Use of Ultivue InSituPlex® Multiplex Immunofluorescence to Localize and Quantify Regulatory T Lymphocytes in Crohn's Disease and Ulcerative Colitis

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SCIENCE FOR LIFE-CHANGING IMPACT

Abstract

The inflammatory bowel diseases ulcerative colitis (UC) and Crohn's disease (CD) are chronic, relapsing inflammatory disorders of the gastrointestinal tract (GIT) that affect millions of individuals worldwide.[1] The pathogenesis of these disorders is thought to involve dysregulation of mucosal immune homeostasis in the GIT in response to environmental factors in genetically susceptible individuals.[2]

Regulatory T cells (Treg) are $CD4^+$ T lymphocytes that play a central role in peripheral immune tolerance, actively inhibiting inflammation upon antigenic stimulation. There are two major populations of Treg: conventional Treg and TR1 cells.[3] Conventional Treg arise from the thymus (tTreg) or can be induced in the periphery (pTreg). Both tTreg and pTreg constitutively express FoxP3 and CD25 (IL-2Rα).

An imbalance in conventional Treg and effector T cells in the GIT microenvironment is thought to play a part in the pathogenesis of inflammatory bowel disease (IBD).[4] Thus, we sought to quantify conventional Treg and CTL populations in GIT tissue sections from IBD patients versus normal individuals by multiplex immunofluorescence.

Conventional Treg are typically defined as lymphocytes with a $CD3^+/CD4^+/CD25^+/FoxP3^+$ immuno-phenotype. This complex antigenic signature has made it difficult to definitively label Treg populations in tissue sections by immunohistochemistry. In this study we combined a 5-plex (CD3, CD4, CD8α, CD25, FoxP3) immunofluorescence assay using Ultivue InSituPlex® multiplex technology with image analysis using Indica Labs Halo™ software to identify, localize and enumerate: 1) total $CD3^+$ T cells, 2) $CD8\alpha^+$ cytotoxic T lymphocytes (CTL) and 3) $CD3^+/CD4^+/CD25^+/FoxP3^+$ conventional Treg in GIT sections from patients with UC and CD versus controls. Using this approach, we were able to definitively identify and enumerate these immune cell populations on single tissue sections from each specimen.

We found no differences in the frequencies of Treg or CTL in colon from CD and UC patients versus controls; however, higher frequencies of Treg and lower frequencies of CTL were measured in small intestine from patients with CD versus controls.

Methods: Study Design

Tissue Specimens

Formalin-fixed, paraffin-embedded (FFPE) blocks of IBD and normal GIT tissues were obtained from the National Disease Research Interchange (NDRI) according to prospectively established protocols. IBD tissues (colon from UC patients and colon and small intestine (SI) from CD patients) were procured from patients with UC or CD who had undergone therapeutic bowel resection. Normal tissues were acquired from the uninvolved margins of colon or SI tumor resections. Tissues were immersion fixed in 10% neutral buffered formalin before processing by routine histological methods to paraffin blocks.

Study Cohort (Table 1)

- **CD: 15 patients** (10 colon; 5 SI)
  - CD (colon)
    - 8 F (22-61 yrs of age; median = 39.5 yrs)
    - 2 M (aged 22 and 32 yrs)
    - Total: n=10 (22-61 yrs of age; median = 33 yrs)
  - CD (SI)
    - 4 F (18-67 yrs of age; median = 27 yrs)
    - 1 M (63 yrs of age)
    - Total: n=5 (18-67 yrs of age; median = 29 yrs)
  - CD (all specimens): n=15 (18-67 yrs; median = 32 yrs)
- **UC: 11 patients**
  - 5 F (22 to 58 yrs of age; median = 32.5 yrs)
  - 7 M (30 to 63 yrs of age; median = 43 yrs)
  - Total: n=11 (22-63 yrs of age; median = 41 yrs)
- **Controls: 21 specimens**
  - Colon (n=16; 39 to 69 yrs, median = 56.5 yrs)
    - 8 F (40 to 69 yrs of age; median = 58.5 yrs)
    - 8 M (39 to 62 yrs of age; median = 52.5 yrs)
  - SI (n=5; median = 40 to 62 yrs, median = 50 yrs)
    - 3 F (43 to 62 yrs of age; median = 57 yrs)
    - 2 M (aged 40 and 50 yrs)
  - All specimens: n=21 (39 to 69 yrs; median = 56 yrs)

Methods: Cohort Demographics

TABLE 1: Cohort Demographics

Normal Tissue Controls			Crohn's Disease Specimens				Ulcerative Colitis Specimens			
Tissue	Age (yrs)	Sex	Tissue	Age (yrs)	Sex	Lesion severity*	Tissue	Age (yrs)	Sex	Lesion severity*
Colon	49	M	Colon	45	F	Marked	Colon	51	M	Moderate
Colon	51	F	Colon	34	F	Severe	Colon	30	M	Marked
Colon	56	M	Colon	22	M	Moderate	Colon	54	M	Moderate
Colon	45	M	Colon	29	F	Mild	Colon	41	M	Severe
Colon	57	M	Colon	47	F	Marked	Colon	22	F	Moderate
Colon	48	F	Colon	28	F	Marked	Colon	41	M	Severe
Colon	69	F	Colon	61	F	Moderate	Colon	58	F	Severe
Colon	62	M	Colon	22	F	Moderate	Colon	35	F	Marked
Colon	59	F	Colon	50	F	Severe	Colon	63	M	Marked
Colon	40	F	Colon	32	M	Marked	Colon	30	F	Marked
Colon	65	F	Sm Int	18	F	Marked	Colon	43	M	Severe
Colon	58	F	Sm Int	67	F	Severe				
Colon	39	M	Sm Int	63	M	Mild				
Colon	59	F	Sm Int	29	F	Moderate				
Colon	59	M	Sm Int	25	F	Marked				
Colon	48	M								
Sm Int	50	M								
Sm Int	57	F								
Sm Int	62	F								
Sm Int	43	F								
Sm Int	40	M								

* Subjective grades for lesion severity: mild, moderate, marked, severe.

Methods: Ultivue InSituPlex Assay

Using the InSituPlex® technology, a custom antibody panel consisting of CD3, CD4, CD8, CD25, and FoxP3 was developed and the resultant multiplexed IHC assay was applied to de-identified FFPE specimens (**Figure 1**). Imaging was performed on the ZEISS Axio Scan.Z1 slide scanner utilizing only the CY5 and Cy7 channels to avoid autofluorescence in the DAPI, FITC, and TRITC channels. To allow for multiple imaging rounds in the same two channels, a process of DNA-Exchange was employed. DNA-Exchange is a mild and specific removal of the labelled probe from a previous imaging round allowing for the application of a new set of probes and the detection of the next two markers. Image analysis was performed using HALO analysis software.

Figure 1

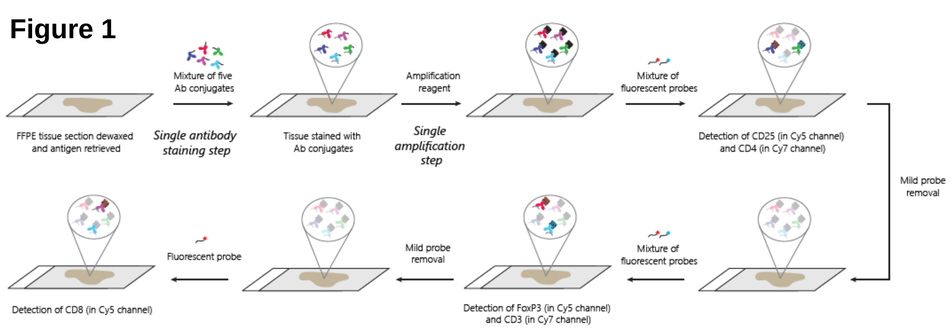

Figure 1: Schematic of the workflow for the Ultivue InSituPlex assay. Three rounds of imaging requiring two DNA-Exchanges were performed to complete the custom 5-plex assay using two fluorophores (Cy5, Cy7) to minimize autofluorescence typically seen in the FITC and TRITC channels.

Methods: Image Analysis

Quantitative image analysis was performed using Indica Labs Halo™ software. For each tissue section, the region of interest (ROI) included the mucosa, submucosa, muscular tunic and serosa. A reference image was used to establish intensity thresholds for each channel, and analysis was performed using the same threshold settings for all images. Numbers of cells expressing each phenotype were recorded in Halo and exported to Excel. Treg and CTL abundance were calculated in Excel and are reported in terms of their frequency within the total $CD3^+$ T cell population (i.e., Treg/$CD3^+$ and $CD8\alpha^+/CD3^+$, respectively). Statistical analysis was performed using GraphPad Prism 8.1.1 software.

Results: Treg and CTL in Colon Specimens

Figure 2: Colon

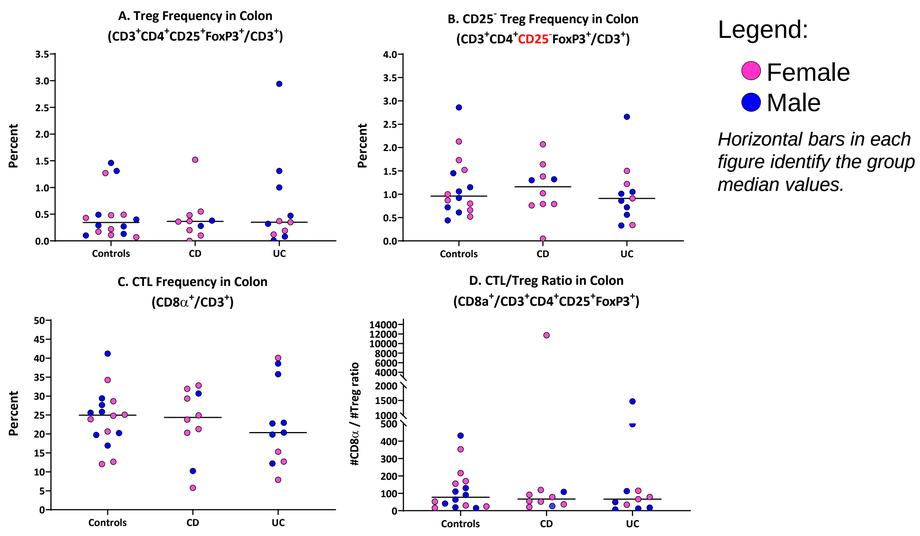

Figure 2: Frequencies of: A) Conventional Treg (CD3+CD4+CD25+FoxP3+); B) CD25- Treg, and C) CTL (CD8α+) within the total CD3+ T lymphocyte population, and D) CTL/Treg ratio in colon sections from patients with Crohn's disease (CD) and ulcerative colitis (UC) versus normal colon controls. There were no significant differences in immune cell populations between specimens from patients with CD and UC versus normal controls.

Results: Treg and CTL in Small Intestine

Figure 3: Small Intestine

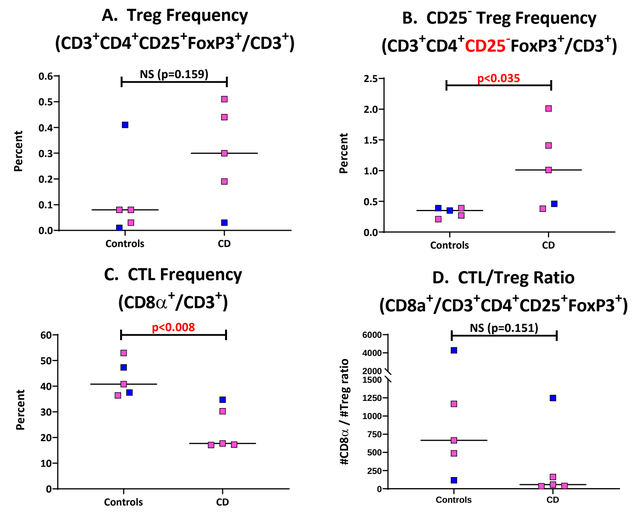

Figure 3: Frequencies of: A) Conventional Treg (CD3+CD4+CD25+FoxP3+); B) CD25- Treg, and C) CTL (CD8α+) within the total CD3+ T lymphocyte population, and D) CTL/Treg ratio in sections of small intestine (SI) from patients with Crohn's disease (CD) versus normal controls. Median values for the frequencies of CD25+ (A) and CD25- (B) Tregs were greater in SI sections from CD patients versus normal controls; the difference was significant for CD25- Treg populations ($p<0035$). The median value for CTL frequency was significantly lower in SI specimens from patients with CD versus normal controls ($p<0.008$). Consequently, the median CTL/Treg ratio was lower for SI specimens from CD patients versus controls, although the difference was not statistically significant.

Figure 4: Multiplex IF example (specimen #2410)

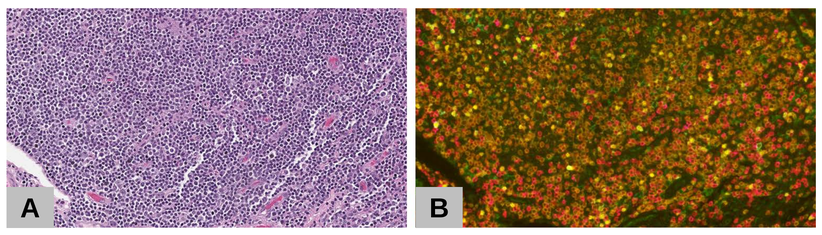

Figure 4: (A) Image of hematoxylin and eosin (H&E) stained section of CD specimen #2410, showing region of mucosal gut-associated lymphoid tissue (GALT) subjacent to inflamed colonic mucosa. (B) Image of serial section to (A), showing 5-plex immunofluorescence for **CD3** (red), **CD4** (green), **CD8α** (magenta), **CD25** (cyan) and **FoxP3** (yellow).

Figure 5: Multiplex Assay on specimen #2410

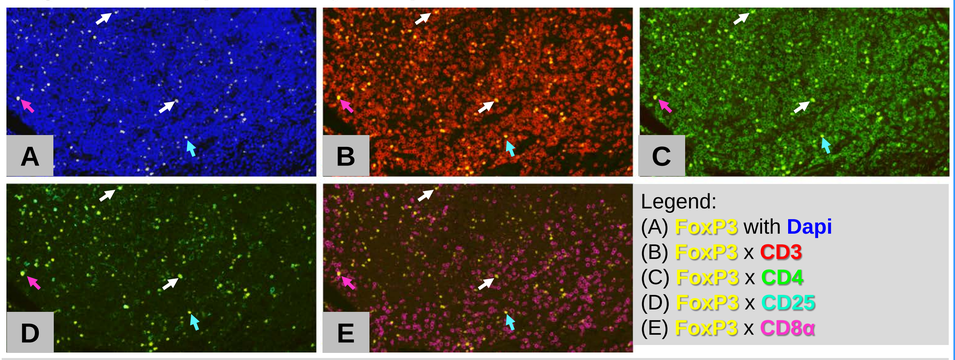

Figure 5: Multiplex IF on single section of specimen #2410. (A) FoxP3 (yellow, nuclear antigen) with Dapi counterstain. (B through E): individual membrane antigens **CD3** (B), **CD4** (C), **CD25** (D) and **CD8α** (E) in combination with nuclear antigen FoxP3. Arrows identify cells expressing different antigenic combinations: white arrows identify two Tregs (CD3+CD4+CD25+FoxP3+); cyan arrows identify a **CD25- Treg** (CD3+CD4+CD25-FoxP3+); magenta arrows identify a **CD8α+ Treg** (CD3+CD8a+CD25+FoxP3+).

Conclusions

- Using the InSituPlex^R multiplex IF platform we localized the nuclear antigen FoxP3 and the membrane antigens CD3, CD4, CD8α and CD25 simultaneously on single sections of GIT tissue.
- This multiplex labelling strategy facilitated definitive identification and spatial localization of conventional Treg (CD3+CD4+CD25+FoxP3+), CD25- Treg (CD3+CD4+CD25-FoxP3+), CD8a+ Treg (CD3+CD8α+CD25+FoxP3+) and CTL (CD8α+) in GIT sections from CD and UC patients vs. normal controls.
- We found no differences in the frequencies of Treg or CTL in colon from CD and UC patients versus controls; however, higher frequencies of Treg and lower frequencies of CTL were measured in small intestine from patients with CD versus controls.
- The frequency of conventional Treg among CD3+ T cells was very low in IBD as well as normal GIT tissue (typically < 2.0%).
- The level of CD25 expression was highly variable among conventional Treg in the GIT. Frequency of $CD25^-$ and $CD25^{lo}$ Treg was generally higher than $CD25^{hi}$ Treg, but still represented < 3.0% of all T cells.
- CD8α+ Treg were definitively identified, but were extremely infrequent among CD3+ T cells in GIT sections from IBD patients and normal controls. CD4+ and CD4- variants of CD3+CD8α+FoxP3+ were observed.

References

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Human tissues were obtained from the National Disease Research Interchange (NDRI). Tissues were collected for research purposes under IRB-approved informed consent and collection procedures and provided to Pfizer in accordance with applicable government regulations and guidelines.

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