Circulating immune cells and immune checkpoints expression in non-Alcoholic SteatoHepatitis and its association with Inflammatory Bowell Disease
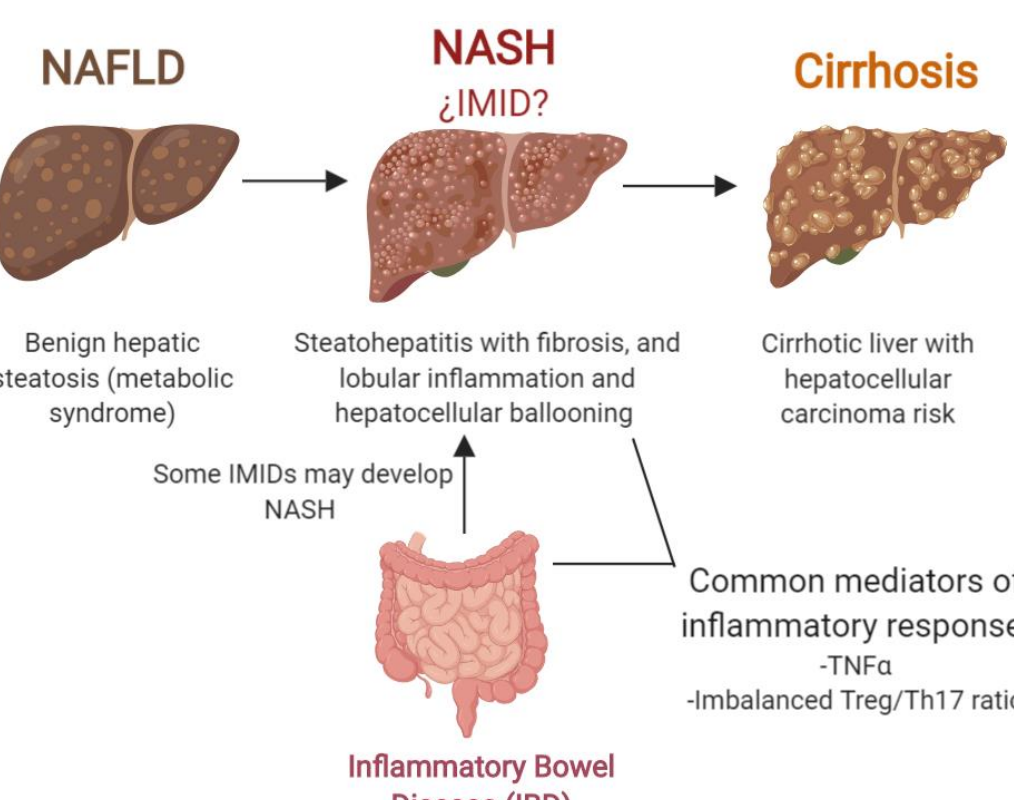
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INTRODUCTION

Non Alcoholic Fatty Liver Disease (NAFLD) occurs in certain immune-mediated diseases such as Inflammatory Bowel Disease (IBD). Non Alcoholic Steatohepatitis (NASH) is its most aggressive form. In both, an inflammatory pathogenic mechanism mediated by TNF-α, Th1 and Th17 is attributed. Immune Mediated Inflammatory Diseases (IMIDs) gather some properties that characterize them as a group: 1) an inappropriate immune response accompanied by a deregulation of cytokines which mediate the inflammatory process, 2) a chronic inflammatory damage whose phenotype depends on the affected organ, 3) susceptibility genes and shared risk factors that may justify both the co-occurrence of several IMIDs in the same individual and the family aggregation of multiple IMIDs, 4) comorbidities and common complications generated by the inflammation chronic effects, 5) and a common response to pharmacological agents with shared therapeutic targets within the inflammatory pathway (deregulated cytokines). Research regarding this issue is certainly scarce and biased. In the current project, a complete study of the number of cell populations of the immune response both innate and adaptive is carried out. A differentiated response may be responsible for the evolution towards NAFLD in patients with IBD.

HYPOTHESIS AND OBJECTIVES

NASH stands for a liver inflammatory disease with an important metabolic risk component, but an exacerbated immune response may be an independent cause of its occurrence. It is possible that the coexistence of another immune mediated diseases, such as IBD, plays a role in the NASH triggering regardless metabolic factors. The study of different cellular populations of the immune response in peripheral blood, both effector and regulatory, can help to differentiate IBD from both NASH and NASH coexisting with IBD, as well as to define cellular markers of clinical utility. The three main objectives are defined as:

- To set up the flow cytometry protocols for diverse cell populations of immune response: effector, memory, naïve T cells, regulatory T cells (Treg) and helper T cell subtypes; B lymphocytes during their different maturational stages, myeloid-derived suppressor cells (MDSC) and innate lymphoid cells (ILC); aside from to measure the expression level of several *immune checkpoints* (ICP).

- To monitorize these blood populations of 3 groups of patients with immunomediated diseases: NASH, NASH coexisting with IBD, and IBD

- To search for disease specific patterns of circulating cell populations of the immune response which may explain possibly related pathogenic mechanisms.

MATERIAL AND METHODS

One hundred and seventy seven patients have been recruited as part of the present study (45 women and 132 men). The study members belong to two different groups: healthy controls (n=149) and patients with inflammatory liver diseases (n=28). The patients group is also divided into three different subgroups: 10 individuals with NASH, 9 with NASH+IBD and 9 with IBD. The control group was recruited from Marqués de Valdecilla University Hospital Blood Bank (Santander, Spain) and the group of patients from the Digestive Service of that hospital. Every member of this study has signed an informed consent. The current study is part of the FIS PI18/01304 project. The following table shows the clinical and demographic characteristics of the 177 individuals studied.

Clinical and demographic patients´s values	NASH (n=10)	NASH+IBD (n=9)	IBD (n=9)	Controls (n=149)	*p* value NASH vs NASH+IBD	*p* value NASH vs IBD	*p* value NASH+IBD vs IBD
%Women	30	33,33	55,56	22,82	1,000	0,3698	0,6372
Age	53 ± 10,41	57,07 ± 12,3	49,4 ± 12,31	46 ± 12	0,2858	0,4841	0,1883 (NS)
Alkaline Phosphatase (AP)	NR	98,78 ± 26,02	65,56 ± 17,72	NR	NA	NA	0,0043
GPT Transaminase (ALT)	NR	36,89 ± 23,51	15,78 ± 9,93	NR	NA	NA	0,0009
C Reactive Protein (CRP)	NR	0,64 ± 0,34	0,47 ± 0,89	NR	NA	NA	0,0368
IgG	NR	1308,11 ± 343,97	1173,33 ± 133,7	NR	NA	NA	0,4283 (NS)
IgA	NR	315,78 ± 168,4	247 ± 105,81	NR	NA	NA	0,293 (NS)
IgM	NR	154,29 ± 78,93	144,01 ± 109,16	NR	NA	NA	0,4799 (NS)
Diabetes (YES/NO)	5/5	2/7	1/8	NR	0,3498	0,1409	1,000
IBD type (Ulcerative C./Crohn D.)	NR	0/9	3/6	NR	NA	NA	0,2059
I.Q (Surgery) (YES/NO)	NR	4/5	2/7	NR	NA	NA	0,6199

NA, not applicable
NR, not realized
NS, not significant

Table 1. Clinical and demographic characteristics of the patients recruited for the current study. The *p* values are also shown by comparing the three groups of patients.

Peripheral blood mononuclear cells (PBMCs) from heparin blood have been isolated by density gradient for immunephenotyping and analysis by flow cytometry. Specific monoclonal antibodies for the study of effector, memory, naïve T cells, Treg, Th cell subtypes, B lymphocytes during their different maturational stages, MDSC and ILC were employed. The expression level of several *immune checkpoints* has been measured twice: basal and at 3rd after PHA stimulation. Stimulation with PMA + ionomycine, and addition of brefeldin for the intracytoplasmic quantification of Th cytokines has been also performed.

The different cell populations have been acquired with the Cytoflex flow cytometer and analyzed with the Kaluza software (Beckman Coulter). Statistical was performed with the statistical package IBM SPSS 22.0 and GraphPad Prism® 6.

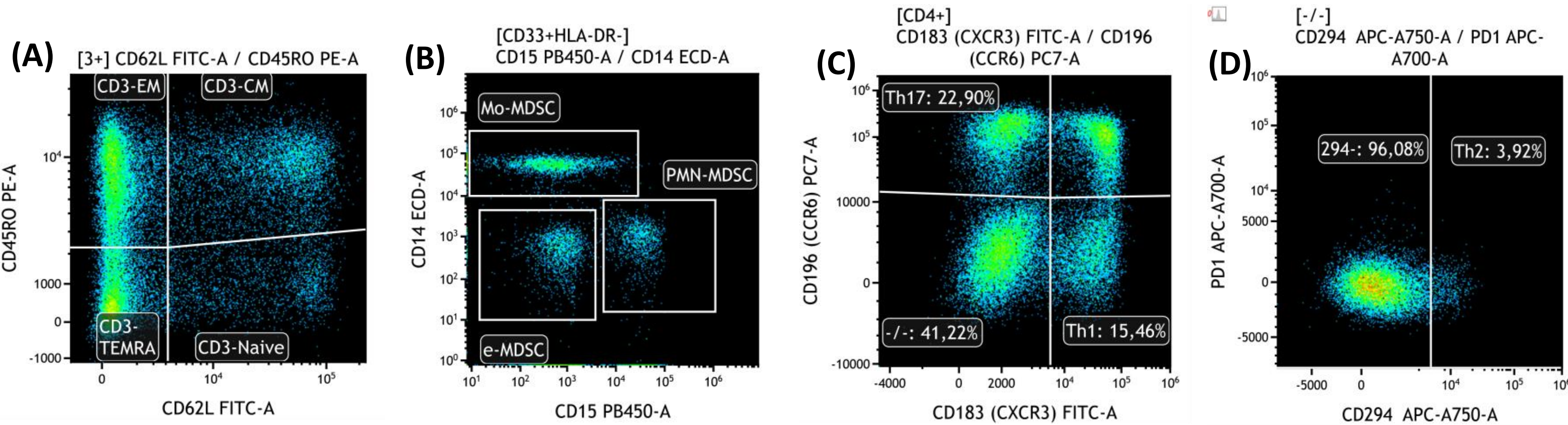### Acknowledgements

RESULTS AND DISCUSSION

Figure 1. Representative flow cytometry analysis of effector, memory and naïve T cells (A), the three suppressive myeloid cells subpopulations of interest (Mo-MDSC, PMN-MDSC and e-MDSC) (B), and helper T cells subtypes (C, D).
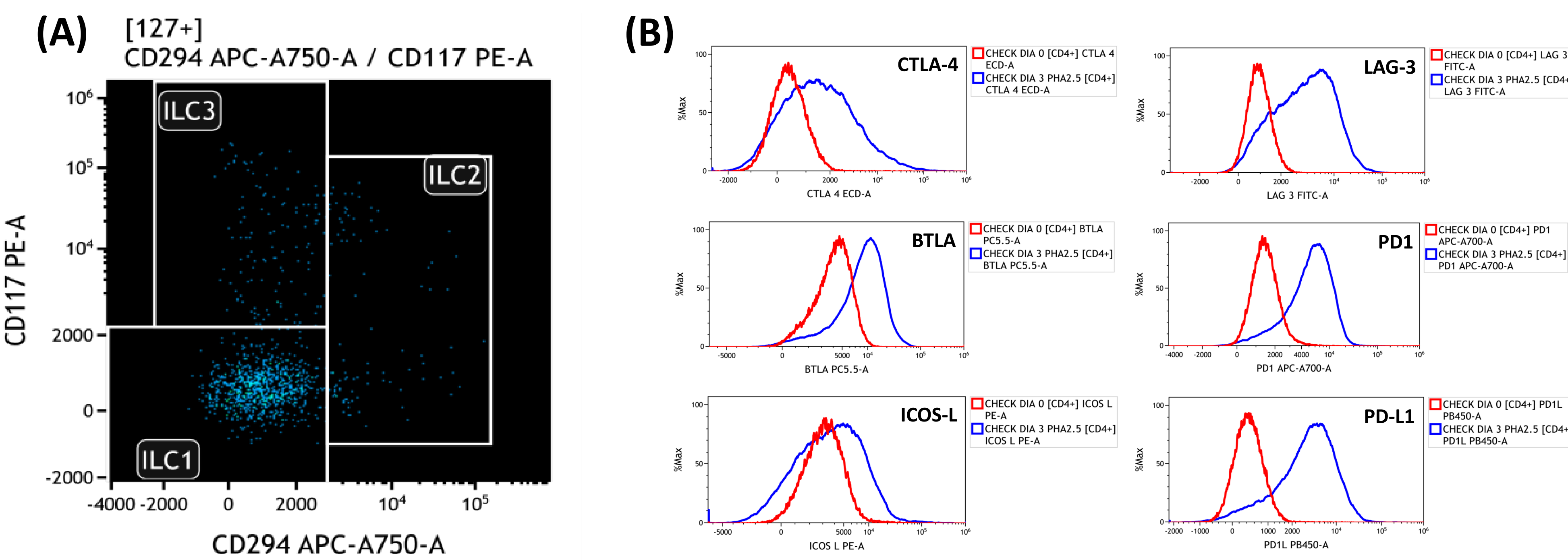

Figure 2. Representative flow cytometry analysis of the three main innate lymphoid cells subpopulations (ILC1, ILC2 and ILC3) (A), and the expression level of several *immune checkpoints* (CTLA-4, LAG-3, BTLA, PD-1, ICOS-L, PD-L1) and its difference between day 0 and after 3 days of PHA stimulation (B).
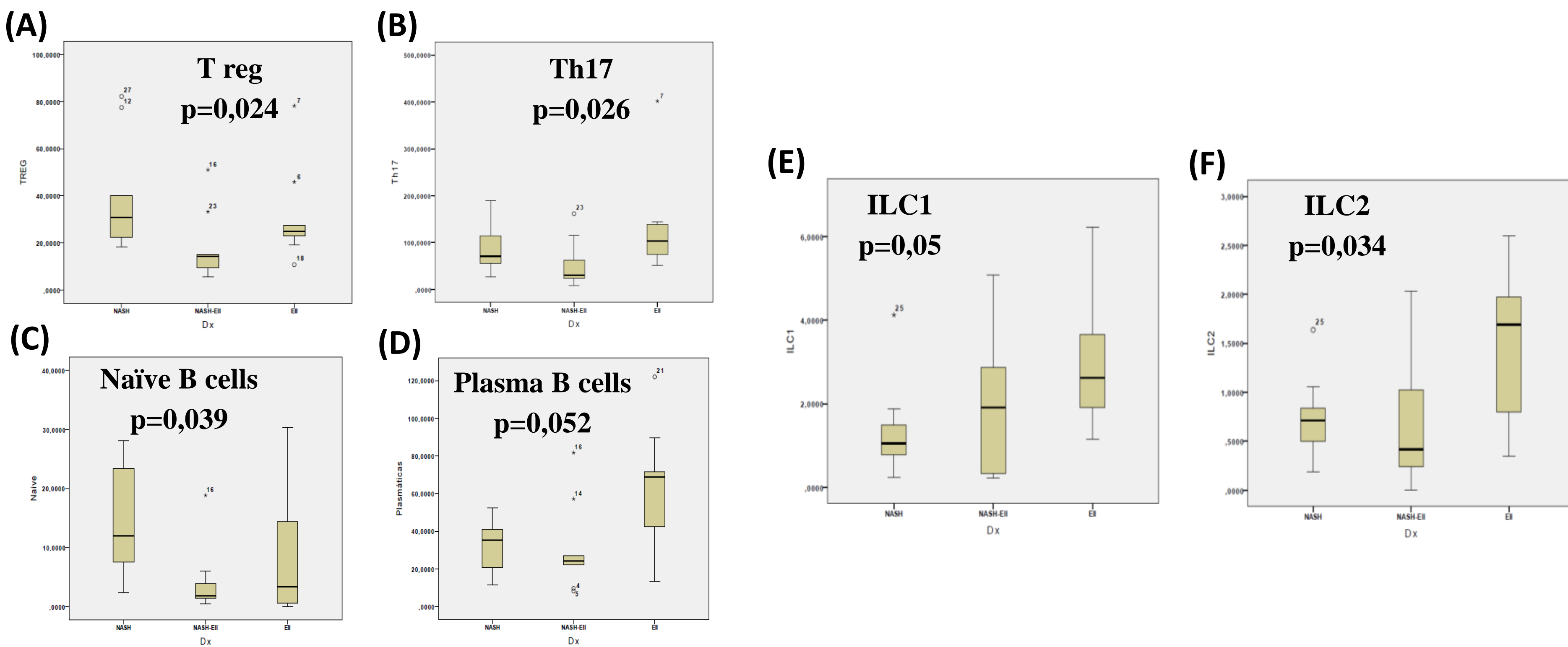

Figure 3. *Box plots* comparing three paciens groups (NASH, NASH coexisting with IBD and IBD) with several subpopulations analysed: Treg (A), Th17 (B), naïve B cells (C), plasma B cells (D), ILC1 (E) e ILC2 (F).

CONCLUSIONS

- The flow cytometry protocols have been set up with several monoclonal antibodies panels for the labeling and quantification of different immunoeffector and regulatory cell populations, from which has been successfully identified effector, memory and naïve T cells, regulatory T cells (Treg) and helper T cell subtypes; B lymphocytes during their different maturational stages, myeloid-derived suppressor cells (MDSC) and innate lymphoid cells (ILC); as well as the expression level of several *immune checkpoints* and its difference after three days of PHA stimulation.

- **In patients with NASH**, a large T activation (TEMRA) and increased B cells in immature stages have been highlighted.

- **In IBD patients**, a decreased T activation (T naïve) with a noticeable inflammatory environment defined by Th17 and ILC1, and increased B cells in mature stages (memory and plasma B cells) have been showed.

- **The coexistence of both pathologies in the NASH+IBD group** has showed T activation as in NASH, a marked inflammatory environment (decreased ratio Treg/Th17, increased ILC1 and decreased ILC2) and a generalized B cell populations decrease.

FURTHER RESEARCH

This is a proof of concept small study to set up the methodology to characterize the main immune cell subtypes and to implement it in a larger study in the context of IMID project at HUM Valdecilla.

References

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