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Title:

Histopathologic Features of Inflammatory Bowel Disease are Associated with Different CD4$^+$ T Cell Subsets in Colonic Mucosal Lamina Propria

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ABSTRACT

Background: Inflammatory bowel disease (IBD) results particularly from aberrance of $CD4^+$ helper and regulatory T cells and is histopathologically chronic active enterocolitis with features reflecting both activity and chronicity of mucosal inflammation. The exact immunologic-histologic correlation is not understood.

Methods: We studied the correlation between colonic mucosal $CD4^+$ T cell subsets (Th1, Th2, Th17, Th22, and Treg) and mucosal histologic changes in ulcerative colitis (UC) and Crohn's disease (CD). $CD4^+$ T cell subtyping and enumeration were achieved by flow cytometry. Histologic features were categorized and semi-quantitated using three validated histological scoring schemes (ECAP, RHI, and D'Haens). Correlations between prevalence (%) of $CD4^+$ T cell subsets and histologic scores were analyzed.

Results: Treg cells were correlated with ECAP category A (activity) as well as RHI scores. Treg was particularly increased in mucosa with severe neutrophilic infiltration in cryptal/surface epithelium and in lamina propria, and with basal plasmacytosis. Th17 cells were also increased in cases with extensive neutrophil infiltrate in lamina propria, whereas $RORc^+$ cells were increased in cases with severe lymphoplasmacytic infiltration in lamina propria. In both UC and CD, the mucosa with marked crypt architectural alteration had increased $IL\text{-}22^+$ and Th22 cells. UC with Paneth cell metaplasia had higher Th17 cells. CD with granuloma had increased $IL\text{-}22^+$ and $IL\text{-}22^+IFN\text{-}\gamma^+$ cells.

Conclusions: Treg appears to be associated with the overall severity of IBD histopathology, particularly with active inflammation. Th17 is also associated with activity. Whereas $IL\text{-}22^+$ cells are associated with chronicity and granuloma formation in CD.

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1. Introduction

The histopathology of inflammatory bowel disease (IBD), ulcerative colitis (UC) and Crohn's disease (CD), is a chronic active inflammation of intestinal mucosa (enterocolitis), with shared and distinctive features. From the biologic perspective and for the purpose of practical clinical-histopathological assessment, the various histologic changes in IBD are broadly categorized into '*activity*' and '*chronicity*'. *Activity* is characterized by active/acute inflammation, *i.e.*, neutrophil infiltration, of the glandular/cryptal epithelium (cryptitis and crypt abscess) and surface epithelium (erosion and ulceration), usually causing destructive damage of the epithelium, along with mixed neutrophilic and lymphoplasmacytic (mononuclear cell) infiltration in lamina propria with the latter one tending to persist (and thus regarded as chronic inflammation). *Chronicity* is primarily the subsequent histoarchitectural and cytologic alterations of glands secondary to epithelial injury following a prolonged and repetitive active and chronic inflammation, and it is featured mainly by the cryptal architectural alteration/distortion (including disorganized arrangement, variable shape and size, shortening/dropout, and branching of crypts), along with villiform configuration of the surface epithelium as well as Paneth cell metaplasia (in left colon) and/or Paneth cell hyperplasia (in right colon and/or terminal ileum). None of these features is pathognomonic *per se* for IBD though.

The key pathogenetic mechanism of IBD is the aberrant immunity of bowel mucosa, including both innate immunity and adaptive immunity, with involvement of gut microbiota and defects in gut barrier. The aberrant adaptive immunity is primarily T cell mediated, with fundamental defects of the $CD4^+$ helper T (Th) cells and regulatory T (Treg) cells. Treg cells and different Th cell subsets, including Th1, Th2, Th17, Th22, defined by their production of

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lineage-specific cytokines and functions, play a discriminatory role in translating certain antigen-specific immune response into different tissue functions or pathology. In general, Th1 and Th2 are primarily pro-inflammatory effectors, Treg helps to keep balance between inflammation and tolerance,[1,2] Th17 has both pro-inflammatory and protective effects, whereas Th22 plays a role in epithelial regeneration and repairing.[3] All of these $CD4^+$ T cells also interplay with each other.

It is reasonable to believe that the different histologic patterns of the mucosal inflammation and associated histomorphologic alterations of glandular epithelium in IBD may be associated with different immunologic mechanisms that involve different subsets/patterns of immune cells and cytokines. However, the exact histologic-immunologic correlation in IBD is not understood yet and has not been specifically studied. This study aims to explore the association between the key histopathological features of the activity and chronicity in IBD and the prevalence of $CD4^+$ T cell subsets in lamina propria, which would help us further understand the pathologic basis of IBD and possibly appreciate the pattern of histologic improvement in response to different targeted therapies in future studies. Such a study needs histopathological scoring that reflects both activity and chronicity as well as lamina propria T lymphocyte immunophenotyping.

2. Materials and Methods

2.1. Patient Cohort and Colonic Mucosal Tissue Samples

53 patients with IBD (27 UC, 26 CD) were enrolled into the study from the University of Calgary IBD Clinic. For CD cases, only those with colonic involvement (*i.e.*, Crohn's colitis or

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ileocolitis) were included and only the colonic mucosa tissues were studied, considering that there are differences between ileal and colonic mucosal immunity. The diagnoses and classification of UC or CD were made by specialized IBD clinicians (SG, MI) based upon the overall clinical, radiographic, endoscopic and histological findings, according to widely accepted criteria.[4]

The patients' data were extracted from their medical records and included demographic characteristics, extent of disease, endoscopic features, clinical and endoscopic assessments for the disease activity, and medication use (Table 1).

The clinical disease activities of the patients' symptoms were assessed using the total Mayo score for UC[5] and Harvey-Bradshaw Index (HBI) for CD.[6] UC patients were categorized by the total Mayo score into 3 groups: remission (score 0 to 2), mild (score 3 to 5), and moderate to severe (score ≥ 6). CD patients were categorized by HBI into 3 groups: remission (score 0 to 4), moderate (score 5 to 8), and severe (score ≥ 9). Furthermore, the endoscopic activity of UC and CD was assessed using Mayo endoscopic subscore[5] and Simple Endoscopic Score for CD (SES-CD),[7] respectively (assessed by MI). UC patients was categorized by Mayo endoscopic sub-score into 3 groups: remission/mild (score 0 and 1), moderate (score 2), and severe (score 3), while CD patients were categorized by SES-CD into 4 groups: remission (score 0 to 2), mild (score 3 to 6), moderate (score 7 to 16), and severe (score ≥ 17). The extent of disease involvement was assessed using the Montreal classification.[8] UC patients were classified into 3 groups as proctitis (E1), left-sided colitis (E2), and pancolitis (E3). CD patients were categorized into 2 groups as colitis (L2) and ileo-colonic CD (L3).

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This study has been approved by the Conjoint Health Research Ethics Board of the University of Calgary (Control ID: REB14-2429). All enrolled patients were consented.

2.2. Histopathological Assessment

Colonic mucosal biopsies were taken from the areas with visibly inflamed mucosa on endoscopy (for the majority of patients) or taken randomly when there was no visible inflammation, where the endoscopic scoring of disease activity was made. All endoscopic scoring and biopsies were taken by an expert IBD endoscopist (MI). The histopathologic changes seen in the biopsies were carefully evaluated on routine Hematoxylin and Eosin (H&E) stained slides, with all features being categorized and semi-quantitated independently by a gastrointestinal pathologist (XG), who was blinded to the clinical and endoscopic findings, using a newly developed (by XG) comprehensive histological scoring system built based on several published schemes[9-14] with modification, which reflects all common histologic features of both active and chronic inflammation seen in IBD. The grading/scoring scheme, coined **ECAP** (***E****xtent*, ***C****hronicity*, ***A****ctivity* and ***P****lus*) system,[15-17] covers the following aspects of IBD pathology: (1) ***E****xtent* (E) of mucosal inflammation (focal, multifocal, or diffuse), (2) ***C****hronicity* (C): crypt architectural alteration and Paneth cell metaplasia or hyperplasia, (3) ***A****ctivity* (A) of colitis: degree of neutrophilic inflammation of cryptal/surface epithelium as well as lymphoplasmacytic and neutrophilic infiltration of lamina propria, and (4) ***P****lus* several other common but less understood and unclassified findings (P) including lamina propria eosinophilic infiltration, lymphoid follicles/aggregates, and granuloma (for CD patients only). The scheme for UC and CD are slightly different only on the assessment of disease extent (E). The details of the ECAP

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system are shown in Table 2, including the specific criteria for inclusion into each of these grades. Examples of the key histologic features are displayed in Figures 1.

The ECAP system has been successfully used by us in studies involving assessment of UC disease activity and severity, in which the ECAP scores showed nice concordance with high-definition endoscopic findings and sensitivity in detecting minor histologic abnormalities in apparently normal mucosa.[15-17] A preliminary validation was recently conducted on a separate group of 20 colonic biopsies of UC by three experienced gastrointestinal pathologists in different countries and showed a very good inter-observer agreement, with the intraclass correlation coefficients (ICC) being 0.8889 (95% CI 0.7621-0.9481) for the total ECAP scores. (unpublished data, please refer to Acknowledgments section.)

As a comparison and further confirmation, two other well-known and widely accepted histological grading systems for IBD were also employed in parallel with ECAP scoring. For UC, the Robarts Histologic Index (RHI), another recently proposed and validated UC activity scoring scheme,[18] was also determined, which can actually be easily derived from the ECAP-category A scores (parameters of activity) in our ECAP scoring results [*by using the formula*: **RHI = (A_1-1)** *(when A_1 >1)* **or A_1** *(when A_1=1)* **x 5 + A_2 x 3 + A_5 + A_7 x 2)**]. For CD patients, the D`Haens score [19] was also obtained.

2.3. Immunologic Profiling of $CD4^+$ T Lymphocyte Subsets in Colonic Lamina Propria

The profiling and enumeration of the subsets of $CD4^+$ T lymphocytes in colonic mucosa were achieved by employing multicolor flow cytometry. Using the fresh tissue samples of colonic

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mucosa that were half splatted from the same biopsies (taken from the same area and at the same time in the same colonoscopy procedure) that were sent to pathology laboratory for histological evaluation, the lamina propria mononuclear cells (LPMNCs) were isolated as previously described.[20] Briefly, the epithelial cells were removed by agitated incubations with Ca^{++} and Mg^{++} free Hanks' balanced salt solution (HBSS, Life Technologies Inc, Burlington, ON, Canada) containing 1 mM EDTA (Sigma-Aldrich, Burlington, ON, Canada) at 37℃. Then, the tissues were incubated in RPMI1640 cell culture media with L-glutamine (Life Technologies) containing 2.5% fetal bovine serum (Life Technologies), 1 mM dithiothreitol (Life Technologies), 100U/mL penicillin and 100µg/ml streptomycin (Life Technologies), and later incubated in the culture media containing 100IU/mL collagenase type IV (Life Technologies) for 1 hour before mechanical dissociation using GentleMACS Dissociator (Miltenyi Biotec Inc, San Diego, CA). In the end, LPMNCs were collected after passing through a 40 µm cell strainer for flow cytometric analysis.

The isolated LPMNCs (500,000 cells) were cultured in the presence of phorbol-12-myristate-acetate (PMA, 5 µg/mL, Sigma-Aldrich) and ionomycin (5 nM, Sigma-Aldrich) for 1 hour followed by the addition of monensin (GolgiStop; BD Biosciences, Mississauga, ON, Canada) for another 16 hours. The harvested cells were incubated with LIVE/DEAD Fixable Dead Cell Stain Dye (Life Technologies) and fluorochrome-conjugated antibodies against the following surface markers: CD3 (clone OKT3, eBioscience, San Diego, CA, USA), CD4 (clone SK3, BD Biosciences), CD8 (clone RPA-T8, BD Biosciences), and CD25 (clone M-A251, BD Biosciences). After washing with PBS, cells were fixed and permeabilized using a Foxp3 staining kit (eBioscience) according to the manufacturer's instructions, followed by staining with

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fluorochrome-conjugated antibodies against the following intracellular components: IFN-γ (clone 4S.B3, eBiosciences), IL-13 (clone JES10-5A2, BD Biosciences), IL-17A (clone N49-653, BD Biosciences), IL-22 (clone 22URTI, eBioscience), T-bet (clone eBio4B10, eBioscience), Gata3 (clone TWAJ, eBioscience), RORc (clone AFKJS-9, eBioscience), FoxP3 (clone 259D/C7, BD Biosciences) and isotype controls. The different **distinct CD4$^+$ T cell subsets were defined by** their expression of specific cytokines and master transcription factors: (1) IFNγ$^+$ or T-bet$^+$ for **Th1**, (2) IL-13$^+$ or Gata3$^+$ for **Th2**, (3) IL-17A$^+$ or RORc$^+$ for **Th17**, (4) a combination of IL-22$^+$/ IFN-γ$^-$ / IL-17A$^-$ for **Th22**, and (5) CD25$^+$ / Foxp3$^+$ for **Treg**.

A LSR II flow cytometer (BD Biosciences) was used to acquire samples and post-acquisition analysis was performed using FlowJo software (TreeStar, Inc., Ashland, OR) using a gating strategy with doublet and dead cell discriminations as previously described[20] and exampled in Figure 2. The flow cytometry data were initially treated blindly from all clinical-endoscopic-histologic diagnoses.

2.4. Statistical Analysis

Continuous variables are shown as the mean (±SD). The probability of significant difference between two groups was calculated using Mann-Whitney test and chi−squared test for continuous and categorical variables, respectively. Statistical significances of ECAP total scores and subcategory scores were determined by performing ANOVA analysis and Bonferroni comparisons test and Kruskall-Wallis test for post-tests comparing each severity group. Spearman's rank order correlation coefficients (Spearman's rho) and the *p* values were

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calculated to compare likelihoods of ECAP score and T helper cell subsets. Data were collected in a Microsoft Excel database (Microsoft Excel 2010; Microsoft Corp., Seattle, WA, USA) and statistically analyzed using SPSS software for Windows, release 19.0 (SPSS Inc., Chicago, IL, USA) and Graphpad Prism 5.0 (Graphpad Inc., La Jolla, CA, USA). Significance was defined as a two-sided *p* value < 0.05.

3. Results

3.1. Correlation between ECAP and RHI and between ECAP and D'Haens in Histological Scoring

In the same group of UC cases, an excellent in-parallel correlation was shown between the total ECAP score and RHI score ($\gamma = 0.893$, $p < 0.001$) as well as between ECAP-category A score and RHI score ($\gamma = 0.955$, $p < 0.001$) (Figure 3). ECAP-category C score was not compared with RHI score, since the latter represents only the activity.

In the same group of CD patients, an equally excellent correlation was demonstrated between the total ECAP score and D'Haens score ($\gamma = 0.947$, $p < 0.001$) as well as between ECAP-category A score and D'Haens score ($\gamma = 0.914$, $p < 0.001$) and between ECAP-category C score and D'Haens score ($\gamma = 0.519$, $p = 0.007$) (Figure 3).

3.2. Association between Histological and Clinical-Endoscopic Assessments of Disease Activity

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Based on the total Mayo clinical disease activity scores (which includes endoscopy), UC cases were categorized into 3 groups for further analysis. As compared with those in remission, the cases with moderate to severe activity showed significantly higher total ECAP scores ($p < 0.05$) and category A scores ($p < 0.01$).

Similar relationship also existed between the ECAP scores and the Mayo endoscopic subscores, the patients with Mayo endoscopic score 3 group had significantly higher total ECAP scores ($p < 0.05$) as well as higher category A scores ($p < 0.01$) in their colonic biopsies, as compared with those in Mayo endoscopic score 0-1 group.

However, there was no significant correlation between the clinical or endoscopic severity of disease activity and the ECAP category C and category P scores in UC. (Data not shown). This suggests that the commonly used total Mayo score and Mayo endoscopic subscore reflect essentially the histologic activity but *not* the chronicity of UC.

In CD patients, there was no significant correlation between total ECAP or any subcategory scores and either Harvey-Bradshaw disease activity index or SES-CD scores. In agreement with the ECAP scoring, CD D'Haens histology scores also failed to show correlation with the clinical and endoscopic scores in the same cases.

3.3. Association between Histologic Changes and Prevalence of Specific $CD4^+$ T Cell Subsets

By analyzing the correlation between the ECAP scores and the prevalence/proportion of certain $CD4^+$ T lymphocyte subsets in lamina propria, including Th1, Th2, Th17, Th22, and Treg cells, a

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number of histologic-immunologic correlations were found to be significant. The different patterns of histopathology appeared to be correlated with different subsets of $CD4^+$ T cells.

First, *Treg cells were associated with the active (i.e., neutrophilic) inflammation and the overall severity of histopathology*. When looking at the total ECAP scores, as shown in Figure 4 (A, B, D, E), the proportion of Treg cells (*i.e.*, $CD25^+FoxP3^+CD4^+$ T cells), but not any other T cell subsets, was weakly correlated with total ECAP scores in both UC ($\gamma = 0.281$, $p < 0.01$) and CD patients ($\gamma = 0.274$, $p < 0.01$). Similarly, the prevalence of Treg cells was also significantly correlated with RHI score in UC patients ($\gamma = 0.562$, $p = 0.003$), and with D'Haens score in CD cases ($\gamma = 0.501$, $p = 0.019$). These suggest a positive correlation between the prevalence of Treg cells and the overall severity of histologic abnormalities.

When exploring the ECAP subcategory scores, the overall category A score and the proportion of Treg cells was correlated in both UC ($\gamma = 0.276$, $p < 0.01$) and CD patients ($\gamma = 0.223$, $p < 0.05$), as shown in Figure 4 (C and F). More specifically, the correlation was particularly seen between Treg and several elements of category A (activity), as shown in Figure 6. In UC, the proportion of Treg cells was particularly increased in the mucosa with more severe neutrophilic cryptitis (A2), crypt abscess (A3), and crypt destruction (A4), neutrophil infiltrate in lamina propria (A7), and with basal plasmacytosis (A6) ($p < 0.05$ for each). In CD, the prevalence of Treg was increased in mucosa with neutrophil infiltration in surface epithelium (A1) as compared with those with non-inflamed surface epithelium ($p < 0.05$). All of the results indicate that the positive correlation between Treg and the disease severity primarily lies in the association of Treg with active inflammation (as also showed with RHI).

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Second, *Th17 cells (IL-17A$^+$ or RORc$^+$ CD4$^+$ T cells) were also associated with histologic activity and chronicity*. In UC, the proportion of RORc$^+$CD4$^+$ T cells was increased in cases with more severe lymphoplasmacytic cell infiltration of lamina propria (A5) ($p < 0.05$). Moreover, the mucosa with Paneth cell metaplasia (C2) had a higher prevalence of IL-17A$^+$CD4$^+$ T cells as compared with the cases without Paneth cell metaplasia ($p < 0.05$), as shown in Figure 7.

Third, *IL-22$^+$CD4$^+$ T cells and Th22 cells were associated with the histologic features of chronicity and the presence of granuloma in CD.* As shown in Figure 8, in UC patients the mucosa with diffuse crypt architectural alteration (C1) had significantly increased proportion of IL-22$^+$CD4$^+$ T cells as well as Th22 (IL-22$^+$IFN-γ$^-$IL-17A$^-$) cells, as compared with those with only focal crypt architectural alteration ($p < 0.05$, respectively). In CD patients the mucosa with diffuse crypt architectural alteration also had increased proportion of Th22 cells in lamina propria, as compared with those with only focal alteration ($p < 0.05$). Interestingly, CD cases with presence of epithelioid granuloma in colonic mucosa (P3) had significantly increased percentage of IL-22$^+$CD4$^+$ T cells as well as IL-22$^+$IFN-γ$^+$CD4$^+$ T cells (a type of crossover CD4$^+$ T cells as result of plasticity), as compared with those with absence of granuloma ($p < 0.01$ for both).

4. Discussion

In the immunopathology of IBD, one of the key mechanisms is the imbalance of CD4$^+$ T cells that include primarily the IFN-γ-expressing Th1 cells, IL-4-secreting Th2 cells, Th17 cells that express IL-17A, IL-17F, and IL-22, and Th22 cells that express IL-22 but no IL-17A/F or IFN-γ. Some fundamental differences exist between UC and CD with regard to their characteristic

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different patterns of cytokines and subsets of $CD4^+$ T cells. UC has been defined as Th2-type disease and CD as Th1-like, based on their different abundance of Th1- or Th2-associated effector cytokines (predominantly IFN- γ and IL-12 for Th1, IL-4, IL-5, and IL-13 for Th2) in gut mucosa, as shown by us and other groups.[20,22-24] Additionally, Th17 and Foxp3-expressing Treg cells, as well as IL-17A/Foxp3 double expressing $CD4^+$ T cells, are also enriched in the lamina propria of IBD patients.[1,21,25,26] Th22, defined as IL-22 $^+$IFN-γ^-IL-$17A^-$ $CD4^+$ T cells, was found to be depleted in gut mucosa of severe UC patients.[27] Certain $CD4^+$ T cell subsets are associated with the disease activity in IBD. Our own previous studies demonstrated that Th1 cells in bowel mucosa and Treg cells in peripheral blood were positively correlated with the IBD disease activity assessed by clinical and endoscopic scores.[18,26] Smids *et al* has revealed that the intestinal T-cell infiltrate associated with active IBD is composed of increased proportion of $CD4^+$ cells, Tregs and central memory T cells (TCM).[28] Our present study specifically and systemically correlates the histopathologic features of IBD with the lamina propria $CD4^+$ T cell subsets, and we had some interesting observations regarding the histologic-immunologic association. Among the $CD4^+$ T cells in mucosal lamina propria the Treg cells appeared to be positively associated with the increasing severity of active inflammation (*i.e.*, neutrophilic inflammation) in both UC and CD patients. Th22 and Th17 cells appeared to be associated with the features of chronicity, with Th22 correlating with crypt architecture alteration and Th17 with Paneth cell metaplasia/hyperplasia. Furthermore, $IL\text{-}22^+$ Th cells seemed to be associated with the occurrence of epithelioid granuloma in CD patients.

Our novel histological scoring scheme, ECAP system, was designed to fully reflect almost every component of the histopathology of IBD, including both active inflammation and chronic histocytologic changes. This approach has been proven in several of our studies to be concordant

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with the finest endoscopic technology in assessment of the mucosal inflammation and mucosal healing in UC [15-17]. Although it has not been widely validated, a preliminary validation process has shown an excellent inter-observer agreement. The parameters included in the category A contain all elements of the recently proposed and validated Robarts Histologic Index (RHI),[18] and can be easily converted to RHI. Our ECAP total scores and subcategory A scores were found to be in excellent correlation with RHI, as shown in this and the other studies.[17] Additionally, the ECAP scores, including the subcategory A and C scores, are in parallel to the D'Haens score in our study. The reliability and usefulness of our ECAP system was again proven. Admittedly, for CD cases the ECAP scores failed to show good concordance with the currently used clinical and endoscopic scoring schemes, as did the D'Haens score. The reasons may include the characteristic patchiness and focal and transmural nature of inflammation in CD, and the currently used HBI and SES-CD for assessment of CD may not be sensitive or reliable by the histological standard. Previous studies, using several different histological indices, also failed to demonstrate a histologic correlation with clinically or endoscopically assessed disease activity in CD.[29-31] Nevertheless, we believe that the ECAP system still provides a reasonable method to semi-quantitate the major changes and the degree of mucosal inflammation in CD at the histologic level.

Treg cells are crucial for the homeostasis of normal gut mucosal immunity. In active UC and CD, they are abundantly recruited to lamina propria and, in contrast, depleted in peripheral circulation. The pivotal role of Treg in the control of pro-inflammatory effects of effector T cells was found to be maintained in the setting of IBD, but meanwhile their protective function is compromised due to the gain of pro-inflammatory effect in IL-17$^+$ FoxP3$^+$ CD4$^+$ T cells under certain cytokine environment,[32] as it is well known that Treg cells under certain inflammatory conditions can lose suppressive function and acquire pathogenicity.[33] We previously demonstrated

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the abundance of the crossover population of Th17/Treg in peripheral or lamina propria compartments in IBD patients.[21] Others found that the abundance of Treg cells in the mucosa with active UC was consistent with the disease activity assessed clinically and endoscopically and it could be modulated by the immunosuppressive medications and biologic agents.[1,34, 35, 36]) In this study, we further demonstrated that the Treg cells were closely associated with the overall histological abnormalities in IBD, primarily the active inflammation (*i.e.*, neutrophil infiltration in glandular epithelium and lamina propria). Our study was not large enough to explore differences due to different drugs and this will require longitudinal studies which are ongoing.

Th17 cells are mainly responsible for IL-17A production, which is known to exert dual effects in IBD, as a pro-inflammatory cytokine as well as a protective factor in the mucosal healing. Th17 and its cytokines IL-17A, IL-21, IL-22 were found increased in the involved gut mucosa in active IBD, and their increase were correlated with the disease activity assessed clinical-endoscopically.[37] Moreover, T17 plasticity, particularly along the Th1 - Th17 and Th17 - Treg axes, plays an important role in the pathogenesis of IBD. [38] In the current study, Th17 cells were found to be associated with lymphoplasmacytic infiltration in lamina propria in UC, one of the key histopathologic features of activity. This result demonstrates again the pro-inflammatory nature of Th17 cells, which is usually restrained by $RORc^{+}FoxP3^{+}$ regulatory T cells (iTreg) in the state of host defense, as revealed in animal studies.[39] The concurrent increase of both $IL\text{-}17A^{+}CD4^{+}$ T cells and $RORc^{+}CD4^{+}$ T cells and their equal correlation with active inflammation, as seen in our results, are likely indicative of their changes toward a new balance between the two at a higher level.

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Among Th subsets, Th1, Th17, and Th22 all produce IL-22, and subsets of Th cells co-expressing IL-22/IFN-γ (as seen in our observation) and/or IL-22/IL-17 exist. Studies found depletion of IL-22$^+$CD4$^+$ T cells in colonic lamina propria in UC.[27] We have also reported previously that the IL-22$^+$CD4$^+$ T cells and Th22 cells decreased in UC.[20] IL-22 also plays a crucial role in maintaining the epithelium integrity of mucosa and skin, while participating in inflammation,[3] as its receptor is expressed only in epithelial cells.[40] IL-22 and its downstream transcription factor STAT3 activation (IL-22/STAT3 signaling) were found to be two important mediators of intestinal epithelial host defense and wound healing during mucosal inflammation, which could induce the proliferation and reconstitution of mucosal epithelial cells, enhance goblet cells to regenerate and produce mucus-associated proteins, promote the remodeling of extracellular matrix and cell migration, and stimulate the production of antimicrobial peptides.[41,42] Some investigators even proposed to consider the IL-22/STAT3 signaling as a potential therapeutic approach in promoting mucosal healing in IBD.[43] In addition, a recent study found that human microbe-responsive γδ T cells can polarize colonic CD4$^+$ T cells to become IL-22$^+$ cells, thus promote IL-22 production and IL-22 inducible antimicrobial protein calprotectin secretion, as a mechanism of promoting mucosal barrier defence.[44] In this study we observed that the crypt architectural alternation, a key feature of chronicity as well as reflection of glandular/cryptal epithelium restitution/regeneration, was associated with the IL-22$^+$CD4$^+$ T cells and Th22 cells. From the biological perspective, our finding may reflect the role of IL-22 and Th22 cells in bowel epithelial regeneration and mucosal healing.

Paneth cells are unique specialized secretory epithelial cells known to play an important role in the innate host defense in normal intestinal mucosa through their secretion of antimicrobial peptides and proteins, *e.g.*, α-defensins. Paneth cell metaplasia, defined as aberrant presence of any number

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of Paneth cells in the distal colon (descending colon, sigmoid and rectum), is also a histologic indicator of chronic mucosal injury, particularly chronicity of IBD. Paneth cell hyperplasia (abnormally prominent, enlarged, in aggregates, and with prominent degranulation) in the proximal colon and terminal ileum seem to represent the same significance in the setting of IBD,[41,45] and it was frequently regarded as being equivalent to, and was grouped together with Paneth cell metaplasia,[45-47] considering their comparable biologic significance, although inaccurate in the strict sense. Paneth cell metaplasia/hyperplasia was found to be positively associated with crypt distortion and lymphoplasmacytic infiltration in lamina propria and related to the epithelial regeneration and repair.[45,48] Here we noticed that UC patients with Paneth cell metaplasia had a significantly higher number of Th17 cells in colonic mucosa as compared with those without Paneth cell metaplasia. To our knowledge, the association or interaction between Paneth cells or Paneth cell metaplasia/hyperplasia and the adaptive immunity of gut mucosa is little known. Kamal *et al* reported Paneth cell increase in the small bowel of *T. spiralis*-infected mice, as a feature of innate mucosal defense, and the increase was modulated by a thymic-independent population of mucosal T cells.[49]Forsberg et al reported a correlation between the mRNA levels of IFN-γ in the intraepithelial lymphocytes and Paneth cell defensins in the duodenal mucosa in celiac disease. [50] Recent studies also suggested a link between Paneth cells and Th17 cells. Salzman found in mice that the expression of human defensin (HD)-5 in small intestine resulted in a reduction of the Th17 cells in lamina propria following the loss of segmented filamentous bacteria, suggesting that Paneth cell defensins can regulate the acquired immune responses through regulating the commensal microbiome.[51, 52] On the other hand, Th17 cells in lamina propria were found to induce mucosal antimicrobial peptide expression, notably RegIIIγ, via IL-22 secretion.[53,54] Moreover, Paneth cell *per se* can produce IL-17 in response to TNF challenge.[55] Based on all of the findings, it is our

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hypothesis that our current finding regarding the association of Th17 cells with Paneth cell metaplasia may be explained by the disruption of intestinal microbiota, which occurs in IBD, that induce Paneth cell metaplasia and in the meantime require the increase of Th17 cells in lamina propria.

The presence of epithelioid granuloma, a collection of macrophages and other inflammatory cells, is a relatively specific histologic feature of CD, although it is only seen in less than half of CD patients. The etiology and significance of granuloma in CD are still unclear. *Mycobacterium paratuberculosis* has been suggested by some observations to be the pathogen related to Crohn's granuloma formation, but it has never been confirmed. On the other hand, limited studies have suggested that the granuloma formation is associated with Th1-shifted immune response, by showing immunohistochemically the increased expression of Th1-type cytokines (IFN-γ, IL-12) and accumulation of $CCR5^+$ and $CXCR3^+$ Th1 cells in colonic mucosa with Crohn's granuloma.[56,57] In our study, it was surprisingly revealed that the intestinal mucosa with Crohn's granulomas had significantly increased prevalence of IL-22^+ $CD4^+$ T cells as well as IL-22^+IFN-γ^+ $CD4^+$ T cells, but not the typical Th1 cells, compared to that without granuloma. One possible explanation for the different results is the methodologic bias. The aforementioned studies used immunohistochemistry to reveal the abundance of Th1 cells in granuloma, which gave the absolute number of Th1 cells, not the percentage as in our study. Also, as mentioned above, IL-22 could be produced by Th1 cells as well. The association of granuloma with both IL-22^+ and IL-22/IFN-γ double expressing $CD4^+$ T cells may partly reflect the predominance and the pathogenic role of Th1 cells in the granuloma formation in CD.

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The present study took the advantage of being the first study systemically analyzing the association between the histopathologic features and the immunologic abnormalities in colonic mucosa in IBD. However, there are some limitations in our study. First, the study focused on the large intestine only. Some biopsies were randomly taken, not site specific. Concurrent medications are likely to change the prevalence of $CD4^+$ T cells in lamina propria. All these are confounding factors that should be taken into account in future study. Second, the immunological study was done by flow cytometry, which although a favored method to provide more information about the detailed immunotypes, was performed on different (though adjacent) pieces of mucosal tissue, instead of by direct immunohistochemical staining on the exactly same tissue as that for histological evaluation. Lastly, the possible relationship of certain Th subsets with certain histologic changes could only be postulated based on the quantitative correlation analysis.

In conclusion, aberrant adaptive immunity appears to be associated with the patterns of histopathologic changes in intestinal mucosa in IBD. Treg cells is associated with the active inflammation, Th22 with the chronic crypt architecture alteration, and Th17 with the Paneth cell metaplasia/hyperplasia. These findings are novel and provide us with more insights about the pathogenesis of IBD.

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Disclosure/conflict of interest

None.

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Author Contributions

X.G.: study concept and design, study supervision, acquisition of data, analysis and interpretation of data, drafting and revision of the manuscript

J.L.: acquisition of data, analysis and interpretation of data, statistical analysis, drafting of the manuscript

A.U.: acquisition of data, analysis and interpretation of data

M.I.: patient enrollment, revision of the manuscript for clinical content

J.Q.: study supervision

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S.G.: study concept, study supervision, patient enrollment, critical revision of the manuscript for important intellectual content

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Figure Legends

Figure1. **Examples of key histologic features of 'Activity' and 'Chronicity' of IBD**. A. Neutrophil infiltration of surface epithelium. B. Neutrophilic cryptitis. C. Neutrophilic crypt abscess causing crypt destruction. D. Crypt architecture distortion. E. Paneth cell hyperplasia in the proximal colon. F. Epithelioid granuloma in CD.

Figure 2. **Examples of flowcytometry gating strategy for defining different $CD4^+$ T cell subsets**.

Figure 3. **Correlation of ECAP scores with RHI scores and D'Haens scores**. Both total ECAP scores and ECAP category A scores are in parallel to the RHI scores in UC (A) and (B) as well as to D'Haens scores in CD (C to E).

Figure 4. **Correlation of Treg cells in lamina propria and histologic disease severity assessed in various histological scores.** The proportion (%) of Treg ($CD25^+FoxP3^+CD4^+$ T cells) in colonic lamina propria of both UC and CD patients were closely correlated with either total ECAP scores or RHI scores or D'Haens scores (A, B, D, E), as well as with the ECAP category A scores (C, F). The solid line in the middle in each graph is the 95% confidence interval.

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Figure 5. Correlation of Treg cells in colonic lamina propria and individual histologic features of activity. The proportion of $CD25^{+}FoxP3^{+}CD4^{+}$ Treg cells were associated with features of the subcategory A2 (neutrophilic cryptitis) (5-A), A3 (crypt abscess) (5-B), A4 (crypt destruction) (5-C), A6 (basal plasmacytosis) (5-D), A7 (lamina propria neutrophilic infiltration) (5-E) in UC patients, and A1 (surface epithelial neutrophilic inflammation) (5-F) in CD patients. * $p < 0.05$.

Figure 6. Correlation of IL-17^{+} CD4^{+} cells and histologic features of UC. The proportion of $IL17^{+}$ Th cells in colonic lamina propria was correlated with the presence of neutrophil infiltration in lamina propria (A7) (6-A) and Paneth cell metaplasia (C2) (6-C), whereas $RoRc^{+}$ Th cells were correlated with the lamina propria mononuclear cell cellularity (A5) (6-B). * $p < 0.05$.

Figure 7. Correlation of IL-22^{+} cells and Th-22 cells and histologic features of chronicity. Certain $CD4^{+}$ T cell subsets in lamina propria were associated with some histologic features of chronicity. UC with diffuse crypt architectural alteration (C1) had a higher percentage of IL-$22^{+}CD4^{+}$T cells (7-A) as well as Th22 (IL-22^{+}IL-17^{-}IFN-$\gamma^{-}CD4^{+}$ T) cells (7-B) in lamina propria. Similarly, CD with diffuse crypt architectural alteration had a higher percentage of Th22 cells (7-D and 7-E), and CD with granuloma had higher percentage of IL-$22^{+}CD4^{+}$ T cells (7-F). * $p < 0.05$, ** $p < 0.01$.

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Table 1. Demographic Data and Clinical Features of the Enrolled Patients

	Ulcerative colitis	Crohn's disease
Male (n,%)	12 (44.4)	19 (73.1)
Age (Mean± SD)	37.59 ± 11.96	43.9 ± 15.6
Family history of IBD	6 (22.2)	1 (3.8)
Disease type, n (%)		
UC Procitis	4 (14.3)	
Left side colitis	12 (42.9)	
pancolitis	11 (39.3)	
CD Colitis		21 (80.8)
Ileocolitis		5 (19.2)
Disease Activity, n (%)		
UC Remission	6 (21.4)	
Mild	11 (39.3)	
Moderate	8 (28.6)	
Severe*	2 (7.1)	
CD Remission		17 (65.4)
Moderate		5 (19.2)
severe		4 (15.4)
Endoscopic Index, n (%)		
Mayo 0-1	12 (48.1)	
Mayo 2	10 (35.7)	
Mayo 3	4 (14.3)	
SES-CD 0-2#		2 (7.7)
SES-CD 3-6		17 (65.4)
SES-CD 7-15		6 (23.1)
SES-CD >16**		1 (3.8)
Medications, n (%)		
5-ASA	10 (35.7)	7 (26.9)
Corticosteroid	3 (10.7)	1 (3.8)
AZA or MTX	7 (25.9)	5 (19.2)
Anti-TNFα	7 (25.9)	5 (19.2)
None	6 (22.2)	11 (42.3)
Biopsy Sites, n (%)		
Rectum	11 (39.3)	4 (15.4)
Sigmoid	11 (39.3)	11 (42.3)
Descending colon	3 (10.7)	1 (3.8)
Splenic flexure	0 (0)	0 (0)
Transverse colon	0 (0)	2 (7.7)
Hepatic flexure	0 (0)	2 (7.7)
Ascending colon	2 (7.1)	5 (19.2)

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Cecum	0 (0)	1 (3.8)

* The moderate and severe active UC were later combined into 'moderate to severe active UC' for further analysis

The patients with SES-CD 0-2 and those with SES-CD 3-6 were later combined for further analysis

** The patients with SES-CD >16 and those SES-CD 7-15 were later combined for further analysis

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Table 2. IBD Histological Scoring Scheme (ECAP system)

Histopathologic Features	Score
Extent of Inflammation (E) (*for UC*)	
None	0
Focal	1
Multifocal (Patchy)	2
Diffuse	3
Extent of Inflammation (E) (*for CD*)	
(% of Affected Biopsies)	
None	0
≤25%	1
>25% to ≤50%	2
>50% to ≤75%	3
>75%	4
Chronicity (C)	
C1. Crypt Architecture Alteration	
None	0
Focal Alteration	1
Patchy Distortion (<50%)	2
Diffuse Distortion (>50%)	3
C2. Paneth Cell Metaplasia / Hyperplasia	
None	0
Present	1
Activity of Inflammation (A)	
A1. Surface Epithelium	
Normal	0
Reactive Changes (Mucin Depletion / Villiform)	1
Neutrophilic infiltration / Probable Erosion	2
Erosion	3
Ulceration	4
A2. Neutrophilic Cryptitis	
None	0
<5%	1
<50%	2
>50%	3
A3. Crypt Abscess	
None	0
Present	1
A4. Crypt Destruction	
None	0
Present	1
A5. Lamina Propria Mononuclear Cellularity	
Normal	0
Mild Increase	1
Moderate Increase	2
Severe Increase	3
A6. Basal Plasmacytosis	
None	0
Focal	1
Diffuse	2
A7. Lamina Propria Neutrophilic Infiltration	
None	0
Rare	1
Scattered	2
Extensive	3
Plus / others (P)	
P1. Lamina Propria Eosinophilic Infiltration	
None	0
Mild	1
Moderate	2
Severe	3
P2. Lymphoid Follicles / Aggregates	
None	0
Rare	1
Prominent	2
P3. Granuloma (for CD only)	

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None	0
Present	1
Total ECAP Score	

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Figure 1.

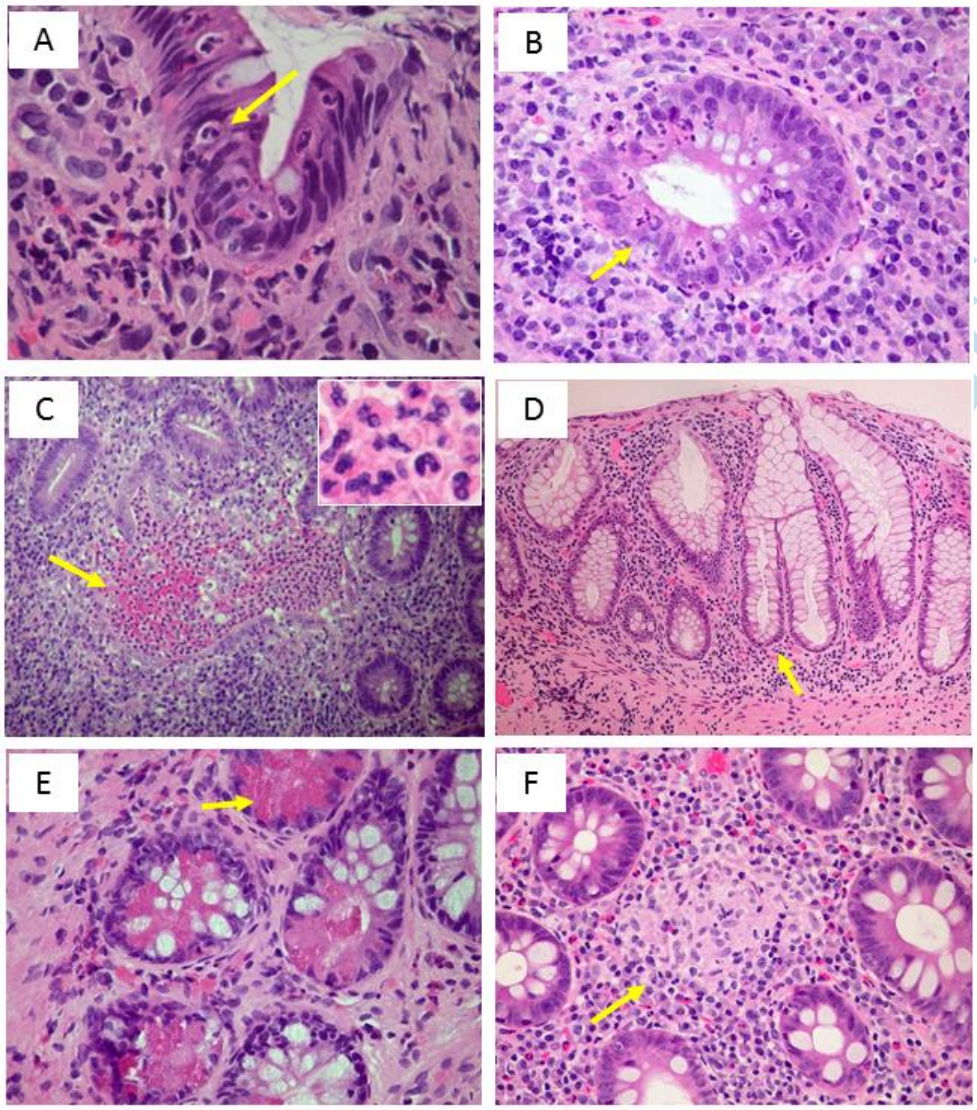

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Figure 2.

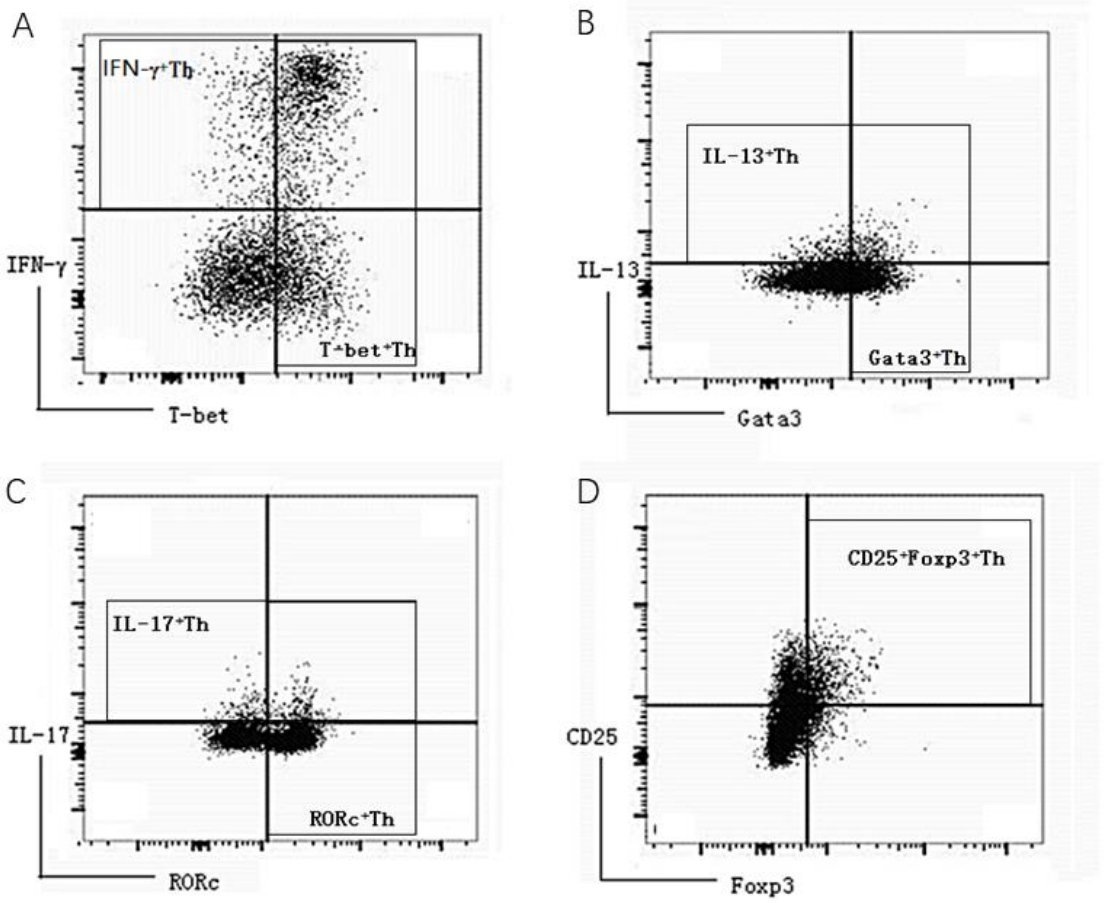

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Figure 3.

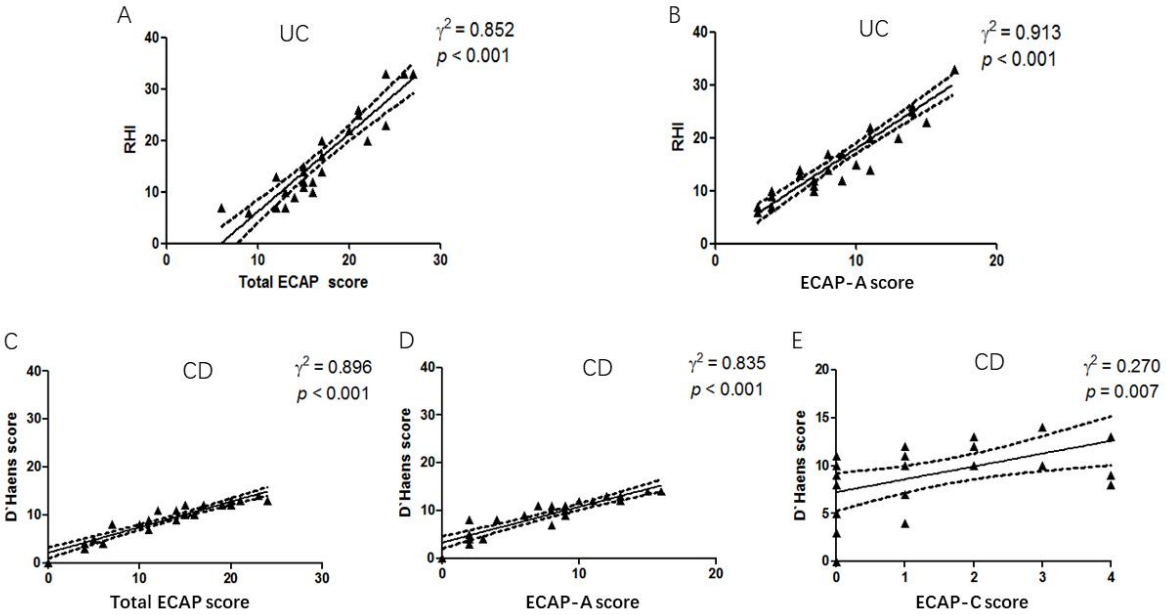

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Figure 4.

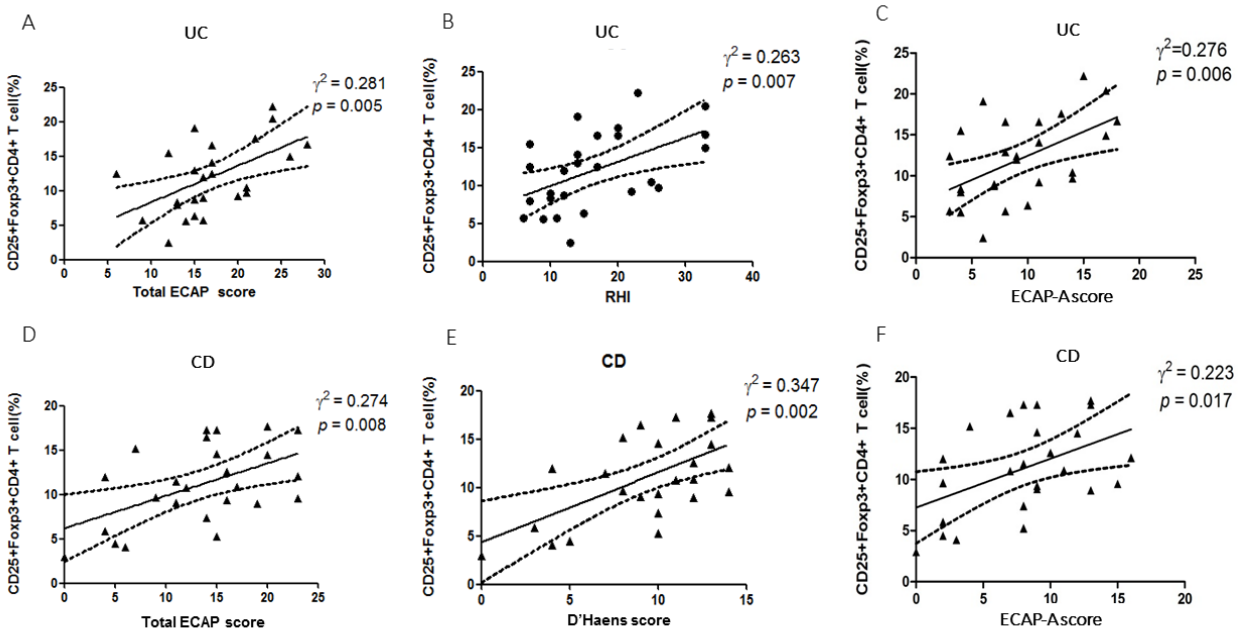

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Figure 5.

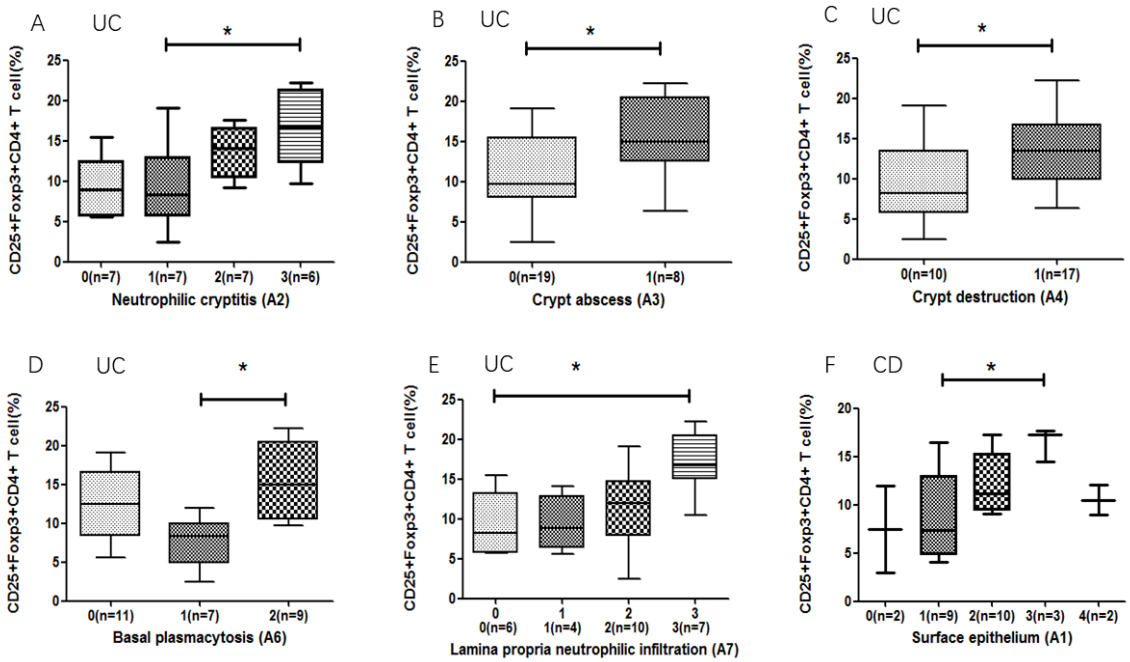

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Figure 6.

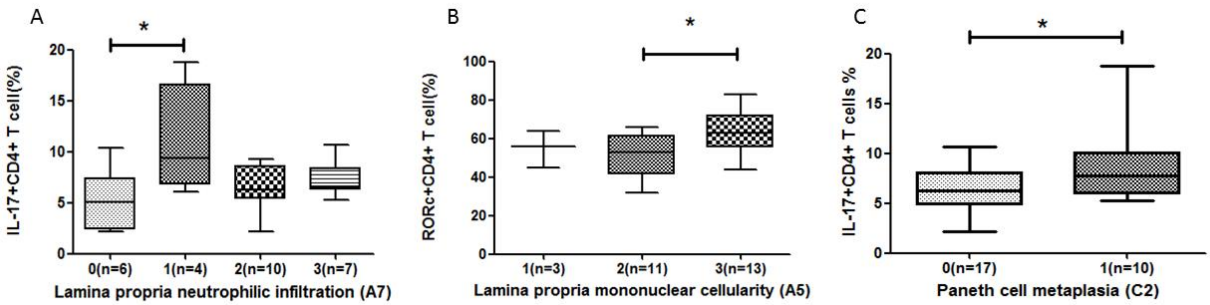

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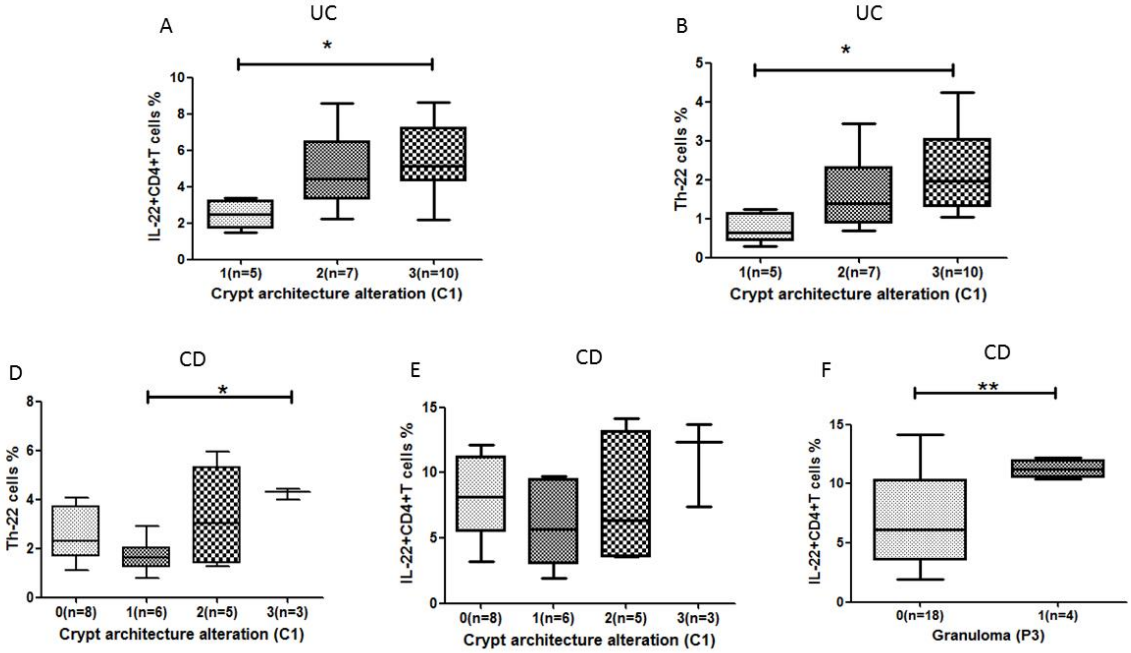
Figure 7.



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