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1 Brief Conclusive Report

2

3 **Spatial heterogeneity for APRIL production by eosinophils in the lower intestine**

4

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25 Summary sentence: heterogeneous expression of the plasma-cell survival factor APRIL in the
26 lower intestine

27

28 Authors declare no conflicts of interest

29

30 **Abstract**

31 Eosinophils may reside in the lower intestine to play several homeostatic functions. Regulation
32 of IgA⁺ plasma-cell (PC) homeostasis is one of these functions. Here, we assessed regulation
33 of expression for a proliferation-inducing ligand (APRIL), a key factor from the TNF
34 superfamily for PC homeostasis, in eosinophils from the lower intestine. We observed a strong
35 heterogeneity, since duodenum eosinophils did not produce APRIL at all, while a large majority
36 of eosinophils from the ileum and right colon produced it. This was evidenced both in the
37 human and mouse adult systems. At these places, the human data showed that eosinophils were
38 the only cellular sources of APRIL. The number of IgA⁺ PCs did not vary along the lower
39 intestine, but ileum and right colon IgA⁺ PC steady state numbers significantly diminished in
40 *APRIL*-deficient mice. Use of blood cells from healthy donors demonstrated that APRIL
41 expression in eosinophils is inducible by bacterial products. Use of germ-free and antibiotics-
42 treated mice confirmed the dependency on bacteria for APRIL production by eosinophils from
43 the lower intestine. Taken together, our study shows that APRIL expression by eosinophils is
44 spatially regulated in the lower intestine with a consequence on the APRIL dependency for
45 IgA⁺ PC homeostasis.

46

47 **Introduction**

48 Eosinophils are best known for their effector functions in immunity against some helminthic
49 parasites ¹. However, most circulating mature eosinophils produced by the bone marrow
50 extravasate in tissues to become resident ². There, they may play diverse functions. One tissue
51 well characterized for the presence of resident eosinophils is the lamina propria of the lower
52 intestine ³. This eosinophil colonization is an early process in life starting before birth and any
53 colonization of the lower intestine by commensal bacteria ^{4,5}. There, resident eosinophils fulfill
54 several homeostatic functions, including maintenance of the mucus layer and interaction with

55 the enteric nervous system. In the lower intestine, eosinophils also have immune functions with
56 a role in the homeostasis of IgA⁺ PC mostly achieved by provision of survival signals to IgA⁺
57 PC ⁶.

58 A proliferation inducing ligand (APRIL), a member from the TNF superfamily (TNFSF13), is
59 a key factor positively regulating PC homeostasis ⁷. The APRIL protein is first produced as a
60 transmembrane protein, and early cleaved in the Golgi apparatus during its secretory path by
61 furin proteases ⁸. Myeloid cells constitute the main cellular sources of APRIL with a
62 constitutive production, which may be further upregulated by inflammatory signals sensed by
63 Toll-like receptors ^{9,10}. We and others previously reported APRIL-production by eosinophils
64 ^{6,11,12}, but the regulation of APRIL production by eosinophils is still poorly characterized. Here,
65 we studied APRIL expression by eosinophils in the gut of adult mice and humans.

66

67 **Material and methods**

68 **Human experimentation**

69 Approval for research on human samples were obtained from the Ethics committee of the
70 Geneva university hospital. Human intestinal samples were obtained from patients with Merkel
71 cell carcinoma outside tumor lesions between 2003 and 2010 after patients' informed consent
72 according to the declaration of Helsinki. The presence of vili with Brunner glands identified
73 the duodenum. The presence of vili without Brunner glands but Peyer's patches identified the
74 ileum. The right colon was identified by the absence of vili.

75

76 **Bacterial cultures**

77 *Escherichia Coli* (*E. Coli*) was grown in standard Luria Broth medium. *Bifidobacterium*
78 *bifidum* was grown anaerobically at 37°C in a Hungate tube with degassed Yeast Casitone Fatty
79 Acids, medium 26 supplemented with antifoam 0.05% (Sigma). Cultures were harvested,
80 washed in PBS and resuspended in Krebs-Ringer glucose (KRG) buffer to the desired
81 concentrations after density measurement at 600 nm.

82

83 **Cell purification and activation**

84 Peripheral blood eosinophils were purified from buffy coats of healthy adult donors. After
85 centrifugation on a Ficoll gradient (Pharmacia) and hypotonic lysis, eosinophils were purified
86 from the granulocyte fraction by FACS-sorting the CD16-SSC^{high} population with a FACS-Aria
87 (Becton-Dickinson). The purity of the eosinophils was routinely >95%. Human blood
88 neutrophils were purified as previously described⁹. Viability assessed by trypan blue exclusion
89 was above 80%. 10⁵ Eosinophils resuspended in KRG were incubated in microwell plates with
90 bacteria at a ratio of 1/1. Human IL-5, GM-CSF and IL-3 used at 25 ng/ml were from Peprotech.
91 Cultures were harvested after 18h of incubation at 37°C for mRNA analyses. Production of

92 reactive oxygen species were determined as previously described ⁹. Mouse lamina propria and
93 purified eosinophils were prepared as previously described ⁶. Purity of eosinophils was above
94 90%.

95

96 **Mouse experimentation**

97 All animal care and procedure were approved by Geneva veterinary office and the French
98 national AFiS veterinary office. C57Bl/6 WT and germ-free mice were obtained from Charles
99 River. WT C57Bl/6 mice were maintained in our animal facility at least 6 weeks before use.
100 *APRIL*^{-/-} mice on a C57Bl/6 background were previously described ¹³. Antibiotics treatment
101 consisted in the addition of 333 mg/l of tylosin tartrate (Sigma) in the drinking water for 2
102 consecutive days. Intestinal samples were collected 1 week after treatment. Duodenum samples
103 originated from the area right distal to the stomach. Ileum samples originated for the small
104 intestine harboring Peyer's patches. Right colons were analyzed only.

105

106 **RT-PCR**

107 Total RNAs were prepared using Trizol (Thermo Fisher Scientific), treated with RQ1 DNase
108 (Promega), and reverse transcribed with Superscript II and oligo-dT₁₂₋₁₈ (Thermo Fisher). The
109 intron spanning for human and mouse *APRIL* have been described elsewhere ¹⁴. Denaturation
110 was performed at 94°C, annealing at 55°C, and extension at 72°C, 1 minute each. Forty cycles
111 were applied. Amplified PCR products were visualized on agarose gels and ethidium bromide
112 staining. Quantitative RT-PCR was done with a light cycler System (Roche Diagnostics) and
113 the Quantitect SYBR Green PCR kit solution (Qiagen). Expression levels were normalized to
114 human 18S rRNA and to mouse actin. Amplification was done in duplicates and experiments
115 were performed twice

116

117 **Immunohistochemistry**

118 Formalin-fixed paraffin-embedded intestine sections of 3µm were stained with Stalk-1 (rabbit
119 polyclonal) detecting APRIL-producing cells and Aprily-2, -6, -8 detecting secreted APRIL as
120 previously described ¹⁵. Eosinophil staining was performed with a mouse anti-major basic
121 protein antibody (clone BM13, Thermofisher) after a citrate-based antigen retrieval. In the
122 human system, IgA⁺ CD138⁺ PC were stained with a goat anti-human IgA (Vector laboratories)
123 and the anti-human CD138 (clone Mi15, Agilent) after citrate-based antigen retrieval. In the
124 mouse system, a rabbit anti-mouse IgA (Thermofisher) and anti-mouse CD138 (Life
125 technologies) were used after citrate-based antigen retrieval. Fluorochrome-conjugated
126 secondary antibodies were from Life technologies. DAPI (Thermofisher) was used for nucleus
127 staining. Stainings were visualized as previously described ⁹.

128

129 **Flow cytometry**

130 Surface and total cell staining was performed as previously described ¹⁵. All fluorescent-
131 conjugated primary antibodies were from Becton-Dickinson. Fluorescence was measured with
132 a FACSCanto and analyzed with the CellQuest software (Becton-Dickinson).

133

134 **Statistical analysis**

135 Statistical analysis was performed using GraphPad Prism software. D'Agostino and Pearson
136 test (normality), Anova and *t*-tests were performed. Significant differences were defined as $p <$
137 0.05.

138

139 **Results and discussion**

140 **1-Spatial heterogeneity for APRIL-production in eosinophils from the lower intestine.**

141 We assessed in the human lower intestine APRIL-production with the Stalk-1 antibody
142 recognizing the stalk fragment left in producing cells after APRIL processing. Notably, we
143 found no APRIL-producing cells in the duodenum region, while ileum and right colon
144 contained numerous cells (figure 1A, upper panel). The highest was the right colon. High
145 magnification of a stained cell revealed an eosinophil morphology with a characteristic
146 bilobated nucleus (figure 1A, upper panel, inserts). We confirmed eosinophil identification for
147 these APRIL-producing cells by a costaining with the major-basic protein, a protein specifically
148 contained in eosinophil granules. These cells were never present in Peyer's patches, the site of
149 T-cell dependent production of IgA⁺ PC in the gut (figure sup1A)¹⁶. Occasionally, they
150 infiltrated the epithelium (figure sup1B). Eosinophil infiltration of a mucosal epithelium is at
151 the bases of several pathologies, but also occurs in healthy small intestines¹⁷. Eosinophils are
152 known to preferentially infiltrate the lower intestine with an increase in distal zones¹⁸. Our
153 eosinophil quantification based on MBP staining were similar with an infiltration of 20 cells
154 +/- 6 per mm² in the duodenum, 22 +/- 8 in the ileum and 43 +/- 4 in the right colon. APRIL
155 and MBP costaining showed that all eosinophils infiltrating the duodenum did not produce
156 APRIL. In the ileum, APRIL-negative eosinophils were reduced down to 9% +/- 6, and all
157 eosinophils produced APRIL in the right colon. We also tested secreted APRIL reactivity with
158 the Aprily-2 antibody. Duodenum was negative, while a faint reactivity was detected in the
159 extracellular space of the duodenum close to APRIL-producing cells (Figure 1C). Some
160 eosinophils were also positive intracellularly for secreted APRIL, and PC could occasionally
161 be detected with surface bound secreted APRIL (inserts in figure 1C). Similar reactivities were
162 obtained with Aprily-6 and -8 antibodies also detecting secreted APRIL. The latter constituted
163 an evidence of APRIL secretion in the lower intestine and an on-target activity. IgA⁺ cells were
164 obviously numerous in the lower human intestine, adjacent to APRIL producing cells (figure
165 1C, upper panel). Among these cells, we quantified IgA⁺ CD138⁺ PCs with two-color

166 immunofluorescence (figure 1C, bottom left panel). We excluded from the quantification the
167 few IgA⁺ CD138⁻ cells present (arrow in figure 1C, bottom left panel) likely being memory B
168 cells present in the lamina propria as reported by others ¹⁹. Despite the heterogeneity in APRIL
169 expression between duodenum and ileum, we did not observe a significant difference in the
170 density of IgA⁺ CD138⁺ PCs at these locations (Figure 1C, bottom right panel).

171 We next assessed APRIL expression in mouse gut at the mRNA level, since the Stalk-1
172 antibody did not cross-react with mouse APRIL. RT-PCR confirmed the human data with an
173 almost undetectable amplicon in the mouse duodenum, while the lamina propria from ileum
174 and right colon were positive (figure 2A, left panel). Analysis of purified eosinophils from these
175 lamina propria gave identical results with negativity and positivity for duodenal and
176 ileal/colonic cells, respectively (figure 2A, right panel). IgA⁺ cells were also numerous in the
177 mouse lower intestine (figure 2B, upper panel). We also quantified the number of IgA⁺ CD138⁺
178 PCs among these cells (figure 2B, bottom left panel). Again, we found no quantitative
179 differences in duodenum, ileum and right colon (figure 2B, bottom right panel). However, we
180 found significantly lower numbers for these cells specifically in the ileum and right colon when
181 we performed our analysis in *APRIL*^{-/-} mice. Taken together, our results showed that the human
182 and mouse small intestine harbor a similar expression pattern for APRIL production with an
183 absence of expression in the duodenum. The absence of APRIL expression in the duodenum
184 was confirmed in the mouse study by the lack of IgA⁺ PC number variation in *APRIL*^{-/-} mice.

185

186 **2-APRIL production in eosinophils is induced by bacterial stimulation**

187 We next assessed the regulation of APRIL expression in order to understand the heterogeneity
188 described above. We used human blood eosinophils. Intracellular staining with the Stalk-1
189 antibody did not detect the APRIL protein in these cells, while blood neutrophils were positive
190 as previously described (figure 3A, left panel)¹⁴. We obtained a similar result at the mRNA

191 level (figure 3A, right panel). Eosinophils may be activated by cytokines ²⁰. The common IL-
192 5, GM-CSF and IL-3 did not induce *APRIL* mRNA in human blood eosinophils, while they
193 were potent inducer of an oxidative burst in these cells (figure 3B). Bacterial products may also
194 activate eosinophils ²¹. Coculture of eosinophils with *E. Coli* potently induced *APRIL* mRNA
195 in these cells. We also detected intracellular APRIL in *E. Coli*-activated eosinophils (figure
196 3C). In this experiment, *Bifidobacterium bifidum* was also able to induce intracellular APRIL.
197 *In vivo*, Gut eosinophils are exposed to products from commensal bacteria. We observed that
198 detection of *APRIL* mRNA by RT-PCR in the lamina propria of ileum and right colon vanished
199 in germ-free mice (figure 4A). Furthermore, modulation of the commensal bacterial load post
200 antibiotics treatment significantly reduced *APRIL* gene transcription in ileum and right colon
201 in the lamina propria (figure 4B). We observed a similar reduction in mRNA with purified
202 eosinophils. Taken together, these show that bacterial products from the commensal bacteria
203 induce production of APRIL by eosinophils from the lower intestine.

204 The phenotype observed in mice genetically deficient for APRIL was impairment of IgA
205 switching ²². Several groups confirmed this role with recombinant APRIL, and showed that it
206 is a T-cell independent process ^{23,24,25,26}. A role for APRIL has also been observed in T-cell
207 dependent humoral responses ^{27,28}. The APRIL receptor involved in this switch process is the
208 transmembrane activator and CAML interactor ^{29,30}. Beside a role in IgA switching, APRIL
209 also has a role later on during B-cell differentiation at the level of PC survival. This second role
210 has been identified in the bone marrow and mucosa ^{13,14,31}. Identification of the first patient
211 deficient in APRIL largely confirmed all these preclinical studies ³². Our present study shows
212 that eosinophils and their production of APRIL are important for IgA⁺ PC homeostasis. This is
213 highly consistent with a report from Chu *et al.* showing that mice deficient for eosinophils
214 harbored reduction in gut IgA⁺ PCs ⁶. However, Beller *et al.* recently reported that microbiota
215 but not eosinophils is required for IgA production³³. In the latter study, IgA class switching

216 occurred due to the production of transforming growth factor β upon bacteria colonization of
217 the gut. One should note that quantification of IgA⁺ PC was performed quite shortly after
218 microbiota transfer (21 days), so that the study likely focused on PC generation rather than on
219 long-term survival as Chu et al. did. Regarding the cellular source of APRIL in the human gut,
220 we did not find evidences for APRIL production in epithelial cells from the lower intestine and
221 right colon. This is contradicting a report from He et al. ²³, despite the fact that the Stalk-1
222 antibody used here identified epithelial cells producing APRIL from several mucosa ^{13,34}. The
223 discrepancy might be due to different zones studied from the gut. We studied the right colon,
224 while He et al. did not precise the region of the human colon they analyzed. Regarding
225 eosinophils, we further found that their APRIL production is heterogeneous along the lower
226 intestine. Such heterogeneity is due to the fact that APRIL production by eosinophils is not
227 constitutive as shown by the negativity of blood eosinophils, but inducible depending on the
228 tissue the cells extravasate in, at least for the gut. We observed that *E. Coli* and *bifidobacterium*
229 *bifidum*, facultative and strictly, respectively, anaerobic strains well present in the lower
230 intestine, induced APRIL production in blood eosinophils. Hence, the spatial heterogeneity
231 observed may be explained by either the different bacteria composition along the lower intestine
232 or the higher bacterial density within the ileum that contains over three Log10 more cells than
233 the duodenum ^{35,36}. This observation is consistent with the fact that gut eosinophils change their
234 phenotype once extravasated in the gut as compared to their circulating blood counterpart ³⁷.
235 Most recent studies further indicated that they do so by responding to the gut microenvironment
236 including the microbiota ^{38,39}. The absence of APRIL in the duodenum is puzzling if one
237 considers that duodenum IgA⁺ PC may persist for decades ⁴⁰. Neverthelless, one should note that
238 duodenum constitutes a second organ known to sustain PC survival in which APRIL is absent
239 in addition to inflamed salivary glands from Sjögren syndrome ⁴¹. Further studies are warranted
240 to identify the PC survival factor(s) substituting to APRIL.

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343

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347 **Authors’ contribution**

348 NS, BH, PL, MG, MRM, FP performed experiments and analyzed data. BH designed the
349 study and wrote the article

350

351 **Figure legends**

352

353 **Figure 1: site-specific production of APRIL by eosinophils from the lower intestine.**

354 A) Duodenum (duo.), ileum (ile.) and right colon (col.) of a human gut were stained for APRIL-
355 producing cells (APRIL p., left upper panel). Arrows indicate cells shown in inserts. A
356 quantification with mean +/- SD of APRIL-p.⁺ cells per surface is shown (right upper panel).
357 Costaining for APRIL-p. (green) and major basic protein (MBP, red) is shown for the three
358 zones (bottom panel). The merged staining includes nuclear DAPI staining. B) Duodenum and
359 ileum were stained for secreted APRIL (APRIL s.) and APRIL p. Right and left inserts show
360 an eosinophil with intracellular APRIL s. reactivity and a CD138⁺ (green) PC with secreted
361 APRIL (red) bound onto its surface, respectively. C) Serial sections of a right colon were
362 stained for IgA and APRIL p. (upper panel). The same small intestine was costained for CD138
363 and IgA (bottom left panel). A quantification is shown for IgA⁺ CD138⁺ PCs (bottom right
364 panel). Ten healthy intestines were tested, and stainings were performed twice. For A, a one-
365 way Anova test was performed. ***: p<0,001, ****: p<0,0001.

366

367 **Figure 2: site-specific dependency on APRIL for mouse IgA⁺ plasma-cell homeostasis.**

368 A) Lamina propria from the indicated parts of the mouse lower intestine was analyzed for
369 *APRIL* mRNA expression (left panel). Samples from two mice were used. Purified eosinophils
370 were also analyzed (right panel). Results are representative of three independent experiments.
371 B) Samples from WT and *APRIL*^{-/-} mice were stained for IgA. Representative images are shown
372 (upper panel). Costaining for CD138 and IgA is also shown (bottom left panel). Arrows
373 indicated excluded cells from the quantification of IgA⁺ CD138⁺ cells with mean +/- SD per
374 surface shown in bottom right panel. Ten mouse healthy intestines were studied. Experiment
375 was performed twice. A two-way Anova test was performed. *: p<0,05, ***: p<0,001.

376 **Figure 3: bacterial products induce APRIL production by human blood eosinophils.**

377 A) The gating used to analyze eosinophils and neutrophils is shown (upper left panel).
378 Intracellular staining for APRIL (thick line) and a control Ig (thin line) analyzed by flow
379 cytometry is shown (upper middle panel). Results are representative of five independent healthy
380 subjects tested once. *APRIL* mRNA expression in purified eosinophils and neutrophils from
381 human and mouse blood (upper right panel). Results are representative of three independent
382 healthy subjects tested once. B) *APRIL* mRNA analysis by qRT-PCR (left panel) and oxidative
383 burst (middle panel) for purified eosinophils stimulated with the indicated reagents for 18hr are
384 shown. A value of 1 was arbitrarily assigned to untreated eosinophils. A Dunn's multiple
385 comparison analysis was performed. Results are representative of ten independent healthy
386 subjects tested once. C) Intracellular staining of APRIL by flow cytometry is shown for
387 untreated and eosinophils stimulated by the indicated bacteria (left panel). Mean fluorescence
388 intensity (MFI) with mean +/- SD is shown (right panel). A Kruskal-Vallis test was performed.
389 Results are representative of five independent healthy subjects tested once. *: $p < 0,05$; **: $p < 0,01$;
390 ***: $p < 0,001$; ****: $p < 0,0001$.

391

392 **Figure 4: microbiota-dependent regulation of APRIL-production by mouse eosinophils**

393 A) Same analysis of *muAPRIL* mRNA as in figure 2A except that germ-free mice were used.
394 Two mice were studied. B) *MuAPRIL* mRNA analysis by qRT-PCR in the indicated parts of
395 the lower intestine from control-treated (Ctrl.) and antibiotics-treated (Tx.) mice (left panel).
396 Experiment was performed once on ten mice. A Tukey's multiple comparison test was
397 performed to compare all samples. Comparison between control and Tx. mice per tissue was
398 performed with a parametric unpaired t-test. *MuAPRIL* mRNA analysis by qRT-PCR of
399 purified lamina propria eosinophils from ileum and right colon of control-treated and

400 antibiotics-treated mice (right panel). Experiment was performed once on five mice. A two-
401 way Anova test was performed. *: $p < 0,05$; **: $p < 0,01$; ****: $p < 0,0001$.

402

Figure 1

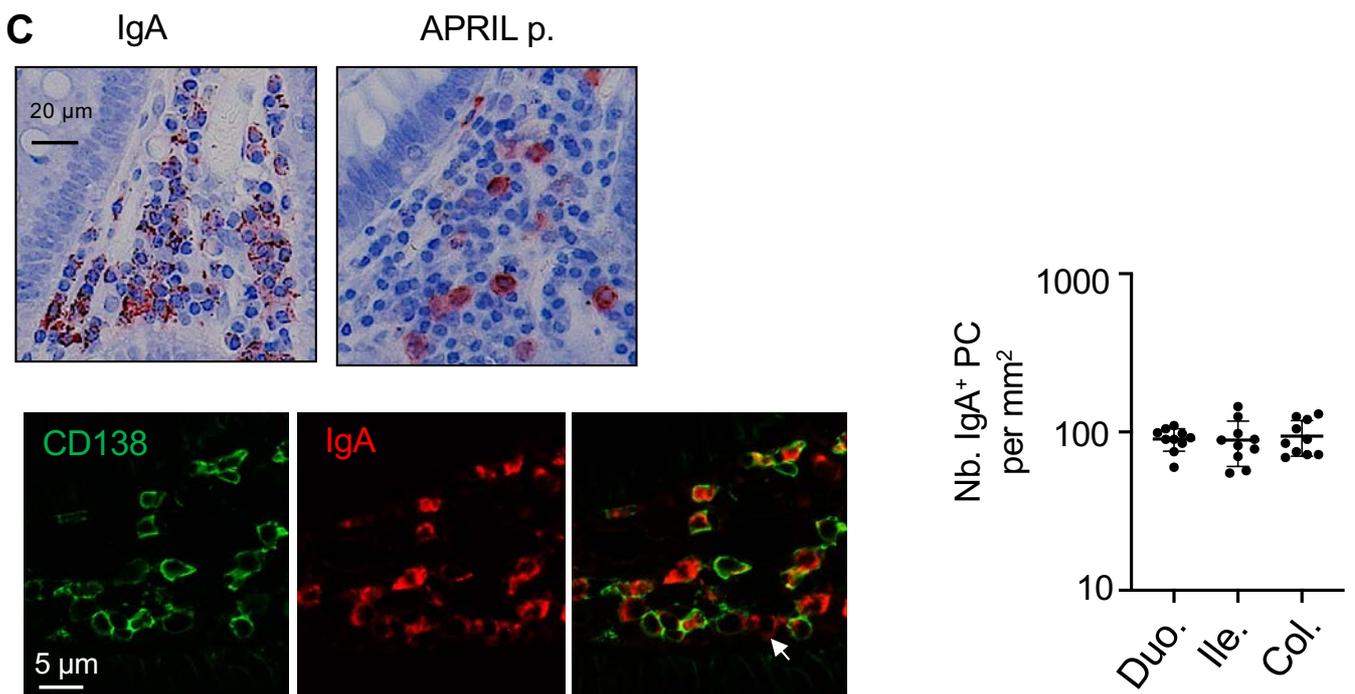
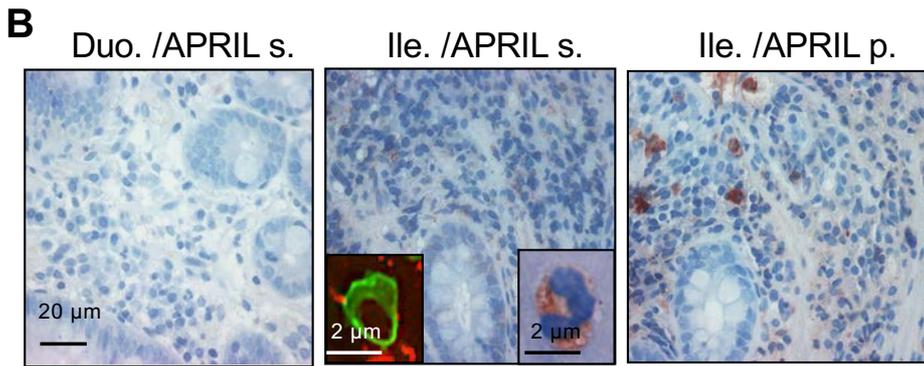
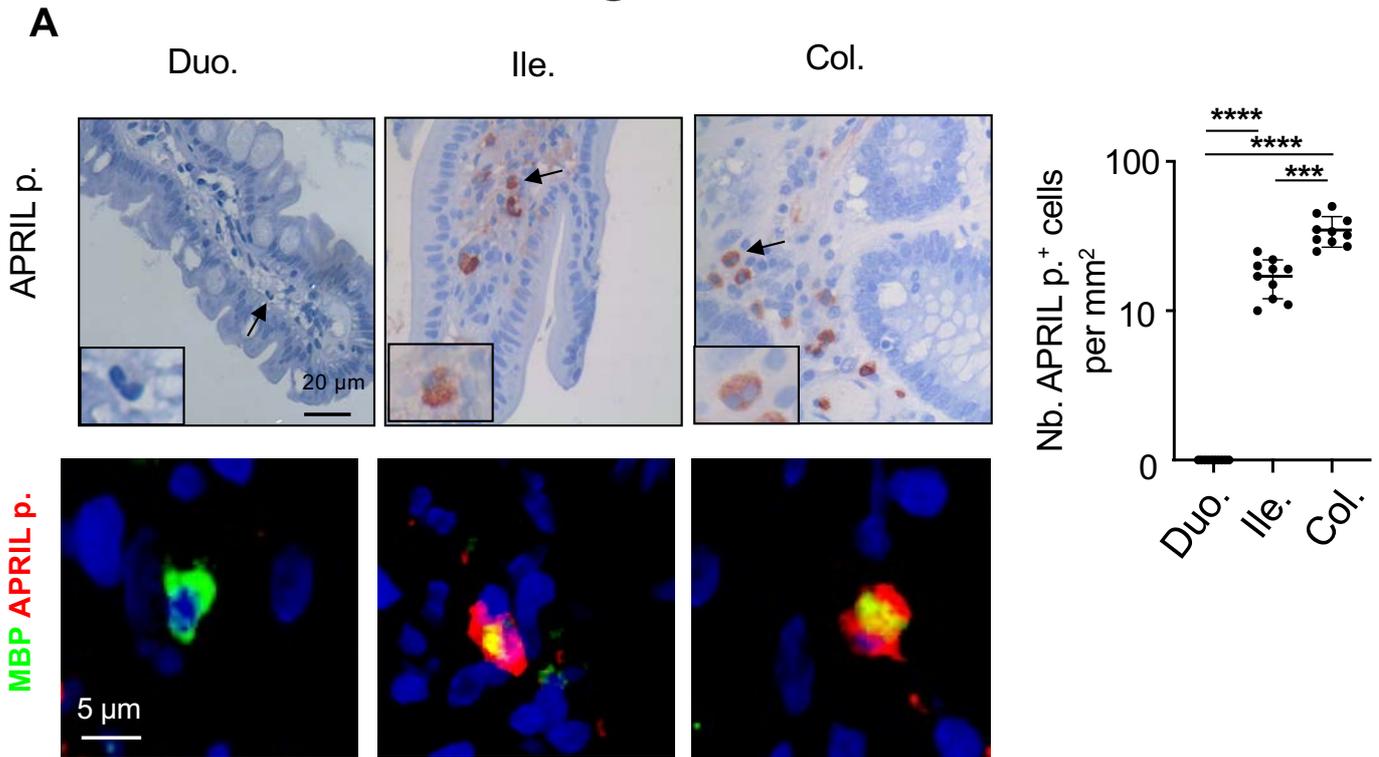


Figure 2

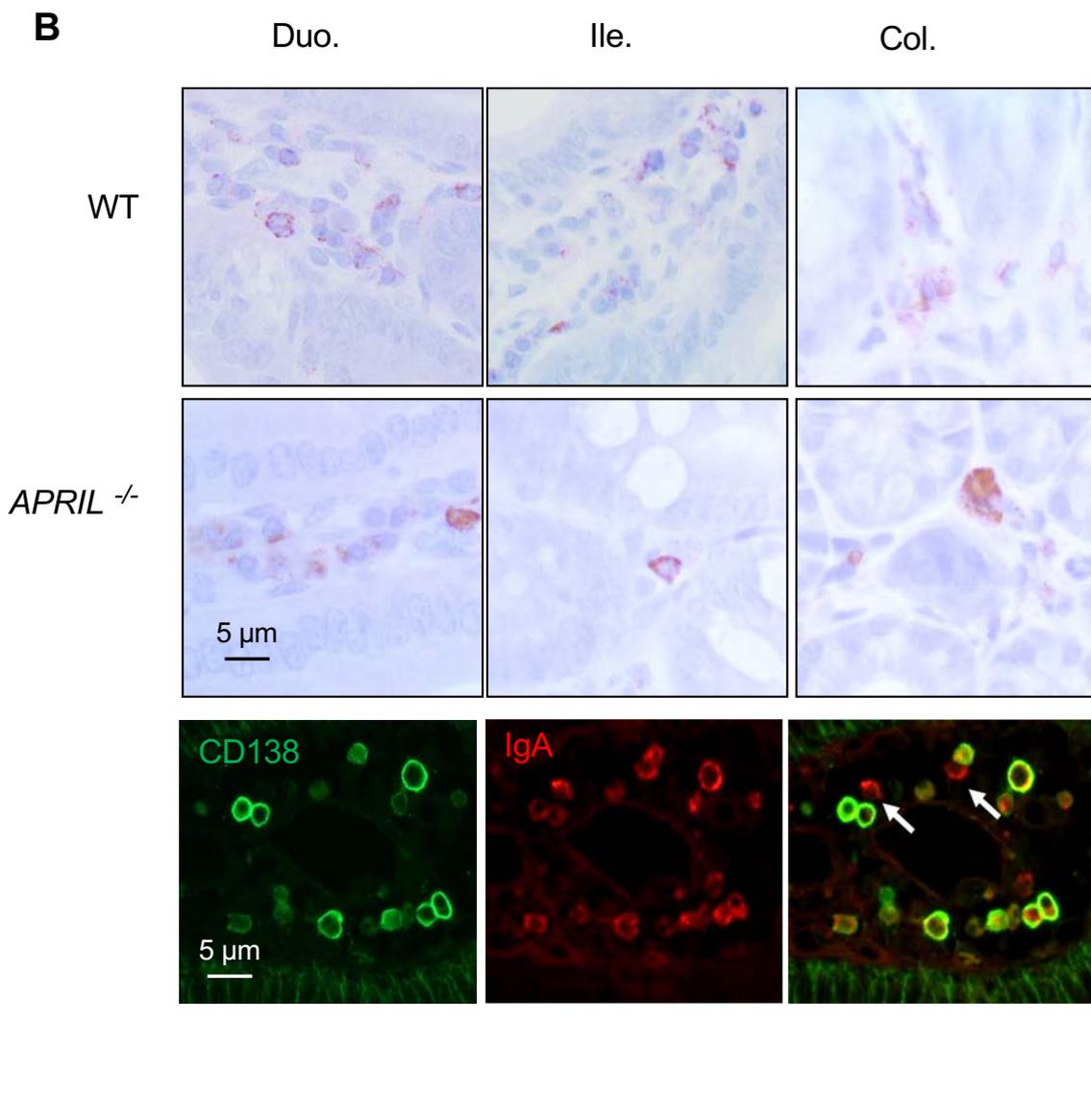
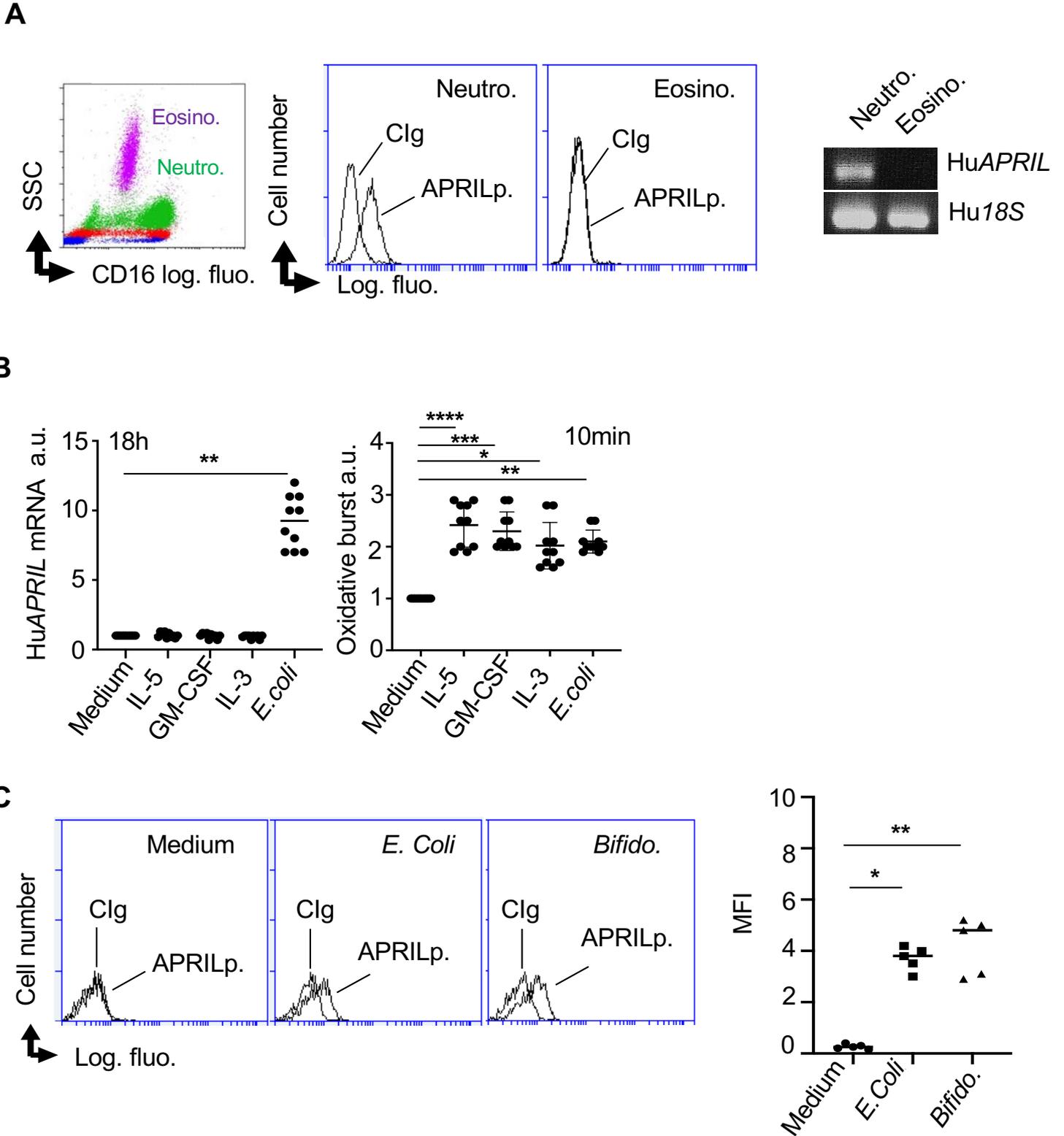


Figure 3



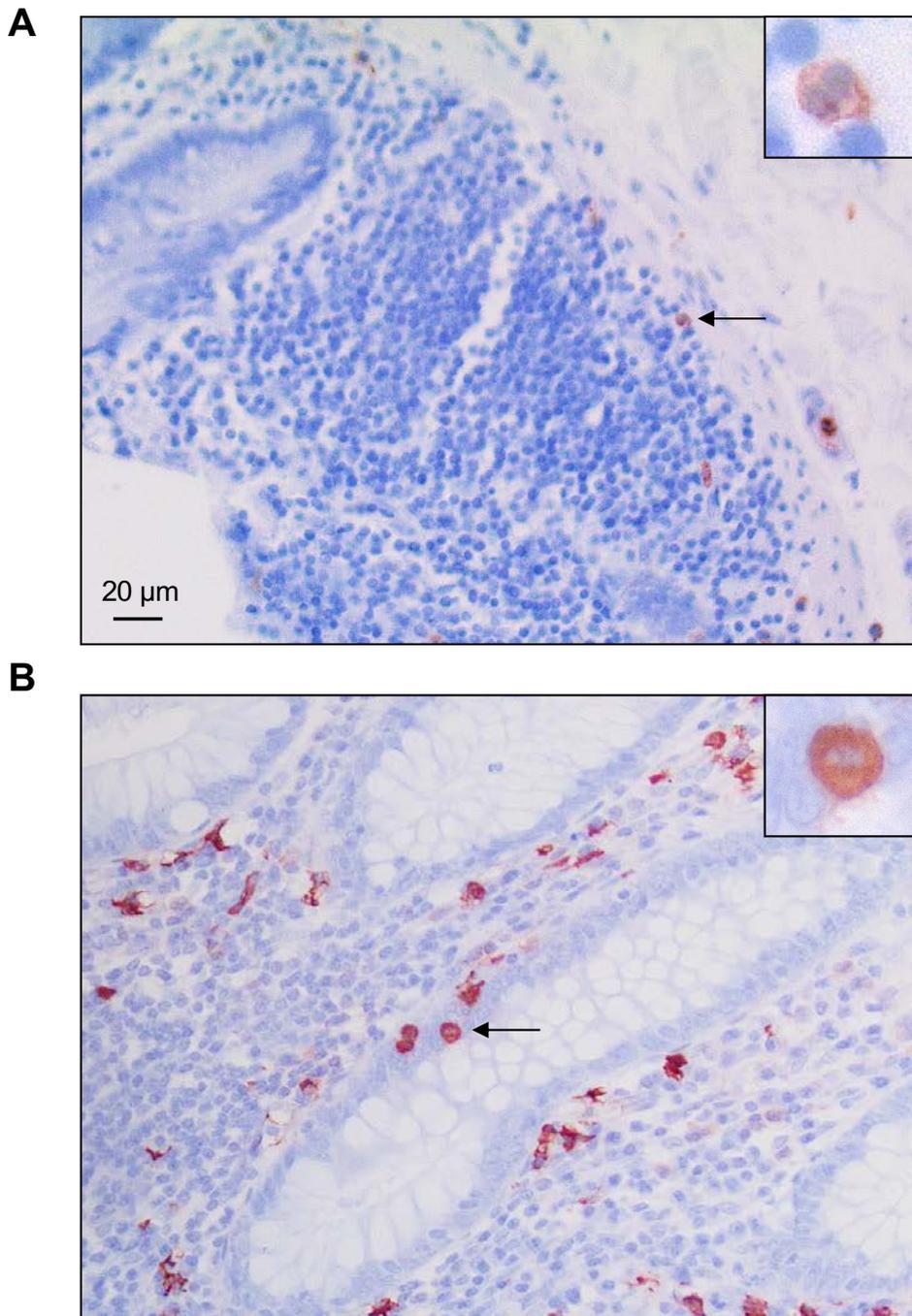


Figure 1sup: localization of APRIL-producing eosinophils in the intestine

A) Stained eosinophils are located outside the Peyer's patches present in the ileum.

B) B) A stained eosinophil has infiltrated the epithelium in the right colon. Scale bars are shown. Inserts are high magnifications of arrowed stained cells.