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Immune Profile of Severe Refractory Crohn's Disease Patients Candidates to Autologous Hematopoietic Stem Cell Transplantation

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1. Abstract

Crohn's disease is a chronic relapse - remitting inflammatory bowel disease with a wide range of incapacitating symptoms, and significant health care costs. The complex pathophysiology of Crohn's disease is not always solved by conventional and biologic therapies and thus, some patients evolve with severe refractory disease. T and B cells reacting to luminal antigens have been linked to the chronic intestinal inflammation. Our aim was to determine the immune profile of Crohn's disease patients, candidates to autologous hematopoietic stem cell transplantation in order to predict the response to this therapy and in turn lead to a personalized therapy with cost-effective benefits.

Peripheral blood mononuclear cells were phenotyped for T (CD4 and CD8), and Myeloid-derived suppressor cells from eight patients (5 males and 3 females), average age of 28 years and disease duration from one to eighteen years, and healthy controls (5 males and 5 females, 20 to 30 years old).

It was observed increased percentage of CD8+ T cells and decreased percentage of CD4+ T cells, with reduced CD4/CD8 ratio. Patients presented reduced percentage of Naive T cells (CD4+ and CD8+) and increased percentage of Effector Memory T cells (CD4+ and CD8+) suggesting a prominent inflammatory process. Myeloid-derived suppressor cells were significantly higher in Crohn's disease patients. The immune profile previously to hematopoietic stem cell transplantation and the longitudinal analysis post-transplant may lead to a further understanding of Crohn's disease pathogenesis, mechanism(s) of remission, cause(s) for relapse, and approaches to improve long-term treatment-free remission.

2. Introduction

Crohn's Disease (CD) is a chronic relapse - remitting inflammatory bowel disease that can affect any site of the digestive tract [1]. The incidence and prevalence of CD has increased worldwide [2]. Symptoms include abdominal pain, diarrhea, hematochezia, bloody stools, fatigue, weight loss, fever, recurrent fistulas and manifestations in other organs [3]. Extraintestinal manifestations comprise arthropathy (both axial and peripheral), ocular involvement (uveitis, scleritis, and episcleritis), dermatological (including pyoderma gangrenosum and erythema nodosum), nephrolithiasis, hepatobiliary involvement (primary sclerosing cholangitis), cholelitiasis, venous or arterial thromboembolism, and other immune-mediated disease [4]. Even though there exists heterogeneity in the affected patients, the common pathophysiology is immune dysregulation. The intestinal mucosa manifests inappropriate immune responses against resident intestinal flora or its antigens that in turn leads to tissue lesions and symptoms. This disturbance in the intestinal mucosa occurs when genetically predisposed patients are exposed to environmental triggers [5].

Cells from the innate and adaptive immune system have been shown to play a role in CD. Macrophages and T cells can produce TNF-α (tumor necrosis factor) and thus contribute for the inflammatory process and intestinal lesions [6]. Colon samples show that resident Memory CD4+ T cells (TRM) are the most abundant cell population

and main mucosal source of TNF during CD. In addition, a unique population of CD4+ T cells producing TNF-α and IL-17A was observed in CD patients but not in healthy controls [7]. The central role played by T cells in the maintenance of intestinal inflammation, is in agreement with secondary non-response to anti-TNF therapy. Thus, T cells are a possible target for resolution of the inflammatory process and could be explored as therapy for CD. In peripheral blood, Dulic et al. observed in CD patient's lower percentage of Naive (CD45RA+) and higher percentage of activated (CD69+ and HLA-DR+) CD4+ T cells compared to healthy controls. Similar results were found in the CD8 compartment plus the higher percentage of Memory cells (CD45R0+). Regarding to the efficacy of anti-TNF therapy it was observed that the lower prevalence of CD4+CD45R0+ (memory phenotype) T cell predicted the long-term remission [8]. Another cell subtype, Myeloid-derived supressor cell (MDSC), has recently been reported as increased in patients with autoimmune disorders [9-12]. In addition, Haile et al. observed in peripheral blood of active CD patients an increased frequency of Myeloid-derived suppressor cells (MDSC) in contrast to healthy controls, suggesting that these cells might be used as therapy for IBD patients [13].

Hematopoietic stem cell transplantation (HSCT) is a potential therapy for patients with severe refractory Crohn's disease [14-17]. Nevertheless, there are few studies focused on the effects of HSCT in the elimination of inflammatory immune cells and subsequent reconstitution of the immune system. In this study our aim was to determine the immune profile of severe refractory Crohn's disease patients, candidates to autologous hematopoietic stem cell transplantation. Determining cellular parameters that correlates with Crohn's disease might predict the response to HSCT therapy and in turn lead to a personalized therapy with cost-effective benefits.

3. Methods

The clinical trial has been registered at http://clinicaltrials.gov (identifier: NCT03000296), conducted in accordance with good clinical practice guidelines and approved CAAE: 49304915.0.0000.5629. Crohn's disease patient's candidates to HSCT met the criteria of refractoriness to anti-inflammatory, immunosuppressant, and at least two biological agents. Refractoriness was defined as a Crohn's Disease Activity Index (CDAI) greater than 150, intestinal lesions detected by colonoscopy or capsule endoscopy, and lack of surgical options due to risk of a permanent stoma, short gut syndrome, or rectal amputation. All patients with significant comorbidities were excluded. Exclusion criteria included cancer (e.g. Hodgkin's lymphoma, chronic myeloid leukemia), liver disease, coexisting psychiatric disorders, active infections, and fistulae. Healthy controls were voluntary students from UNIFESP (20 to 30 years old, 5 males and 5 females).

3.1. Cell Phenotyping

Blood (5mL) was collected in EDTA tubes for the isolation of Peripheral Blood Mononuclear Cells (PBMCs) via Ficoll–Hypaque density gradient (Amersham Biosciences, Uppsala, Sweden) and centrifugation. Viable cells were counted, adjusted for 2 x 106/100μL in 80% fetal bovine serum and 20% dimethylsulfoxide (Sigma, St. Louis, MO, USA), and frozen stored (−80°C) until cell phenotype

Peripheral blood mononuclear cells were used to phenotype T (CD4 and CD8), and myeloid-derived suppressor cells (MDSC) using flow cytometry. The cells were stained with monoclonal antibodies to T-cell phenotype CD3 APC, CD4 PerCP Cy 5.5, CD8 APC Cy7, CD27FITC, CD45RA PE, CD31 PE Texas Red (eBioscience, CA, USA). For MDSC phenotype cells were stained with CD3 APC, CD19 APC, CD56 APC, HLA-DR APC e-fluor 780, CD33 PerCP Cy5.5, CD11b PE (eBioscience, CA, USA).

After 30 min of incubation with monoclonal antibodies, in the dark and at 4°C, the cells were washed with PBS and centrifuged. Living cells (based on forward and side scatter) were acquired in the FACS Canto II using the DIVA software (Becton Dickinson, USA). Further analyses of FACS data were performed using the 9.3 FLOWJO software (Tree Star, USA).

3.2. T Lymphocytes Were Characterized

Naive: CD3+CD4+CD45RA+CD27+ or CD3+CD8+CD45RA+CD27+

Central Memory: CD3+CD4+CD45RA−CD27+ or CD3+CD8+CD45RA−CD27+ (TCM)

Effector Memory: CD3+CD4+CD45RA−CD27− or CD3+CD8+CD45RA−CD27− (TEM)

Effector Memory re-expressing CD45RA: CD3+CD4+CD45RA+CD27− or CD3+CD8+ CD45RA+CD27− (TEMRA).

3.3. Myeloid-Derived Suppressor Cells Were Characterized As

CD3−CD19−CD56−(Lin−) HLA−DR−/lowCD33+CD11b+

4. Statistics

Data are expressed as mean ± STD and Mann–Whitney was used for comparisons (parameters of the immune system) between patients with Crohn's disease and healthy controls. Values of $p<0.005$ were considered statistically significant. All data were analysed with the aid of the Graph Pad PRISM software (Graph pad, La Jolla, CA, USA).

5. Results

5.1. Demographics (Table 1)

For this study, five males and three female's patients from 15 to 39 years were enrolled with Crohn's disease duration from one to eighteen years. Irrespective of the elapsed time from symptoms to diagnosis, they presented severe disease (perianal diseases, extraintestinal diseases, fistulas). In addition, patients were refractory to therapies such as anti-TNF, anti-inflammatory drugs, corticosteroids, anti-integrin α4β7, anti- IL-23/IL-12.

Healthy controls from 20 to 30 years old (5 males and 5 females) were voluntary students from UNIFESP.

Table 1: Demographics of Crohn's disease patients

Patients	1	2	3	4	5	6	7	8
Age	35	28	24	15	28	38	39	20
Sex	M	M	M	F	F	M	F	M
Time elapsed - symptoms/diagnosis (years)	17	6	3	10	0	0	18	2
Montreal								
Age diagnosis	A2	A2	A1	A1	A1	A2	A2	A1
Localizations disease	L3	L1	L2	L3	L3	L3	L3	L4
Behavior	B3	B1	B3	B3	B3	B3	B3	B1
Perianal diseases	YES	NO	NO	NO	NO	YES	NO	YES
Extraintestinal diseases	YES	NO	YES	YES	YES	NO	YES	YES
Articular	YES	NO	YES	YES	YES	NO	YES	YES
Skin	YES	NO	NO	NO	NO	NO	YES	YES
Eye	NO	NO	NO	NO	NO	NO	YES	NO
Others	NO	NO	NO	NO	NO	NO	NO	NO
Auto immune concomitant or previous disease	NO	NO	NO	NO	NO	NO	NO	YES
Multiple sclerosis	NO	NO	NO	NO	NO	NO	NO	NO
Systemic lupus erythematosus	NO	NO	NO	NO	NO	NO	NO	NO
Ankilosant spondylitis	NO	NO	NO	NO	NO	NO	NO	NO
Behçet Syndrome	NO	NO	NO	NO	NO	NO	NO	YES
Rheumatoid arthritis	NO	NO	NO	NO	NO	NO	NO	NO
Fistulas	YES	NO	NO	NO	NO	YES	YES	YES
Ostomia	NO	NO	NO	NO	NO	NO	NO	NO
Familial history	NO	NO	NO	NO	NO	NO	YES	NO
Previous therapy								
Anti-inflammatory	YES	YES	YES	YES	YES	YES	YES	NO
Corticosteroids	YES	YES	YES	YES	YES	YES	YES	YES
Azathioprine	YES	YES	NO	NO	YES	YES	YES	YES
Methotraxate	NO	NO	NO	NO	NO	NO	YES	YES
Cyclosporine	NO	NO	NO	NO	NO	NO	NO	NO
Infliximab	YES	YES	YES	YES	NO	YES	YES	YES
Adalimumab	NO	NO	YES	YES	NO	YES	YES	YES
Certolizumabe pegol	NO	NO	NO	NO	NO	NO	NO	NO
Vedolizumabe	YES	NO	YES	YES	NO	NO	NO	NO
Ustekinumabe	NO	NO	NO	NO	YES	NO	YES	NO
Natalizumabe	NO	NO	NO	NO	NO	NO	NO	NO
Previous Surgeries	YES	NO	NO	YES	NO	YES	YES	YES
CDAI	353	179	170	279	105	210	322	272
HBI	15	7	8	16	10	13	27	13
CRAIG CSI	25	18	17	25	15	27	28	25

5.2. Immune Profile (Table 2)

Percentages of T cells were obtained as exemplified in (Figure 1A and B). In patients with Crohn's disease, the mean of CD8+ T cells in peripheral blood was higher in percentage (54.3%) than the mean of CD4+ T cells (37.5%) and six out of eight presented a CD4/CD8 ratio lower than 1. There was an increased percentage of T CD4+ Central Memory (TCM, mean 42.1%) and Effector Memory (TEM, mean 40.9%) cells at the expense of a decreased percentage of Naive cells (mean 14.1%). In the compartment CD8+, there was an increased percentage of Effector Memory cells (TEM, mean 45.9%) and Effector Memory RA cells (TEMRA, mean 18.6%) at the expense of a decreased percentage of Naive cells (mean 17.6%).

The strategy for Myeloid-derived suppressor cells (MDSC) gate is exemplified in (Figure 2A). MDSC percentage (range 0.83% to 10.7%) was higher than 1 in seven out of eight patients.

For comparison of the immune profile, ten healthy controls from 20 to 30 years (5 males and 5 females) were enrolled and results are presented in (Table 2). In comparison with healthy controls, patients with Crohn's disease showed significant increased percentage of CD8+ T cells (54.3% versus 25.1%), decreased CD4/CD8 ratio (0.73 versus 1.9), diminished percentage of Naive CD4+ (14.1% versus 51.5%) and CD8+ (17.6% versus 54.3%) T cells, and increased percentage of Effector Memory CD4+ (40.9% versus 13.6%) and CD8+ (45.9% versus 11.4%) T cells. We observed an elevated frequency of MDSC (4.4% versus 0.07%) in patients in comparison with healthy controls.

Table 2: Peripheral blood immune profile of Crohn's disease patients and healthy controls

	% T cells		Ratio	% $CD4^+$ T cells				% $CD8^+$ T cells				
Patient	$CD4^+$	$CD8^+$	CD4/CD8	Naive	TCM	TEM	TEMRA	Naive	TCM	TEM	TEMRA	%MDSC
1	29	43.5	0.67	13.4	27.7	48.8	10.1	22.8	6.11	20.9	50.2	1.6
2	30.3	73.8	0.41	3.8	33	59.4	3.8	4.5	15.3	56.4	23.8	3.8
3	45.6	42	1.09	16.9	58.3	23.9	0.84	27.2	32.2	32	8.6	1
4	51.2	43.6	1.18	7.6	43.9	47.2	1.3	4.5	22.4	64.4	8.7	6.1
5	46.2	53.8	0.86	15.9	60.3	21.2	2.6	25.3	31	31.7	12	0.83
6	35.8	66.4	0.54	4.5	24.5	69.6	1.4	3.5	6.4	68.4	21.7	9.2
7	31.4	63.6	0.49	13	47.7	37.3	1.9	22.1	20.4	47.9	9.6	2.1
8	30.6	47.5	0.64	37.8	41.3	19.8	1.1	31.3	8.9	45.5	14.4	10.7
Control	$CD4^+$	$CD8^+$	CD4/CD8	Naive	TCM	TEM	TEMRA	Naive	TCM	TEM	TEMRA	%MDSC
1	35.1	31.5	1.11	54.2	31.7	11.6	2,43	54.83	18.7	8.86	17.6	0.103
2	50.5	21.2	2.38	58.6	18,13	19.1	4.14	70.03	5	3.58	21.4	0.021
3	47.8	34.2	1.4	47.9	36.06	14.4	1.56	50.6	24.2	18.9	6.2	0.035
4	51.6	24.5	2.1	47.9	29.57	14.1	8.47	46.8	24.4	8.87	19.7	0.08
5	47	20.4	2.3	64.5	20.73	10.7	4.11	38.4	25.6	15	20.9	0.044
6	29.8	16.9	1.76	63.7	27.37	7.8	1.19	80.9	11.5	2.08	5.5	0.179
7	54.5	22.8	2.39	35.5	45.4	18.2	0.89	58.6	22.3	13.5	6.1	0.032
8	38.6	33.4	1.15	53.9	25.9	13.9	6.38	41.3	14	19,9	24.7	0.048
9	56.5	25.4	2.22	60.2	29.8	8.9	1.11	61.2	22.5	8.97	7.31	0.017
10	47.2	20.8	2.27	29	52.8	17.3	0.86	40.9	35.6	14.5	8.95	0.114
Patient												
Mean	37.5	54.3	0.73	14.1	42.1	40.9	2.9	17.6	17.8	45.9	18.6	4.4
STD	8.8	12.2	0.28	10.8	13.2	18.5	3.1	11.5	10.4	16.8	14	3.8
Control												
Mean	45.8	25.1	1.9	51.5	31.7	13.6	3.11	54.3	20.4	11.4	13.8	0.07
STD	8.66	5.97	0.51	11.7	10.6	3.9	2.6	13.7	8.5	6	7.6	0.05
p value	0.117	0.00012	0.00002	0.000003	0.095	0.004	0.87	0.00001	0.59	0.00047	0.41	0.0150
STD - standard deviation, TCM - T central memory, TEM - T effector memory, TEMRA - T effector memory repressing CD45RA, MDSC - myeloid-derived suppressor cells												

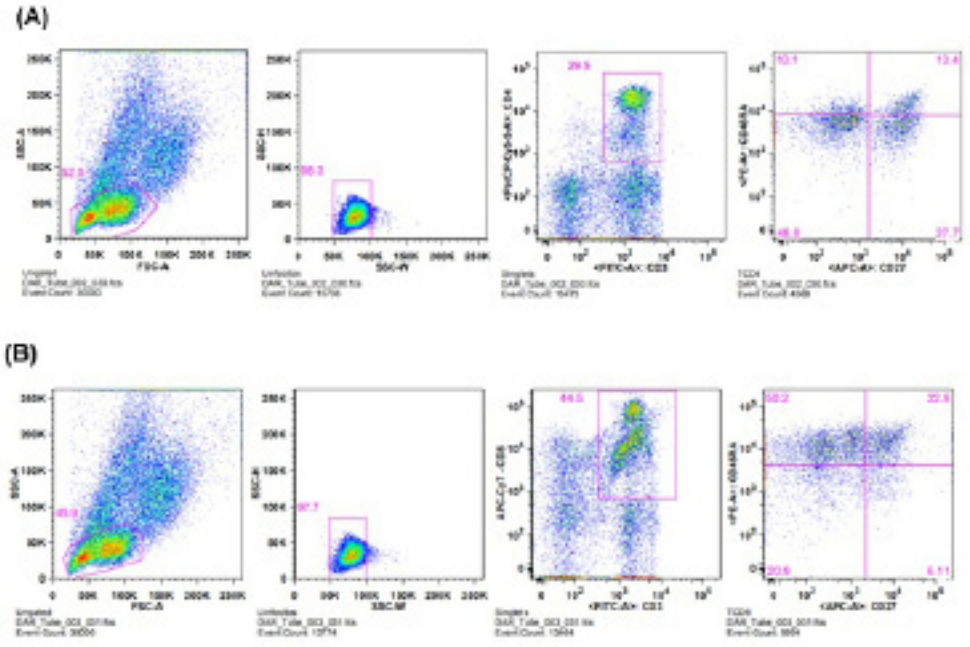

Figure 1: Representative flow cytometry showing gate strategy for the frequency of: CD4+ T cells phenotype. Gate in live lymphocytes (FSC-A × SSC-A), doublets exclusion (SSC-H × SSC-W), gate in CD3+CD4+ T cells, gate in CD45RA+ CD27+ (Naïve), gate in CD45RA−CD27+ (Central Memory, TCM), gate in CD45RA−CD27− (Effector Memory, TEM), gate in CD45RA+CD27− (Effector Memory re-expressing CD45RA, TEMRA) (A). CD8+ T cells phenotype. Gate in live lymphocytes (FSC-A × SSC-A), doublets exclusion (SSC-H × SSC-W), gate in CD3+CD8+ T cells, gate in CD45RA+CD27+ (Naïve), gate in CD45RA−CD27+ (Central Memory, TCM), gate in CD45RA−CD27− (Effector Memory, TEM), gate in CD45RA+CD27− (Effector Memory re-expressing CD45RA, TEMRA) (B).

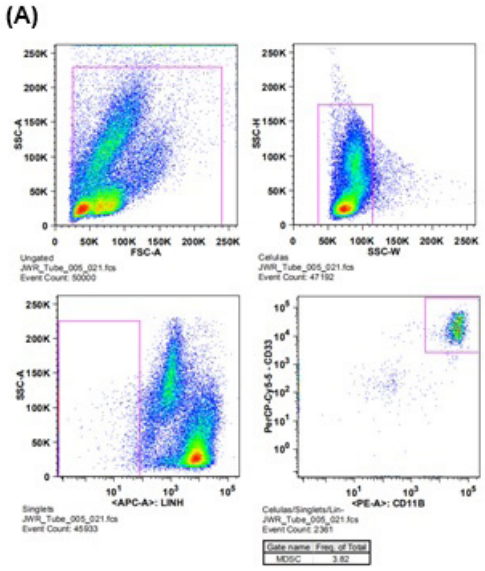

Figure 2: Representative flow cytometry showing gate strategy for the frequency of Myeloid-derived suppressor cells (MDSC), gate in live cells (FSC-A × SSC-A), doublets exclusion (SSC-H × SSC-W), gate in lineage negative cells (CD3−CD56−CD19−), gate in HLA-DRlow/neg cells, gate in CD33+CD11b+ cells (A).

6. Discussion

Crohn's Disease (CD) is characterized by immune response against luminal antigens, wide range of incapacitating symptoms, and significant health care costs. In addition, the pathophysiologic heterogeneity of CD is not always solved by conventional and biologic therapies and thus, a considerable percentage of patients evolves with severe refractory disease [18]. These facts highlight the importance of further knowledge about the CD-associated immune process in order to offer individualized and more adequate therapy.

The evaluation of intestinal lymphocytes by Smids et al. [19] showed that CD patients presented increased percentage of Naive and Central Memory T cells (TCM), along with reduced Effector Memory T cells (TEM) in comparison with healthy controls. The study by Kredel et al. [20] observed in biopsies from ileal a colonic tissues an increased percentage of CD4+ TCM and TEM cells at the expense of a decreased percentage of CD4+ Naive T cells. In the CD8+ compartment it was found an increased percentage of TEM cells and a decreased frequency of Naive cells. Kredel's findings from the intestinal inflammatory process were similar to our results in blood. In addition, we observed increased percentage of CD8+ T cells and decreased percentage of CD4+ T cells, resulting in reduced CD4/CD8 ratio. In comparison with healthy controls we found reduced percentage of Naive T cells (CD4+ and CD8+) and increased percentage of Effector Memory T cells (CD4+ and CD8+). Regarding to B lymphocytes, we observed an increased frequency of Memory cells (CD27-IgD-) in CD patients (data not shown) in agreement with results showed by Pararasa et al. in gut-associated lymphoid tissue from patients with CD and UC (ulcerative colitis) (21). Our findings suggest that a less invasive (peripheral blood) analysis of the immune profile corresponds to the phenotype displayed by intestinal infiltrating T and B cells in CD and UC [20, 21]. In addition, our results also point to a prominent inflammatory process depicted by the increased percentage of Memory Effector cells. A possible reversion of this phenotype could be achieved by modulatory/suppressive actions of T regulatory (Treg) or Myeloid-derived suppressor (MDSC) cells.

Myeloid-derived suppressor cells (MDSC) are a very heterogeneous cell population present mainly in inflammatory processes and with the capacity to suppress immune activation [22, 23]. Whitfield-Larry et al. identified MDSC in Type 1 diabetes mellitus patients but despite increased frequency of these cells, their efficacy to suppress T cell proliferation was reduced. The authors suggested that to control autoimmune disease progression the goal is to have MDSCs with optimal suppression function [24]. Haile et al. (13) observed that patients with CD displayed higher percentage of MDSC in peripheral blood compared to healthy controls (27.3% versus 3.1%). In addition, purified MDSC from patients caused a dose-dependent inhibition of proliferation and cytokine production by autologous-stimulated PBMCs in vitro. Authors suggested that the inflammatory environment generated by CD induced an increase in the frequency of MDSC. In turn, MDSC suppressive properties could prevent the progression for a more severe inflammatory process. In the present study, we found higher percentage of MDSC in patients with Crohn's disease compared to healthy controls. However, as we did not measure the suppressive action of MDSC, it was not possible to confirm a role played by MDSC in the progression of Crohn's disease.

Autologous hematopoietic stem cell transplantation (AHSCT) offers an opportunity of long-term disease remission for refractory patients with systemic lupus erythematosus [25, 26], multiple sclerosis [27], neuromyelitis optica [28], type 1 diabetes [29], and Crohn's disease [16]. Patients' outcome after AHSCT has been paralleled with the immune profile before and during the follow-up in Multiple Sclerosis (MS), Systemic Lupus Erythematosus (SLE), and Type 1 diabetes. While there is some variation in results, T cell subsets after AHSCT generally show an initial decrease in Central Memory CD4+ T cells (TCM), increase in CD4+ Treg, and a gradual increase in CD4+ Naïve T cells [26, 27, 29, 30]. A non-myeloablative condition regimen followed by AHSCT in patients with Type 1 Diabetes showed that lower anti-islet Cytotoxic T Cells (CTL) correlated with insulin free remission. In addition, Effector Memory CD4+ T cells (TEM) were increased at early times post-transplant, while an increase in the absolute numbers of CD8+CD28-CD57+ suppressor T cells and CD4+ Treg cells persisted during long-term remission in insulin independent patients [29].

In Crohn's disease, there are few studies that correlate immune phenotype and outcome after AHSCT. Clerici et al. observed after AHSCT, an increase in CD4+ Treg cells and a decrease in the expression of TNF-α and IL-10 by CD14+ monocytes in patients with clinical and endoscopic remission [31]. Corraliza at al, demonstrated that the

response to AHSCT is associated with expanded Naive B cells and decreased CD4+ Memory T cells [32].

Our findings in severe refractory CD patients with increased experienced T cells (Memory Effector CD4+ and CD8+) and decreased Naive T cells suggest that modulation/suppression of the immune system is essential to obtain disease remission. We hypothesize that monitoring the immune profile via peripheral blood, before and after AHSCT, may instruct how to optimize the conditioning regimen, and post-transplant care to facilitate restoration of the immune system homeostasis and improve HSCT's efficacy. Therefore, the immune profile may lead to a further understanding of CD's pathogenesis, mechanism(s) of remission, cause(s) for relapse, and approaches to improve long-term treatment-free remission.

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