Phenotype and polyfunctionality of iNKT cells in different forms and treatments of multiple sclerosis

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Introduction. Associations between Multiple Sclerosis (MS) and defects in invariant natural killer T cells (iNKT) have been reported, but data are constrasting, mainly due to methodological issues. iNKT cells are potent cytokine producers, have immunoregulatory potential, and can be divided into functionally distinct subsets. A more detailed characterization of iNKT cell subsets is needed to better clarify their role in MS, particularly in the different forms of the disease or during different treatments. Aim of our study was not only to **evaluate the amount of these cells in different forms** of MS, but also **to characterize their phenotype and functional activities**.

Methods. We studied **165 MS patients:** 17 untreated, newly-diagnosed patients with Active1 Relapsing-Remitting MS, (ARR), 19 untreated patients with not-active1 RRMS (NARR), 20 untreated Primary Progressive (PP) and 24 untreated Secondary Progressive (SP) MS patients (classified according to Lublin *et al.*, Neurology 83: 278-286, 2014). Furthermore, we enrolled RR patients who had been treated for at least six months with either glatiramer acetate (GA) (29 patients), beta-Interferon-1a (IFN) (31 patients) or Natalizumab (NAT) (25 patients). Fifty-five healthy subjects were enrolled as controls. In particular, because of different ages in MS forms, we recruited 39 CTR <46 years of age (younger CTR, Y CTR) and 16 CTR >46 years of age (Adult CTR, A CTR). The control group was divided in order to eliminate any potential effect of aging on iNKT cells. Therefore, YCTR were compared to ARR patients while ACTR were compared to NARR and progressive patients. For the quantitative analysis of iNKT cells, CD3+ T cells were counted using a CyFlow Counter (Partec, Germany). Fresh PBMC were stained with different mAbs and analyzed on a 6-color acoustic focusing Attune flow cytometer (Thermofisher, USA). We used anti-Vα24Jα18Vβ11 TCR, -CD4, -CD8, -CD161, -CD3, -CD19 and -CD14 mAbs. A minimum of 5 million cells per sample were acquired (Figure 1). We detected the polyfunctionality of iNKT cells by analyzing their capacity to produce up to 4 cytokines simultaneously in 41 RR (11 treated with GA, 12 treated with IFN, 13 treated with NAT, 5 NARR), 4 PP, 12 SP and 26 CTR. PBMC were stimulated for 4 hours with PMA plus ionomycin, then fixed and stained with Live Dead dye, anti-Vα24Jα18Vβ11 TCR, -CD4, -CD8, -CD3, -IL-17A,-TNF-α, -IFN-γ, and IL-4, according to standard methods. Up to 20 million cells were acquired on a 14-color high speed acoustic focusing Attune NxT flow cytometer (Figure 2). Data were analyzed by FlowJo 9.8.5 and Stata 11.0 softwares using Ranksum and Kruskal Wallis test. With linear regression, we analyzed the associations between clinical and immunological parameters. Bonferroni correction for multiple testing was applied when necessary.

Gating strategy for iNKT cells and their main subsets

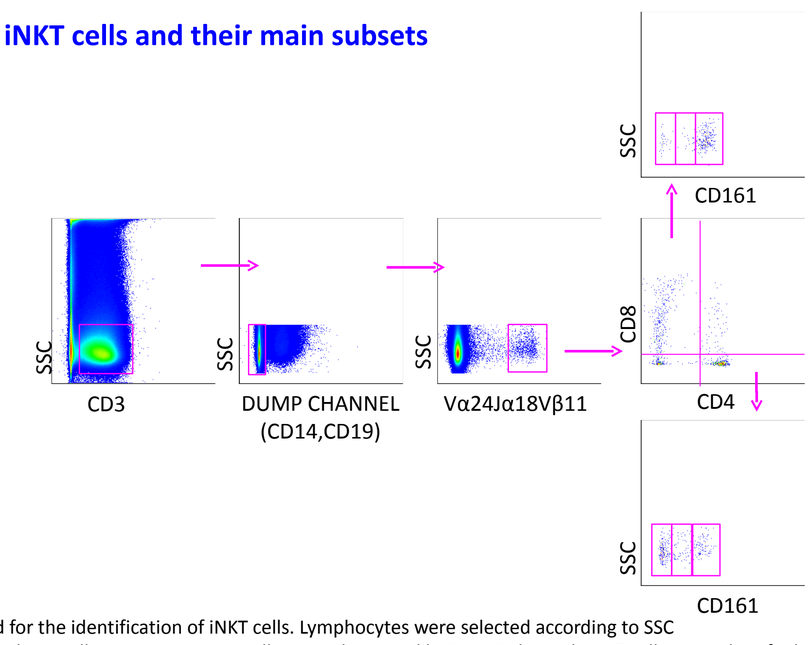

Figure 1. Gating strategy used for the identification of iNKT cells. Lymphocytes were selected according to SSC and positivity for CD3. Among these cells, monocytes or B cells were eliminated by DUMP channel. iNKT cells were identified according to the positivity for TCR-Vα24Jα18Vβ11. CD4 and CD8 expression was analyzed among iNKT cells; CD161 was evaluated in each subpopulation.

Gating strategy for iNKT polyfunctionality

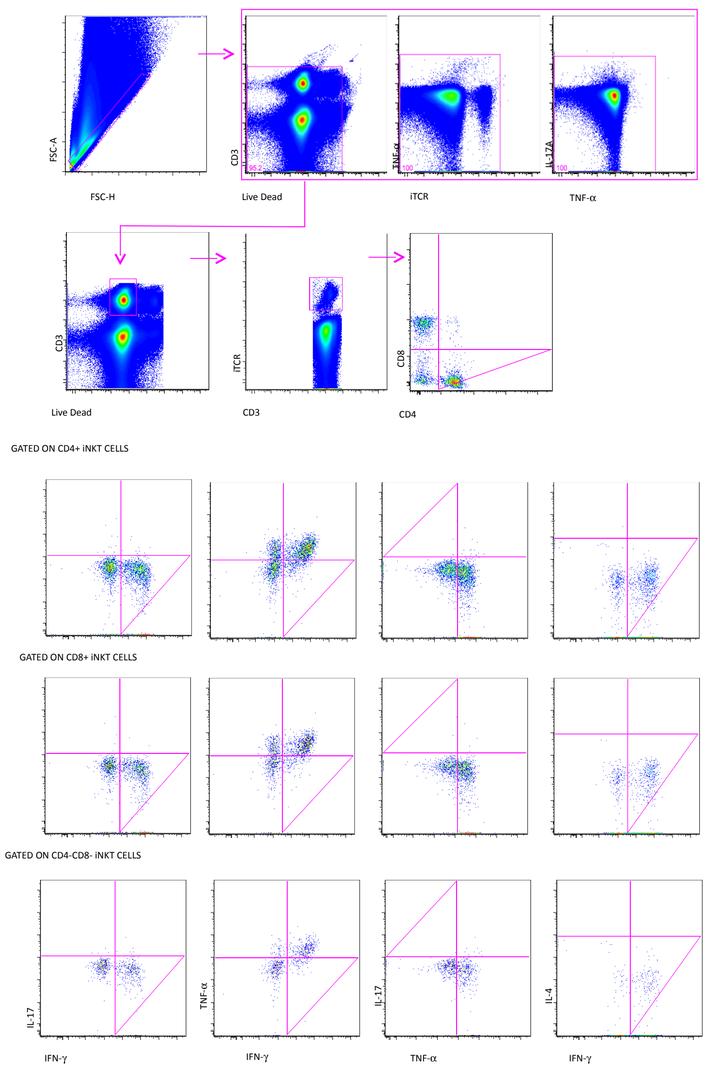

Figure 2. Gating strategy used for the identification of cytokine production by iNKT cells. Doublets and fluorochrome aggregates were removed. Living T lymphocytes were selected according to negativity for Live Dead and positivity for CD3. Among these cells, iNKT cells were identified according to the positivity for TCR-Vα24Jα18Vβ11. CD4 and CD8 expression was analyzed among iNKT cells; production of IFN-γ, IL-17, TNF-α and IL-4 were evaluated in each subpopulation of iNKT cells.

Results. No differences were found in the amount of iNKT and their subsets among different forms and treatments of MS (Figure 3). However, in MS patients, the polyfunctional response of iNKT cells mainly showed Th-1 and Th17 profiles. In SP patients, iNKT cells expressing CD4+ produced the highest level of TNF-α or TNF-α plus IL-17 (Figure 4). Then, CD8+ iNKT cells expressed high levels of TNF-a associated to IFN-g or to IL-17 (Figure 5). In these patients, also CD4-CD8- iNKT cells produced high levels of TNF-α, and of IFN-γ plus TNF-a. Concerning IL-4, no differences were found between MS patients and controls. Among RR patients, those treated with IFN-1b showed a higher production of TNF-α in all subsets of iNKT cells when compared with patients treated with Nat (Figure 6, Figure 7, Figure 8).

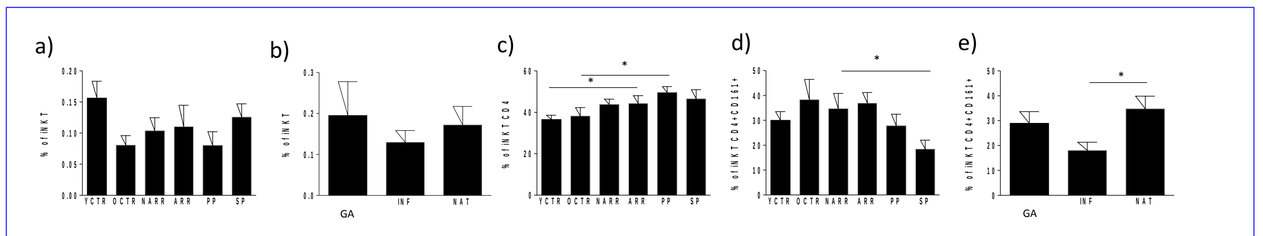

Figure 3. Percentages of iNKT cells and their subpopulations among the different groups of MS patients and controls. **a)** percentage of iNKT cells among patients with different MS form; **b)** percentage of iNKT cells among different RR treated patients. **c)** percentage of CD4+ iNKT cells among patients with different MS form **d)** percentage of CD4+ iNKT cells among different RR treated patients. **e)** percentage of CD4+ iNKT cells expressing CD161+ among patients with different MS form ARR: Active Relapsing-Remitting, NARR: untreated patients with not-active RRMS, PP: untreated Primary Progressive (PP), SP: Secondary Progressive; GLA: RR patients who had been treated for at least six months with either glatiramer acetate (GLA), or with beta-Interferon-1a (IFN) or with natalizumab (NAT). YCTR: donors whose age was <46 years; OCRT: older than 46 years. Data are represented as the mean ± SEM.

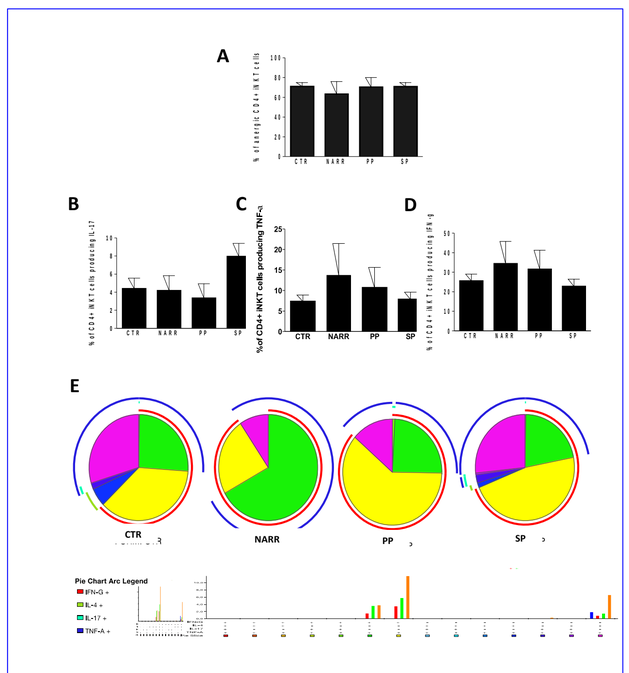

Figure 4. Cytokine production by CD4+ iNKT cells a): Percentage of CD4+ iNKT cells not responding after in vitro stimulation; b) total IL-17 production by CD4+ iNKT cells; c) total TNF-a production by CD4+ iNKT cells; d) total IFN-g production by CD4+ iNKT cells; e) Polyfunctionality of CD4+ iNKT cells after in vitro stimulation. Data shown in the cakes are presented as arc (total production of a given cytokine) and slices (production of one or more molecules). Bars are related to (f) the percentage of cells producing only TNF-alpha but not other cytokines. *P<0.05. Bar graphs are represented as mean ± SEM.

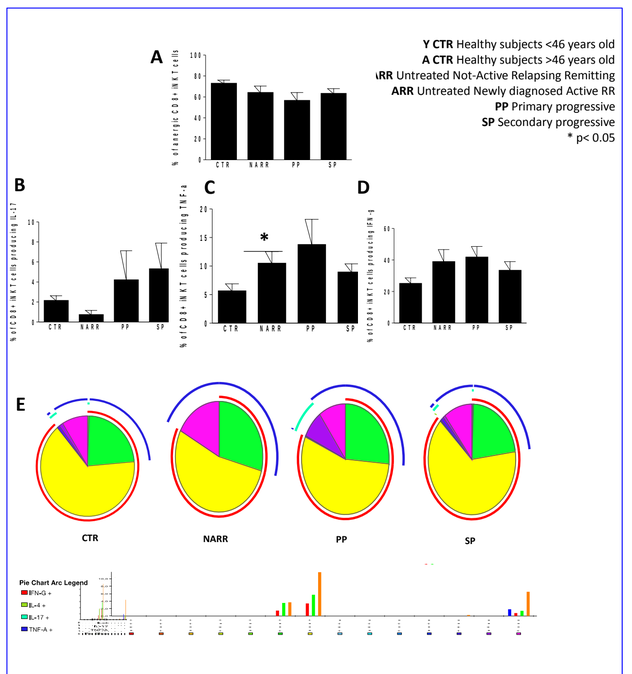

Figure 5. Cytokine production by CD8+ iNKT cells a): Percentage of CD8+ iNKT cells not responding after in vitro stimulation; b) total IL-17 production by CD8+ iNKT cells; c) total TNF-a production by CD8+ iNKT cells; d) total IFN-g production by CD8+ iNKT cells; e) Polyfunctionality of CD8+ iNKT cells after in vitro stimulation. Data shown in the cakes are presented as arc (total production of a given cytokine) and slices (production of one or more molecules). *P<0.05. Bar graphs are represented as mean ± SEM.

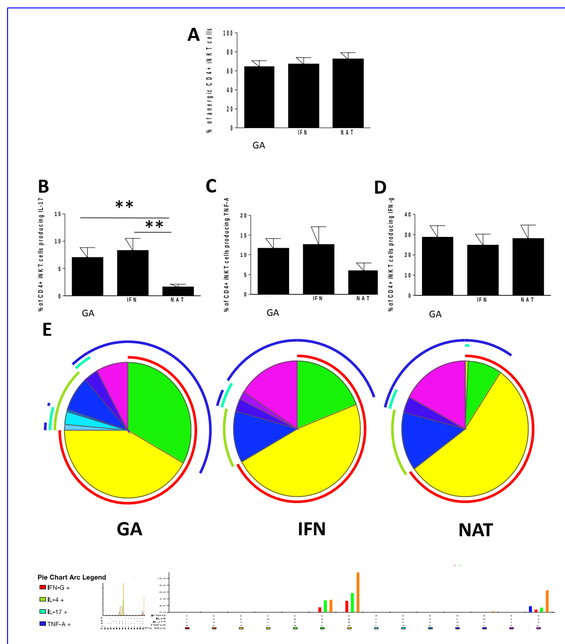

Figure 6. Cytokine production by CD4+ iNKT cells of RR patients treated with different immunomodulatory drugs. a): Percentage of CD4+ iNKT cells not responding after in vitro stimulation; b) total IL-17 production by CD4+ iNKT cells; c) total TNF-a production by CD4+ iNKT cells; d) total IFN-g production by CD4+ iNKT cells; e) Polyfunctionality of CD8+ iNKT cells after in vitro stimulation. Data shown in the cakes are presented as arc (total production of a given cytokine) and slices (production of one or more molecules). *P<0.05. Bar graphs are represented as mean ± SEM.

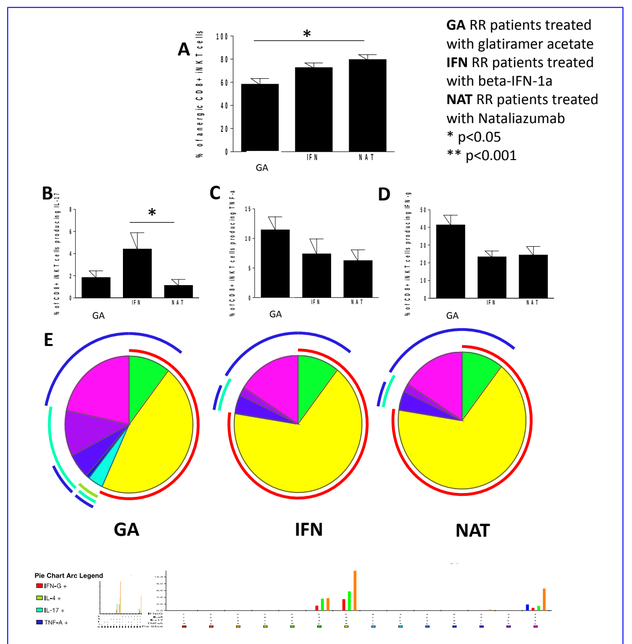

Figure 7. Cytokine production by CD8+ iNKT cells of RR patients treated with different immunomodulatory drugs. a): Percentage of CD8+ iNKT cells not responding after in vitro stimulation; b) total IL-17 production by CD8+ iNKT cells; c) total TNF-a production by CD8+ iNKT cells; d) total IFN-g production by CD4+ iNKT cells; e) Polyfunctionality of CD8+ iNKT cells after in vitro stimulation. Data shown in the cakes are presented as arc (total production of a given cytokine) and slices (production of one or more molecules). Bars are related to (f) the percentage of cells producing only IFN-g but not other cytokines. *P<0.05. Bar graphs are represented as mean ± SEM.

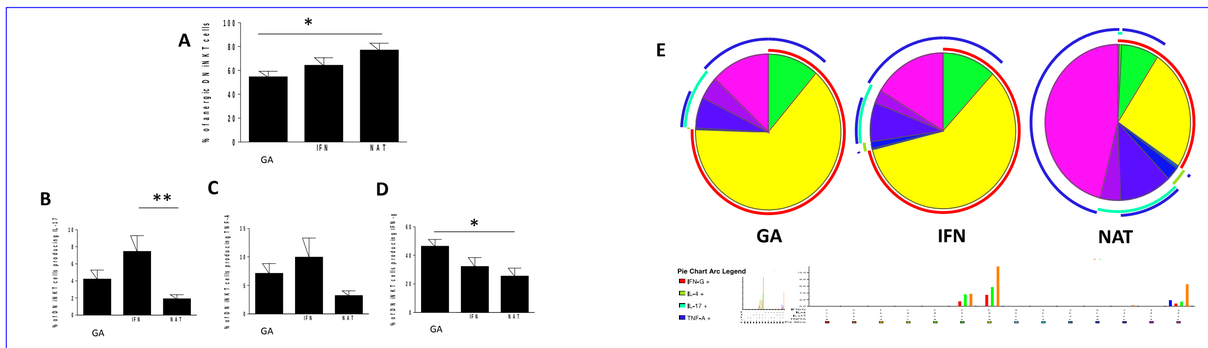

Figure 8. Cytokine production by CD4-CD8- iNKT cells of RR patients treated with different immunomodulatory drugs. a): Percentage of CD4-CD8- iNKT cells not responding after in vitro stimulation; b) total IL-17 production by CD4-CD8- iNKT cells; c) total TNF-a production by CD4-CD8- iNKT cells; d) total IFN-g production by CD4+ iNKT cells; e) Polyfunctionality of CD4-CD8- iNKT cells after in vitro stimulation. Data shown in the cakes are presented as arc (total production of a given cytokine) and slices (production of one or more molecules). Bars are related to (f) the percentage of cells producing only TNF-alpha but not other cytokines. *P<0.05. Bar graphs are represented as mean ± SEM.

Conclusion. SP patients exhibited lower percentages of iNKT CD4+CD161+ cells and **a sustained increase in the production of Th1 and Th17 cytokines by iNKT cells**. This could suggest that the progressive phase of the disease is characterized by a permanent iNKT activation. RR patients treated with Natalizumab showed a significantly lower production of IL-17 and TNF-α compared to patients treated with IFN or glatiramer acetate, which could reflect its higher level of efficacy on disease activity in RRMS.