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Immunometabolic profiling of T cells from patients with relapsing-remitting multiple sclerosis reveals an impairment in glycolysis and mitochondrial respiration

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ABSTRACT

Background. Metabolic reprogramming is shaped to support specific cell functions since cellular metabolism controls the final outcome of immune response. Multiple sclerosis (MS) is an autoimmune disease resulting from loss of immune tolerance against central nervous system (CNS) myelin. Metabolic alterations of T cells occurring during MS are not yet well understood and their studies could have relevance in the comprehension of the pathogenetic events leading to loss of immune tolerance to self and to develop novel therapeutic strategies aimed at limiting MS progression.

Methods and Results. In this report, we observed that extracellular acidification rate (ECAR) and oxygen consumption rate (OCR), indicators of glycolysis and oxidative phosphorylation, respectively, were impaired during T cell activation in naïve-to-treatment

Abbreviations: MS, multiple sclerosis; RR, relapsing remitting; CNS, central nervous system; PBMCs, peripheral blood mononuclear cells; IFN, interferon; Treg, regulatory T cells; Tconv, conventional T cells; iTreg, inducible regulatory T cells; TCR, T cell receptor; sCD40L, soluble CD40 ligand; sICAM-1, soluble ICAM-1; MCP-1, monocyte chemoattractant protein-1; MPO, myeloperoxidase; OPG, osteoprotegerin; sTNF-R, soluble tumor necrosis factor receptor; sLeptinR, soluble leptin receptor; ECAR, extracellular acidification rate; OCR, oxygen consumption rate; DLST, dihydrolipoamide succinyltransferase; DLAT, dihydrolipoamide S-acetyltransferase; RANKL, receptor activator of nuclear factor kappaB ligand; EAE, experimental autoimmune encephalomyelitis; CIA, collagen-induced arthritis; T1D, type 1 diabetes; Th, T helper; E2, exone2; BMI, body mass index.

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relapsing remitting (RR)MS patients when compared with healthy controls. These results were also corroborated at biochemical level by a reduced expression of the glycolitic enzymes aldolase, enolase 1, hexokinase I, and by reduction of Krebs cycle enzymes dihydrolipoamide-S-acetyl transferase (DLAT) and dihydrolipoamide-S-succinyl transferase (DLST). Treatment of RRMS patients with interferon beta-1a (IFN beta-1a) was able to restore T cell glycolysis and mitochondrial respiration as well as the amount of the metabolic enzymes to a level comparable to that of healthy controls. These changes associated with an up-regulation of the glucose transporter-1 (GLUT-1), a key element in intracellular transport of glucose.

Conclusions. Our data suggest that T cells from RRMS patients display a reduced engagement of glycolysis and mitochondrial respiration, reversible upon IFN beta-1a treatment, thus suggesting an involvement of an altered metabolism in the pathogenesis of MS.

1. Introduction

Multiple Sclerosis (MS) is a demyelinating disease mediated by pathogenic T cell responses against myelin antigens followed by a broader neurodegenerative process [1]. Hyperactivity of T cells observed during MS is also associated with the failure of local regulatory mechanisms mainly mediated by regulatory T (Treg) cells [2]. The balance between protective immunity and inflammatory response requires that T cells properly activate and differentiate into either Treg or conventional T (Tconv) cells. In this context, it has been reported that Tconv and Treg cells require distinct metabolic programs to support their own functions, suggesting that metabolism drives the outcome of immune response. While Tconv are quiescent *in vivo*, Treg cells represent the most actively proliferating and glycolitic compartment *in vivo* [3–5].

The close relationship between the systemic metabolic asset and immune function was also suggested by data showing an altered immunometabolic profile in patients with autoimmune diseases. Indeed, the evaluation of the serum biomarkers involved in the control of both obesity and immune system function (ie. leptin, soluble CD40 ligand (sCD40L), soluble leptin receptor (sLeptinR)), revealed their unbalanced expression in autoimmune diseases such as type 1 diabetes (T1D), MS and Behcet's syndrome [6–8].

Metabolic alterations of T cells occurring during MS, have not been investigated so far, and could have relevance in the comprehension of the pathogenic mechanisms leading to the loss of immune tolerance to self. In this context, the role of interferon (IFN) beta-1a in the metabolic control of immune cells function has not been elucidated [9]. Here we report that T cells from naïve-to-treatment relapsing remitting (RR)MS patients displayed an impaired engagement of glycolysis and mitochondrial metabolism upon T cell receptor (TCR) activation, reversible upon IFN beta-1a treatment. These data provide novel evidence for an involvement of metabolism in the pathogenesis of RRMS and suggest a novel mechanism of action for IFN beta-1a through the modulation of $CD4^+$ T cell metabolic pathways.

2. Methods

2.1. Subjects and Study Design

We obtained peripheral blood from healthy individuals (n = 57), naïve-to-treatment (patients with clinically definite MS without treatment, either cortisone or other drugs) (n = 32) and IFN beta-1a (Rebif®-44; Merck-Serono) treated (n = 39) RRMS patients after they signed an informed consent approved by the Review Board of the Università degli Studi di Napoli "Federico II". RRMS patients had relapsing-remitting disease with a Kurtzke Expanded Disability Status Scale (EDSS) score between 0 and 7; we excluded subjects with concomitant endocrine and metabolic disorders. We included in the study healthy donors that had no history of inflammatory, endocrine or autoimmune diseases, and were gender-, age- and body mass index- matched with RRMS, (baseline characteristics of healthy controls and RRMS patients are shown in Table S1). We collected all blood samples at 9:00 a.m. in heparinized vacutainers (BD Biosciences) and processed them within the following 4 h.

2.2. Immunophenotypic Analysis

Immunophenotypic analysis of peripheral blood of RRMS patients and healthy controls was performed as previously described [6]. Briefly, triple combinations of different anti-human mAbs (*e.g.*, FITC- and phycoerythrin (PE)-anti-CD3, PE- and PC-5-anti-CD4, PC5-anti-CD8, PE-antiCD16, PC5-anti-CD19, PE-anti-CD25, FITC-anti-CD45, and PEanti-CD56 all from Coulter Immunotech), were used for immunofluorescence staining and to identify different cell populations. Analysis was performed with an EPICS XL flow cytometer (Beckman Coulter) using the Beckman Coulter software program XL system II.

2.3. Fluorescent Bead-based Immunoassay

All serum samples from RRMS patients and controls were obtained *via* centrifugation and stored at −80 °C before the analysis. The bead-based analyte detection system Human obesity 9plex kit (Bender MedSystems) was used to perform the quantitative detection of leptin, soluble CD40 ligand (sCD40L), soluble ICAM-1 (sICAM-1), monocyte chemoattractant protein-1 (MCP-1), myeloperoxidase (MPO), osteoprotegerin (OPG) and soluble tumor necrosis factor (sTNF)-R by Flow Cytometry.

2.4. sLeptinR Measurement

Circulating soluble leptin receptor (sLeptinR) was determined in serum samples using human Leptin sR Immunoassays (R&D System).

2.5. Metabolic Seahorse Assays

The metabolic profile has been evaluated in 12 h cultured peripheral blood mononuclear cells (PBMCs) stimulated with OkT3 (mouse anti-human CD3) 0.1 μg/ml. Cells were plated in XF^e-96 plates (seahorse Bioscience) at the concentration of 4×10^5 cells/well and cultured for 12 h in RPMI-1640 medium supplemented with 100 UI ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Thermo scientific) and 5% autologous serum. Real-time measurements of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were made with an XF^e-96 Extracellular Flux Analyzer (Seahorse Bioscience). OCR was measured in XF medium (non-buffered DMEM medium containing 10 mM glucose and 1 mM sodium pyruvate) under basal conditions and in response to 5 μM oligomycin, 1.5 μM of FCCP (carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone) and 1 μM of antimycin and rotenone (Sigma-Aldrich). ECAR was measured in XF medium in basal conditions and in response to 10 mM glucose, 5 μM oligomycin and 100 mM of 2DG (all from Sigma-Aldrich). Experiments with the Seahorse system were done with the following assay conditions: 3 min of mixture; 3 min of waiting; and 3 min of measurement.

2.6. Western Blot Analyses

Total cell lysates from CD4$^+$ T cells, were obtained through incubation of cells for 20 min at 4 °C in a solution of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl and 1.0% Triton X-100, plus SigmaFast protease inhibitor (S8820; Sigma-Aldrich) and Sigma phosphatase inhibitor (P5726; Sigma-Aldrich), and immunoblot analyses were performed using the following antibodies: anti-aldolase, anti-enolase 1, anti-hexokinase I, anti-DLAT, anti-DLST (all 1:1000 dilution and from Cell Signaling Technology, Beverly, MA) anti-Glut-1 (1:500 dilution and from Abcam) and anti- VDAC (1:1000 dilution and from Santa Cruz Biotechnology). The filters were also probed with an ERK1/2 antibody (1:1000 dilution from Santa Cruz Biotechnology) to normalize for the amount of loaded protein.

2.7. Statistical Analysis

Comparison between healthy controls, naïve-to-treatment and IFN beta-1a treated RRMS patients were evaluated using non-parametric one-way ANOVA test (Kruskal-Wallis test) with Dunn's post-hoc test or chi-square test. We verified the Gaussian distribution with appropriate test, D'Agostino-Pearson omnibus normality test. Statistical analyses were performed with GraphPad program (Abacus Concepts).

3. Results

3.1. Immunometabolic Profiling of RRMS Patients Reveals a Specific Effect of IFN Beta-1a Treatment on Immune Cell Populations and Circulating Inflammatory Adipocytokines

We analyzed the immunological and metabolic profile of naïve-to-treatment and IFN beta-1a treated RRMS patients respectively. We measured peripheral blood immune cell subpopulations (Fig. 1A–H) and the plasma levels of different immunometabolic markers such as leptin, soluble leptin receptor (sLeptin-R), sCD40 ligand (sCD40L), osteoprotegerin (OPG), myeloperoxidase (MPO) and sICAM-1 (Fig. 1I–N). Naïve-to-treatment RRMS patients had a higher number of CD4$^+$ T cells with a memory phenotype (CD4$^+$CD45RO$^+$) with respect to healthy controls ($P \leq 0.05$) while their number was lower in IFN beta-1a treated when compared to naïve-to-treatment RRMS patients ($P \leq 0.05$) (Fig. 1G). IFN beta-1a treated RRMS patients were characterized by a reduced number of lymphocytes, CD3$^+$ and CD4$^+$ T cells when compared with naïve to treatment RRMS patients ($P \leq 0.05$) (Fig. 1A–C). In addition, patients treated with IFN beta-1a displayed a lower number of total lymphocytes, CD3$^+$ T cells, CD8$^+$ T cells and NK cells, T cells with a naïve phenotype (CD3$^+$CD45RA$^+$) and CD8$^+$CD11b$^+$ cells (recently activated effector CD8$^+$ T cells) as compared with healthy control subjects ($P \leq 0.05$ and $P \leq 0.001$) (Fig. 1A and B, D–F and H).

Parallel analysis of the plasma immunometabolic biomarkers revealed that RRMS patients, at diagnosis, showed significant higher levels of the circulating adipocytokine leptin ($P \leq 0.05$) (Fig. 1I) and OPG ($P \leq 0.05$) (Fig. 1L), and a lower concentration of MPO ($P \leq 0.05$) and sICAM-1 ($P \leq 0.05$) (Fig. 1M and N) as compared with those in control subjects. IFN beta-1a treatment resulted in a serum leptin downtrend as testified by the loss of significant difference in leptin levels between controls and treated patients (Fig. 1I). In addition, IFN beta-1a treated patients showed a significant higher concentration of circulating sLeptin-R ($P \leq 0.05$) and a lower level of sCD40L ($P \leq 0.05$) and OPG ($P \leq 0.0001$) with respect to naïve-to-treatment RRMS patients (Fig. 1J–L).

3.2. Reduced Engagement of T Cell Glycolysis and Mitochondrial Respiration in naïve-to-treatment RRMS Patients are Recovered by IFN Beta-1a Treatment

We analyzed the extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR), indicators of glycolysis and oxidative phosphorylation, respectively, in PBMCs isolated from healthy controls, naïve-to-treatment and IFN beta-1a treated RRMS patients upon T cell receptor (TCR) anti-CD3 stimulation (OKT3) (Fig. 2). We observed that, naïve-to-treatment RRMS patients had a lower glycolytic rate (Fig. 2A) compared to healthy controls as testified by impaired, basal, maximal glycolysis (oligomycin-stimulated) and glycolytic capacity ($P \leq 0.05$ and $P \leq 0.001$) (Fig. 2B-D). In agreement with these findings also the O_2 consumption rate (OCR), an indicator of oxidative phosphorylation (OXPHOS), was lower in naïve-to-treatment RRMS patients as compared to healthy controls (Fig. 2E) as indicated by a decreased basal, maximal respiration and spare capacity (ability to respond to ATP demand) ($P \leq 0.05$, $P \leq 0.001$ and $P \leq 0.0001$) (Fig. 2F–H). Surprisingly, in IFN beta-1a treated patients we detected a higher glycolysis, as testified by higher maximal glycolysis and glycolytic capacity when compared with naïve-to-treatment RRMS patients ($P \leq 0.05$) (Fig. 2A–D). IFN beta-1a treatment was able to improve mitochondrial respiration in RRMS patients, since basal and maximal respiration and the spare capacity were higher in IFN beta-1a treated with respect to naïve-to-treatment RRMS patients ($P \leq 0.05$) (Fig. 2E–H).

3.3. Key Enzymes of Glycolysis and Mitochondrial Respiration are Reduced in Naive-to-treatment RRMS Patients and Restored by Treatment with IFN Beta-1a

Next, we evaluated whether the above observed functional impairment of ECAR and OCR in RRMS subjects associated

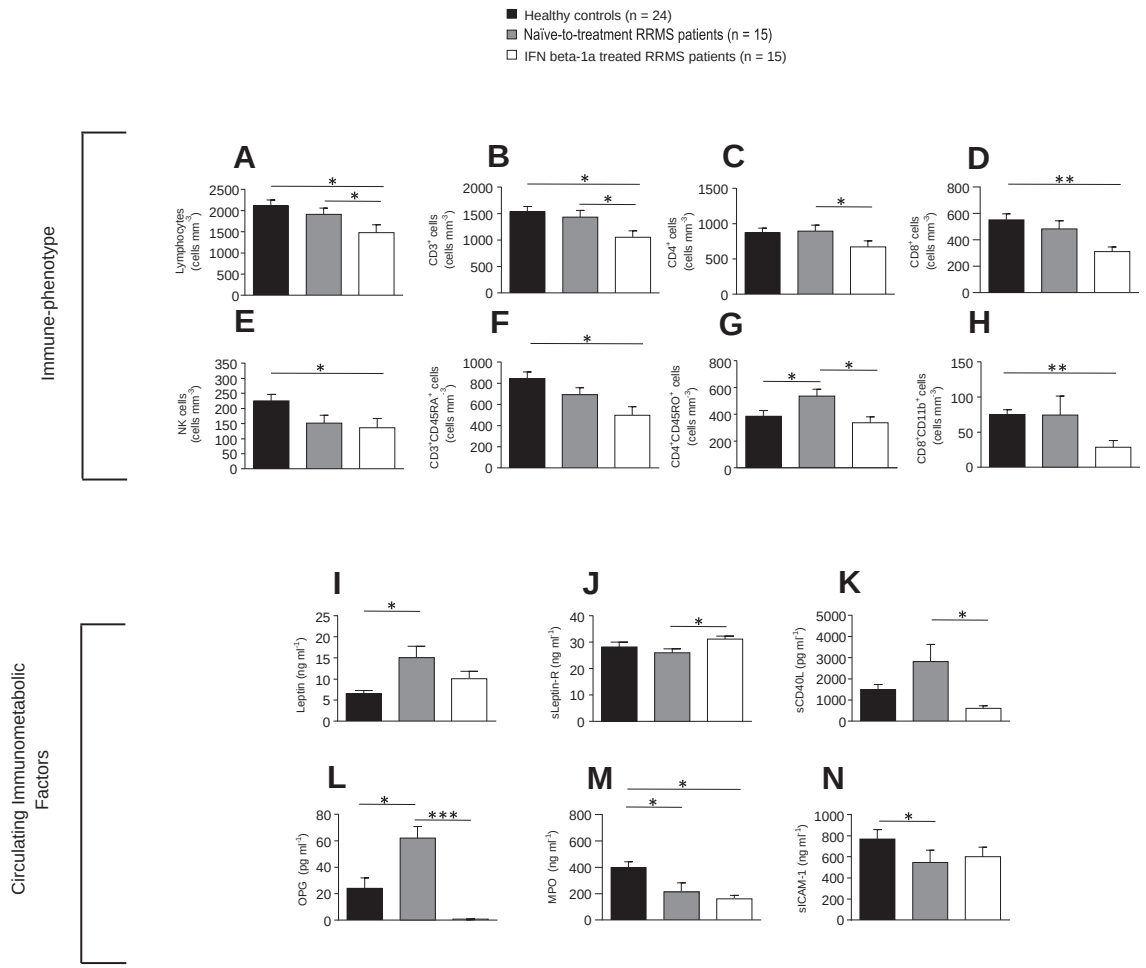

Fig. 1 – Peripheral immunometabolic signature of RRMS patients before and after IFN beta-1a treatment. The graphs show the number per mm^3 of several peripheral blood immune cell subpopulations and the serum concentration of several cytokines implicated in the control of metabolism and immune system functions in healthy controls (n = 24), naïve-to-treatment (n = 15) and IFN beta-1a treated (n = 15) RRMS patients. Data are shown as mean ± s.e.m. Comparisons were evaluated using non-parametric one-way ANOVA test (Kruskal-Wallis test) and Dunn's post-hoc test *P ≤ 0.05, **P ≤ 0.001, ***P ≤ 0.0001.

with a parallel alteration in the expression of key enzymes involved in glycolysis and mitochondrial respiration, respectively. Western blotting analyses on purified CD4$^+$ T cells from naïve-to-treatment RRMS patients stimulated for 12 h with anti-CD3/CD28 beads, revealed a statistically significant reduction in aldolase, enolase 1, hexokinase I, and dihydrolipoamide S-acetyltransferase (DLAT), and a trend towards a lower expression of the glucose transporter Glut-1, and of dihydrolipoamide succinyltransferase (DLST) (Fig. 3A and B). To rule out that these changes were to be ascribed to a reduction in the mitochondrial mass, we evaluated the expression of Voltage-Dependent Anion Channel (VDAC), a marker of reflecting the amount of mitochondrial proteins (Fig. S1). We observed that the naive-to-treatment RRMS patients showed an higher level of VDAC compared with healthy controls and IFN beta-1a treated MS patients, thus suggesting that the deficit in the metabolic mitochondrial enzymes level in naïve-to-treatment RRMS patients was not secondary to a reduced mitochondrial mass and number (Fig. S1A and B). Interestingly, IFN beta-1a treatment restored the amount of glycolitic and mitochondrial proteins to a level comparable to that of healthy controls (Fig. 3A and B).

4. Discussion

Disturbed metabolic pathways could lead to alterated functions of immune cells resulting in loss of immune tolerance to self [10]. In this context, several studies have demonstrated that cell metabolism is critical to regulate T cell fate since distinct metabolic programs may direct towards either effector or regulatory functions [11], and that important inhibitors of metabolism can regulate immune response in healthy and pathogenic cells [12]. Little is known about the systemic and cellular metabolic changes of immune cells occurring in MS patients [6] and, in addition, the effect of immunomodulating therapies on T cells metabolism (ie. IFN beta-1a) still remains unclear.

In this study, we investigated the systemic immunometabolic signature and the metabolic profile of T cells from patients with RRMS before and after immunomodulating therapy with IFN beta-1a. Specifically, we performed a comprehensive peripheral blood immuno-phenotype of healthy controls, naïve-to-treatment and IFN beta-1a treated RRMS patients to evaluate the effect of pharmacological treatment on immune cells populations number.

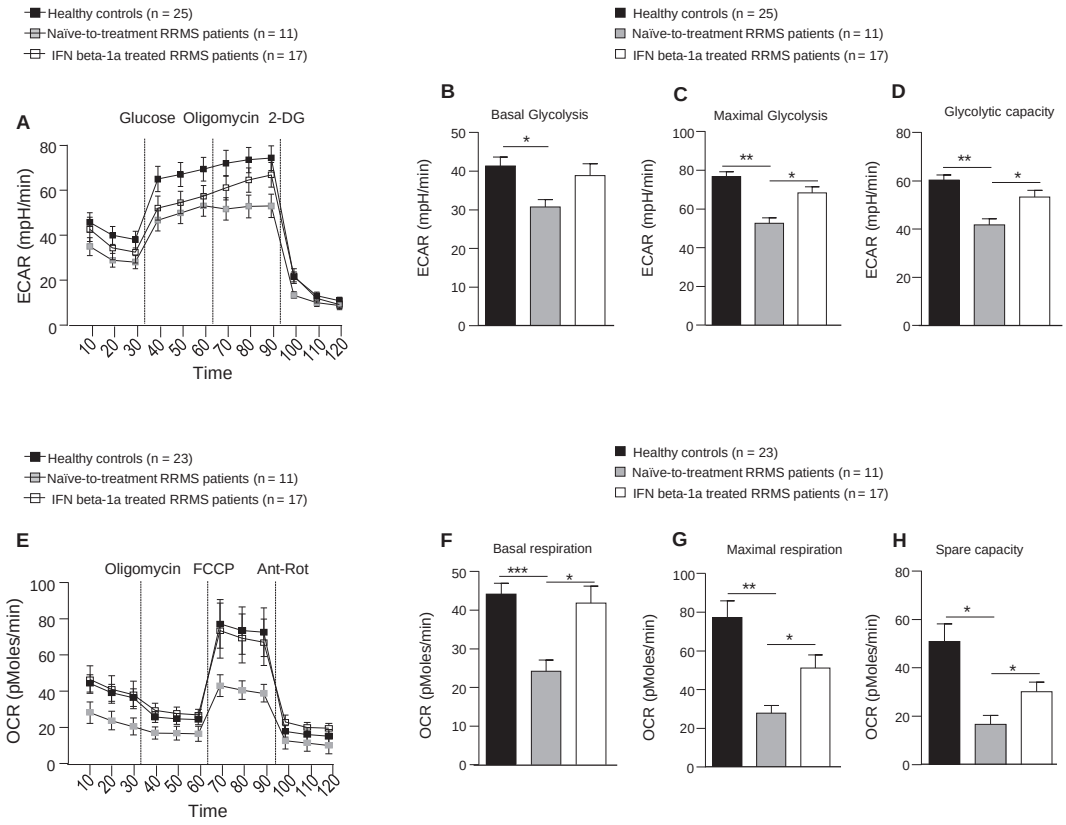

Fig. 2 – Impaired engagement of glycolysis and mitochondrial respiration of T cells from RRMS is reversed by IFN beta-1a treatment. The graphs represent the metabolic profile of PBMCs isolated from healthy controls, naïve-to-treatment and IFN beta-1a treated RRMS patients. (A) Kinetic profile of ECAR in PBMCs stimulated with OKT3 for 12 h. Healthy controls *n* = 25, naïve-to-treatment *n* = 11 and IFN beta-1a treated *n* = 17 RRMS patients. The data are shown as mean ± s.e.m. ECAR was measured in real time, under basal conditions and in response to glucose, oligomycin and 2-DG. Indices of glycolytic pathway activation, calculated from PBMCs ECAR profile: (B) basal, (C) maximal glycolysis and (D) glycolytic capacity. Data are expressed as mean ± s.e.m. (E) Kinetic profile of OCR in PBMCs stimulated with OKT3 for 12 h. Healthy controls *n* = 23, naïve-to-treatment *n* = 11 and IFN beta-1a treated *n* = 17 RRMS patients. The data are shown as mean ± s.e.m. OCR was measured in real time, under basal conditions and in response to oligomycin, FCCP, Antimycin A and Rotenone. Indices of mitochondrial respiratory function, calculated from PBMCs OCR profile: (F) basal, (G) maximal respiration and (H) spare capacity in PBMCs stimulated with OKT3 for 12 h. Data are expressed as mean ± s.e.m. Comparisons were evaluated using non-parametric one-way ANOVA test (Kruskal-Wallis test) and Dunn's post-hoc test $^{*}P \leq 0.05$, $^{**}P \leq 0.001$, $^{***}P \leq 0.0001$.

Naïve-to-treatment RRMS patients showed a higher number of $CD4^+$ T cells with memory phenotype ($CD45RO^+$) probably ascribed to chronic inflammatory state that leads to an increase of memory population and a decrease in the pool of naïve T cells, thus confirming also other studies that found an increased frequency and activation of the memory $CD4^+$ T cell compartment in MS [6,13]. In our experimental setting, IFN beta-1a treatment reduced $CD4^+$ memory T cell population, likely reflecting the effect of therapy on abnormally persistent systemic activation occurring in MS patients. This result is in agreement with other reports showing that both either IFN beta-1a or glatiramer acetate were able to restore the imbalance in the number of memory $CD4^+$ T cells in MS subjects [14]. At metabolic level, we confirmed that RRMS patients had higher levels of the adipocytokine leptin [15], known to be increased during inflammation and autoimmunity. IFN beta-1a treatment induced a trend, even if not statistically significant, towards a decrease in serum leptin. sLeptinR represents the main leptin-binding protein in human blood and biologically it can modulate leptin effects on cells target either by inhibiting the binding of leptin to its membrane receptors or by increasing the availability of circulating leptin, delaying its clearance [16]. IFN beta-1a therapy resulted in an increase of sLeptinR levels that could act as a decoy molecule and inhibit leptin activity in the periphery. OPG, a protein member of the tumor necrosis factor (TNF) receptor family and its ligand, namely receptor activator of nuclear factor kappaB ligand (RANKL), are principally involved in the regulation of osteoclastogenesis [17]; however abnormalities of the OPG/RANKL system have been reported in various immune-mediated human diseases, such as rheumatoid arthritis, periodontal disease, autoimmune thyroid disease, coronary artery disease and myeloma bone disease [18,19]. In line with this evidence, OPG levels were higher in naive-to-treatment RRMS patients and IFN beta-1a treatment was able to decrease significantly OPG levels in RRMS patients. In addition, we also measured the amount of sCD40L, known to be involved in the pathogenesis of

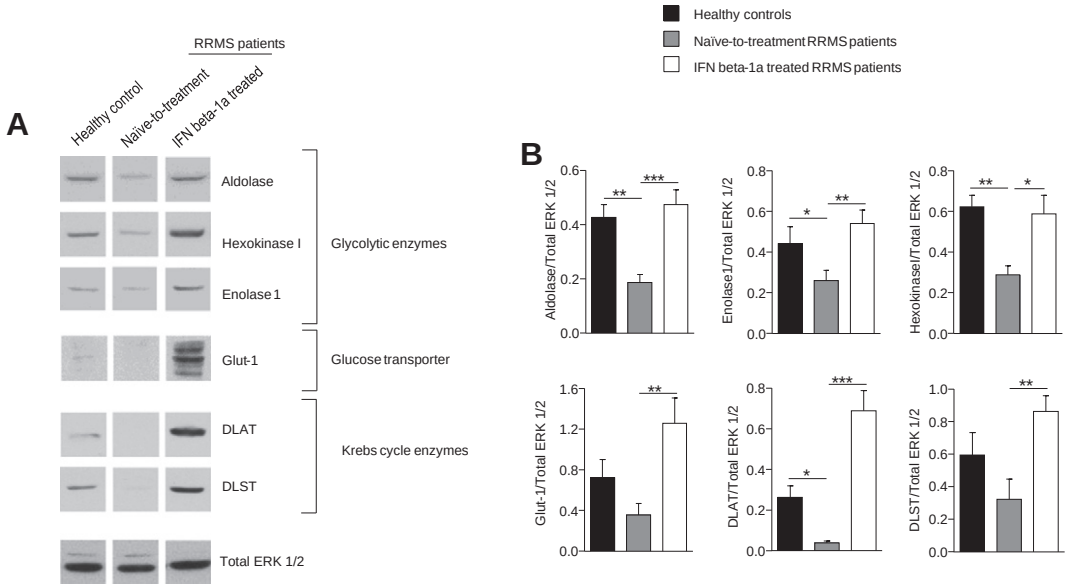

Fig. 3 – Biochemical pathways of CD4⁺ T cells from RRMS patients before and after IFN beta-1a treatment. (A) Immunoblot for aldolase, hexokinase I, enolase 1, Glut-1, DLAT and DLST on CD4⁺ T cells from healthy control, naïve-to-treatment and IFN beta-1a treated RRMS patient upon 12 h anti-CD3/CD28 stimulation. Total ERK 1/2 served as a loading control. One representative out of at least three independent experiments. (B) The graphs show the relative densitometric quantitation of aldolase, hexokinase I, enolase 1, Glut-1, DLAT and DLST normalized on total ERK1/2 in healthy controls, naïve -to-treatment and IFN beta-1a treated RRMS patients. One representative experiment is shown, from one individual for each condition; in total we runned al least 3 healthy controls, 3 naive-to-treatment and 3 RRMS IFN beta-1a treated patients. We scanned 3 times at least three films with different exposures from each subject, and averaged values were utilized as densitometry to reduce variations among samples. Comparisons were evaluated using non-parametric one-way ANOVA test (Kruskal-Wallis test) and Dunn's post-hoc test *P ≤ 0.05, **P ≤ 0.001, ***P ≤ 0.0001.

autoimmunity such as MS, experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA) and T1D by inducing costimulation and T helper (Th)1 responses [20–22]. Also, since CD40/CD40L mRNAs have been previously reported to be increased in non-stimulated PBMCs from MS patients compared with healthy controls [23], we measured plasma sCD40L and observed that IFN beta-1a treatment reduced significantly its leves. Finally, we evaluated MPO and observed that its levels are lower in RRMS patients and remained unchanged after IFN beta-1a treatment.

One of the immune abnormalities observed in RRMS is a reduction in the suppressive function and/or the peripheral frequency of the anti-inflammatory $CD4^+CD25^+Foxp3^+$ Treg cells [24]. Recent studies demonstrated that human Treg cells *in vivo* represent the highly proliferative and metabolically active fraction of $CD4^+$ T cells, whose suppressive function tightly depends on glycolysis *via* the induction of specific Foxp3 gene splicing variants containing the E2, Foxp3E2 [5]. Indeed, we have previously demonstrated that glycolysis is indispensable for the generation of human inducible (i)Treg cells from Tconv cells *in vitro* [5,25]. However, in literature there are only apparently contrasting results suggesting that Treg cells rely mainly on lipid oxidation rather than glycolysis. These results could be ascribed to the different experimental setting utilized since lipid oxidative *in vitro* induced Treg cells were obtained with high doses of IL-2 and TGF-beta in the presence of strong TCR ligation [11]. However, more recently also other groups reported that Treg cell proliferation associates with increased PI(3)K-Akt-mTORC1 signaling, induction of glycolysis and expression of Glut1 [25,26]. In this report we found that T cells from RRMS patients were less glycolytic at functional and biochemical level. Since the engagement of glycolytic pathway is crucial for the induction and function of Treg cells [5], we hypothesize that the reduced engagement of glycolysis in Tconv cells from RRMS patients could associate with an impaired generation of Treg cells leading to loss of self immune tolerance. In this context, we also evaluated the peripheral frequency of Treg cells, and we did not observe a significant change in their number in our window of observation (data not shown). This evidence, suggests that the clinical response to IFN beta-1a, in terms of tolerance induction, may be still present since Treg cells could be better induced by IFN beta-1a treatment and migrate into the central nervous system (CNS) thus not necessarily increasing in the periphery.

Our data are in agreement with results showing a glycolytic deficit also in other autoimmune diseases, such as rheumatoid arthritis (RA), in which T cells showed a defect in glycolysis [27]. However, it has also been considered that other studies have reported increased glycolysis in T cells from MS patients [28]. This could ascribed to a different method used for the assessment of glucose metabolism. Indeed, we analyzed functional ECAR/OCR as indicators of glycolysis and mitochondrial respiration, respectively, while in literature reported data mainly rely on the evaluation of the activity of isolated glycolytic enzymes and not on the whole cell. Further, our data are in agreement with previous studies

reporting the capacity of IFN beta-1a to induce glucose uptake (and consequently glycolysis), *via* translocation of glucose transporters on the cell surface [29]. It is important to note that IFN beta-1a effects were observed only during *in vivo* treatment with IFN beta-1a in RRMS patients, while *in vitro* stimulation of PBMCs did not result in an induction of glycolysis and mitochondrial respiration (data not shown), thus suggesting that IFN beta-1a is able to induce glycolysis in T cells only upon chronic treatment. Finally, our data could also be instrumental for creation of novel markers of disease progression and response to therapy, showing a change in the metabolic response to IFN-beta1a treatment in MS subjects.

In conclusion, our data support the hypothesis of a possible involvement of an altered metabolism of Tconv cells in response to TCR stimulation in the pathogenesis of MS. This notion is corroborated by data showing that metabolic manipulation with metformin and pioglitazone has beneficial anti-inflammatory effects in patients with MS [30], as well as a diet with low calorie and protein content [31,32]. For example, a diet with very low calorie and pioglitazone administration reduce the incidence and severity of EAE in C57BL/6 mice [31–33] and treatment with metformin ameliorates clinical score and mitigates the inflammation in CNS by reducing secretion of IL-17 and by enhancing that of IL-10 and TGF-β [34]. Other evidence supporting the involvement of metabolism in MS pathogenesis is the observation that elevated body mass index (BMI) and obesity correlated with an increased susceptibility to MS [35–38].

5. Conclusion

Although it would be necessary to increase the number of patients and follow them over time during IFN beta-1a therapy, these data suggest the presence of metabolic alterations of T cells in RRMS, and highlight an important role for IFN beta-1a in modulating cellular metabolism through the induction of glycolysis and mitochondrial respiration, which could lead to an increased generation of Treg cells and consequent improvement of immune tolerance during disease course.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.metabol.2017.08.011.

Author Contributions

C.L.R., F.C., V.D.R., A.C., M.G., and F.P. performed experiments; R.L., V.B., G.O., I.C., C.F., and G.T.M. recruited patients, contributed to conception and design of the study; M.S., D.C., A.U., S.L., C.L.R., F.C., G.M and A.V. designed the study and wrote the manuscript; G.M. coordinated the study.

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Disclosures

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REFERENCES

[1] Compston A, Coles A. Multiple sclerosis. Lancet 2008;372:1502–17.
[2] Costantino CM, Baecher-Allan C, Hafler DA. Multiple sclerosis and regulatory T cells. J Clin Immunol 2008;28:697–706.
[3] Vukmanovic-Stejic M, Zhang Y, Cook JE, Fletcher JM, McQuaid A, Masters JE, et al. Human CD4+ CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo. J Clin Invest 2006;116:2423–33.
[4] Vukmanovic-Stejic M, Agius E, Booth N, Dunne PJ, Lacy KE, Reed JR, et al. The kinetics of CD4+Foxp3+ T cell accumulation during a human cutaneous antigen-specific memory response in vivo. J Clin Invest 2008;118:3639–50.
[5] De Rosa V, Galgani M, Porcellini A, Colamatteo A, Santopaolo M, Zuchegna C, et al. Glycolysis controls the induction of human regulatory T cells by modulating the expression of FOXP3 exon 2 splicing variants. Nat Immunol 2015;16:1174–84.
[6] Carrieri PB, Carbone F, Perna F, Bruzzese D, La Rocca C, Galgani M, et al. Longitudinal assessment of immuno-metabolic parameters in multiple sclerosis patients during treatment with glatiramer acetate. Metabolism 2015;64:1112–21.
[7] Cantarini L, Pucino V, Vitale A, Talarico R, Lucherini OM, Magnotti F, et al. Immunometabolic biomarkers of inflammation in Behçet's disease: relationship with epidemiological profile, disease activity and therapeutic regimens. Clin Exp Immunol 2016;184:197–207.

[8] Galgani M, Nugnes R, Bruzzese D, Perna F, De Rosa V, Procaccini C, et al. Meta-immunological profiling of children with type 1 diabetes identifies new biomarkers to monitor disease progression. Diabetes 2013;62:2481–91.
[9] Markowitz CE. Interferon-beta: mechanism of action and dosing issues. Neurology 2007;68(24 Suppl. 4):S8–11 [Review].
[10] Pearce EL, Poffenberger MC, Chang CH, Jones RG. Fueling immunity: insights into metabolism and lymphocyte function. Science 2013;342:1242454.
[11] Michalek RD, Gerriets VA, Jacobs SR, Macintyre AN, MacIver NJ, Mason EF, et al. Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4$^+$ T cell subsets. J Immunol 2011;186:3299–303.
[12] Bettencourt IA, Powell JD. Targeting metabolism as a novel therapeutic approach to autoimmunity, inflammation, and transplantation. J Immunol 2017;198:999–1005.
[13] Okuda Y, Okuda M, Apatoff BR, Posnett DN. The activation of memory CD4(+) T cells and CD8(+) T cells in patients with multiple sclerosis. J Neurol Sci 2005;235:11–7.
[14] Praksova P, Stourac P, Bednarik J, Vlckova E, Mikulkova Z, Michalek J. Immunoregulatory T cells in multiple sclerosis and the effect of interferon beta and glatiramer acetate treatment on T cell subpopulations. J Neurol Sci 2012;319:18–23.
[15] Matarese G, Carrieri PB, La Cava A, Perna F, Sanna V, De Rosa V, et al. Leptin increase in multiple sclerosis associates with reduced number of CD4(+)CD25+ regulatory T cells. Proc Natl Acad Sci U S A 2005;102:5150–5.
[16] Zastrow O, Seidel B, Kiess W, Thiery J, Keller E, Böttner A, et al. The soluble leptin receptor is crucial for leptin action: evidence from clinical and experimental data. Int J Obes Relat Metab Disord 2003;27:1472–8.
[17] LaceyDL TimmsE, TanHL Kelley MJ, Dunstan CR, Burgess T, et al. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 1998;93:165–76.
[18] Browner WS, Lui LY, Cummings SR. Associations of serum osteoprotegerin levels with diabetes, stroke, bone density, fractures, and mortality in elderly women. J Clin Endocrinol Metab 2001;86:631–7.
[19] Hofbauer LC, Cepok S, Hemmer B. Osteoprotegerin is highly expressed in the spinal cord and cerebrospinal fluid. Acta Neuropathol 2004;107:575–7.
[20] Gerritse K, Laman JD, Noelle RJ, Aruffo A, Ledbetter JA, Boersma WJ, et al. CD40-CD40 Ligand interactions in experimental allergic encephalomyelitis and multiple sclerosis. Proc Natl Acad Sci U S A 1996;93:2499–504.
[21] Munroe ME, Arbiser JL, Bishop GA. Honokiol, a natural plant product, inhibits inflammatory signals and alleviates inflammatory arthritis. J Immunol 2007;179:753–63.
[22] Sosvorova L, Kanceva R, Vcelak J, Kancheva L, Mohapl M, Starka L, et al. The comparison of selected cerebrospinal fluid and serum cytokine levels in patients with multiple sclerosis and normal pressure hydrocephalus. Neuro Endocrinol Lett 2015;36:564–71.
[23] Huang WX, Huang P, Hillert J. Systemic upregulation of CD40 and CD40 ligand mRNA expression in multiple sclerosis. Mult Scler 2000;6:61–5.
[24] Carbone F, De Rosa V, Carrieri PB, Montella S, Bruzzese D, Porcellini A, et al. Regulatory T cell proliferative potential is impaired in human autoimmune disease. Nat Med 2014;20:69–74.
[25] Procaccini C, Carbone F, Di Silvestre D, Brambilla F, De Rosa V, Galgani M, et al. The proteomic landscape of human ex vivo regulatory and conventional T cells reveals specific metabolic requirements. Immunity 2016;44:406–21.
[26] Gerriets VA, Kishton RJ, Johnson MO, Cohen S, Siska PJ, Nichols AG, et al. Foxp3 And toll-like receptor signaling balance T(reg) cell anabolic metabolism for suppression. Nat Immunol 2016;17:1459–66.
[27] Yang Z, Fujii H, Mohan SV, Goronzy JJ, Weyand CM. Phosphofructokinase deficiency impairs ATP generation, autophagy, and redox balance in rheumatoid arthritis T cells. J Exp Med 2013;210:2119–34.
[28] De Riccardis L, Rizzello A, Ferramosca A, Urso E, De Robertis F, Danieli A, et al. Bioenergetics profile of CD4(+) T cells in relapsing remitting multiple sclerosis subjects. J Biotechnol 2015;202:31–9.
[29] Burke JD, Platanias LC, Fish EN. Beta interferon regulation of glucose metabolism is PI3K/Akt dependent and important for antiviral activity against coxsackievirus B3. J Virol 2014;88:3485–95.
[30] Negrotto L, Farez MF, Correale J. Immunologic effects of metformin and pioglitazone treatment on metabolic syndrome and multiple sclerosis. JAMA Neurol 2016;73:520–8.
[31] Choi IY, Piccio L, Childress P, Bollman B, Ghosh A, Brandhorst S, et al. A diet mimicking fasting promotes regeneration and reduces autoimmunity and multiple sclerosis symptoms. Cell Rep 2016;15:2136–46.
[32] Choi IY, Lee C, Longo VD. Nutrition and fasting mimicking diets in the prevention and treatment of autoimmune diseases and immunosenescence. Mol Cell Endocrinol 2017;7207:30055-2.
[33] Feinstein DL, Galea E, Gavrilyuk V, Brosnan CF, Whitacre CC, Dumitrescu-Ozimek L, et al. Peroxisome proliferator-activated receptor-gamma agonists prevent experimental autoimmune encephalomyelitis. Ann Neurol 2002;51:694–702.
[34] Sun Y, Tian T, Gao J, Liu X, Hou H, Cao R, et al. Metformin ameliorates the development of experimental autoimmune encephalomyelitis by regulating T helper 17 and regulatory T cells in mice. J Neuroimmunol 2016;292:58–67.
[35] Mokry LE, Ross S, Timpson NJ, Sawcer S, Davey Smith G, Richards JB. Obesity and multiple sclerosis: a Mendelian randomization study. PLoS Med 2016;13:e1002053.
[36] Chitnis T, Grag J, Weinstock-Guttman B, Belman A, Olsen C, Misra M, et al. Distinct effects of obesity and puberty on risk and age at onset of pediatric MS. Ann Clin Transl Neurol 2016;4:897–907.
[37] Hedström AK, Lima Bomfim I, Barcellos L, Gianfrancesco M, Schaefer C, Kockum I, et al. Interaction between adolescent obesity and HLA risk genes in the etiology of multiple sclerosis. Neurology 2014;82:865–72.
[38] Hedström AK, Olsson T, Alfredsson L. Body mass index during adolescence, rather than childhood, is critical in determining MS risk. Mult Scler 2016;22:878–83.