies modulating IL-22 bioactivity in CD, especially in the context of disease location.

52 ## Introduction

53 Inflammatory bowel disease (IBD) is a group a chronic inflammatory conditions of the
54 gastrointestinal tract, the main clinical entities of which are Crohn's disease (CD) and ulcerative
55 colitis (UC) (1). IBD results from dysregulated mucosal immune responses against the
56 microbiota, triggered by environmental factors in genetically susceptible individuals (2).
57 Inflammatory responses in IBD alter the intestinal epithelial barrier, further amplifying immune
58 pathogenicity. The cytokine interleukin-(IL) 22 is proposed to play central roles during IBD
59 inflammation (3,4). IL-22 is induced during IBD flares, as a result of increased production by
60 $CD4^+$ T cells and group 3 innate lymphoid cells (ILC3) (5). IL-22 receptor (IL-22R) expression
61 is mostly limited to epithelial cells and protective functions for IL-22 on the gut epithelium
62 have been suggested in several rodent models of intestinal inflammation (6,7). IL-22 supports
63 gut epithelial barrier properties by inducing the secretion of antimicrobial peptides (6) and
64 mucins (7), as well as intestinal epithelial cell (IEC) survival and proliferation (8). When
65 dysregulated, however, IL-22 actions on IEC can promote tumor cell proliferation (9,10). IL-
66 22 binding protein (IL-22BP ; encoded by the gene *IL22RA2*) is a soluble inhibitor specific for
67 IL-22 (11), which regulates the level of IL-22 bioactivity *in vivo* (12,13). Concordantly, we
68 have observed increased IL-22-dependent protection against DSS-induced colitis in IL-22BP-
69 deficient rats (14). In mice, IL-22BP worsens T cell-dependent colitis (15) but prevents
70 tumorigenic long-lasting pro-proliferative actions of IL-22 (12). We and others have shown that
71 the expression of *IL22RA2* is down-regulated during infectious colitis but not in IBD inflamed
72 tissues, hence suggesting possible pathophysiological relevance for the IL-22BP-dependent
73 modulation of IL-22 bioactivity (14,15). IL-22BP is produced by various cell types, which
74 include subsets of intestinal dendritic cells (DCs) and macrophages in the gut lamina propria
75 (LP) and secondary lymphoid structures (12,15–18). In human, we have revealed that IL-22BP
76 is also produced by gut eosinophils in the LP of both healthy and inflamed IBD tissues (14).

77 Finally, $CD4^+$ T cells have also been proposed to contribute to IL-22BP levels detected in IBD
78 (15,19).
79 Several layers of disease heterogeneity exist in CD. It has so far remained unclear
80 whether the general findings about IL-22BP regulation in IBD discussed above can be
81 extrapolated homogenously to patients with CD, especially when considering intestinal
82 segments of disease location. In the light of therapeutic strategies modulating IL-22 bioactivity
83 in IBD that are currently under evaluation, we thus sought to extend further the characterization
84 of IL-22BP production and distribution in intestinal tissues from a cohort of CD patients.
85

Material and Methods

Patients

Patients were recruited, from January 2016 to October 2019, from the Institut des Maladies de l'Appareil Digestif (IMAD), the gastroenterology department and the digestive endoscopy unit at CHU Nantes Hospital. The protocol was approved, and informed consent was obtained from all participants in accordance with the institutional review board (DC-2008-402). A cohort 1 of CD patients was constituted to explore cytokine secretions in supernatants from *ex vivo* biopsy cultures. Cohort 1 included a total of 43 CD patients for whom endoscopic biopsies in involved areas were collected during colonoscopies planned for routine care (**Supplementary Table 1**). Control biopsies were also analyzed and collected from surgical specimens of colon cancers, on unaffected areas of the colon or the ileum, at least 10 cm distant from the tumor. A cohort 2 that included mesenteric lymph nodes and intestinal mucosa obtained from ileocecal resections of 6 patients with active CD was constituted for gene expression analyses in sorted immune cell subsets (**Supplementary Table 2**).

Ex vivo cultures of gut biopsies.

During the colonoscopy procedure, biopsies were collected with forceps directly in ice cold RPMI and processed within 2h. Biopsies (controlled for weight) were put into 4-well Petri dishes filled with in 500µL serum-free medium (RPMI 1640, Gibco™) supplemented with BSA (0.01%), 200µg/mL Penicillin/Streptomycin (Gibco ™; ref 15140-122) and 0.25µg/mL Fungizone (Gibco™; ref 15290-026), and cultured *ex vivo* during 6 h at +37°C in a 95% O_2 / 5% CO_2 atmosphere on a low-speed rocking platform. Supernatants were collected and stored at -80°C until use.

ELISA assay for 22BP

IL-22BP quantification was performed with the Human IL-22BP ELISA DuoSet kit (R&D System, ref DY-1087-05) according to manufacturer's instruction. Briefly, a 96-well microplate

111 was coated with a rabbit monoclonal anti-IL-22BP [4µg/mL] and incubated at room
112 temperature (RT) overnight. The next day, after blocking with Reagent Diluent (R&D System,
113 ref DY006), 100-µL standard dilutions and samples were added to each well and incubated 2
114 hours at RT. Then, 100µL/well of goat anti-IL-22BP [70ng/mL] were added and 2 h incubation
115 at RT was performed. Finally, ELISA was revealed and plates were read at 450 nm with
116 TECAN Spark® instrument.

117 **Multiplex assay**

118 Levels of soluble cytokines (CCL4, CXCL10, CXCL2, CCL24, CCL11, IFNγ, IL-10, IL-17A,
119 IL-22, IL-27, IL-6, IL-8, CCL2, OSM, CXCL1, CXCL11, CXCL5, CCL26, IL-1β, IL-18, IL-
120 23, IL-33, IL-7, CXCL9, TNF) were quantified in supernatants obtained after *ex vivo* biopsy
121 cultures with a multiplex assay from Biotechne (Rennes, France) and a Luminex MAGPIX®
122 instrument (**Supplementary Table 3**).

123 **Eosinophil Counts**

124 Eosinophil counts were scored in a blinded fashion by a trained pathologist. Hematoxylin and
125 eosin-stained slides from intestinal tissues of 36 CD patients were analyzed with an Olympus
126 BH2 microscope. A high-power field (HPF) included an area of 0.196mm^2. For each slide,
127 eosinophil counts were defined by averaging the results obtained in 5 different HPFs selected
128 randomly. Results of eosinophil counts were reported as the number of eosinophils/mm^2.

129 **Isolation of intestinal lamina propria cells**

130 Tissues from surgical resections were collected in ice cold RPMI 1640 (Gibco^{TM}) and processed
131 within one hour after the end of the surgery. The mucosa was stripped and cut into small pieces
132 before transfer into complete RPMI media. Epithelial cells were dissociated in an EDTA-
133 enriched dissociation medium (HBSS w/o Ca^{2+} Mg^{2+} (Gibco^{TM}) - HEPES 10mM (Gibco^{TM}) -
134 EDTA 5mM at +37°C through two 15 min cycles of agitation at 100 rpm. After each cycle,
135 intestinal fragments were hand-shaken for 30 s and vortexed vigorously for another 30 s.

136 Epithelium-free fragments were washed in PBS, and transferred in the digestion medium
137 (HBSS with Ca^{2+} Mg^{2+} - Fetal Calf serum (FCS) 2% - DNase I 0.1mg/mL (Sigma-Aldrich, ref
138 11284932001) – Collagenase IV 0.5mg/mL (Sigma-Aldrich, ref C5138) for 40 min at +37°C
139 under 100 rpm agitation; and vortexing every 20 min. Cell suspensions were filtered through
140 70µm pore size cell strainers (BD Biosciences) and washed with FACS-buffer (with EDTA
141 1mM) twice before being processed for cell sorting.

142 **Intestinal mesenteric lymph nodes isolation**

143 Tissues were collected in ice cold RPMI 1640 (Gibco™) from the resected specimens and
144 processed within one hour after the end of the surgery. After removing the adipose tissue, lymph
145 mesenteric nodes were cut in small pieces and transferred into the digestion medium (10mL of
146 complete RPMI with Collagenase D (2mg/mL- Sigma-Aldrich, ref 1108882001) and DNase I
147 (0,1 mg/mL)). After 30 min of incubation at +37°C 100 rpm, EDTA (1mM) was added to block
148 the reaction. Cell suspensions were filtered through 70µm pore size cell strainers (BD
149 Biosciences) and processed for cell sorting.

150 **Cells sorting**

151 Single cell suspensions were incubated in PBS containing the flow cytometry antibody cocktail
152 (**Supplementary Table 4**) for 20 min at 4°C in the dark. Dead cells were excluded by gating
153 on 4',6-diamidino-2-phenylindole (DAPI)-negative cells. Cell sorting was performed on a BD
154 FACS Aria Cell sorter (BD Biosciences) using the gating strategies shown in Figure 2.

155 **Real-time quantitative PCR**

156 Sorted cells were suspended in TRIzol reagent (Thermo Fisher Scientific) and frozen at -80°C.
157 Total RNA was extracted using an RNeasy mini kit (Qiagen,Valencia, CA) according to
158 manufacturer's instructions. Reverse transcription was performed using Murine Moloney
159 Leukemia Virus Reverse Transcriptase (Thermo Fisher Scientific) following manufacturer's
160 instructions. Gene expressions were assessed with the TaqMan Fast Advanced Master Mix

161 reagent (Applied Biosystems, Foster City, Calif). Primers and probes were from Applied
162 Biosystems (**Supplementary Table 5**). Real-time PCR was performed using the StepOne Plus
163 System (Applied Biosystems). Relative expression was normalized to hypoxanthine-guanine
164 phosphoribosyltransferase and calculated using $2^{\wedge -ddct}$ method. Results were expressed in
165 arbitrary units (a.u.).

166 **Monocyte-derived dendritic cells**

167 Monocytes from healthy volunteers were isolated either by elutriation of PBMCs (Clinical
168 Development and Transfer Platform, Nantes, France) or by magnetic labelling (untouched cells,
169 Human monocyte Isolation kit II). To obtain monocyte-derived DC (moDC),
170 $2.5x10^6$ monocytes were incubated in a 6-well plate in 5 mL of complete medium (RPMI 1640
171 medium containing 10% FCS, 1% L-glutamine, 1% antibiotics, 1mM Sodium Pyruvate, 1mM
172 HEPES, 1% non-essential amino acids) supplemented with recombinant human IL-4
173 (200U/mL) and recombinant human GM-CSF (100U/mL) for 6 days at 37°C with 5%
174 CO_2. When indicated, cells were treated with retinoic acid (RA) (100nM, Sigma Aldrich), LPS
175 (1 µg/mL, Sigma Aldrich) and TNF (50 ng/mL, Miltenyi). After 6 days, moDC and
176 supernatants were collected and frozen at −80°C until use.

177 **Immunofluorescence staining from mesenteric lymph nodes**

178 Lymph nodes isolated from 3 CD patients were frozen in tissue-Tek. Sections were fixed for
179 15 min in paraformaldehyde. After rehydration, a 10 min step saturation was performed with
180 H2O2 (3%). For double staining, mouse IgG anti-hIL-22BP (MAB 1087 from R&D System,
181 [1/800]) or isotype control (mouse IgG1, DDXCMO1P from Dendritic products [1/800] were
182 incubated at room temperature (RT) for 1 hour. After washing, several steps are carried out in
183 order to amplify the IL-22BP signal: polymer enhancer (M023 from ImPath) then HRP-2
184 (M024 from ImPath) and finally Opal 650 (FP1496A from Akoya) following manufacturer's
185 instruction. Then, after washing and a second step of saturation with goat serum 1.5%, a second

186 purified antibody: rabbit anti-hCD3 (A0452 from Dako, [1/800]) or isotype control rabbit IgG
187 (I-1000 from Vector) was added and incubated at RT during 1 hour. Purified antibody was
188 revealed with adapted secondary antibody labelled with-Alexa488 (goat anti-rabbit, A11008)
189 from Life Technologies. After washing, DAPI (Molecular Probes, D1306) was incubated 30
190 min. Slides were mounted with Vectashield® Vibrance™ Antifade Mounting Medium (H-1700
191 Vector Laboratories). Images were obtained with A1 R Si Confocal microscope (MicroPICell).

192 **Cytokine correlation plots**

193 Pairwise correlations between cytokines were calculated and visualized as a correlogram using
194 R function corrplot. Spearman's rank correlation coefficient (ρ) was indicated by heat scale;
195 significance was indicated by *P < 0.05, **P < 0.01, and ***P < 0.001.

196 **Statistical analysis**

197 Statistical analysis was performed with GraphPad Prism Software (GraphPad Software, San
198 Diego, CA). Means comparisons of unpaired samples were performed using the Mann–Whitney
199 U-test or the Kruskal–Wallis test with Dunn's post-test. The Wilcoxon signed-rank test was
200 used for paired samples. P-values <0.05 were considered statistically significant.

201 # Results

202 **IL22BP levels are higher in the ileum than in the colon of Crohn's disease patients**

203 Both IL-22 and IL-22BP mediate their biological functions as secreted soluble proteins
204 (11,20). To explore IL-22BP production in a biologically relevant manner in CD, we thus
205 quantified secreted levels released after short *ex vivo* culture of intestinal biopsies. Soluble IL-
206 22BP was consistently detected in supernatants from both controls and CD biopsies (see
207 **Supplementary Table 1** for patient characteristics), and though means were not statistically
208 significant, the highest levels were detected in a subset of CD patients (**Figure 1A**). To begin
209 explore what factors would preferentially associate with IL-22BP heterogenous distributions in
210 CD, we ran a multiple linear regression analysis that included several disease-relevant clinical
211 parameters such as age, sex, disease location, disease phenotype and medication (**Table 1**). This
212 analysis revealed disease location as the only factor reaching statistical significance (Student t-
213 test t=2.693; P=0.012). Accordingly, higher levels of IL-22BP were detected in the supernatants
214 of ileal vs. colonic CD biopsies (**Figure 1B**). IL-22 levels in turn, were similar between biopsy
215 supernatants from both locations, which translated into IL-22/IL-22BP ratios, a surrogate to
216 infer IL-22 bioactivity, higher in the colon as compared to the ileum of CD patients (**Figures**
217 **1C&1D**). In fact, the analysis of IL-22BP and IL-22 in control patients indicated that both
218 proteins were produced at higher levels in ileal vs. colonic biopsies (**Figures 1E&F**), an
219 observation confirming previous reports about the physiological distribution of IL-22 across
220 intestinal segments (21). Remarkably, however, IL-22 induction was largely limited to CD
221 colons (**Figures 1G&H**). IL-22BP mean levels in turn, remained similar between controls and
222 CD patients when controlling for gut segments (**Figures 1I&J**). Concordantly, in comparison
223 to controls, IL-22/IL-22BP ratios were unchanged in the ileum but increased significantly in
224 CD colons (**Figures 1K&L**).

225 Altogether, these results suggest that regional specialization in the intestine are
226 characterized by higher levels of IL-22BP production in the ileum as compared to the colon in
227 both controls and CD patients.

228 **The highest levels of IL22BP expression are detected in CD MNP and eosinophils**

229 Various cell types can produce IL-22BP in IBD intestinal tissues, including populations
230 of mononuclear phagocytes (MNP), which encompass dendritic cells (DCs) and macrophages,
231 $CD4^+$ T cells and eosinophils (14,15,19). So far, however, no systematic direct comparison of
232 *IL22RA2* levels of expression by these different immune cell subsets has been performed
233 specifically in intestinal tissues of CD patients. We thus analyzed *IL22RA2* expression in
234 FACS-sorted populations of MNP ($CD45^+$ HLA-DR^+ $CD11c^+$), eosinophils ($CD45^+$ SIGLEC-
235 8^+), $CD4^+$ T cells ($CD45^+$ $CD3^+$ $CD4^+$), $CD4^-$ T cells ($CD45^+$ $CD3^+$ $CD4^-$), and B cells ($CD45^+$
236 $CD3^-$ $CD19^+$) isolated from surgically resected intestinal tissues of CD patients (**Figures**
237 **2A&B, Supplementary Figure 1A**). Compared to the three lymphocytes fractions, MNP and
238 eosinophils expressed higher levels of IL-22BP mRNA (**Figure 2B**). We observed similar
239 trends between MNP and T cells isolated from CD mesenteric lymph nodes (MLN) (**Figures**
240 **2C&D, Supplementary Figure 1B**). Of note, similar mRNA levels were detected between
241 populations of naïve and non-naïve T cells. Using IFI approaches, we previously verified IL-
242 22BP protein expression in major basic protein $(MBP)^+$ eosinophils and HLA-DR^+ MNP in the
243 gut LP but not in $CD3^+$ T cells (14). We could not detect IL-22BP in $CD3^+$ MLN cells either
244 (**Supplementary Figures 1C**), hence possibly reflecting a lack of method sensitivity to capture
245 lower expression levels in T cells. Finally, while we could observe increased expression levels
246 of activation-induced *IL2RA* (encoding for CD25) in sorted gut and MLN T cells upon
247 simulation with PMA and ionomycin, no difference existed for *IL22RA2* (**Supplementary**
248 **Figures 1D-G**).

249 Taken together, these data suggest that in a cell-intrinsic fashion, MNP and eosinophils
250 express IL-22BP at the highest levels in intestinal tissues of CD patients.

251 **High levels of IL22BP in the ileum associate with higher proportions of eosinophils**

252 In inflamed CD tissues, the strongest factor linked to IL-22BP variability was disease
253 location (**Table 1**), and as for controls, IL-22BP secretion in biopsy supernatants was higher in
254 the ileum than in the colon (**Figure 1**). Interestingly, the levels of CCL11 (aka. eotaxin-1), the
255 main driver of eosinophil homeostatic recruitment in the intestine (22), were also higher in
256 biopsy supernatants from the ileum than from the colon of controls (**Figure 2E**), concordant
257 with the reported increased abundance of gut resident eosinophils in the small intestine (23–
258 25). This suggested that the higher levels of IL-22BP we detected in inflamed CD ileums as
259 compared to CD colons could in part be explained by the heterogenous distribution of resident-
260 eosinophils across intestinal segments. Accordingly, we confirmed the existence of higher
261 eosinophil numbers in CD ileums than in CD colons (**Figures 2F&G**). Blood-derived
262 eosinophil infiltration has been reported during colonic inflammation, including in CD (26–28),
263 and CCL11 levels were indeed moderately increased in CD colons as compared to controls
264 (**Figure 2E**). Median-based stratification of colonic CD patients into eosinophil^{high} and
265 eosinophil^{low}, however, did not reveal differences in IL-22BP production between the two
266 subgroups (**Figure 2H**). Importantly, we previously reported that *IL22RA2* expression was
267 undetectable in human peripheral blood eosinophils (14). This suggested that the contribution
268 of eosinophil-derived IL-22BP detected in CD lesions could in fact reflect a pre-established
269 production by tissue-imprinted gut-resident eosinophils but not by recently recruited
270 eosinophils in inflamed tissues.

271 In consequence, our interpretation of these data is that homeostatic enrichment of tissue-
272 resident eosinophil contributes to create an IL-22BP-rich environment that is maintained upon
273 inflammation induction in CD ileums and participate to limit the extent of IL-22 bioavailability.

274 **IL22BP levels correlate with CCL24 produced by monocyte-derived dendric cells in the**
275 **colon of CD patients**

276 In the colon of CD patients, IL-22BP detected in biopsy supernatants spanned a wide
277 range of concentrations, and while means did not differ from controls, a subset of patients
278 nevertheless exhibited levels reaching those observed in the ileum (**Figures 1B**). As described
279 above, this variability could not be explained by colonic eosinophil abundance (**Figure 2H**),
280 which suggested a role for other IL-22BP sources (i.e. MNP and/or T cells). We were unable
281 to examine IL-22BP production by flow cytometry because in our hands none of the anti-IL-
282 22BP antibodies commercially available could be confidently validated. To infer possible
283 sources, we hypothesized that IL-22BP secretion should be correlated with other cytokines in
284 biopsy supernatants, in part as a consequence of shared cellular origins. We characterized the
285 cytokine milieu associated with IL-22BP secretion in the CD colon through a multiplex analysis
286 on the same biopsy supernatants as used for IL-22BP quantification. We then realized a
287 correlation matrix to identify possible cytokines sharing similar regulation of expression as IL-
288 22BP (**Figure 3A**). The strongest correlate of IL-22BP secreted levels was CCL24 (aka.
289 eotaxin-2; spearman r=0,7; P<0,0001). Accordingly, the median-based stratification of CD
290 patients based on their CCL24 levels in the colon showed that almost all $CCL24^{low}$ patients had
291 IL-22BP levels below the lower limit of quantification, while it was the opposite for $CCL24^{high}$
292 patients (**Figure 3B**). We thus compared *CCL24* expression between T cells and MNP isolated
293 from the CD lamina propria. The highest levels were detected in MNP (**Figure 3C**), which
294 suggested they could be the privileged source responsible for IL-22BP heterogeneous secreted
295 levels across colonic CD patients.

296 In an attempt to further refine the nature of a possible MNP subset producing both IL-
297 22BP and CCL24, we reanalyzed a CD single-cell RNA-sequencing (scRNA-seq) dataset we
298 published recently (29). While the contribution of eosinophils could not be evaluated in these

299 data because of scRNAseq technical limitations, our analysis confirmed the highest expression
300 levels of *IL22RA2* were present in MNP populations of DCs followed by lymphocyte
301 populations including Type 3 cytokine-producing T cells, while *CCL24* was preferentially
302 detected in macrophages (**Figure 3D**) (see (29) for detailed description about cluster
303 annotations). Interestingly, only monocyte-derived dendritic cells (moDC) displayed shared
304 detectable expression of both genes (**Figure 3D**), and we validated that moDC differentiated *ex*
305 *vivo* co-secreted IL-22BP and CCL24 (**Figures 3E-G**). Importantly, we previously showed that
306 retinoic acid (RA) was a potent inducer of *IL22RA2* expression in moDC (16), which we could
307 further confirm at the protein level in moDC supernatants (**Figures 3E&F**). Rather
308 interestingly, the same held true for CCL24 (**Figure 3G**), suggesting IL-22BP and CCL24
309 inductions could share similar regulatory pathways, such as RA activity in moDC. In turn, the
310 activation of moDC with LPS for 24h had no effect on CCL24 secreted levels but decreased
311 the production of IL-22BP (**Figures 3E&F**), hence supporting ours and others previous
312 observations (16,30).

313 Other myeloid-derived cytokines showed significant positive correlations with IL-
314 22BP, though weaker than for CCL24 (Spearman; $P < 0{,}05$) (**Figure 3A**). These included IL-6,
315 IL-27 and IL-23, the most potent inducer of IL-22 in $CD4^+$ T cells and ILC3 (31,32).
316 Concordantly, IL-22 correlated with IL-22BP and was also increased in $CCL24^{high}$ patients,
317 hence leading to similar IL-22/IL-22BP ratios between the two subgroups of $CCL24^{high}$ and
318 $CCL24^{low}$ patients (**Figures 3H&I**). The co-regulation of IL-22/IL-22BP secretion in the colon
319 of CD patients was in agreement with previous results we and others reported in IBD (14,15).
320 Because $CD4^+$ T cells also produce IL-22BP in IBD tissues (15), it is thus possible that Type 3
321 cytokine-producing T cells contribute to increase both IL-22 and IL-22BP levels in the colon
322 of CD patients.

323 **Higher levels of IL-22BP are detected in the colon of actively smoking CD patients**

324 Our multiple linear regression analysis suggested tobacco smoking as a possible
325 additional factor associated with IL-22BP production in CD, though not reaching statistical
326 significance (Student t-test t=1.813; P=0.08) (**Table 1**). We thus analyzed IL-22BP secretion in
327 the colon of CD patients based on their smoking status. The production of IL-22BP was
328 significantly higher in actively smoking patients (**Figure 3J**). A role for tobacco smoking has
329 been suggested for the reprogramming of macrophages into the production of more
330 immunoregulatory molecules, including CCL24 among others (corresponding to *in vitro* so-
331 called "M2" macrophages) (33–35). Because a strong association existed between IL-22BP and
332 CCL24 secretions in CD colons, we compared the proportions of smokers and non-smokers
333 between $CCL24^{high}$ and $CCL24^{low}$ CD patients and verified an enrichment of smokers in the
334 $CCL24^{high}$ subgroup, though not reaching statistical significance (**Figure 3K**). While this would
335 deserve further investigations, it is thus possible that tobacco-derived metabolites be part of the
336 signals shaping moDC toward higher production of IL-22BP and CCL24 in the CD colon.
337

338 ## Discussion

339 Our understanding of the pathophysiological functions assumed by the IL-22/IL-22BP
340 axis in IBD is currently described in a rather homogenous way, frequently intermixing
341 conclusions obtained from mouse and human studies, and including results generated in both
342 CD and UC. By providing new insights focused on a more detailed characterization of IL-22BP
343 biology in tissues from CD patients specifically, our study unravels a more complex picture
344 arguing against a such generalization. Our work reveals that the distribution of IL-22BP
345 production is not homogenous between CD patients and we suggest this could be the product
346 of multiple factors related to IL-22BP complex biology in the human gut. First, we show that
347 IL-22BP levels are higher in inflamed ileums when comparing to inflamed colons of CD
348 patients, and we suggest this could be explained by distinct homeostatic distributions of gut

349 resident-eosinophils between the two segments. This is of particular relevance as the injection
350 of recombinant IL-22 is currently under evaluation in IBD clinical trials (NCT03650413), and
351 it is thus unclear whether the IL-22BP-rich environment created in the ileum could lead to more
352 interference with the biological actions of the drug in involved ileum vs. colon of CD patients.
353 In addition, our data suggest that a raise of IL-22 bioactivity is more likely to be achieved in
354 inflammatory responses developing in the colon, hence indicating that greater clinical benefits
355 of IL-22 injections may nevertheless be expected in involved ileums of CD patients. More
356 human studies will be needed to address this important question, as we previously showed that
357 lL-22BP expression in gut resident-eosinophils is a feature not conserved in rodents (14). The
358 reason why IL-22 is not induced in inflamed ileums despite higher homeostatic production in
359 control tissues would also deserve more investigations. Beyond the difference of IL-22BP
360 production created by regional specializations across gut segments, we also uncover additional
361 heterogeneity in inflamed colons of CD patients. In particular, we observe a strong correlation
362 between IL-22BP and CCL24 secretion and suggest this could be the consequence of a shared
363 production by moDC with variable intensity across subsets of patients. Importantly, while,
364 CCL24 was initially described as an eosinophil chemoattractant trough the receptor CCR3,
365 experiments in rodents suggested it was not involved in eosinophil infiltration during colitis
366 (28,36). Our data further suggest that IL-22BP variability among inflamed CD colons was likely
367 not explained by the level of infiltration of blood-derived eosinophils but relied more on other
368 IL-22BP cellular sources such as moDC. CCR3 is also expressed by subsets of T cells (37) and
369 it is possible that CCL24-mediated T cell infiltration participates to the total production of IL-
370 22BP in the colon of CD patients (15). Supporting the existence of similar regulatory pathways
371 for both IL-22BP and CCL24 in inflamed CD colons, we report that retinoic acid increases the
372 secretion of both proteins by moDC. As suggested by the higher IL-22BP levels we noticed in
373 tobacco smokers, it is likely that additional factors contribute to differential regulations of IL-

374 22BP production across patients. In that regard, it is interesting to note that IL-22BP levels also
375 correlated with those of IL-22 and IL-23, the main driver of IL-22 expression in lymphocytes.
376 Recent evidence suggests that lymphotoxin (LT)α1β2 upregulates IL22BP via the noncanonical
377 NF-κB pathway in moDC (19). Because NF-κB is involved in the generation of IL-22-
378 producing T cells, in part indirectly through the induction of IL-23 secretion in MNP (38), one
379 may speculate that privileged cellular circuits exist in inflamed colons of a subset of CD
380 patients, in which LTα1β2 from CD4 T cells drives both IL-22BP and IL-23 expression in
381 moDC (39,40). The latter in turn can promote IL-22 production in CD4 T cells. While this
382 would deserve further investigations, specific cellular niches in subsets of CD patients could
383 thus participate to increase both IL-22 and IL-22BP global levels associated with a more
384 localized impact on epithelial cell biology (19, 41). Clarifying the existence, tissue distribution
385 and pathophysiological actions of such cellular circuits would be especially relevant in light of
386 the upcoming IL-23 blockers in the CD therapeutic armamentarium (42,43).

387 In conclusion, our work provides new important information about the biology of IL-
388 22BP in CD, which can open the way to therapeutically relevant future studies with regard to
389 the modulation of the IL-22/IL-22BP axis in patients.

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Author contributions

JCM, RJ and AF designed experiments and interpreted the data; JCM wrote the manuscript; AF, RJ and JCM edited the manuscript; AF, EL, TL, LD, SB and GB performed experiments and analyses; CB and NS performed cytokine multiplex assays; EM and AM performed *in vitro* moDC experiments; JP and AB managed the biobank and selected the patients included in the study.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors at the following e-mail addresses: Jerome.martin@univ-nantes.fr; regis.josien@univ-nantes.fr

Disclosure

The authors declared no conflict of interest.

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560

561 # Figure Legends

562 **Figure 1: IL22BP levels are higher in the ileum than in the colon of Crohn's disease**
563 **patients**

564 (**A**) Levels of soluble IL-22BP were quantified by ELISA in the supernatant of gut endoscopic
565 biopsies from controls (Ctrls) (n=16) and Crohn's disease (CD) (n=43) patients after 6 hours of
566 ex vivo culture. (**B-C**) Quantification of soluble IL-22BP (B) and IL-22 (C) in culture
567 supernatants of biopsies from CD patients with inflamed lesions in the ileum (n=11) or in the
568 colon (n=32). (**D**) Comparison of IL-22/IL-22BP ratios in the supernatants of CD patients with
569 inflamed ileum and colon. (**E-F**) Quantification of soluble IL-22BP (E) and IL-22 (F) levels in
570 the supernatant of gut biopsies from Ctrls ileum (n=4) and colon (n=12). (**G-H**) Comparison of
571 IL-22 levels in the ileum (G) and the colon (H) of Ctrls and CD patients. (**I-J**) Comparison of
572 IL-22BP levels in the ileum (I) and the colon (J) of Ctrls and CD patients. (**K-L**) Comparison
573 of IL-22/IL-22BP ratios in the ileum (K) and the colon (L) of Ctrls and CD patients. Statistical
574 significance for mean comparisons was assessed by the Mann-Whitney U-test in a two-sided
575 manner, using a nominal significance threshold of $P < 0.05$.

576

577 **Figure 2: The highest levels of IL22BP expression are detected in CD MNP and**
578 **eosinophils**

579 (**A**) Representative dot plots of the gating strategy used to sort indicated cell subsets from the
580 lamina propria (LP) of intestinal surgical resections from Crohn's disease (CD) patients. (**B**)
581 *IL22RA2* expression was analyzed by RT-qPCR in indicated FACS-sorted cell subsets from
582 CD patient gut LP samples (n=6). Grey and red dots refer to cells sorted from surgical resections
583 respectively defined as non-inflamed and inflamed after pathology examination. (**C**)
584 Representative dot plots of the gating strategy used to sort indicated cell subsets from
585 mesenteric lymph nodes (MLN) of CD patients. (**D**) *IL22RA2* expression was analyzed by RT-

586 qPCR in indicated FACS-sorted cell subsets from CD patient MLNs (n=5). (**E**) Levels of
587 soluble CCL11 were quantified in gut endoscopic biopsy supernatants from controls (Ctrls)
588 (n=12) and CD (n=37) patients after 6 hours of ex vivo culture. Dots and squares refer to ileum
589 and colon tissues respectively. (**F**) Representative pictures of slides from formalin-fixed,
590 paraffin-embedded (FFPE) sections of CD tissues stained with standard hematoxylin and eosin
591 (HES) coloration. Original magnification x 200. (**G**) Eosinophil counts were scored in a blinded
592 manner by a trained pathologist on HES slides from CD patients (n=36). Each count was
593 obtained by averaging the results obtained from the analysis of different 5 fields and was
594 expressed as eosinophils per mm^2. (**H**) Comparison of soluble IL-22BP levels in the supernatant
595 of endoscopic colonic biopsies of CD patients stratified into eosinophils $(EOS)^{high}$ and EOS^{low}
596 based on the median value. Mean comparisons of unpaired samples were performed using the
597 Kruskal Wallis (for > 2 conditions) or the Mann-Whitney U-test. P-value < 0.05 were
598 considered statistically significant.
599
600 **Figure 3: IL22BP levels correlate with CCL24 produced by monocyte-derived dendric**
601 **cells in the CD colon**
602 (**A**) Spearman correlation and hierarchical clustering of indicated cytokines quantified in
603 culture supernatants of endoscopic colonic biopsies from 28 CD patients. (**B**) Comparison of
604 soluble IL-22BP levels in CD patients stratified into $CCL24^{low}$ and $CCL24^{high}$ patients based
605 on the median level. (**C**) *CCL24* expression was analyzed by RT-qPCR on FACS-sorted cells
606 from CD lamina propria, as in Figure 2A. (**D**) Heatmap representing the expression of *IL22RA2*
607 and *CCL24* in indicated cell types using the clustering analysis of a previously published single-
608 cell RNA-sequencing (scRNA-seq) dataset (Martin JC, Cell, 2019). (**E-G**) Human blood
609 classical monocytes from healthy donors were differentiated into monocyte-derived DC
610 (moDC) with GM-CSF and IL-4 for 6 days in the presence of indicated ligands. At day 6 of

611 culture *IL22RA2* mRNA expression was analyzed by RT–qPCR in moDC (E), and levels of IL-
612 22BP (F) and CCL24 (G) were quantified in culture supernatants. (**H-I**) Comparison of soluble
613 IL-22 (H) and IL-22/IL-22BP ratios (I) in culture supernatants of colonic CD biopsies from
614 $CCL24^{high}$ or $CCL24^{low}$ patients. (**J**) Comparison of soluble IL-22BP levels in culture
615 supernatants of colonic CD biopsies (n=32) from patients stratified according to their smoking
616 status. (**K**) Pie charts representing the proportion of active smokers vs. non-smokers in the colon
617 of $CCL24^{high}$ or $CCL24^{low}$ CD patients.
618
619 **Supplementary Figure 1**
620 (**A-B**) Representative dot plots showing the post-sort purity of indicated cell subsets sorted from
621 the lamina propria of intestinal surgical resections (A) and mesenteric lymph nodes (MLN) (B)
622 of CD patients. (**C**) Representative IFI pictures of slides from formalin-fixed, paraffin-
623 embedded (FFPE) sections of CD MLN stained with mAbs against IL-22BP (red) and CD3
624 (green), as well as with DAPI (blue) (n=3 CD patients). Original magnification x 630. (**D-G**)
625 Indicated T cell subsets were sorted from CD lamina propria intestinal surgical resections (n=5)
626 (D, F) and MLN (n=3) (E, G) and cultured with or without PMA (50ng/mL) and Ionomycin
627 (250ng/mL) (PMA/Iono). After 20 hours, expressions of *IL2RA* (D-E) and *IL22RA2* (F-G) were
628 analyzed by RT-qPCR. IL22RA2 expression levels of freshly isolated DC/Macrophages and
629 eosinophils are indicated for reference. Means comparisons were performed with the Kruskal–
630 Wallis test for unpaired samples and with the Wilcoxon test for paired samples. P-value < 0.05
631 were considered statistically significant. CD: Crohn's disease; DC: dendritic cells, EOS:
632 eosinophils; Mθ: macrophages.
633

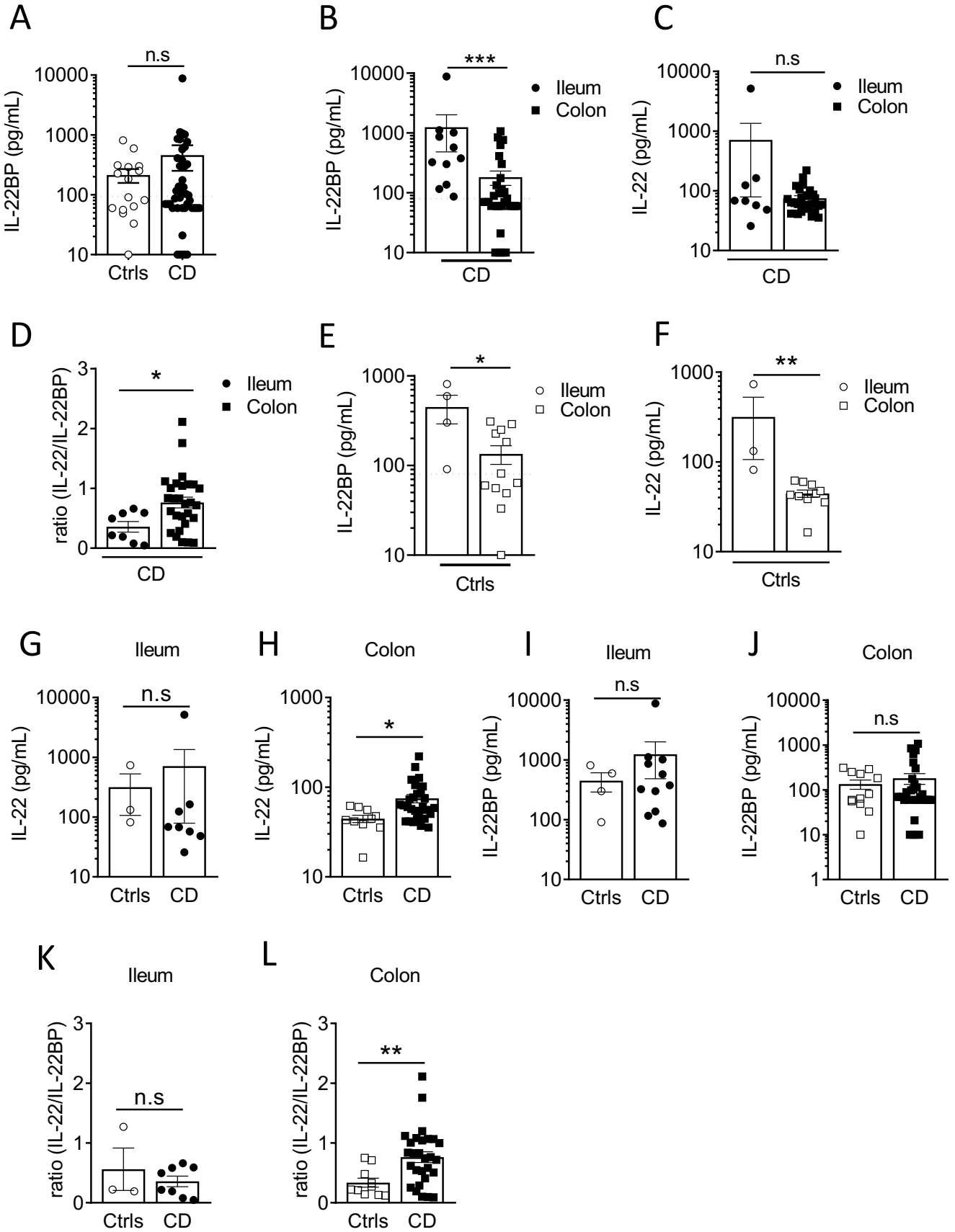
634 **Table 1: Multiple linear regression to assess associations between clinical parameters of**

635 **CD and IL-22BP levels**

Variable	|t|	P value
Sex	0.7929	0.43
Age	0.0998	0.92
Localization	2.693	**0.012**
Tobacco	1.813	0.08
Disease evolution	0.0809	0.94
Phenotype	0.0845	0.93
Perianal involvement	0.8205	0.42
Treatment: anti-TNF	0.4463	0.66
Treatment: IS	0.5842	0.56
Surgical resection	1.108	0.28
Clinical score	0.033	0.97

636

Figure 1


A
n.s
IL-22BP (pg/mL)
10000
1000
100
10
Ctrls
CD
B
***
Ileum
Colon
IL-22BP (pg/mL)
CD
C
n.s
Ileum
Colon
IL-22 (pg/mL)
CD
D
*
Ileum
Colon
ratio (IL-22/IL-22BP)
3
2
1
0
CD
E
*
Ileum
Colon
IL-22BP (pg/mL)
Ctrls
F
**
Ileum
Colon
IL-22 (pg/mL)
Ctrls
G
Ileum
n.s
IL-22 (pg/mL)
Ctrls
CD
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Colon
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IL-22 (pg/mL)
Ctrls
CD
I
Ileum
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IL-22BP (pg/mL)
Ctrls
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n.s
IL-22BP (pg/mL)
1
Ctrls
CD
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Ileum
n.s
ratio (IL-22/IL-22BP)
Ctrls
CD
L
Colon
**
ratio (IL-22/IL-22BP)
Ctrls
CD

Figure 2

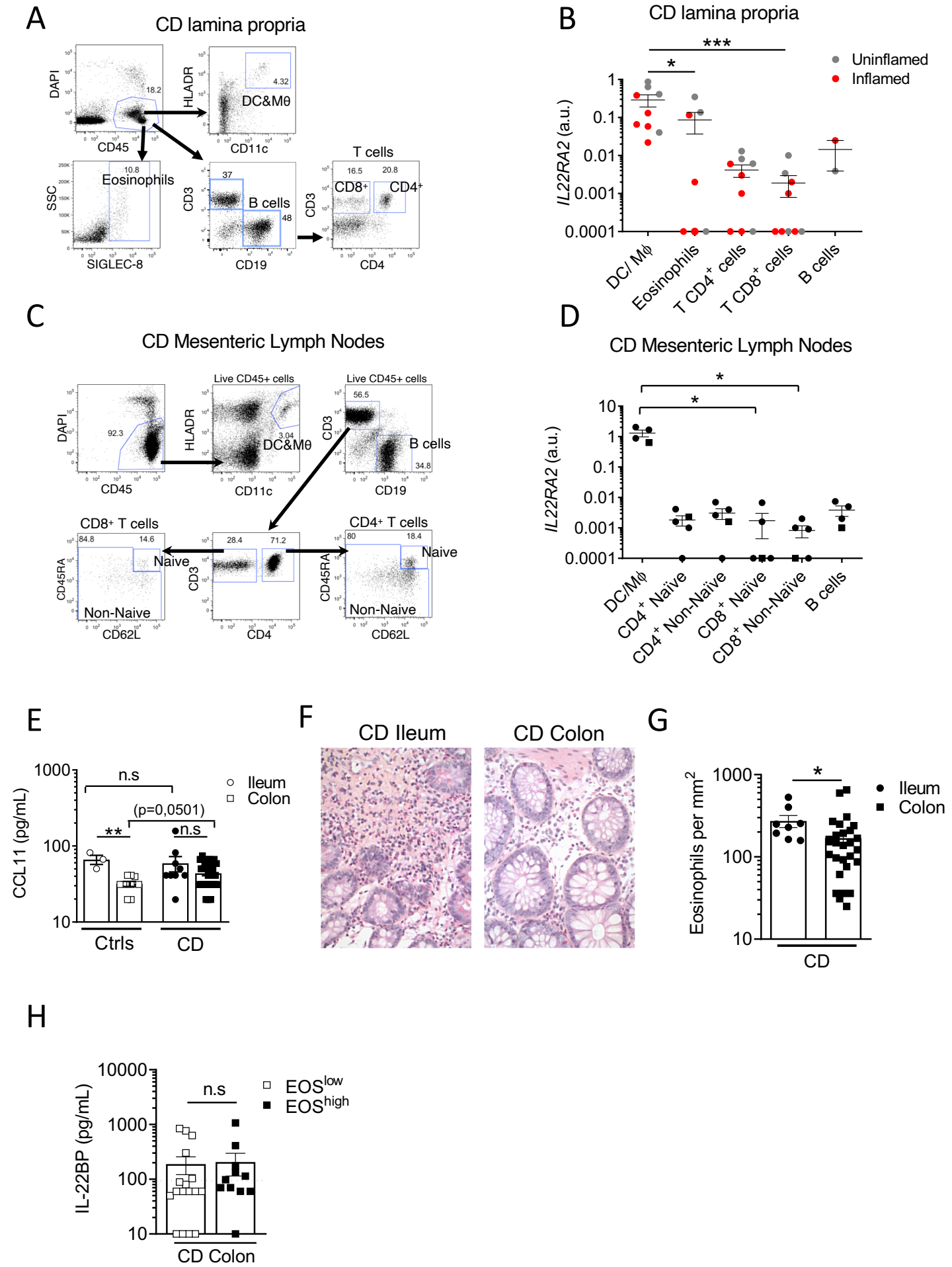
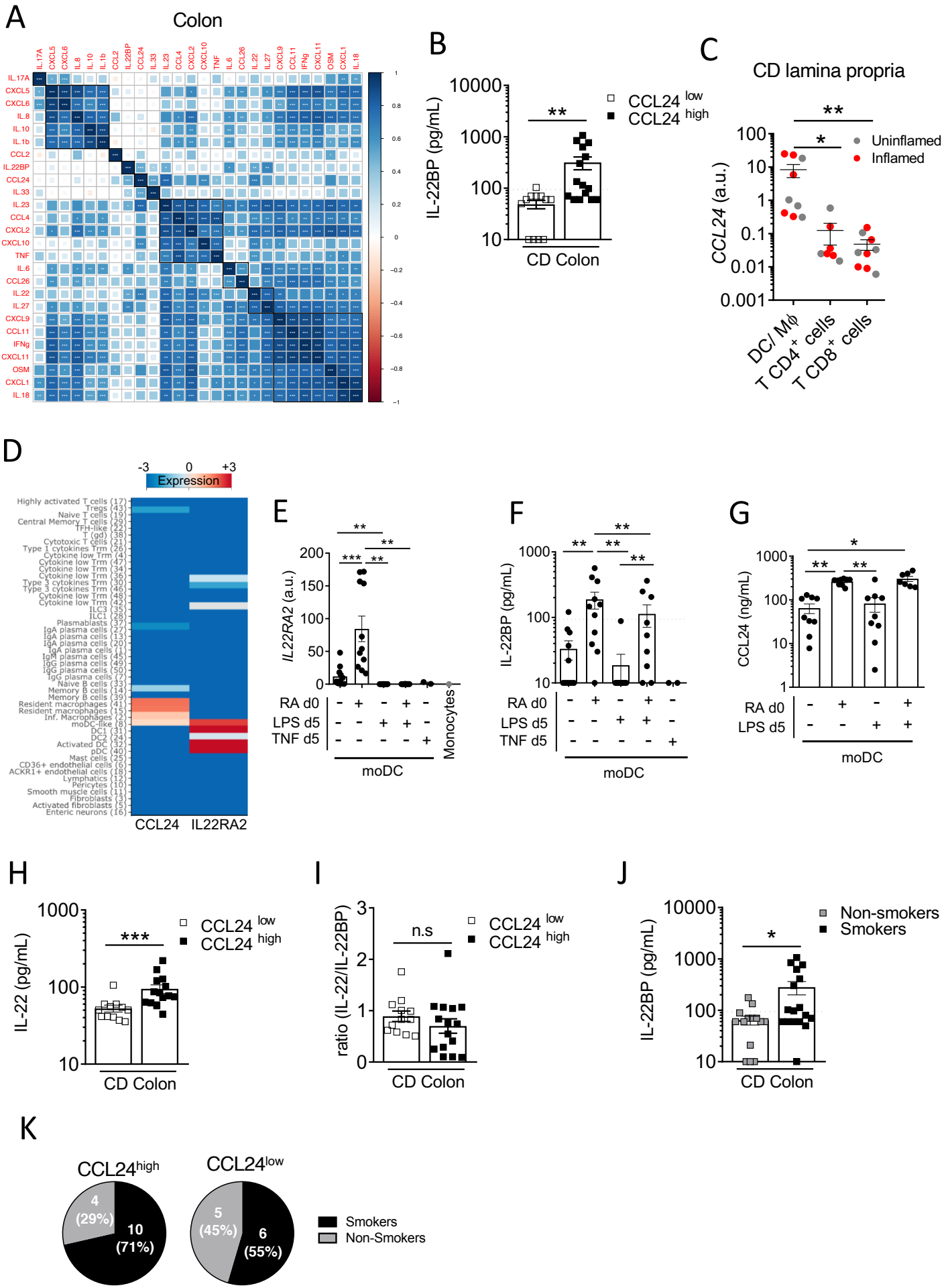
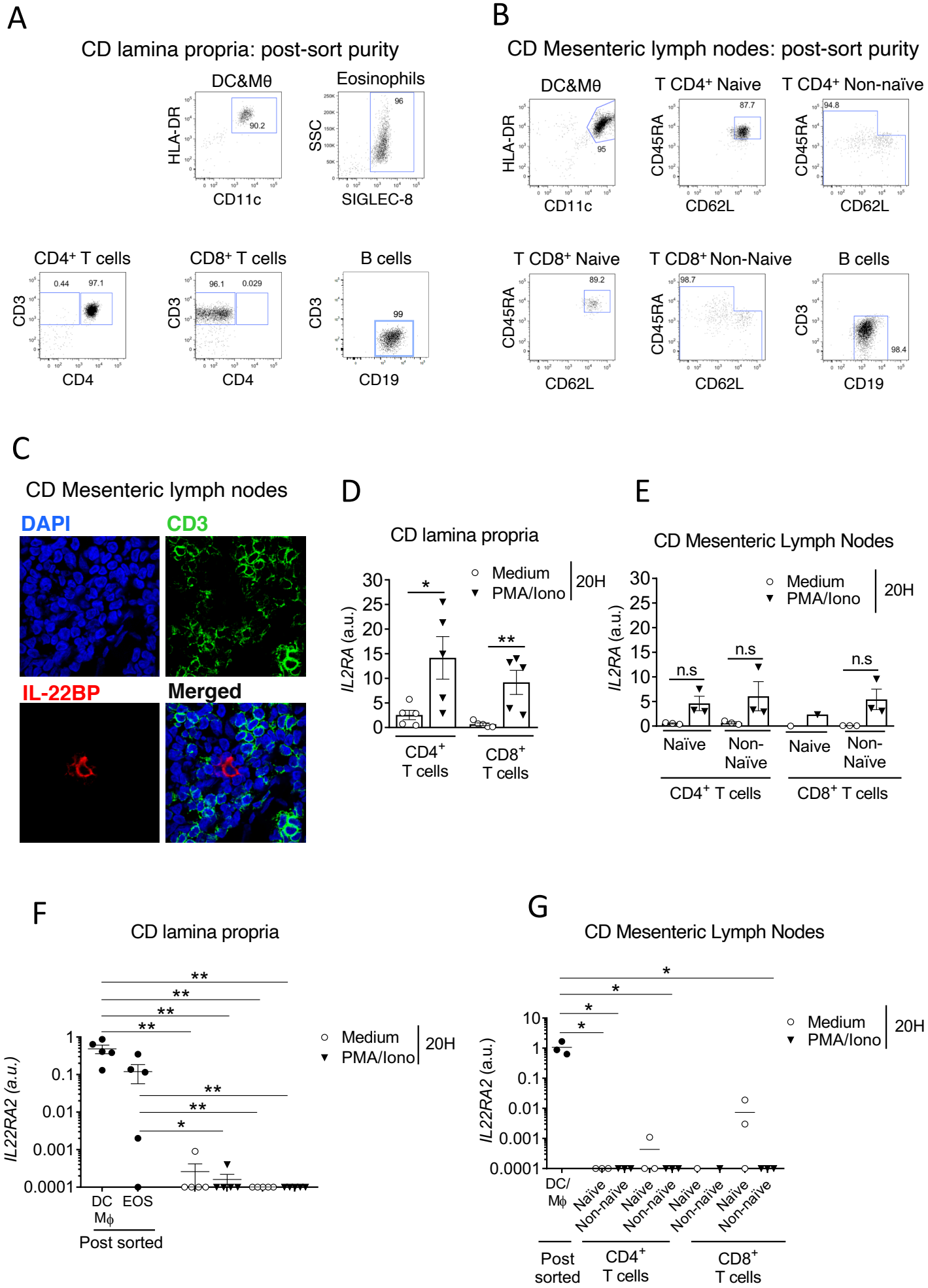

A
CD lamina propria
DAPI
CD45
18.2
HLADR
CD11c
4.32
DC&Mθ
SSC
SIGLEC-8
10.8
Eosinophils
CD3
CD19
37
B cells
48
T cells
CD4
16.5
CD8+
20.8
CD4+
B
CD lamina propria
Uninflamed
Inflamed
IL22RA2 (a.u.)
DC/ Mϕ
Eosinophils
T CD4+ cells
T CD8+ cells
B cells
C
CD Mesenteric Lymph Nodes
Live CD45+ cells
92.3
3.04
56.5
34.8
CD8+ T cells
84.8
14.6
Naive
Non-Naive
CD62L
CD45RA
28.4
71.2
CD4+ T cells
80
18.4
D
CD Mesenteric Lymph Nodes
DC/Mϕ
CD4+ Naïve
CD4+ Non-Naïve
CD8+ Naïve
CD8+ Non-Naïve
B cells
E
CCL11 (pg/mL)
n.s
(p=0,0501)
**
n.s
Ileum
Colon
Ctrls
CD
F
CD Ileum
CD Colon
G
Eosinophils per mm2
*
Ileum
Colon
CD
H
IL-22BP (pg/mL)
n.s
EOSlow
EOShigh
CD Colon

Figure 3


A
Colon
B
IL-22BP (pg/mL)
CCL24 low
CCL24 high
CD Colon
C
CD lamina propria
CCL24 (a.u.)
Uninflamed
Inflamed
DC/ Mϕ
T CD4+ cells
T CD8+ cells
D
Expression
CCL24
IL22RA2
E
IL22RA2 (a.u.)
RA d0
LPS d5
TNF d5
moDC
Monocytes
F
IL-22BP (pg/mL)
G
CCL24 (ng/mL)
H
IL-22 (pg/mL)
I
ratio (IL-22/IL-22BP)
n.s
J
Non-smokers
Smokers
K
CCL24high
CCL24low
4 (29%)
10 (71%)
5 (45%)
6 (55%)
Smokers
Non-Smokers

Supplementary Figure 1