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Local and Systemic Imbalance of CD4+ T Cells in Granulomatous Colitis: A Comparative study

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Abstract:
Background and study aims: *T helper cells and the various cytokines secreted by them play a crucial role in the pathogenesis of Intestinal tuberculosis (ITB) and Crohn's disease (CD). We aimto study the levels of five cytokines (IL-4, IL-6, IL-17, IFN-ϒ & TGF-β1) in serum of patients with CD & ITB which indirectly reflects the levels of CD4+ T cells.*
Methodology*: An observational, cross-sectional study done in a tertiary care hospital for 2 years. Patients with features of CD or ITB were included, with 12 cases of CD&13 cases of ITBand 20 controls. Colonoscopic biopsies were taken from the ulcerated and normal mucosa along with the patient's peripheral blood. The tissues were processed for histopathological examination. Serum levels of five cytokines (IL-4, IL-6, IL-17, IFN-ϒ & TGF-β1) were estimated by ELISA along with flow cytometric estimation of T helper cell populations in the peripheral blood.*
Results: *We found a significant imbalance among T helper cells in CD and ITB. This correlated with the levels of cytokines in the peripheral blood, of which mean IL-6 and IL-17 levels showed a statistically significant increase in CD. In addition, IFN- γ was also significantly increased, probably as a result of the increase in Th1 and Th17 cells. There was no change in IL4 levels.*
Conclusion: *Cytokines have an important role in the pathogenesis of granulomatous inflammatory bowel diseases. The role of cytokines in their pathogenesis might be helpful for future therapy based on anti-cytokine antibodies.*

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I. Introduction

Tuberculosis is an infectious disease caused by Mycobacterium tuberculosis, commonly affecting extra pulmonary sites. [1]The gastrointestinal tract is often susceptible to an inflammatory response due to its constant exposure to bacterial antigens in food and other toxic factors. [2] In view of the socioeconomic conditions in a developing country like India, the risk of exposure to the tubercle bacilli is considerable. However, exposure to M. tuberculosis alone does not lead to a clinically significant disease in almost 90-95% cases, due to the integrity of the host's cell mediated immunity (CMI). [1, 3]

Inflammatory bowel disease (IBD) predominantly includes two forms: Crohn's Disease (CD) & Ulcerative Colitis (UC). The pathogenesis of this group of diseases is yet to be fully understood. Although, it is known that an inflammatory response, sometimes leading to granuloma formation (in case of Crohn's disease), is found. Interestingly, Tuberculosis also manifests as a granulomatous colitis, dominated by cells of the immune system from the intestinal mucosa represented by the neutrophils, macrophages and cytotoxic T-cells. These cells attack and destroy the cells in the vicinity, either by direct contact or indirectly by releasing soluble factors like reactive oxygen species, cytotoxic proteins, lytic enzymes or cytokines. [2, 3]

Loss of immune tolerance to intestinal flora has been an established fact in the pathogenesis of IBD. This is mediated by various substances, which include cytokines. Cytokines are small peptides secreted by activated dendritic cells and macrophages. Their functions include intercellular signal transmissions, cell proliferation stimulation and mediating the autocrine, paracrine and endocrine effects of inflammation. [1]

The prevalence of Tuberculosis in India makes it highly relevant to study the immunological pathogenesis of intestinal tuberculosis to compare and contrast it from that of Crohn's disease, especially due to

their morphological similarities. Thus, we have studied the serum levels of a few cytokines, namely IL-4, IL-17, IL-6, IFN-ϒ & TGF-β1 as well as the CD4+ T helper cell population in the blood of patients with ITB and CD in a tertiary care center in eastern India to compare them with a cohort of patients free from either disease.

II. Methodology

This study was a single-center, observational, cross-sectional study conducted over a period of 18 months. Samples were collected from patients attending the Gastroenterology clinic with evidence of colitis, ileitis or ileo-colitis due to Crohn's disease or intestinal tuberculosis and undergoing clinical, radiological and endoscopic evaluation. A total of 50 cases were taken, with 15 cases of Crohn's disease and intestinal tuberculosis each and 20 controls. Controls were defined as people suffering from any other disease other than CD or ITB and undergoing endoscopic-biopsy.

Patients with positive serological markers for hepatitis, rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis or any other autoimmune condition were excluded from the study. Other criteria for exclusion from the study included history of organ transplantation or treatment with steroids, immunomodulators or biological agents. The patients diagnosed with CD or Intestinal Tuberculosis (ITB) based on history, clinical examination, radiology and histopathological examination following the appropriate guidelines. [4] Crohn's disease activity was monitored by the CD activity index (CDAI), with a CDAI score ≥ 150 defined as active disease.

At least 5 biopsies were taken from the ulcerated areas & 3 from normal looking mucosa. Haematoxylin and eosin stain was done for confirmation of histopathological diagnosis (Figs 1 &2). Samples were collected from the peripheral blood of these patients and serum levels of five cytokines (IL-4, IL-17, IL-6 IFN-ϒ & TGF-β1) were estimated by ELISA, strictly following the guidelines in the user manual supplied with the ELISA kits. Each sample was run in duplicate along with a control and standard samples. The mean absorbance for each set was calculated. The average zero standard optical density was then subtracted from that value.

To evaluate the T helper cell subpopulations, mononuclear cell flow cytometric analysis was done from peripheral blood samples. Ficoll-Paque density centrifugation was done to isolate peripheral blood mononuclear cells (PBMCs). The mononuclear cells were then suspended in RPMI 1640 culture medium, supplemented with penicillin (100 U/ml), streptomycin (100 μg/mL), L-glutamine and 10% heat inactivated fetal bovine serum (Gibco, Invitrogen Inc., Carlsbad, CA, USA).

T helper regulatory cells (Treg) were identified using fluorescein isothiocyanate (FITC)-labelled anti-CD4, phycoerythrin (PE)-cyanine (Cy) 7-labelled anti-CD25 and PE-labelled anti-Foxp3 (all from BD Biosciences, Franklin Lake, NJ, USA). For the analysis of T helper 1 (Th1), T helper 2 (Th2) and T helper 17 (Th17) subgroups, the sample was stimulated in an incubator set at 37°C for 4 hours under a 5% CO2 atmosphere along with 50 ng/ml of phorbolmyristate acetate and 1 mMionomycin (both from Sigma, St Louis, MO, USA) in the presence of monensin (BD Biosciences).

Flow cytometry was done using a BD FACScan (BD Biosciences) collecting 300 000–500 000 events. Lymphocytes were gated based on their forward and side scatter properties. Data was analyzed using the CellQuest analysis program (BD Biosciences). Treg proportion was determined based on CD4+, CD25+ and Foxp3+ cell surface expression. CD4+, IFN-γ+; CD4+, IL-10+, and CD4+, IL-17+ phenotypes were defined as Th1, Th2, and Th17 cells, respectively.

Statistical forms were used to record the relevant demographic, clinical, laboratory data for each patient before uploading to a database maintained to track the clinico-pathological progress of the cohort. Microsoft Access, Excel 2013 and SPSS 20.0 (SPSS, Inc., Chicago, IL, USA) were used when appropriate for analysis. Data was presented as mean ± standard deviation (SD) normally distributed data, or as median and inter-quartile range for non-normally distributed data.

Correlation between CDAI, cytokines levels and flow cytometric data were done using Pearson correlation coefficient giving a value between +1 and −1 inclusive, with +1 representing total positive linear correlation, 0 being no linear correlation, and −1 representing total negative linear correlation. Comparisons between the two groups were performed using an independent sample t-test &Levene's Test for Equality of Variances. A P-value <0.05 was considered indicative of statistically significant differences.

III. Results & Analysis

A total of 15 CD & 15 ITB cases along with 20 healthy age-matched volunteers were included in the study. In our study group, a marginal female predominance was observed. Age distribution shows that these two diseases predominantly involved the middle age group with patients of CD showing a mean age of 42.2 years compared to patients of ITB, who had a mean age of 37.6 years.

Table 1 - Mean Levels of various cytokines in serum obtained by ELISA in CD, ITB and control group (levels of cytokines are in pg/ml except TGF-β1 which is in ng/ml).

SUBJECT	Mean IL4	Mean IL17	Mean IFN-γ	Mean TGF-β1	Mean IL6
CD (n=15)	6.9	47.72	22.31	9.52	49.10
ITB (n=15)	6.61	54.56	17.64	7.25	6.86
CONTROL (n=20)	6.55	25.70	10.37	16.80	1.77

Table 1 describes mean levels of various cytokines in serum obtained by ELISA in patients with CD, ITB and normal controls (levels of cytokines are in pg/ml except IFN-γ & TGF-β1 which is in ng/ml). Application of Levene's test for Equality of variance & independent sample t-test in CD & ITB cases reveals an increase of IFN-ϒ, IL6 and IL17 levels compared to the control population. No change was observed in IL4 levels.

In CD, increase of IFN-ϒ was statistically significant (p = 0.002), as with IL6, which was highly significant (p = <0.001). Decreased TGF-β1 also showed statistical significance (p=0.003). However, increase in IL17 was statistically insignificant. In ITB, there is an increase in the concentration of IFN-γ but it is not statistically significant but that of IL6 is statistically significant (p=0.000). IL17 level increase in ITB compared to control without statistical significance. Decrease of TGF-β1 was not statistically significant. There is no change in IL4 levels.

Table 2 - Mean Levels of various T helper cells in peripheral blood obtained by flow-cytometry in CD, ITB and controls (levels of T helper cells are in %)

SUBJECT	Th1	Th2	Th17	Treg
CD (n-15)	30.91	7.68	1.44	6.00
ITB (n=15)	25.31	6.88	1.68	6.98
CONTOL (n=20)	13.83	8.46	0.72	7.57

Table 2 describes mean levels of various T helper cells in peripheral blood obtained by flow-cytometry in CD, ITB and controls (levels of T helper cells are in %). Except the level of Th2, all the other cytokines Th1, Th17 and Treg show changes in CD and ITB that are statistically significant compared to the control group.

Table 3 - Correlation of flow cytometric data and ELISA data with CDAI score in the CD patient group by Pearson Correlation (n=15)

	ELISA					Flow-cytometry			
	IFN-γ	IL4	IL6	IL17	TGF-β1	Th1	Th2	Th17	Treg
Pearson Correlation	.065	-.058	.542*	.720**	.205	-.079	.258	.094	.322
Sig. (2-tailed)	.818	.838	.037	.002	.464	.781	.354	.738	.242

*. Correlation is significant at the 0.05 level (2-tailed).
**. Correlation is significant at the 0.01 level (2-tailed).

Table 3 describes correlation of flowcytometric data and ELISA data with CDAI score in the CD patient group by Pearson Correlation. Except IL4, all cytokines show positive correlation, with highest significance shown by IL17.

IV. Discussion

Over the past decades, several works have contributed to significant progress in understanding the immunogenesis of CD, especially following the discovery of T regulator and Th17 cells. It is postulated that cellular interactions in IBD are modulated by the action of cytokines like Tumor Necrosis Factor α (TNF-α), Gamma Interferon (INF-γ), Interleukins (IL-1, IL-6, IL-4, IL-5, IL-10), Transforming Growth Factor β (TGF-β) or recently described cytokines like IL-13, IL-12, IL-18 and IL-23 that can have a pro-inflammatory as well as an anti-inflammatory effect. [5]

These factors seem to be mediated by Th1 and the more recently discovered Th17 cell populations, which result in increased production of TNF- α and IFN- γ. It is hypothesised that cytokines such as IL-12 and IL-23 control the differentiation and other cytokines such as IL-15, IL-18 and IL-21 bring about stabilization of Th1 cell populations. Other notable cytokines include IL-6, which induces anti apoptotic factors like Bcl-2 and Bcl-xl, resulting in a resistance to apoptosis in T cells, eventually leading to inflammation. It is also said to play a role in Th17 pathway activation. [2] A few studies have focussed on the therapeutic effects of antagonising these cytokines in the treatment of CD.Antibodies against IL-6 suggesting promising applications in clinical research. [6] However, antibodies against the primary Th17 cytokine IL-17 failed to show benefits in CD

patients. [7] Hence, although the importance of IL-17 has been established in the process of chronic inflammation, there is controversy regarding the exact role of Th17 cells in CD [8,9] and about a possible competition between Th17 and Th1 subsets in CD. [10] We have also included IL-4 in our panel of cytokines. IL-4 produces the differentiation of naive T helper cells (Th0 cells) to Th2 cells & results in a defective immunosuppressive effect. [11]

To explain the role of CD4+ T-cell subgroups in CD patients, we included CD patients without any history of immunosuppressive therapy or any other immunological diseases. Importantly, a majority of patients included in this study were newly diagnosed and had not received any treatment. Therefore, we hope that the results thus obtained would illuminate correctly the real immunogenesis of CD, at least in our population.

We find that compared to healthy controls, Th1 and Th17 subsets are increased, while Treg cells are decreased in CD patients. The serum concentrations of CD4+ T-cell related cytokines and transcription factors correlate well with these observations. This is in accordance with previous studies, [12, 13] suggesting cooperation between CD4+ T-cell groups in its pathogenesis. However, therapeutic regimens in CD are always based on disease activity and it has not still not been well understood whether CD4+ T-cell subgroups are associated with disease activity. Previous studies have observed that the circulating Treg subgroup was lower in active disease compared to inactive CD and that a Treg/Th17 imbalance existed in CD remission. [13, 14, 15] However, all these studies investigated only one or two subsets of CD4+ Tcells or included only patients in remission. We found a trend that Th1 and Th17 subgroup populations were increased, and the Treg subgroup was decreased in active versus inactive CD patients, but these differences were not significant. This might be due to the small sample sizes of enrolled CD patient groups.

Measurement of the various subtypes of CD4+ T cells in the systemic circulation was done directly by flow cytometry and indirectly through evaluating levels of cytokines by ELISA. Amongst the five cytokines we tested, IFN-ϒ was secreted by Th1 cells, IL4 was secreted by Th2 cells, IL17 was secreted by Th17 cells & TGF-β1 was secreted by Treg cells. IL6 is released by both Th1 and Th17 cells. In our cohort, IL-17 levels showed the highest significance in correlation with disease activity (CDAI). Concentration of IL6 by ELISA showed a significant increase in CD group compared to control group. Not only that, IL6 achieved second highest significance in co-relation with disease severity. This increase in IL6 value in CD correlated well with the findings of Pugazhendhi et al. [16] In our study the increase of IL17 in CD showed statistical significance, and this finding correlated well with the findings of Sahin et al. [17] We also found that the increase of IFN-ϒ was statistically significant (p=0.002) and this correlated with the findings of Kang Chao et al in the Chinese population. [18]

ITB is a morphological differential diagnosis of Crohn's disease. It is an infectious disease characterised by chronic inflammation and granuloma formation. This leads to some extent of immunological similarity with CD. As was observed in patients with CD, there was upregulation of Th1 and Th17 cells along with downregulation of Treg cells. This is in agreement with the findings of Pugazhendhi et al., who conclude that ITB involves a Th1-Th17 driven inflammation along with strong innate immune activation. [16]There was significant decrease in the Treg cell population in ITB compared to the control group, which correlates with the finding of significantly increased IL-6 levels.

Detection of the levels of other cytokines secreted by CD4+ T cells showed significant increase in the concentration of IFN-γ (p=0.023). This finding of upregulation of IFN-γ in ITB correlated with the findings of Pugazhendhi et al. [16] The concentration of IL17 secreted by Th17 cells showed a statistically significant increase compared to the control group, which corroborated with the data of other authors. [16, 17] Decrease in IL4 concentration in our ITB patient group did not achieve statistical significance compared to the control group. IL4 concentration secreted by Th2 cells was similar to the control group in our population.

To summarise, we found that a significant imbalance among Th1, Th17 and Treg cells existed in CD and ITB. Amongst all the cytokines tested in this study, the only statistically insignificant change was observed in the level of IL-4 in patients with ITB. Elevation of the levels of IL-6 and IL-17 also showed the highest concordance with disease activity in cases of CD. IFN- γ was significantly increased, probably as a result of the increase in Th1 and Th17 cells. Further studies should be conducted in a larger cohort to elucidate the precise mechanistic roles of diverse CD4+ T-cell subsets in CD development and progress. As this study was conducted in a tertiary care hospital, the difficulty in getting newly diagnosed and untreated cases of CD and ITB patient in the absence of other immunological disorder was considerable.Finally, we have evaluated these cytokines and T cell subpopulations at a systemic level. It is essential to also look at the local mucosal imbalances of T cell populations to gain better perspective regarding these changes. However, it is technically difficult to isolate enough mucosal lymphocytes for experiments, unless large surgical pieces were taken.

V. Conclusion

Cytokines have an important role in the pathogenesis of granulomatous inflammatory bowel diseases. Our study has found a distinct correlation between irregularities in T helper cell populations with cytokine

imbalance, especially IL-6 and IL-17. The identification of newer cytokines & identification of their role in the pathogenesis might be helpful for future therapy.

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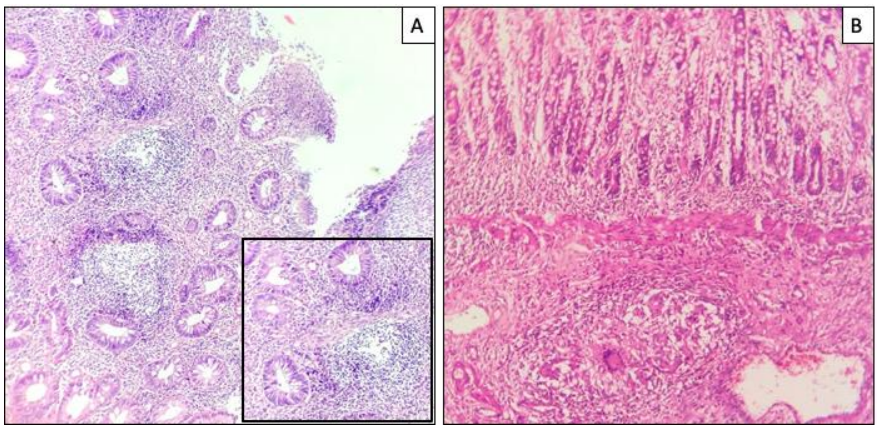

LEGENDS

Figure 1: A. Low power view (100x) of hematoxylin & eosin stained endoscopic biopsy showing the presence of granuloma and chronic inflammatory infiltrate in the submucosa. Inset – High power view of granuloma (400x).
B. Low power view (100x) of epithelioid granuloma with presence of caseous necrosis below the mucosa, with a central Langhan giant cell.

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