Involvement of CD40-CD40L and ICOS-ICOSL pathways in the development of Chronic Rhinosinusitis by modulating eosinophil function

Qingqing Jiao[1], Aina Zhou[1], Chenxi Shi[1], Yuhui Fan[1], Yushuang Zheng[1], Jue Wang[1], Zhichen Liu[1], Huanxia Xie[1], and Jisheng Liu[1]

[1]First Affiliated Hospital of Soochow University

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Abstract

Background: Whether the CD40-CD40 ligand (CD40L) and inducible co-stimulatory molecule (ICOS)-ICOS ligand (ICOSL) signals are involved in chronic rhinosinusitis (CRS) development needs further investigation. **Objectives:** To investigate the association of CD40-CD40L and ICOS-ICOSL expression with CRS and underlying mechanisms. **Methods:** Immunohistology detected the expression of CD40, CD40L, ICOS, and ICOSL. Immunofluorescence was performed to evaluate the co-locations of CD40 or ICOSL with eosinophils. Correlations between CD40-CD40L and ICOS-ICOSL as well as clinical parameters were analyzed. Flow cytometry was used to explore the activation of eosinophils by CD69 expression and the CD40 and ICOSL expression on eosinophils. **Results:** Compared with the Non-eCRS subset, ECRS (Eosinophilic chronic rhinosinusitis) subset showed significantly increased CD40, ICOS, and ICOSL expression. The CD40, CD40L, ICOS, and ICOSL expressions were all positively correlated with eosinophil infiltration in nasal tissues. CD40 and ICOSL were mainly expressed on eosinophils. ICOS expression was significantly correlated with the expression of CD40-CD40L, while ICOSL expression was correlated with CD40 expression. ICOS-ICOSL expression positively correlated with blood eosinophils count and disease severity. rhCD40L and rhICOS significantly enhanced the activation of eosinophils from ECRS patients. TNF-α and IL-5 obviously upregulated CD40 and ICOSL expression on eosinophils, which was significantly inhibited by the p38 MAPK inhibitor. **Conclusions:** Increased CD40-CD40L and ICOS-ICOSL expression in nasal polyps are linked to eosinophils infiltration and disease severity of CRS. CD40-CD40L and ICOS-ICOSL signals enhance eosinophils activation of ECRS. TNF-α and IL-5 regulate eosinophils function by increasing CD40 and ICOSL expression partly via p38 MAPK activation in CRS patients.

Introduction

Chronic rhinosinusitis (CRS) is a chronic inflammatory disease in the nose and paranasal sinus characterized histologically by the infiltration of inflammatory cells, especially eosinophils, with high prevalence, worldwide[1-3]. Based on the extent of tissue eosinophilia, CRS can be classified into eosinophilic chronic rhinosinusitis (ECRS) and non-eosinophilic (Non-eCRS) subtypes[4,5]. Compared with Non-eCRS, ECRS is associated with worse disease severity, a higher risk of comorbid asthma, and a higher ratio of recurrence and revision surgery[6-8]. There are significant geographic and ethnic differences in the tissue eosinophilic infiltration, ECRS is predominant in Western white patients and less common in East Asians[9-11]. However, it has been reported that the proportion of ECRS has increased over time in Korea and China[12,13]. Thus, identifying specific mediators that drive the development of eosinophils and modulating their functions, in particular of ECRS, will be important for developing novel treatment strategies and improving treatment outcomes.

CD40 is a cell surface receptor that belongs to the tumor necrosis factor-R (TNF-R) family[14]. Although the primary function was initially restricted to B and T lymphocytes, CD40 has been explored more extensively

because of its broad expression on non-lymphocytic cell types[15-19]. It has been reported that eosinophils isolated from allergic subjects express CD40 by Yuichi Ohkawara et al., which is biologically functional. Interestingly, they also found that CD40 was detected in nasal polyp tissues but not in normal nasal mucosa (inferior turbinate), and primarily in eosinophils. At the same time, they demonstrated that CD40 expression in eosinophils could be upregulated by exposure to IgA immune complexes and downregulated by interleukin (IL) -10 and the synthetic steroid budesonide[20]. These observations suggest that the CD40-CD40 ligand (CD40L) pathway may contribute to the development of eosinophil-mediated inflammation. It is therefore reasonable to speculate that the CD40-CD40L signal pathway may be involved in the regulation of eosinophils function in CRS.

CRS without nasal polyps (CRSsNP) and chronic rhinosinusitis with nasal polyps (CRSwNP) are the 2 phenotypes of CRS according to the presence or absence of nasal polyp (NP)[1,3]. CRSwNP is often characterized by the local production of polyclonal IgE idiotypes[21-25]. As for the induction and regulation of IgE synthesis, a two-signal model is accepted. The first signal is provided by cytokines IL-4 or IL-13, which are secreted by T cells, mast cells, and basophils. The second signal is CD40-CD40L interaction, which is well established as a key signal for the induction of isotype switching in B-cells[26-29]. Interestingly, inducible co-stimulator (ICOS) ICOS-ICOSL ligation can promote the expression of CD40L, which in turn strengthens CD40-CD40L interaction to provide a co-stimulatory signal for B cell activation. And one very recent study has shown that ICOS co-stimulation induces CD40L expression by human T cells[30,31]. Nevertheless, the role of ICOS-ICOSL and its interaction with CD40-C40L in CRS has not been investigated.

Therefore, in the current study, we investigated patients with CRS for their CD40 and C40L levels, as well as ICOS and ICOSL levels. We characterized the clinical relevance of CD40-CD40L and ICOS-ICOSL, especially with eosinophils, in CRS, and we explored potential mechanisms that underlie their role in the pathogenesis of CRS.

Material and methods

Study subjects

This study was approved by the ethics committee of the first affiliated hospital of Soochow University (No. 215). A total of 31 patients with CRS treated with functional endoscopic sinus surgery (FESS) were included after informed consent from April 2021 to May 2021 in the otolaryngology department of the first affiliated hospital of Soochow University. The basic information and clinical characteristics of these patients were displayed in Table 1. The diagnosis of sinus disease was based on clinical symptoms and related examinations such as nasal endoscopy, and computed tomography (CT), according to the guidelines of the European Position Paper on Rhinosinusitis and Nasal Polyps 2020 (EPOS2020) and Chinese guidelines for diagnosis and treatment of chronic rhinosinusitis (2018). Participants whose age ranged from 18 to 70 were included. Our study excluded patients treated with oral, nasal, or systematic corticosteroids or antibiotics, antileukotrienes 4 weeks preceding the operation, patients suffering from upper respiratory tract infections 4 weeks preceding the operation as well as patients developing immune disorders, pregnancy, malignancy such as nasopharyngeal carcinoma, carcinoid such as inverting papilloma. At the same time, subjects who had CRS because of specific causes, cystic fibrosis, fungal sinusitis, vasculitis, or primary ciliary dyskinesia were excluded.

Preoperative demographic information including sex, age, phone number, and drug allergies was obtained from each patient. Medical history including rhinorrhea, nasal blockage, hyposmia, facial pressure or pain, headache, duration, and prior nasal surgery was recorded carefully. Rhinology specialists classified CRS into CRSwNP and CRSsNP through nasal endoscopy and CT, into ECRS and Non-eCRS through the following HE staining. CT findings were graded according to the Lund–Mackay method. Blood samples were taken to perform complete blood cell counts. Recurrence of CRS was defined as the presence of nasal polyps after nasal endoscopy.

Histological analysis

Mucosal tissues from patients with CRS were obtained from nasal polyps or the uncinate process. Tissues were immediately fixed in 10% formalin, embedded in paraffin, and cut into thin sections. Sections were stained with hematoxylin–eosin to differentiate CRS into various eosinophilic phenotypes. Representative HE staining pictures of Non-eCRS and ECRS were shown in Supplementary Fig.1A. The numbers of eosinophils and total inflammatory cells beneath the epithelial surface per high power field (HPF) (x400) were quantified by 2 independent researchers and the percentage of eosinophils in total infiltrating inflammatory cells (eosinophils percentage) was calculated. Five fields were randomly selected, then the average percentage was analyzed. According to previous studies of ECRS in China, we defined ECRS as eosinophil percentage exceeding 10%, as proposed by *Cao et al*[32].

At the same time, the histological patterns of each patient were evaluated according to histopathological characteristics referring to basement membrane thickening, goblet cell hyperplasia, subepithelial edema, submucous gland formation, eosinophils infiltration, fibrosis and atypical cells by 2 independent researchers. Briefly, there were four main classifications: edematous, eosinophilic CRS with a great number of eosinophils, goblet cell hyperplasia, thickening of the basement membrane; CRS characterized by numerous seromucous glands and ductal structures; fibroinflammatory CRS, lack of stromal edema and goblet cell hyperplasia, frequently showed evident dilated vessels and a great number of fibrocytes; atypical CRS with distinct stromal cells that were bizarre and atypical. Representative HE stainings of the histologic pattern was shown in Supplementary Fig. 1B.

Immunohistochemistry (IHC) analysis

For expression analysis of CD40, CD40L, ICOS, ICOSL, formalin-fixed and paraffin-embedded nasal biopsies were cut into 4 μm thick sections deparaffinized by serial treatment. Deparaffinized sections were subjected to antigen retrieval by heating the sections in sodium citrate buffer, pH 6.0. After blocking the endogenous peroxidase in 3% hydrogen peroxide and with 3% bovine serum albumin, the sections were incubated overnight at 4°C in the presence of rabbit-derived primary antibodies against CD40 (1:100, Affinity Biosciences, AF5336), CD40L (1:200, Abcam, Cambridge, MA, USA, ab65854), ICOS (1:500, Abcam, Cambridge, MA, USA, ab224644) and ICOSL (1:200, Abcam, Cambridge, MA, USA, ab233151). Thereafter, each section was incubated with HRP (Horse Raddish Peroxidase) conjugated anti-rabbit secondary antibody (1:500) for 50 min. After washing, sections were incubated with DAB (3,3'-diaminobenzidine tetrahydrochloride) and immediately washed under tap water after color development. Then, sections were counterstained with hematoxylin and mounted with DPX (dibutyl phthalate xylene). The sections were blindly examined with no awareness of the clinical data with an Olympus CX40 Microscope (Olympus Optical Co, Hamburg, Germany). The number of positive cells was counted in 5 random HPFs (x200) and averaged.

For further analysis of co-location of CD40 and ICOSL with eosinophils, immunofluorescence was performed using TSA (Tyramide signal amplification) technique. Sections were deparaffinized and antigen retrieval was performed in Tris-EDTA (Ethylenediaminetetraacetic acid) buffer, pH=9.0. After blocking the endogenous peroxidase, sections were incubated overnight at 4°C in the presence of primary antibody against PRG2 (1:1000, Abcam, Cambridge, MA, USA, ab236851), which is MBP, major basic protein, the predominant constituent of the crystalline core of the eosinophil granule. Then, HRP conjugated anti-rabbit secondary antibody (1:500) was incubated with sections for 50 min at room temperature. Sections were then incubated with 488-TSA at room temperature for 10 min. Next, antigen retrieval was performed before incubating with primary antibody against CD40 (1:250, Affinity Biosciences, AF5336) or ICOSL (1:200, Abcam, Cambridge, MA, USA, ab233151). After washing, sections were incubated with CY3 conjugated anti-rabbit secondary antibody (1:300). The DNA dye 4',6-diamidino-2-phenylindole (DAPI) was used to visualize the nucleus. Results were captured under fluorescence microscope. Agents not mentioned specifically obtained from Servicebio technology CO, Wuhan, China.

Assessment of blood eosinophils activation

Whole heparinized blood was obtained from 10 ECRS patients. Blood was incubated for 24 h at 37degC with either recombinant human CD40L protein (rhCD40, 5 ug/ml; R&D systems, Minneapolis, MN, USA,

6420-CL-025) or recombinant human ICOS protein (rhICOS, 10ug/ml; R&D systems, Minneapolis, MN, USA, 169-CS-050). IgG (5 ug/ml; R&D systems, Minneapolis, MN, USA, 1-001-A) was used as control. Cells were harvested for further analysis. Leukocytes were stained with an antibody cocktail of CD45-APC (Life Technologies, Calif. USA, 17-0459-42, HI30), CD16-FITC (BioLegend, San Diego, Calif. USA, 360716,B73.1), and CD69-PE (BioLegend, San Diego, Calif. USA, 985202,FN50). Eosinophils were defined as $CD45^{+}CD16^{-}$ and CD69 was determined as its activation marker.

Eosinophils isolation and culture

Peripheral blood eosinophils from healthy controls were purified by using an eosinophil isolation kit (Miltenyi Biotec, San Diego, Calif. USA, 130-092-010). Eosinophil purity was assayed using flow cytometry and Wright-Giemsa staining (Supplementary Fig. 2A). This procedure consistently resulted in a highly purified eosinophil population (95-99%). These eosinophils (¿99% viable by trypan blue exclusion) were cultured in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 50 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF, Novoprotein, Suzhou, China, C003) at 37 in a humidified atmosphere of 5% CO2. Then, eosinophils ($2x10^5$/well in 200uL RPMI) were stimulated in a 96-well plate for 24 or 48 hours with or without the addition of the following agents: 50ng/mL recombinant TNF-α (Novoprotein, Suzhou, China, C008), 50ng/mL recombinant IL-5 (Novoprotein, Suzhou, China, CI59), 3 μM specific p38 MAPK inhibitor SB203580 (MedChemExpress, NJ., USA, HY-10256A). At the end of this incubation, eosinophils were harvested and investigated further by using flow cytometry for the expression of CD40 and ICOSL.

Flow cytometry analysis

Flow cytometry was used to detect CD40 and ICOSL expression on purified eosinophils at 0h, 24h, or 48h. Every time when eosinophils were isolated, CD16 was used to access their purity. Briefly, harvested eosinophils were resuspended in PBS with 1% FBS. 100 μL cell suspension was incubated with the fluorescein-conjugated antibody at 4 in the dark for 20 min. All the antibodies were purchased from Biolegend, San Diego, Calif, the detailed information was as follows: CD16-FITC (360716, B73.1), PE anti-human CD40 (334308, 5C3), APC anti-human ICOSL (309407, 2D3).

Statistical analysis

All data were analyzed using GraphPad Prism 7 software (GraphPad, San Diego, California). Normality of variables was evaluated using Shapiro-Wilk test. Student's unpaired t-test was performed for two-group comparisons of the data with normal distribution, otherwise Mann-Whitney U test was used. In addition, the interaction between variables was assessed by Pearson's /Spearman's correlation test, which was appropriate for normally and abnormally distributed variables respectively. P values of less than .05 indicated statistical significance.

Results

CD40-CD40L and ICOS-ICOSL expression are markedly increased in nasal polyp tissue of ECRS patients

Representative staining of CD40, CD40L, ICOS, and ICOSL on sections from the nasal tissue involved in this study, which varied in density and intensity in patients with ECRS and Non-eCRS (Fig. 1A). The expression levels of CD40 (64.67 ± 13.48 vs 13.12 ± 2.52, p=0.0001), ICOS (63.85 ± 16.8 vs 7.05 ± 2.31, p=0.0039), and ICOSL (81.36 ± 15.88 vs 14.72 ± 2.00, p<0.0001) were significantly higher in the nasal tissue of ECRS patients compared with that in patients of Non-eCRS (Fig. 1B). And the number of CD40L positive cells was also increased in ECRS nasal tissue compared with that in the nasal polyps of Non-eCRS patients, although there was no significant difference (Fig. 1B).

CD40-CD40L and ICOS-ICOSL expression are correlated in nasal polyps of CRS patients

Then, we investigated the correlation of CD40-CD40L and ICOS-ICOSL expression in the nasal tissue of our CRS patients. Our correlation analysis results show that there was a significantly positive correlation

between ICOS and CD40 expression (r=0.7875, p<0.0001, Fig. 2A), ICOSL and CD40 expression (r=0.5232, p=0.0061, Fig. 2B), ICOS and CD40L expression (r=0.5604, p=0.0102, Fig. 2D), as well as ICOS and ICOSL expression (r=0.6389, p=0.0018, Fig. 2F). Similar correlation tendencies were observed between CD40L and CD40 expression (Fig. 2C), as well as CD40L and ICOSL expression (Fig. 2E), whereas there was no significant correlation shown.

ICOSL expression is significantly higher in nasal tissue of CRS patients with edematous pattern

All CRS patients were also classified into different histopathological pattern. We found that the percentage of mere hyperplasia (38.1%) and fibrotic pattern (33.3%) were overwhelming in Non-eCRS, while none of these two types were observed in ECRS (Fig. 3A). In group ECRS, pattern edematous combined with fibrotic accounted for the largest proportion (40.0%), followed by edematous plus hyperplasia pattern (30.0%) and edematous pattern (30.0%), which were all characterized by edema (Fig. 3A). When the six patterns were combined into three types, the edematous pattern was seen in 28.6% of Non-eCRS patients and 100.0% in patients of ECRS. Whereas, the proportions of fibrotic and hyperplasia patterns were both slightly lower in ECRS than Non-eCRS, respectively. (40.0% vs 47.6%, 30.0 vs 47.6%, Fig. 3B).

According to the great difference of the proportion in edematous subtype and merely little variation of that in hyperplasia and fibrotic subtypes between ECRS and Non-eCRS, we thus only examined CD40-CD40L and ICOS-ICOSL expression in the histopathological subtype of edema. Results showed that the expression levels of ICOSL (59.21 ± 12.76 vs 16.16 ± 2.89, P=0.0319, Fig. 3C) were significantly increased in the nasal tissue of CRS patients with edematous pattern compared with that in Non-edematous pattern CRS patients. The number of ICOS or CD40 positive cells was also higher in edematous pattern nasal tissue compared with that in the nasal polyps of Non-edematous pattern CRS patients, but no significant difference was observed (Fig. 3D-E). But in contrast, there was no obvious difference in tissue CD40L expression between edematous and Non-edematous pattern CRS patients (Fig. 3F).

CD40-CD40L and ICOS-ICOSL expression are strongly correlated in nasal tissue of edematous pattern CRS patients

Additionally, we further found a strong positive correlation between the expression of CD40-CD40L and ICOS-ICOSL in nasal polyps of edematous pattern CRS patients. To be specific, there was a significantly positive correlation between ICOS and CD40 expression (r=0.8966, p<0.0001, Fig. 4A), ICOSL and CD40 expression (r=0.6679, p=0.0080, Fig. 4B), CD40L and CD40 expression (r=0.5429, p=0.0391, Fig. 4C), ICOS and CD40L expression (r=0.6300, p=0.0238, Fig. 4D), ICOSL and CD40L expression (r=0.8286, p=0.0003, Fig. 4E), as well as ICOSL and ICOS expression (r=0.8611, p=0.0003, Fig. 4F).

High levels of CD40-CD40L and ICOS-ICOSL expression in nasal tissue are linked to high eosinophil levels and disease activity in CRS patients

Our further findings showed that the expression levels of CD40 (r=0.6291, p=0.0003), CD40L (r=0.5820, p=0.0023), ICOS (r=0.6149, p=0.0030), ICOSL (r=0.5127, p=0.0063) in nasal polyps of CRS patients were all significantly correlated with tissue eosinophil count (Fig. 5A). Consistently, our immunofluorescence co-staining results showed that a great number of CD40-positive cells were eosinophils in ECRS nasal tissue (Fig. 5B). Most eosinophils are also ICOSL-positive in nasal polyps of ECRS patients (Fig. 5C).

Furthermore, both increased tissue CD40 and CD40L expression in our CRS patients were linked to higher blood eosinophil count (r =0.5066, p=0.0059, Fig. 6A; r =3893, p=0.0544, Fig. 6B). Similarly, ICOS and ICOSL-positive cell numbers in tissue were strongly, positively correlated with blood eosinophil count (r=0.6419, p=0.0017, Fig. 6C; r=0.6694, p=0.0001, Fig. 6D). Besides, nasal tissue ICOS and ICOSL expression levels correlated with disease activity assessed by Lund-Mackay score (r=0.4714, p=0.0416, Fig. 6E; r=0.4047, p=0.0498, Fig. 6F).

CD40-CD40L and ICOS-ICOSL interactions enhance the activation of eosinophils from ECRS patients

Since upregulation of CD40 and ICOSL expression on eosinophils in nasal tissue of CRS patients, we investigated whether CD40-CD40L and ICOS-ICOSL interactions involved in eosinophils dysfunction. Considering that ECRS is characterized circulating and histologically high proportion of eosinophils. To determine this, peripheral blood samples from 10 ECRS patients were stimulated by rhCD40L (5ug/mL), rhICOS (10 ug/mL), or control IgG (5ug/mL), respectively. Cells were harvested 24h post-stimulation for flow cytometry. $CD45^{+}C16^{-}$ cells was defined as eosinophils and CD69 was an activation marker of eosinophils. The detailed gating strategy was shown in Fig. 7A. There were notably upregulation CD69 expression on eosinophils in response to CD40L and ICOS protein stimulation compared with that to control IgG group (Fig. 7B). These data indicated that the up-regulation of CD40 and ICOSL on eosinophils mediated their activation in ECRS patients.

TNΦ-α πλυς IΛ-5 ενηανςεδ ˆΔ40 εξπρεσσιον ον εοσινοπηιλς δεπενδινγ ον τηε αςτιατιον οφ τηε π38 MAΠK πατηωαψ

Finally, we investigated the possible inflammatory mediators involved in enhanced CD40 and ICOSL expression on eosinophils in patients with CRS. As CRS is characterized by the increased local tissue levels of TNF-α and IL-5, especially in ECRS[33-35]. Previous studies have reported that TNF-α induces the expression of CD40 on epithelial and endothelial cells as well as the expression of ICOSL expression on fibroblasts, endothelial cells and B cells, monocytes[36-40]. IL-5 is the most potent activator of eosinophils[41-43]. Thus, we investigated the effect of TNF-α and IL-5 on CD40 and ICOSL expression on human eosinophils. At the baseline, purified eosinophils from healthy human peripheral blood (purity>95%) have no CD40 or ICOSL expression (Supplementary Fig. 2B). As shown in Fig. 8, the expression of CD40 was markedly upregulated on eosinophils after rTNF-a (50ng/mL) stimulation (P=0.0014) for 24h, not rIL-5 (50ng/mL). Furthermore, TNF-α plus IL-5 further markedly enhanced CD40 expression on eosinophils compared with TNF-α incubation ($p<0.0001$). However, no time-dependent effect of TNF-α or TNF-α plus IL-5 induced CD40 expression on eosinophils were observed. Whereas, TNF-α alone or TNF-α combined with IL-5 has no significant effect on ICOSL expression on eosinophils (Supplementary Fig. 2C-D).

Activation of p38 MAPK has been shown to partly mediated TNF-α-induced anti-apoptotic signals in human eosinophils[44]. Finally, we sought to determine whether p38 MAPK pathway mediates the up-expression of CD40 here. Purified eosinophils were treated with the specific p38 MAPK inhibitor SB203580 before TNF-a plus IL-5 stimulation. We found that SB203580 highly suppressed the TNF-a+IL-5-induced CD40 expression on eosinophils ($p<0.0001$, Fig. 8).

Discussion

This is the first study to show that both CD40-CD40L and ICOS-ICOSL are upregulated in the nasal polyps of CRS patients. Our results demonstrate that increased expression of CD40-CD40L and ICOS-ICOSL in CRS nasal tissues is linked to high eosinophils infiltration and disease severity. Then, we found CD40-CD40L and ICOS-ICOSL pathways do take effect on the activation of eosinophils from ECRS patients. Additionally, we illustrated that TNF-a induces CD40 expression on eosinophils via the activation of the p38 MAPK signaling pathway, and IL-5 further augments TNF-a stimulated CD40 expression on eosinophils. Our findings indicate that CD40-CD40L and ICOS-ICOSL are potential clinical biomarkers of disease activity in patients with CRS, particularly in the population with high-level eosinophils.

For the first time, our findings show that levels of CD40-CD40L and ICOS-ICOSL are markedly increased in the nasal tissue of ECRS patients compared with that in Non-eCRS patients. Our subsequent correlation analyses showed that high nasal tissue CD40-CD40L and ICOS-ICOSL levels were strongly correlated in CRS. Besides, based on the classification of histopathologic phenotypes, we observed similar upregulation of CD40 and ICOS-ICOSL in nasal polyps of edematous CRS. Consistently, a strong correlation with CD40-CD40L and ICOS-ICSOL levels was observed in edematous CRS nasal tissues. It has been reported that edematous CRS was commonly observed in eosinophilic inflammation[1,45]. However, our findings that groupings based on ECRS/Non-eCRS and groupings based on histopathologic phenotypes do not completely overlap. We think that this is mainly related to the uneven distribution and the small number of patient cases in different

pathological subtypes, so statistical analysis cannot be conducted. Given that the ICOS-ICOSL signal can strengthen CD40-CD40L interaction thus providing a co-stimulatory signal for B cell activation[30,31], as well as the allergic characteristics of CRS. Importantly and novel, our findings indicate that high CD40-CD40L and ICOS-ICOSL expression in nasal tissues are potential immunoregulatory factors for the development of CRS, especially in patients with high eosinophil levels.

Then, our subsequent correlation analyses showed that high CD40-CD40L and ICOS-ICOSL expression was linked to high eosinophils infiltration in the nasal tissue of CRS patients. We further observed that both augmented CD40 and ICOSL expression was primarily on eosinophils in the local tissue of ECRS.

So far, several studies have shown that not only CD40 but also CD40L is expressed on the surface of human eosinophils[20,46,47]. And close to what we found, Ohkawara Y et al. also found that CD40 was mainly expressed on the surface of eosinophils in the nasal polyp tissues of allergic subjects. They only compared the expression of CD40 in nasal polyp tissues[20]. In this study, we further found the different CD40-CD40L expression in Non-ECRS and ECRS nasal polyps and also their correlation with clinical feature of CRS. We assume that the high nasal tissue eosinophils proportion of CRS mainly contributed to the high CD40 expression levels. As CD40L is predominantly expressed on activated $CD4^+$ T cells, it has been shown that there is a large number of T cells infiltrating in nasal tissue of CRS[32,48-50]. Thus, we suspect that there is a "T-eosinophils-centered function" of CD40-CD40L in the nasal tissue of CRS with high-level eosinophils, which is worthy of further study.

As for the expression of ICOS-ICOSL in nasal tissues, we reported it for the first time. Andreas Hutloff et al. reported that there is no ICOS expression on granulocytes using F44 (specific monoclonal antibody to ICOS)[51]. And no research has studied the expression of ICOSL on eosinophils so far. Our co-location staining showed first that ICOSL but no ICOS expression on eosinophils. Considering that ICOS is mainly expressed on activated $CD4^+$T cells, especially activated T_H2 cells[52,53]. ECRS found worldwide is characterized by a type 2 immune response involving T_H2 cells, type 2 innate lymphoid cells, eosinophils, mast cells, and M2 macrophages[50,54-58]. Thus, we speculate that activated $CD4^+$ cells, especially T_H2 cells, can exert influence on eosinophils mono-directionally, through ICOS-ICOSL ligation signal pathway in ECRS. Further studies are still needed.

Then, our clinical correlation analysis shown that blood eosinophils count was significantly higher in ECRS subset compared with that in Non-eCRS subset (Supplementary Fig. 3A), which is consistent with previous studies[59-61]. As shown in Supplementary Fig. 3B-C, we further observed that blood eosinophil count was positively correlated with disease activity assessed by Lund-Mackay score as well as nasal tissue eosinophils count in our CRS patients. Developing from progenitors in bone marrow, eosinophils can be recruited to diseased nasal tissue from peripheral circulation by chemokines and cytokines, which resulting a specific correlation between them. Then, positive correlations between blood eosinophil count and tissue CD40-positive cell numbers as well as CD40L-positive cell numbers were found, and the same findings were with ICOS-ICOSL-positive cell numbers. Importantly, we noticed that high ICOS-ICOSL expression levels was positively correlated with Lund-Mackay score of patients with CRS patients. Recent studies have reported the pathological effect of ICOS-ICOSL signals widely participate in inflammatory responses, particularly $ICOS^+$ T cells, including T_H1, T_H2, T_H17 as well as T follicular helper (Tfh), T follicular regulatory cells (Tfr) and regulatory T cells (Treg), with the increased generation, proliferation, and survival abilities[62-66]. Thus, the ICOS-ICOSL pathway may associate with the local immune microenvironment and then contribute to the development of CRS, especially ECRS. Interestingly, ICOSL positive cells also had positive correlation with blood basophils (Supplementary Fig. 3D). Therefore, our above data indicate that CD40-CD40L and ICOS-ICOSL signals may involve in the pathogenies of CRS by modulating the function of eosinophils.

Next, we confirmed whether CD40-CD40L and ICOS-ICOSL axis function on eosinophils by using CD40 and ICOSL protein in ECRS. We found CD40 protein stimulation upregulated the expression of CD69, which is an important marker of activation for eosinophils. In addition, CD69 levels were also increased in response to ICOSL protein stimulation. These results show that both CD40-CD40L and ICOS-ICOSL signals activate eosinophils, and then contributes to the development of ECRS. Cause recent evidence suggests that

activated eosinophils have an axial role in symptomology of CRS, especially ECRS. Studies have shown the association between activated eosinophil count and the development of ECRS. Moreover, some reports demonstrated a significant drop of blood eosinophils from before to after FESS[61,67-69]. In the advantage of great local cytokines and chemokines production, eosinophils are characterized by increased production, enhanced activation and prolonged survival. These factors promote the eosinophils accumulation, which ultimately contributing to the increased destroy of epithelial barrier and hyper-activity in nasal mucosa[70-72].

TNF-α and IL-5 are closely related to CRS. Previously many researchers have reported the high levels of TNF-a and IL-5 in patients with CRS and positive correlation with disease activity[33,34]. In addition, TNF-a and IL-5 are critical for the function of eosinophils including antigen presentation, cytokine or chemokine production, and secretion of granule mediators[42,73,74]. Furthermore, clinical studies of anti-IL-5 antibody (Ab), anti-IL-5 receptor (IL-5R) Ab have been performed for severe CRSwNP. Several placebo-controlled double-blind study of anti-IL-5 (mepolizumab) and anti-IL-5RA (benralizumab) demonstrated to decrease nasal polyps and to improve CT findings in patients with large nasal polyps, especially in ECRS[75-77]. Then, we observed that TNF-a stimulation significantly upregulated CD40 expression on eosinophils, which was further markedly enhanced by combined incubation with IL-5. However, TNF-a, IL-5, or TNF-a plus IL-5 stimulation feebly affected ICOSL expression on eosinophils, no significant difference was observed compared with that in control groups. These results indicated that TNF-a and IL-5 mainly affected the expression of CD40 on eosinophil. As for the expression of eosinophils derived ICOSL, the specific mechanism needs to be further explored in the future. For example, is there a synergistic effect of cytokines? Or other potential, unknown mediators?

Since previously, it has been described that p38 MAPK is activated in eosinophils by TNF-α[44,73]. Thus, in discerning the individual contributions of specific signaling pathways, we observed that inhibitor that target the pathway mediated by the p38 MAPK. The present study shows that the specific p38 MAPK inhibitor SB203580 could largely inhibit TNF-α and IL-5 induced CD40 expression on eosinophils. These data indicated the important role of the activation of p38 MAPK in the mechanism of TNF-α and IL-5 induced CD40 expression on eosinophils. Therefore, modulation of TNF-a/IL-5/CD40/p38 MAPK pathways might be useful for the treatment of CRS. Besides, we found that SB203580 did not fully inhibit the CD40 expression on eosinophils. These findings indicate that pathways other than p38 MAPK are also involved in TNF-a and IL-5 induced inhibition of CD40 expression on eosinophils. Since p38 MAPK is required for NF-kB-dependent gene expression and CD40 gene expression could partly mediated by NF-kB, it is reasonable that the inhibition of p38 MAPK can down-regulate the expression of CD40.[78-81]. Therefore, it may be possible that the inhibition of p38 MAPK by SB203580 can block TNF-a and IL-5 induced eosinophil-derived CD40 by indirect inhibiting NF-kB activity and subsequently suppress the eosinophil activation. Further investigation is required to explore other signaling pathways involved in TNF-a and IL-5 mediated modulation of CD40 expression on eosinophil.

The limitations of our study are its retrospective, cross-sectional design, the univariate and descriptive nature of the analyses performed, the lack of a large cohort of CRS patients, and the not yet identified relevant mechanisms underlying.

In summary, we observed that the high levels of CD40-CD40L, ICOS-ICOSL in local nasal tissues are closely associated with high eosinophils infiltration and high disease activity in CRS. We demonstrated a previously unrecognized role for CD40-CD40L and ICOS-ICOSL pathways, most remarkably in eosinophil activation of ECRS. Our data has shown that TNF-a and IL-5 mediate CD40 upregulation in human eosinophils in part via activation of p38 MAPK. In view of the above findings, we conclude that blocking of the activation of eosinophils by targeting CD40-CD40L and ICOS-ICOSL pathways, especially manipulation of TNF-a/p38 MAPK pathways targeting eosinophils activation might be useful for the treatment of CRS with high-level eosinophils.

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Tables and Figure legends

Table 1. Demographic and clinical profile of patients involved in the present study

	eCRS	**non-eCRS**
	10(9M/1F)	21(11M/10F)
Age(y, mean±std)	39±13	46±15
Patients with bilateral lesion, n (%)	5(50%)	10(45%)
Lund-Mackay score (mean±std)	13±4	9±4
Eosinophils in PB (10^9/L, mean±std)	0.37±0.25	0.10±0.08
Histological pattern		
edematous	3(30%)	1(5%)
fibrotic	0(0%)	7(33%)
hyperplasia	0	8(38%)
atypical	0	0
edematous+fibrotic	4(40%)	3(14%)
edematous+hyperplasia	3(30%)	2(10%)
CRSwNP, n (%)	9(100%)	17(81%)
Comorbidity*		
atopy	3/7(43%)	5/17(29%)
asthma	1/7(14%)	0/17(0)
aspirin intolerance	1/7(14%)	1/17(6%)

ECRS , eosinophilic chronic rhinosinusitis;

Non-eCRS , non eosinophilic chronic rhinosinusitis;

M , male; **F** , female; **std** , standard deviation;

CRSwNP , chronic rhinosinusitis with nasal polyp.

***** : missing of clinical data

Table 2. Unpaired T-test between Non-eCRS and ECRS patients

	Non-eCRS	**ECRS**	**P value**
Lund-Mackay score	9.18±4.32	12.60±4.09	0.053
Blood neutrophil count (10^9/L)	4.20±1.30	4.45±1.62	0.650
Blood basophil count (10^9/L)	0.03±0.02	0.04±0.02	0.279

Values were expressed as mean±standard deviation

Table 3. Correlation analysis of CD40-CD40 and ICOS-ICOSL expression and clinical parameters in CRS patients

Parameter1	**Parameter1**	**Parameter2**	**R value**	**P value**
Lund-Mackay score	Tissue eosinophil count / HPF	Tissue eosinophil count / HPF	0.3042	0.1229
$CD40^+$ cells / HPF	Lund-Mackay score	Lund-Mackay score	0.2300	0.2795
$CD40^+$ cells / HPF	Blood neutrophil count (10^9/L)	Blood neutrophil count (10^9/L)	-0.2830	0.1445
$CD40^+$ cells / HPF	Blood basophil count (10^9/L)	Blood basophil count (10^9/L)	0.0581	0.7689
$CD40L^+$ cells / HPF	Lund-Mackay score	Lund-Mackay score	0.1168	0.6048
$CD40L^+$ cells / HPF	Blood neutrophil count (10^9/L)	Blood neutrophil count (10^9/L)	0.1431	0.4951
$CD40L^+$ cells / HPF	Blood basophil count (10^9/L)	Blood basophil count (10^9/L)	0.0376	0.8584
$ICOS^+$ cells / HPF	Blood neutrophil count (10^9/L)	Blood neutrophil count (10^9/L)	-0.2859	0.2090
$ICOS^+$ cells / HPF	Blood basophil count (10^9/L)	Blood basophil count (10^9/L)	0.2881	0.2054
$ICOSL^+$ cells / HPF	Blood neutrophil count (10^9/L)	Blood neutrophil count (10^9/L)	-0.0907	0.6529

Figure 1 The expression of CD40, CD40L as well as ICOS, ICOSL in nasal tissues of ECRS and Non-eCRS patients

(A). The representative IHC stainings of CD40, CD40L, ICOS, and ICOSL. Original magnification x400. (B). The mean numbers of $CD40^+$, $CD40L^+$, $ICOS^+$, and $ICOSL^+$ cells in nasal tissues.

Figure 2 The correlation among the levels of CD40, CD40L, ICOS, and ICOSL in nasal tissues of patients with CRS

Figure 3 The expression of CD40-CD40L and ICOS-ICOSL in different histological patterns of CRS patients

The percentage of six different histological patterns (A) and three patterns (B) (edematous: edematous, edematous + fibrotic, edematous + hyperplasia; fibrotic: fibrotic and edematous + fibrotic; hyperplasia: hyperplasia and edematous + hyperplasia) in ECRS and Non-eCRS. (C-F). Expression levels of ICOSL, ICOS, CD40 and CD40L in nasal tissues of edematous and non-edematous CRS patients.

Figure 4 The correlation among the levels of CD40, CD40L, ICOS and ICOSL nasal polyp of edematous CRS patients

Figure 5 Association between levels of CD40-CD40L, ICOS-ICOSL and eosinophil in nasal tissues of CRS

The correlation analysis between the number of $CD40^+$, $CD40L^+$, $ICOS^+$,

$ICOSL^+$ cells and tissue eosinophils levels in CRS. (B-C) The co-location of eosinophils (PRG2, Green) and CD40 (Red) as well as ICOSL (Red) assessed by immunofluorescence in patients with ECRS. Original magnification x400.

Figure 6 The correlation between levels of nasal tissue CD40-CD40L and ICOS-ICOSL and blood eosinophil count (A-D) as well as Lund-Mackay score (E-F)

Figure 7 The effect of CD40-CD40L and ICOS-ICOSL pathways on eosinophil activation

Peripheral blood of ECRS patients was stimulated with medium alone (Control, circle), IgG (5ug/mL, rectangle), rhCD40L (5ug/mL, triangle) or rhICOS (10ug/mL, triangle) for 24h. Then CD69 expression on eosinophils was detected by Flow cytometry. (A). The gating strategy of activated eosinophils ($CD45^+CD16^-CD69^+$). (B). The percentage of activated eosinophils after different stimulation.

Φιγυρε 8 Τηε εφφεςτ οφ ΤΝΦ-α ανδ ΙΛ-5 ον ̃Δ40 εξπρεσσιον οφ πυριφιεδ εοσινοπηιλς

GM-CSF (50 ng/mL) was added as a basic condition (medium) to keep eosinophils culture for all groups. Freshly isolated eosinophils from healthy controls were cultured with medium alone, or rhTNF-α (50 ng/mL), or rhIL-5 (50 ng/mL) or rhTNF-α plus rhIL-5 in the absence or presence of specific p38 inhibitor SB203580 (10 uM) for 24h and 48h. And SB203580 was preincubated with eosinophils for 1h before cytokines stimulation. CD40 expression was subsequently detected by Flow cytometry.

Supplementary figure 1 Representative eosinophilic subtypes and histological patterns in nasal tissues of CRS patients

(A). Eosinophils infiltration in nasal tissue of ECRS and Non-eCRS. Eosinophils were in the bottom of right panel. (B). Different histological change in nasal tissue of 4 patterns: edematous CRS with a great number of eosinophils (thick arrow), goblet cell hyperplasia (triangle), thickening of the basement membrane (arrow), and the loose stroma contains pseudocystic spaces filled with fluid (star); CRS with hyperplasia of seromucinous glands (arrow); Fibroinflammatory CRS with evident dilated vessels(star) and a great number of fibrocytes (arrow); Atypical CRS with bizarre cells in stroma. The nuclei of these "atypical" cells often tend to be hyperchromatic (arrow). Original magnification x200.

Συππλεμενταρψ φιγυρε 2 Τηε εφφεςτ οφ ΤΝΦ-α ανδ ΙΛ-5 ον ΙC̃ΟΣΛ εξπρεσσιον οφ πυριφιεδ εοσινοπηιλς

(A). The purity of isolated eosinophils verified by Flow cytometry (left panel) and Wright-Giemsa staining (right panel, x 1000). (B). The expression level of CD40 and ICOSL on purified eosinophils at 0h accessed by flow cytometry. (C). The effect of TNF-α and IL-5 on ICOSL expression of eosinophils isolated from healthy donators. GM-CSF (50 ng/mL) stimulation used as basic condition (medium) for all groups. Freshly isolated eosinophils were cultured with medium alone, or rhTNF-α (50 ng/mL), or rhIL-5 (50 ng/mL) or rhTNF-α plus rhIL-5. ICOSL expression was then detected by Flow cytometry.

Supplementary figure 3

(A). Correlation of blood eosinophil count with ECRS/Non-eCRS subset. (B). The association between blood eosinophil count and Lund-Mackay score. (C). Correlation between tissue eosinophil count and blood eosinophil count. (D) The correlation between blood basophil count and the levels of ICOSL in nasal tissue of CRS patients.

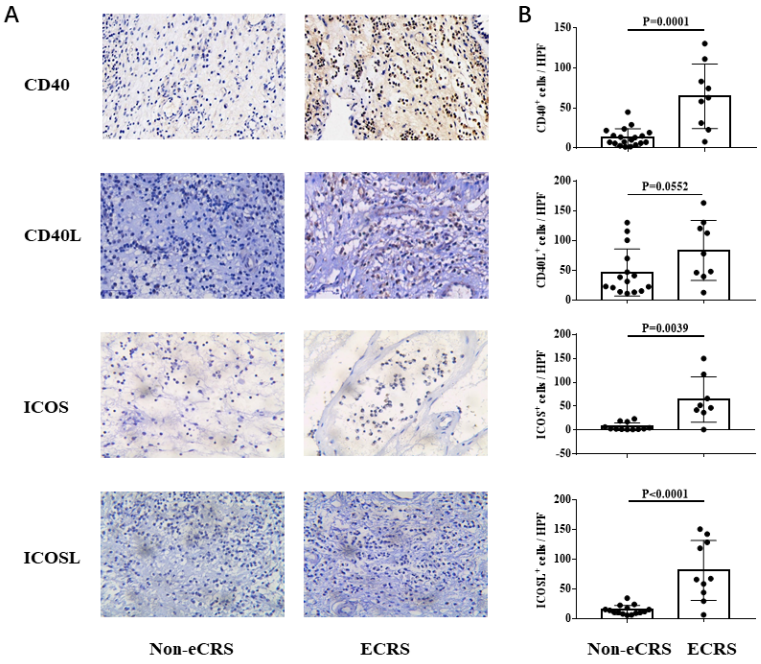
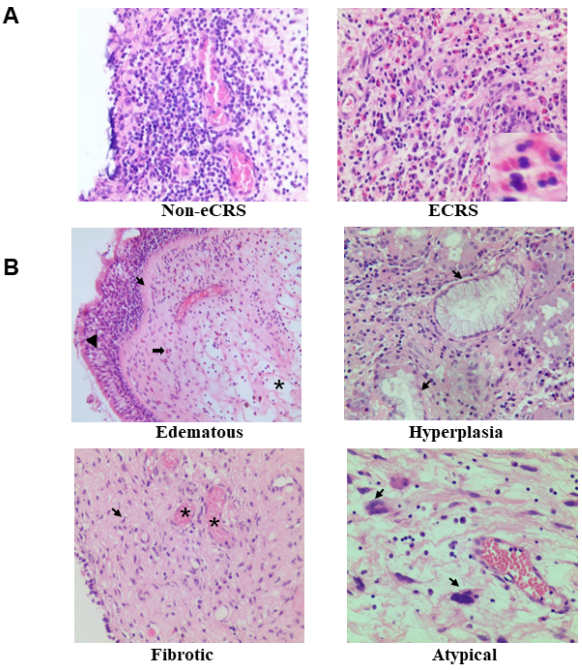


Figure 1



Supplementary figure 1

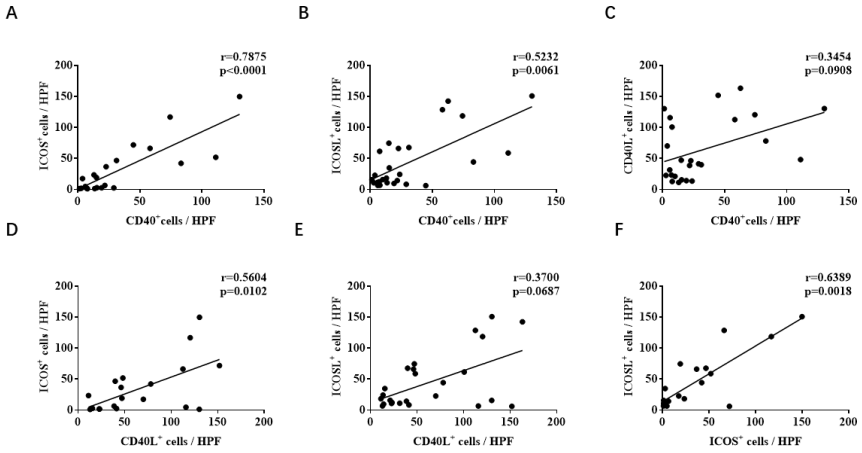
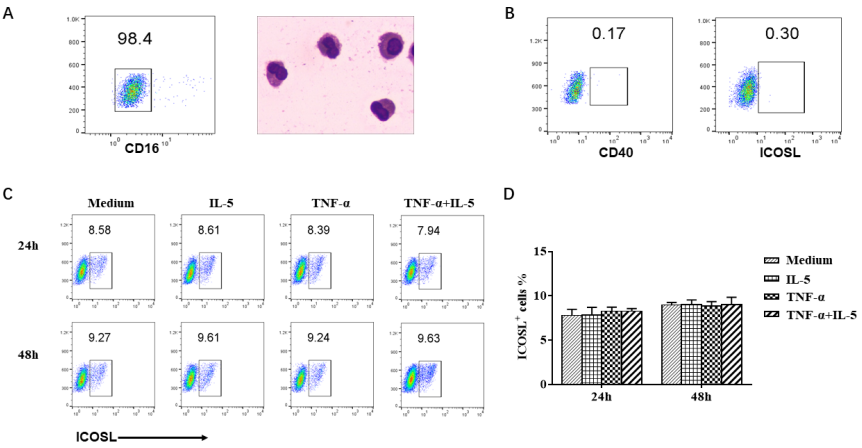


Figure 2



Supplementary figure 2

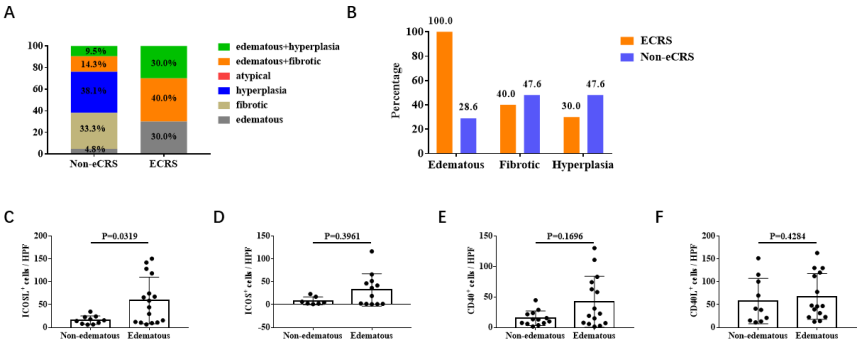
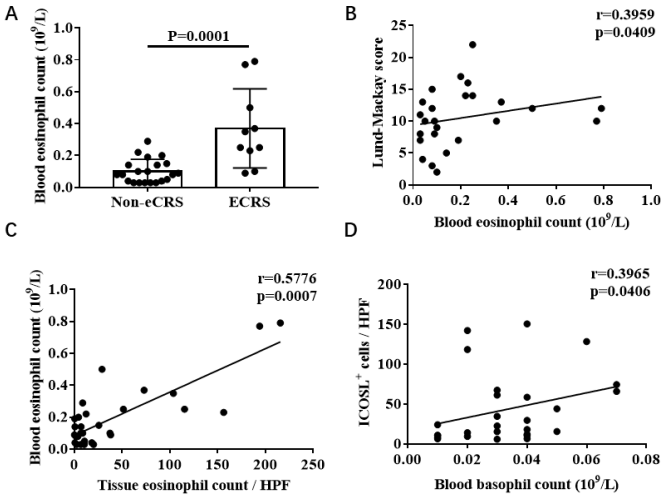


Figure 3



Supplementary figure 3

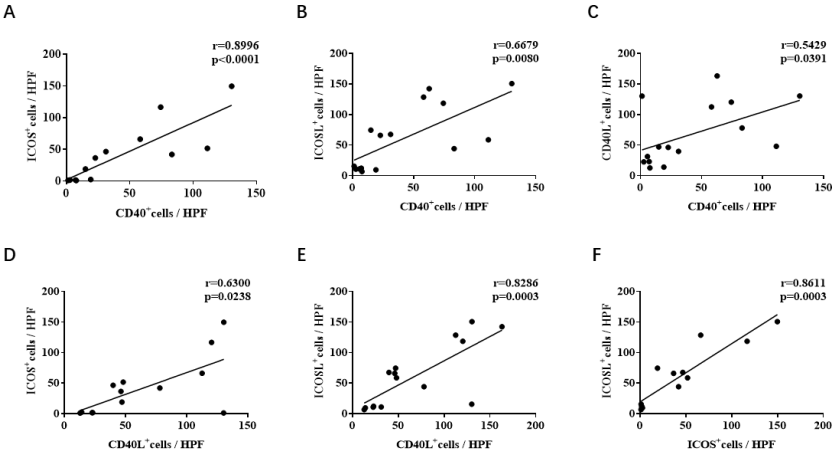


Figure 4

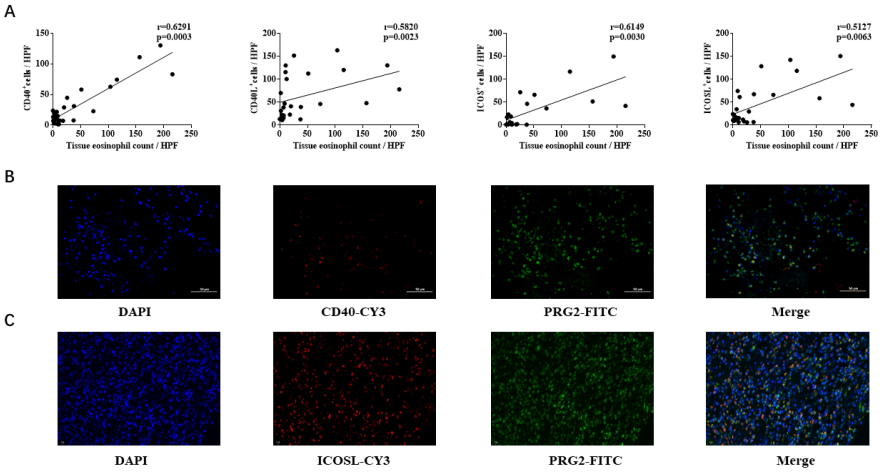


Figure 5

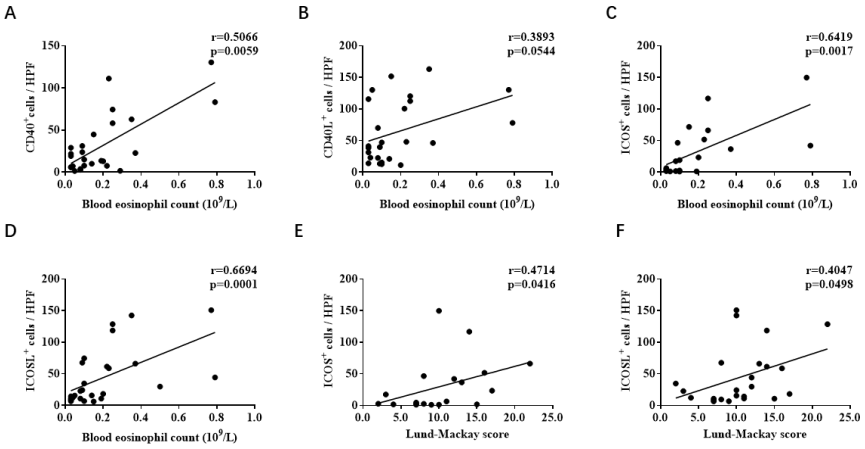


Figure 6

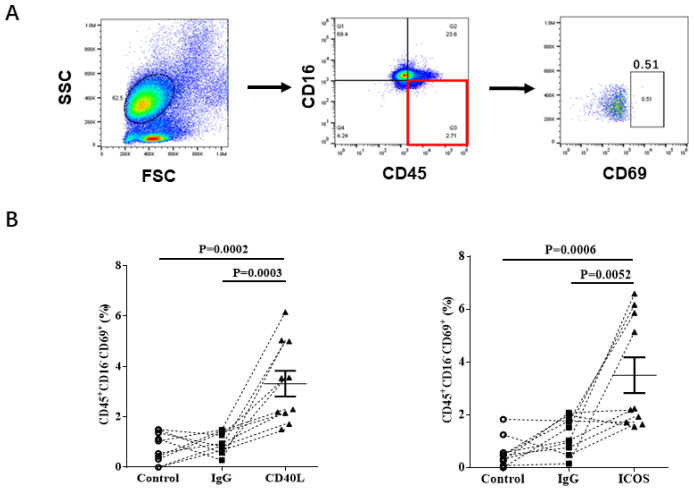


Figure 7

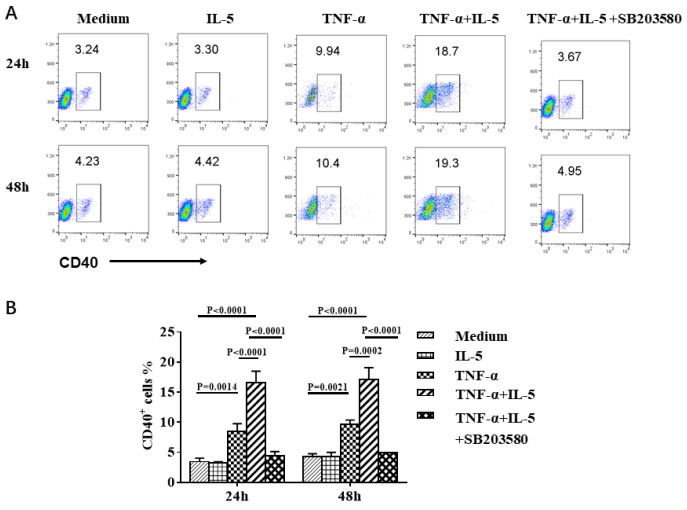


Figure 8

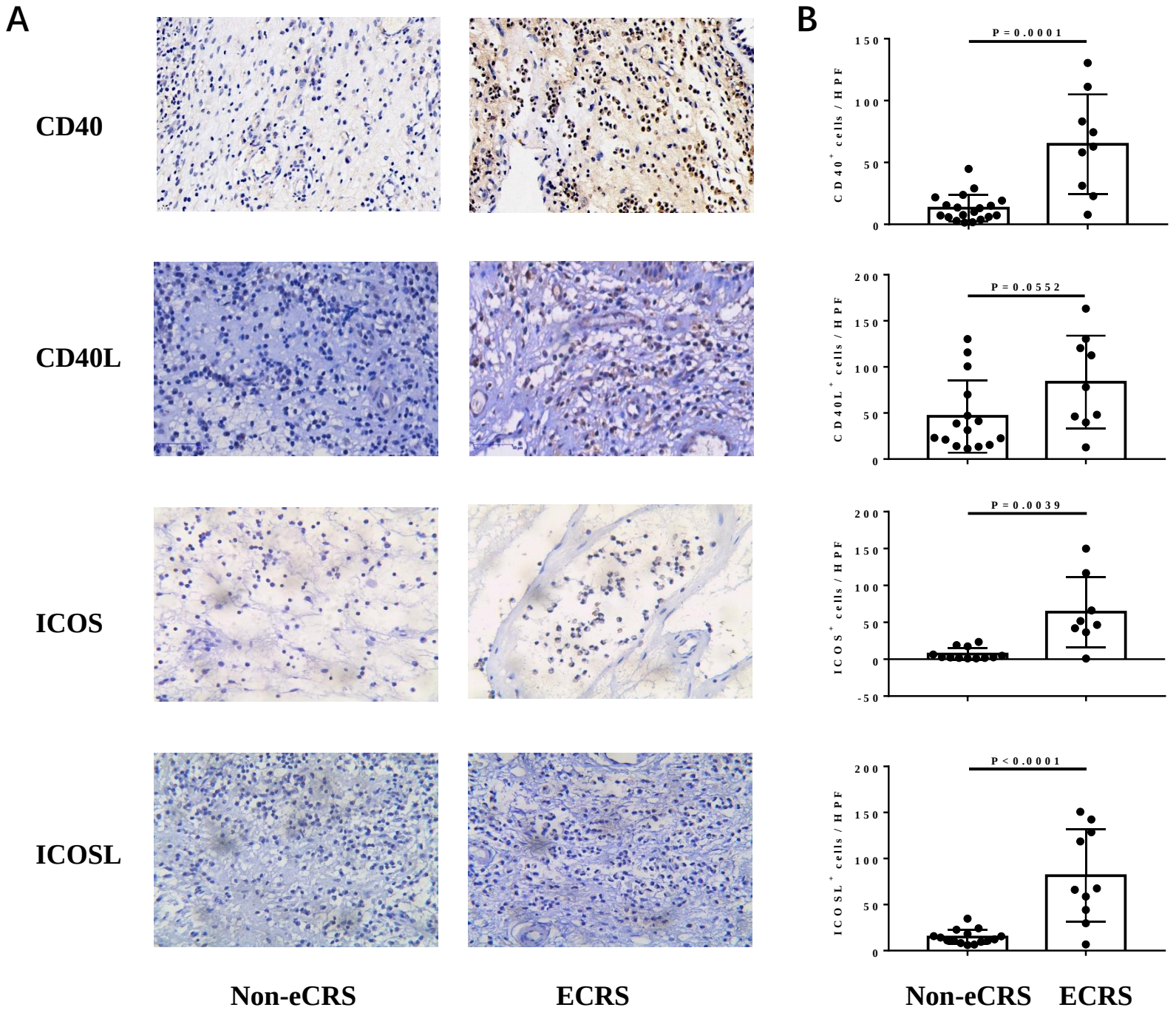

A
CD40
CD40L
ICOS
ICOSL
B
P = 0.0001
CD40+ cells / HPF
P = 0.0552
CD40L+ cells / HPF
P = 0.0039
ICOS+ cells / HPF
P < 0.0001
ICOSL+ cells / HPF
Non-eCRS
ECRS
Non-eCRS
ECRS

Figure 1

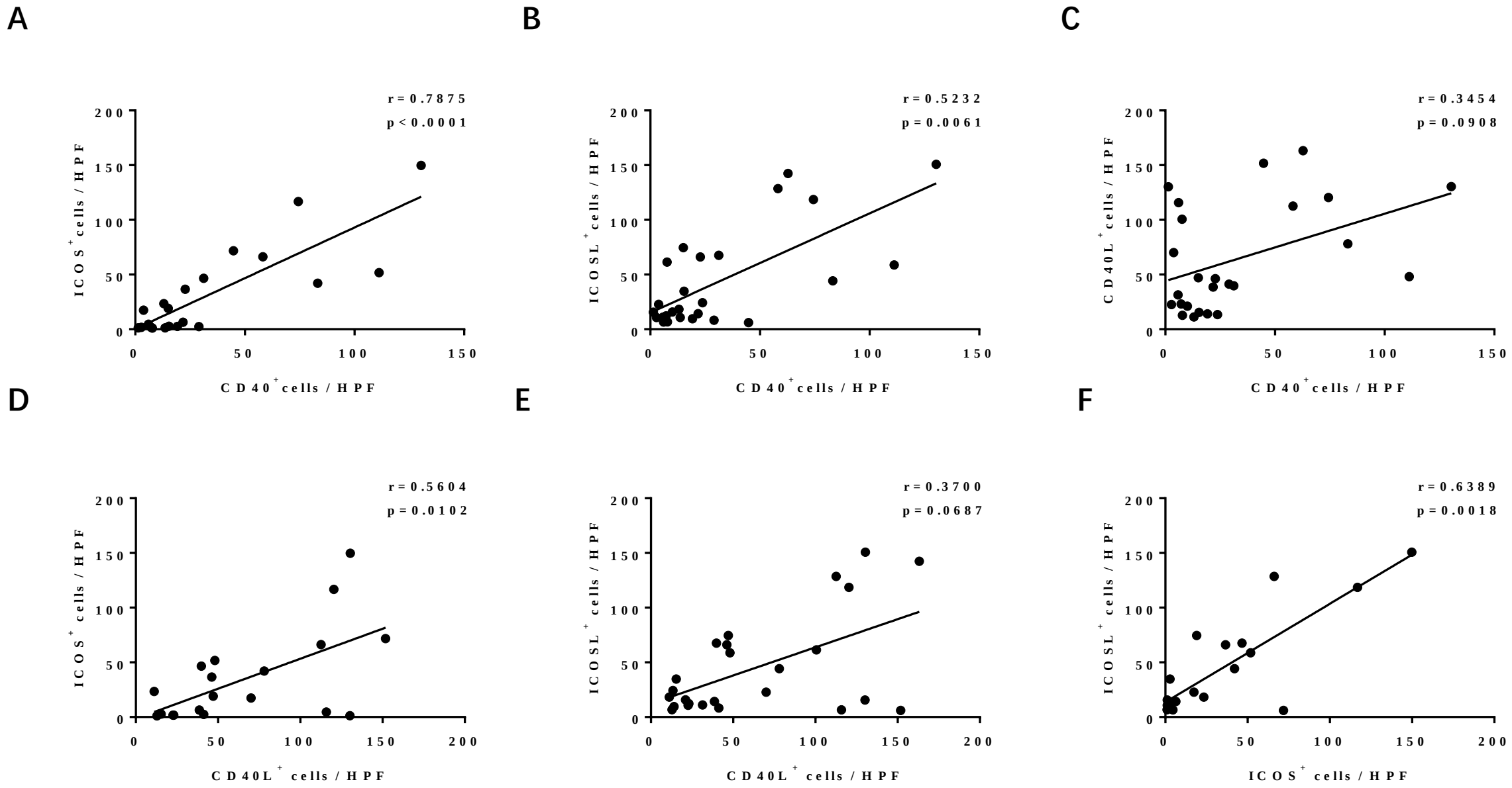

A
r = 0.7875
p < 0.0001
ICOS+ cells / HPF
CD40+ cells / HPF
B
r = 0.5232
p = 0.0061
ICOSL+ cells / HPF
CD40+ cells / HPF
C
r = 0.3454
p = 0.0908
CD40L+ cells / HPF
CD40+ cells / HPF
D
r = 0.5604
p = 0.0102
ICOS+ cells / HPF
CD40L+ cells / HPF
E
r = 0.3700
p = 0.0687
ICOSL+ cells / HPF
CD40L+ cells / HPF
F
r = 0.6389
p = 0.0018
ICOSL+ cells / HPF
ICOS+ cells / HPF

Figure 2

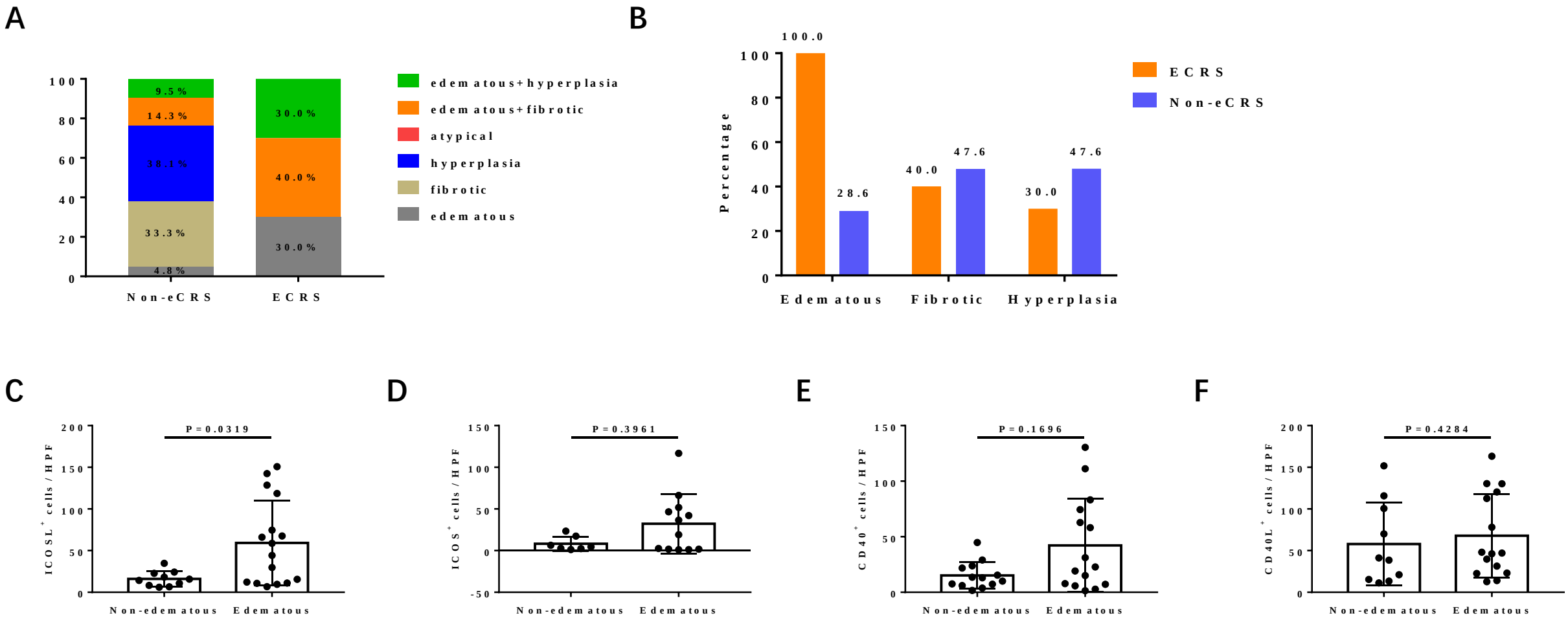

A
100
80
60
40
20
0
9.5%
14.3%
38.1%
33.3%
4.8%
30.0%
40.0%
30.0%
Non-eCRS
ECRS
edematous+hyperplasia
edematous+fibrotic
atypical
hyperplasia
fibrotic
edematous
B
Percentage
100.0
28.6
40.0
47.6
30.0
47.6
Edematous
Fibrotic
Hyperplasia
ECRS
Non-eCRS
C
ICOSL+ cells / HPF
P=0.0319
Non-edematous
Edematous
D
ICOS+ cells / HPF
P=0.3961
Non-edematous
Edematous
E
CD40+ cells / HPF
P=0.1696
Non-edematous
Edematous
F
CD40L+ cells / HPF
P=0.4284
Non-edematous
Edematous

Figure 3

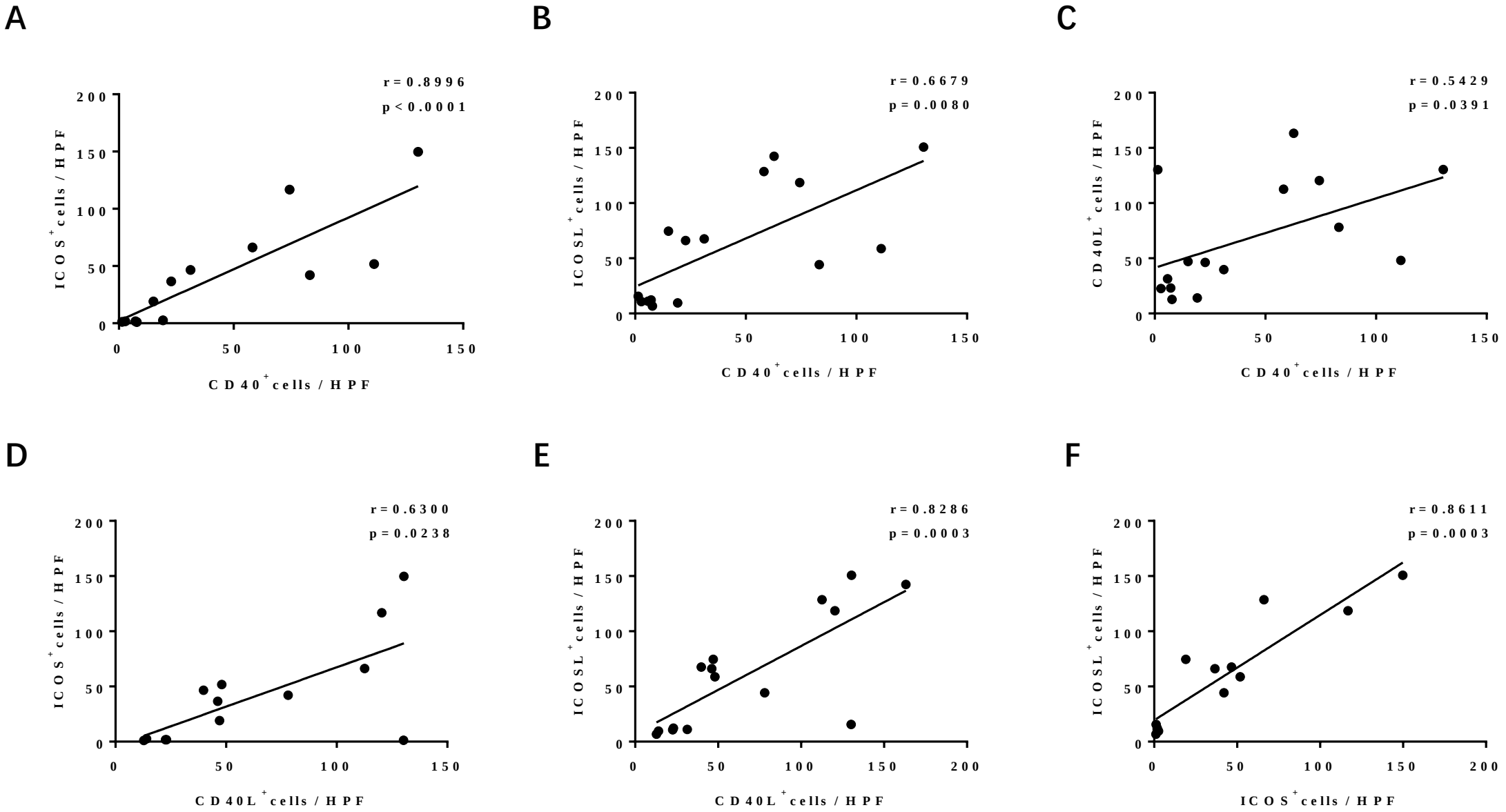
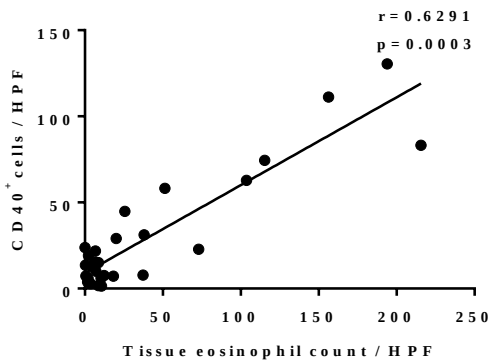
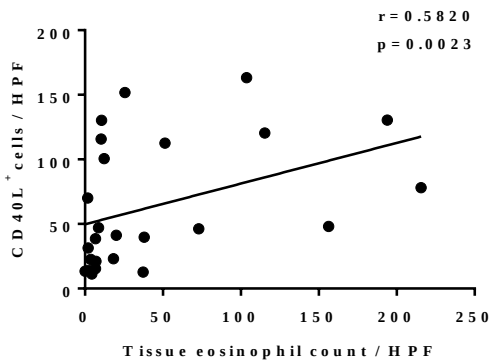
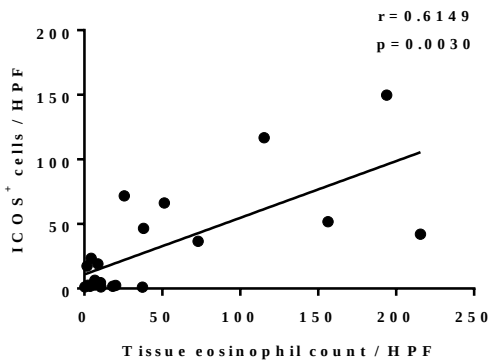

A
r = 0.8996
p < 0.0001
ICOS+cells / HPF
CD40+cells / HPF
B
r = 0.6679
p = 0.0080
ICOSL+cells / HPF
CD40+cells / HPF
C
r = 0.5429
p = 0.0391
CD40L+cells / HPF
CD40+cells / HPF
D
r = 0.6300
p = 0.0238
ICOS+cells / HPF
CD40L+cells / HPF
E
r = 0.8286
p = 0.0003
ICOSL+cells / HPF
CD40L+cells / HPF
F
r = 0.8611
p = 0.0003
ICOSL+cells / HPF
ICOS+cells / HPF

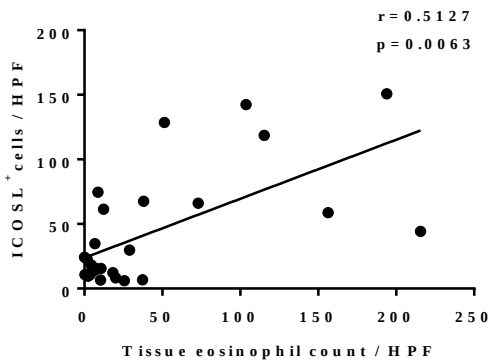
Figure 4

A

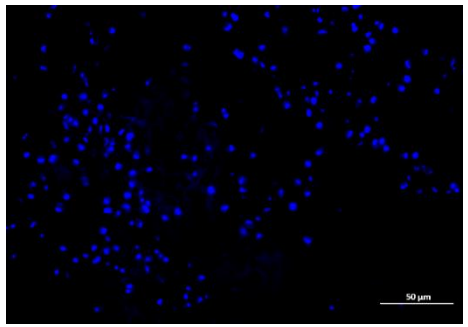




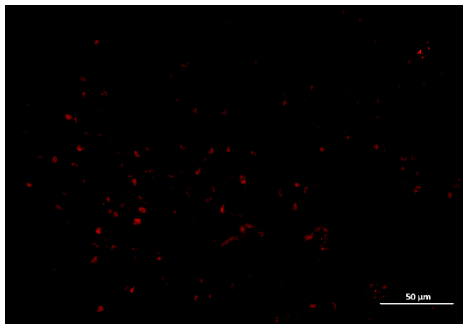




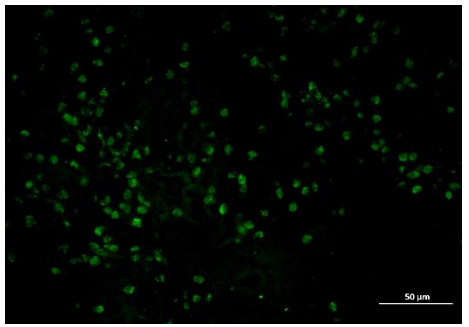
B



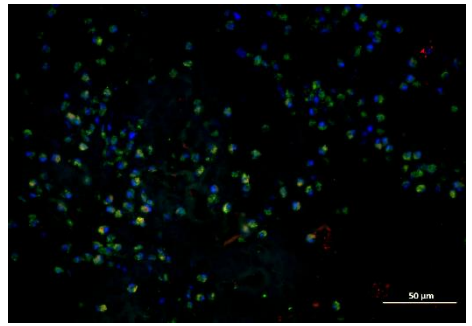
DAPI



CD40-CY3

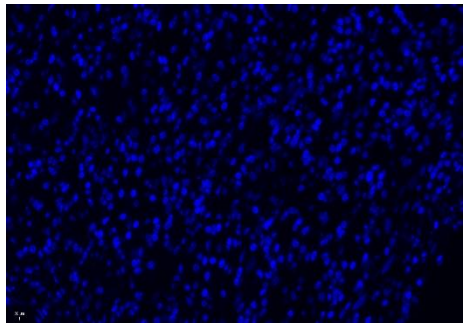


PRG2-FITC

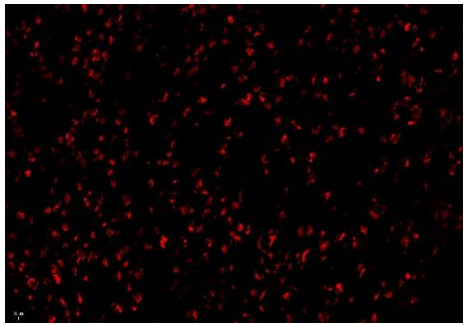


Merge

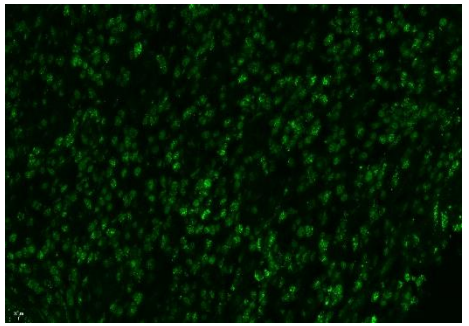
C



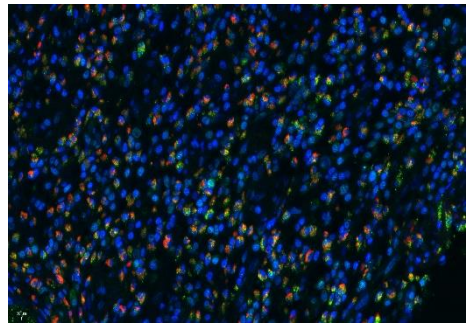
DAPI



ICOSL-CY3



PRG2-FITC



Merge

Figure 5

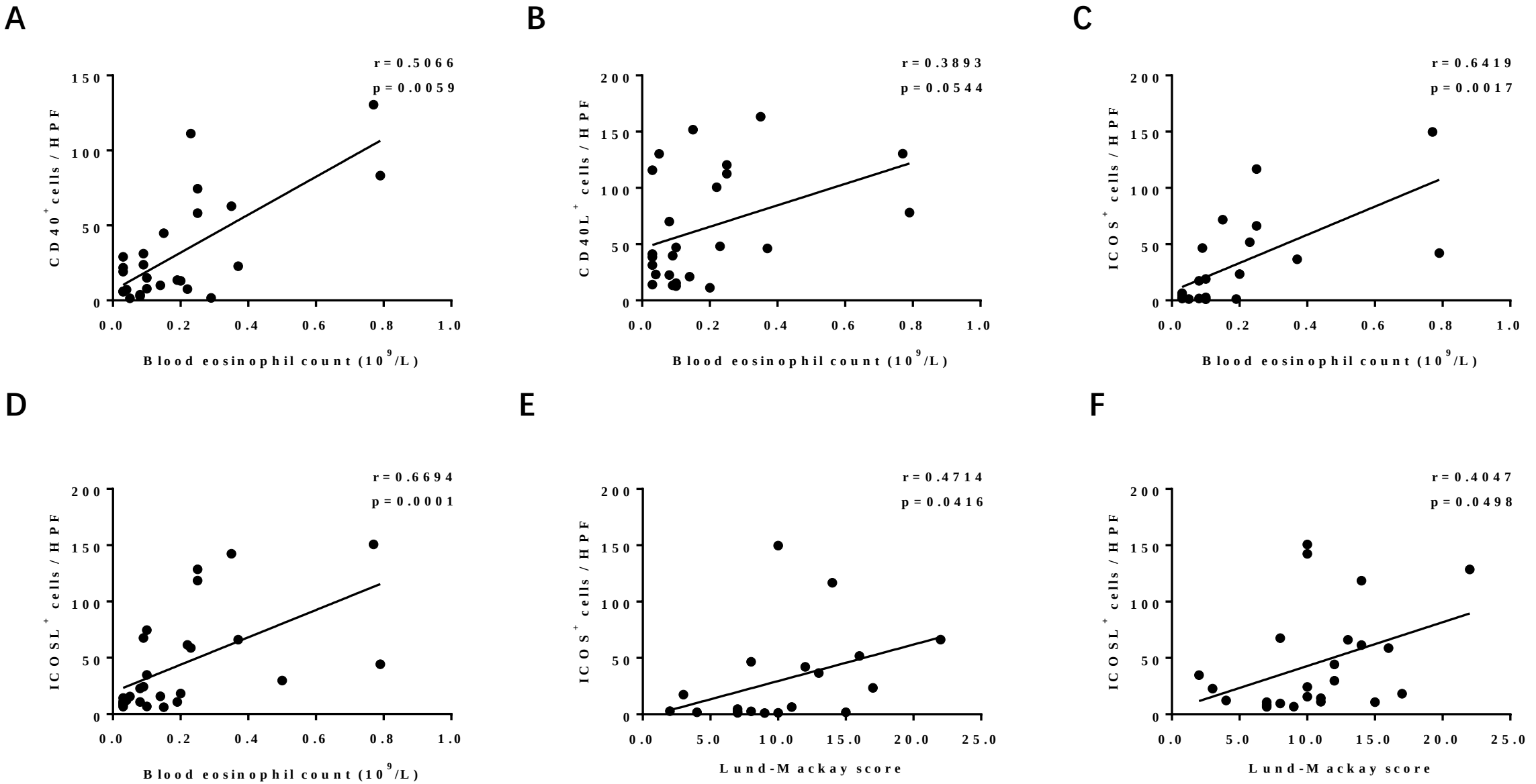
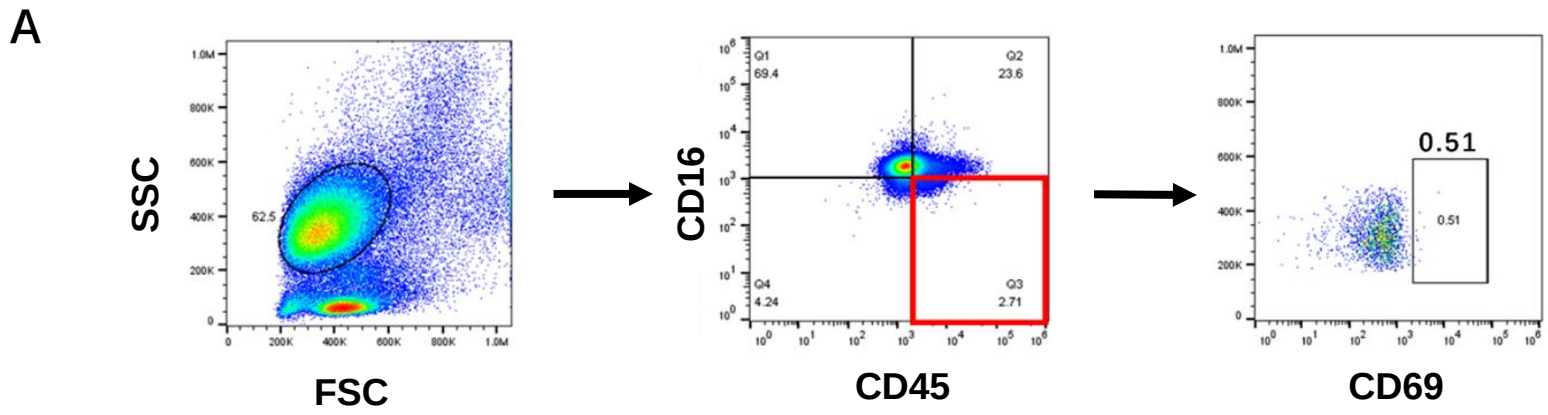

A
r = 0.5066
p = 0.0059
CD40+ cells / HPF
Blood eosinophil count (10^9/L)
B
r = 0.3893
p = 0.0544
CD40L+ cells / HPF
Blood eosinophil count (10^9/L)
C
r = 0.6419
p = 0.0017
ICOS+ cells / HPF
Blood eosinophil count (10^9/L)
D
r = 0.6694
p = 0.0001
ICOSL+ cells / HPF
Blood eosinophil count (10^9/L)
E
r = 0.4714
p = 0.0416
ICOS+ cells / HPF
Lund-Mackay score
F
r = 0.4047
p = 0.0498
ICOSL+ cells / HPF
Lund-Mackay score

Figure 6


A
SSC
FSC
62.5
CD16
CD45
Q1
69.4
Q2
23.6
Q4
4.24
Q3
2.71
CD69
0.51
0.51

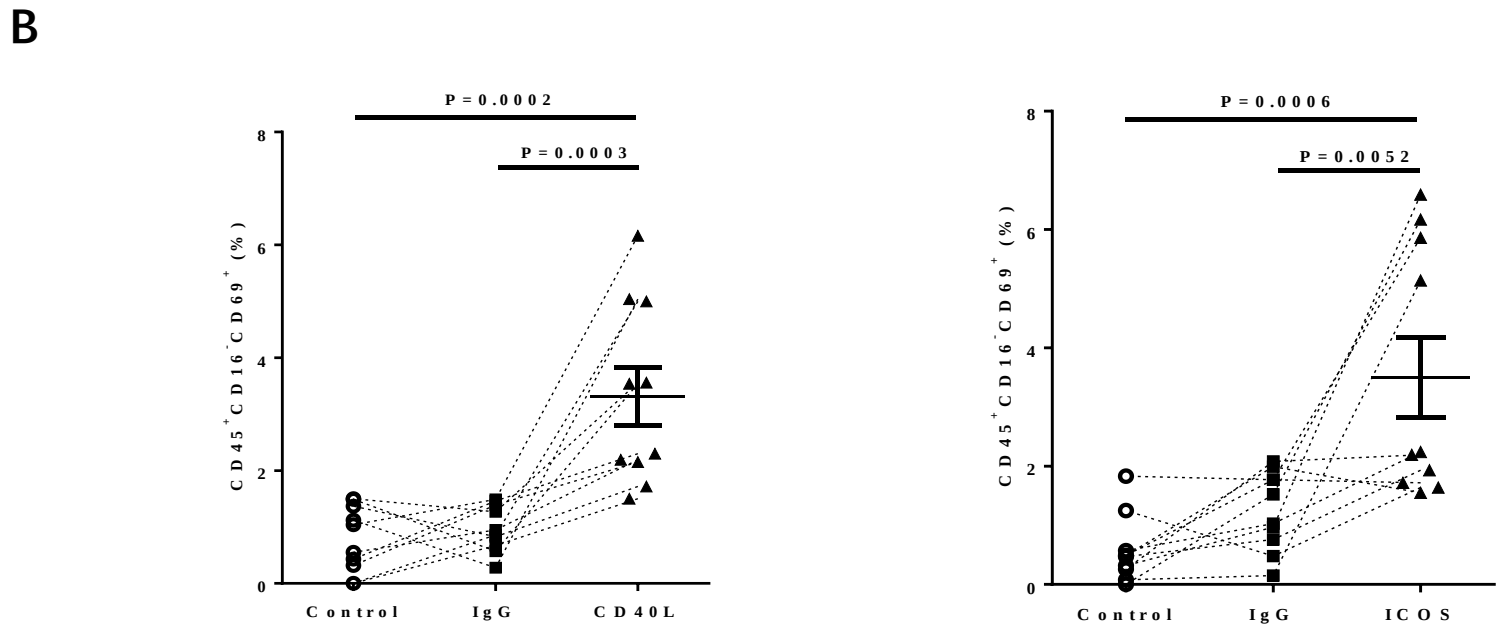

B
P = 0.0002
P = 0.0003
CD45+CD16-CD69+ (%)
Control
IgG
CD40L
P = 0.0006
P = 0.0052
CD45+CD16-CD69+ (%)
Control
IgG
ICOS

Figure 7

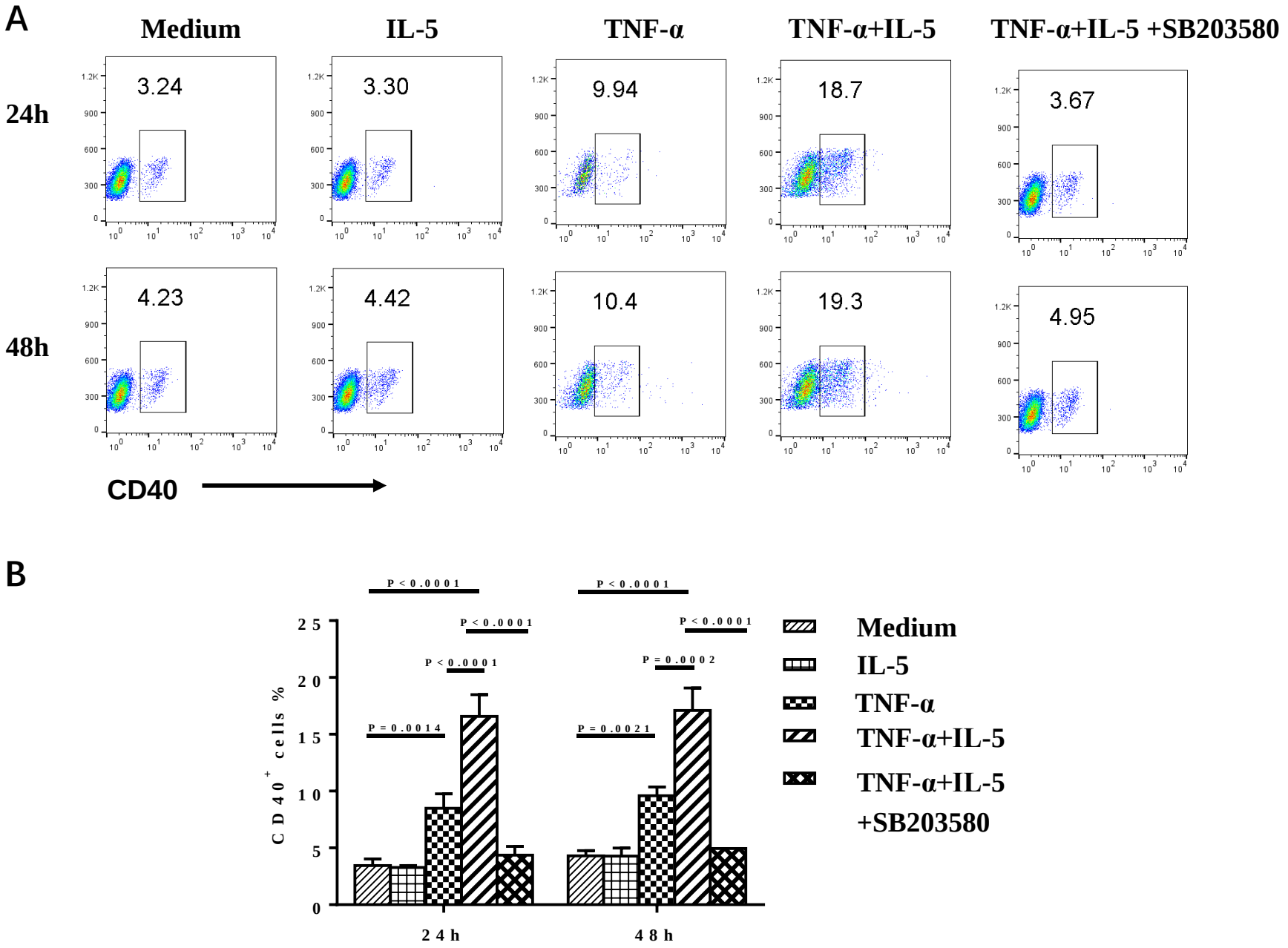
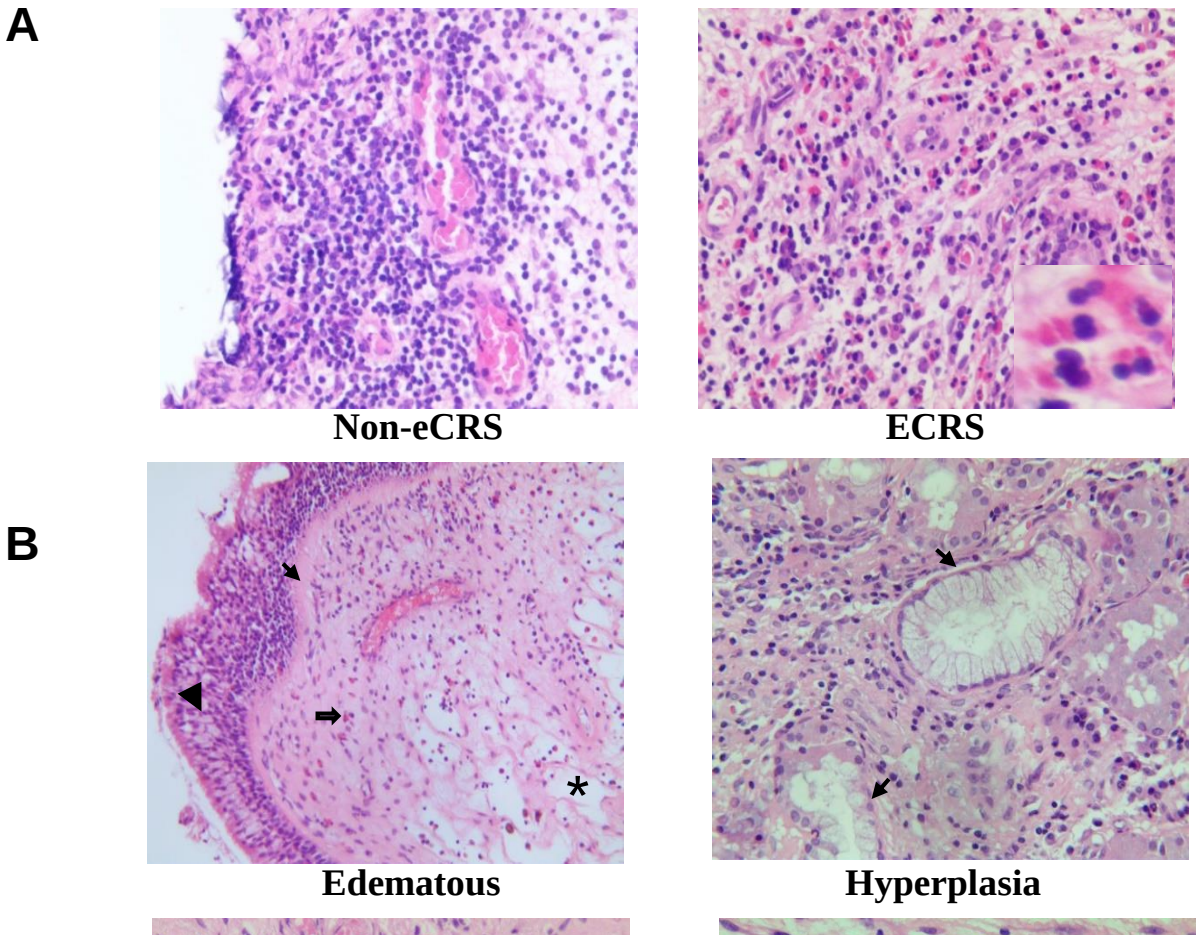
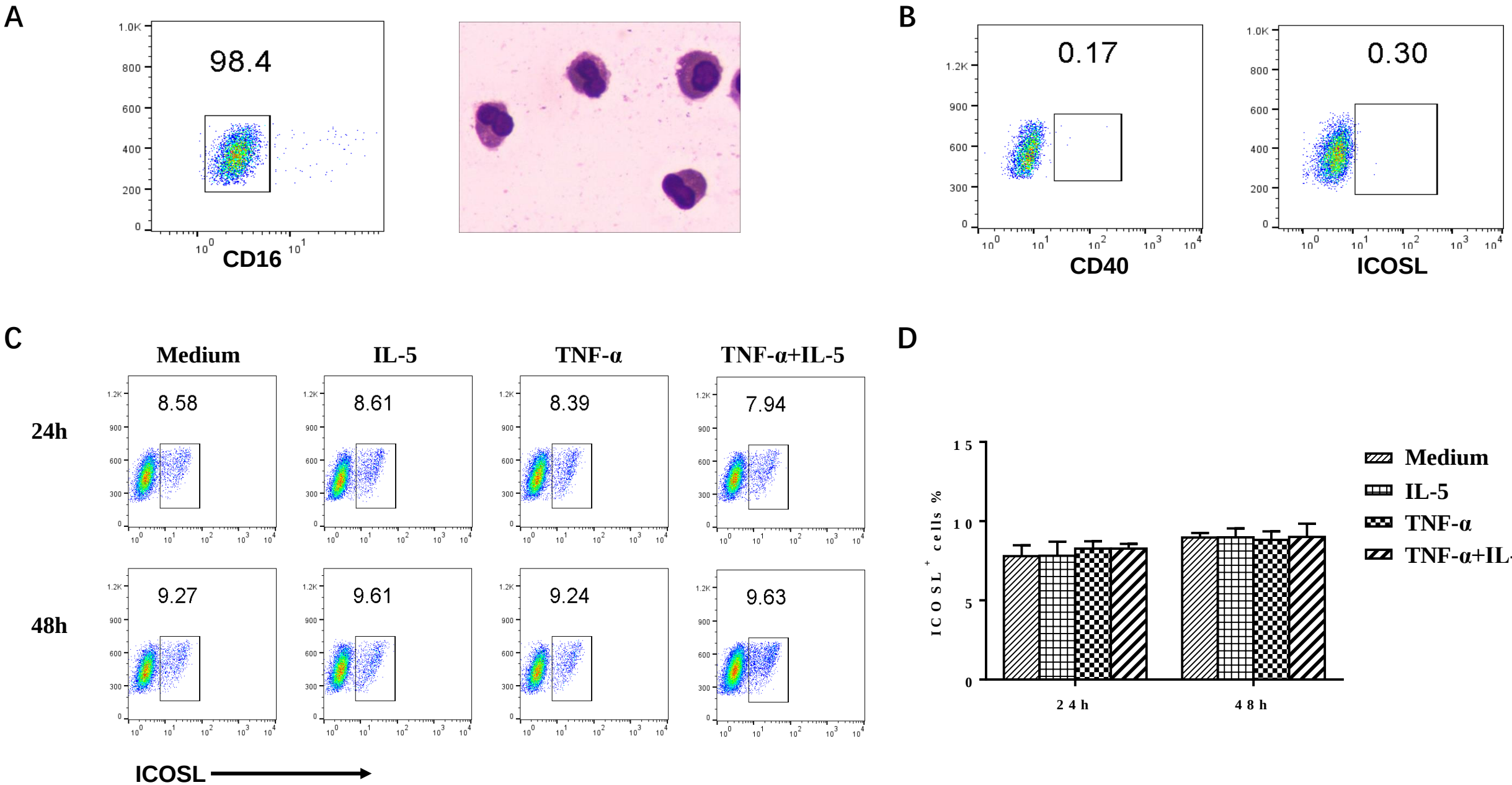

A
Medium
IL-5
TNF-α
TNF-α+IL-5
TNF-α+IL-5 +SB203580
24h
48h
3.24
3.30
9.94
18.7
3.67
4.23
4.42
10.4
19.3
4.95
CD40
B
P < 0.0001
P < 0.0001
P < 0.0001
P < 0.0001
P = 0.0014
P = 0.0021
P = 0.0002
CD40+ cells %
25
20
15
10
5
0
24 h
48 h
Medium
IL-5
TNF-α
TNF-α+IL-5
TNF-α+IL-5 +SB203580

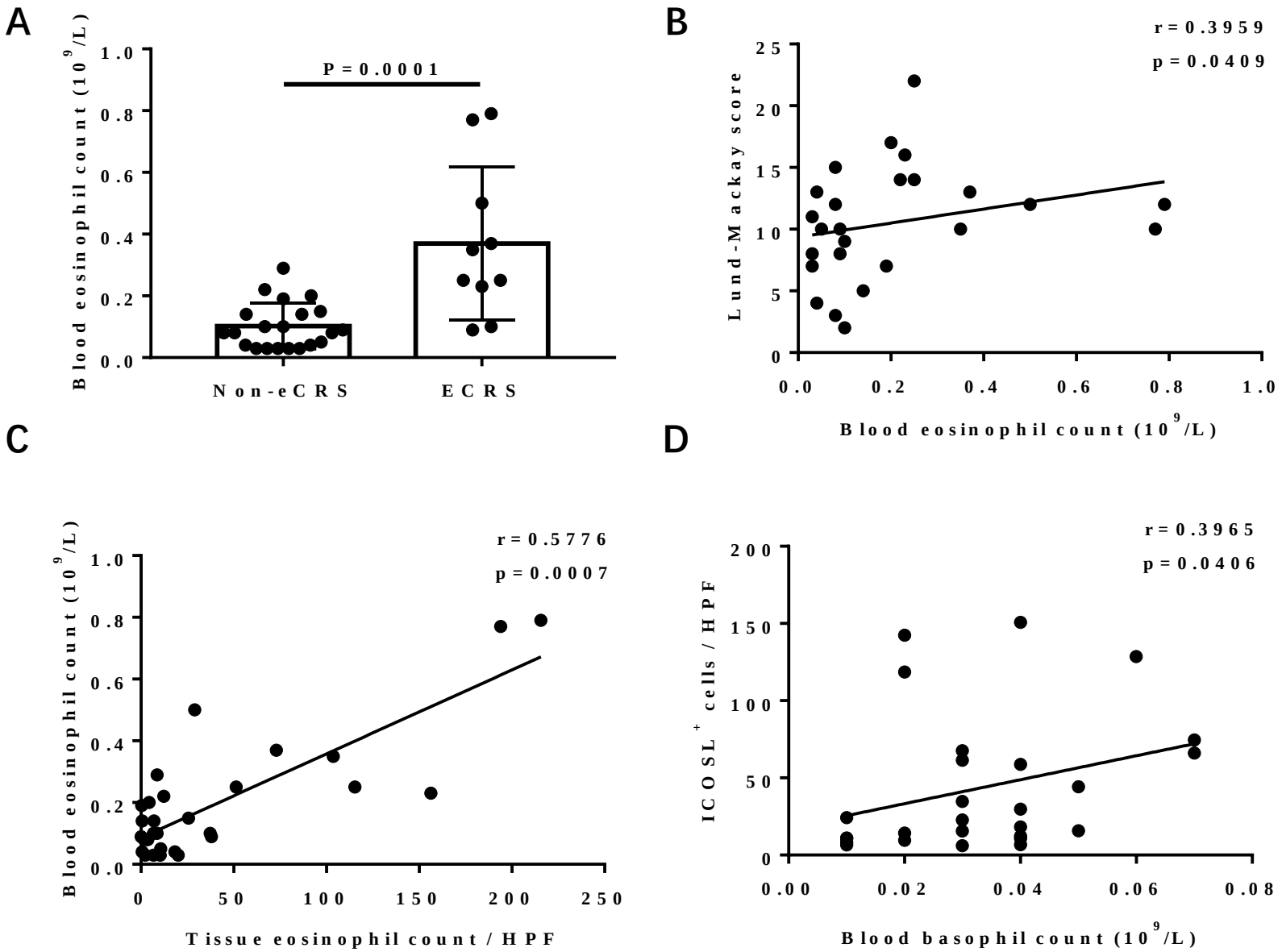
Figure 8



Supplementary figure 1



Supplementary figure 2



Supplementary figure 3