Alterations of Mitochondrial Respiration and Complex I Activity in Mononucleate Cells from Psoriatic Patients: Possible Involvement of GRIM-19-STAT3α/β

Rosella Scrima¹, Claudia Piccoli¹, Giovanni Quarato¹, Maria Ripoli¹, Mario Mastrodonato² and Nazzareno Capitanio**

¹Department of Clinical and Experimental Medicine, University of Foggia, Foggia, Italy
²Medical and Surgical Sciences, University of Foggia, Foggia, Italy

Abstract

Objective: Although the pathogenesis of psoriasis is largely unknown accumulating evidences configure it as an immune-mediated disease determined through cytokines-mediated positive loops between activated lymphocytes subsets and keratinocytes. Mitochondria in addition to their role in the cell bioenergetics are now recognized as a decisional hub in controlling the immunological response. In the present study we compared mitochondria-related functions of PBMC between psoriatic patients and healthy controls.

Methods: Freshly isolated PBMC from eleven psoriatic patients and nine healthy controls were subjected to mitochondria-dependent respiratory activity measurements by high-resolution oxymetry and the specific activity of respiratory chain complexes assessed by spectrophotometric assays. Quantitative RT-PCR and immunoblotting were applied to detect the level of selected transcripts and proteins respectively.

Results: Respiriometric analysis unveiled in patients' cells a significant three-fold increase of oligomycin-sensitive endogenous mitochondria-driven oxygen consumption, which was traceable back to a specific increased activity of the respiratory chain complex I. Analysis by quantitative RT-PCR of transcription factors regulating the mitochondrial biogenesis did not result in significant changes between patients and control cells and was confirmed by the unaffected expression of the complex I subunits. Treatment of either patients’ or control cells with isoproterenol and IBMX ruled out the involvement of a cAMP-PKA-mediated post-transcriptional modification of the respiratory complex. GRIM19 a pleiotropic protein, involved in the structural and functional stabilization of complex I and in the mitochondrial translocation of STAT3 was significantly up-regulated in patients' cells. Phosphorylation at S727 of STAT3 was increased in patients cells, which, in addition, unveiled a shift in the relative expression of the STAT3α/β spliceforms.

Conclusion: Altogether the results obtained suggest the occurrence in circulating mononucleate cells from psoriatic patients of an altered activity of complex I likely mediated by up-regulation of GRIM19/STAT3β, which might lead to a chronic activation of T-lymphocytes thereby contributing to the development of psoriasis.

Keywords: Psoriasis; Peripheral blood mononuclear cells; Mitochondria; Respiratory chain; Complex I; GRIM19; STAT3

Introduction

Psoriasis is one of the most common immune-mediated chronic, inflammatory skin diseases characterized by hyperproliferative keratinocytes and infiltration of T cells, dendritic cells, macrophages and neutrophils. Although the pathogenesis of psoriasis is not fully understood, there is ample evidence suggesting that the dysregulation of immune cells in the skin, particularly T cells, plays a critical role in psoriasis development [1]. Consistent with other autoimmune-type diseases, psoriasis has been traditionally considered a T-helper (Th) 1-type disease [2]. However, several lines of evidence point to Th-17 cells as the main culprit with Th1 having a secondary role [3]. Some reports have also suggested the presence of activated autoreactive T cells in psoriatic [4].

The evolution of a psoriatic lesion entails a complex interplay between environmental and genetic factors, which sets the scene for a cascade of events that activate dendritic cells and T cells. Cross-talk between epithelial cells and immune cells shapes and maintains the inflammatory milieu [5,6]. The cytokine network in psoriasis is mainly characterized by IFN-γ, IL-12 and TNF-α. Major cytokine producers in the lesions are dendritic cells (DCs), CD4+ and CD8+ T cells and keratinocytes. IFN-γ and TNF-α induce keratinocytes to produce IL-6, IL-7, IL-8, IL-12, IL-15, IL-18 and TNF-α besides multitude of other cytokines, chemokines and growth factors. IL-18 acts on DCs synergistically with IL-12, to increase the production of IFN-γ. IL-7 and IL-15 are important for the proliferation and homeostatic maintenance of the CD8+ T cells. IL-17, which is produced by activated CD4+ T cells, synergizes with IFN-γ to elicit further production of pro-inflammatory cytokines by the keratinocytes. In this way the cytokine network in psoriasis can become a self-sustaining process [7].

Mitochondria are intracellular organelles known to contribute as ATP suppliers to the cellular bioenergetics. This is achieved by means of the oxidative phosphorylation (OxPhos), which is the cellular oxidative process with the highest energy yield [8]. In the last decades the interest on mitochondria has been shifted from their cell “powerhouse" to a chronic activation of T-lymphocytes thereby contributing to the development of psoriasis.

*Corresponding author: Nazzareno Capitanio, Department of Clinical and Experimental Medicine, University of Foggia, Via L. Pinto, c/o OO.RR., 71100 Foggia, Italy, Tel: +39 0881 711148; Fax +39 0881 58602; E-mail: nazzareno.capitanio@unifg.it

Received November 18, 2013; Accepted January 07, 2014; Published January 17, 2014


Copyright: © 2014 Scrima R, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
properties to their involvement in cell signalling pathways. Indeed, it is becoming increasingly apparent that mitochondria perform various signalling functions, serving as platforms to initiate cell signalling, as well as acting as transducers and effectors in multiple processes. These include cell death, growth factor signalling, differentiation, hypoxic stress responses, autophagy [9-12]. In general, mitochondria regulate cell signalling through two means: serving as physical platforms on which protein-protein signalling interactions occur, and by regulating the levels of intracellular signalling molecules, including Ca2+ and reactive oxygen species (ROS). More recently the array of cell functions controlled by mitochondria has encompassed innate and adaptive immunity as well as inflammatory response [13-16].

Mitochondrial functions in psoriatic keratinocytes have been studied with specific focus on their resistance to pro-apoptotic stimuli. These researches have led to the development of anti-psoriatic drugs, like anthralin, which accumulates in keratinocyte mitochondria and induces apoptosis through a pathway dependent on respiratory competent mitochondria [17]. However, to the best of our knowledge there is no report in literature aimed to characterize mitochondrial activities in immunocytes of psoriatic patients. In this study we investigated basic properties of the mitochondrial respiratory chain in the peripheral blood mononucleate cells (PBMC) from a cohort of psoriatic patients and show significant alterations at the functional and molecular level as compared with PBMC from healthy subjects.

Materials and Methods

Samples

Eleven patients affected by psoriasis before therapy and nine healthy volunteers were enrolled in this study (Table 1). Venous blood was withdrawn in Vacutainer® and immediately processed for isolation of peripheral blood mononucleonucleate cells (PBMC). Briefly, blood was diluted 1:1 in phosphate-buffered saline (PBS), delicately stratified on half volume of ficoll and centrifuged at 1800 rpm for 30 min; PBMC localized at the plasma-ficoll interface were harvested, washed in phosphate-buffered saline (PBS), counted and immediately assayed or frozen at -80°C. The tests described in this study were carried our serially on groups of the overall cohort according to their availability from the clinical center over a period of 18 months.

Measurement of respiratory activity

Freshly isolated PBMC were washed in PBS, resuspended in 10 mM KH2PO4, 27 mM KCl, 1 mM MgCl2, 40 mM Hepes, 0.5 mM EGTA, pH 7.1 at 4x106 cells/ml and immediately assayed for O2 consumption by high resolution oxymetry (Oxygraph-2k, Oroboros Instruments) at 37°C under continuous stirring. After attainment of a stationary endogenous substrate-sustained respiratory rate, 8 µg/ml of oligomycin was added followed after 5 min by the addition of 2 µg/ml valinomycin. The rates of oxygen consumption (OCR) were corrected for 3 mM KCN-insensitive respiration and normalized to the cell number. The respiratory control ratio (RCR) was obtained dividing the rates of oxygen consumption achieved before and after the addition of oligomycin. To note, frozen PBMC samples were unsuited for this analysis because after thawing ensued non-reproducible results.

Measurement of complex I and complex IV activity

PBMC were spun down, the resulting pellet resuspended in 0.32 M sucrose, 40 mM KCl, 20 mM Tris-HCl, 2 mM EGTA, pH 7.2, frozen at -80°C for 10 min, thawed at room temperature and subjected to 6 cycles of 10 sec sonicatation (>20 kHz). The specific enzymatic activities of the NADH:ubiquinone oxidoreductase (complex I) or the cytochrome c oxidase (complex IV) were assayed spectrophotometrically in 10 mM Tris-HCl, 1 mg/ml BSA, pH 8.0. Complex I was assayed (in the presence of 1 µg/ml of antimycin A plus 2 mM KCN) by following the initial 2 µg/ml rotenone-sensitive rate of 50 µM NADH oxidation (ε340 nm=6.22 mM-1 cm-1) in the presence of 200 µM decylubiquinone (dUQ) as electron acceptor. Complex IV was assayed by following (in the presence of antimycin A) the initial 2 mM KCN-sensitive rate of 20 µM ferro-cytochrome c oxidation under aerobic conditions. The

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Age of onset (years)</th>
<th>PASI</th>
<th>Control #</th>
<th>Sex</th>
<th>Age (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>24</td>
<td>20</td>
<td>20</td>
<td>1</td>
<td>F</td>
<td>38</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>37</td>
<td>17</td>
<td>35</td>
<td>2</td>
<td>M</td>
<td>36</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>20</td>
<td>10</td>
<td>5</td>
<td>3</td>
<td>M</td>
<td>22</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>41</td>
<td>21</td>
<td>10</td>
<td>4</td>
<td>F</td>
<td>44</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>57</td>
<td>27</td>
<td>5</td>
<td>5</td>
<td>M</td>
<td>27</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>33</td>
<td>13</td>
<td>20</td>
<td>6</td>
<td>F</td>
<td>34</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>30</td>
<td>15</td>
<td>10</td>
<td>7</td>
<td>F</td>
<td>26</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>55</td>
<td>30</td>
<td>26</td>
<td>8</td>
<td>M</td>
<td>35</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>36</td>
<td>15</td>
<td>5</td>
<td>9</td>
<td>M</td>
<td>52</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>34</td>
<td>30</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>36</td>
<td>26</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Baseline characteristics of the study population. PASI, Psoriasis Area and Severity Index.

<table>
<thead>
<tr>
<th>Gene product</th>
<th>Forward</th>
<th>Reverse</th>
<th>T_ambient (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>5'-TCCCATGGGTGGTTGGTATTTA-3'</td>
<td>5'-AAGACCAGGGCCATGAATACT-3'</td>
<td>58</td>
</tr>
<tr>
<td>IL-10</td>
<td>5'-GATGCCCTTACAGGAGTGAAG-3'</td>
<td>5'-GCAACGAGTTACCCCTAATA-3'</td>
<td>58</td>
</tr>
<tr>
<td>Rec p2AR</td>
<td>5'-GAGCACAAGCCTCAAGCACC-3'</td>
<td>5'-CTGGAAAGCGACATTGGAATG-3'</td>
<td>58</td>
</tr>
<tr>
<td>NRF1</td>
<td>5'-CTACTCTGTGGTGGAGAGCAAG-3'</td>
<td>5'-AATTCGCTTGATGGTGGAGAG-3'</td>
<td>62</td>
</tr>
<tr>
<td>NRF2</td>
<td>5'-GGCACAGGAAAGAGATGATGAGGC-3'</td>
<td>5'-GTTGGCACTGACTGCTGGTT-3'</td>
<td>62</td>
</tr>
<tr>
<td>TFAM</td>
<td>5'-CGTTCTTCGAGATCGATCAG-3'</td>
<td>5'-GTGGAACCTGCTGCGAGACAG-3'</td>
<td>62</td>
</tr>
<tr>
<td>GRIM-19</td>
<td>5'-ACGGAAAGTGGGATAGTCT-3'</td>
<td>5'-GCTACGTCGTCGTCGCTCT-3'</td>
<td>60</td>
</tr>
<tr>
<td>ND5</td>
<td>5'-CATCAAGGACTGAGCAACT-3'</td>
<td>5'-GCTGTGACTGCTGTGACAC-3'</td>
<td>60</td>
</tr>
<tr>
<td>NDUFS8</td>
<td>5'-CCACATTCAACTCACCCTGTT-3'</td>
<td>5'-AAGCAGAGATTGACACCT-3'</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 2: Primers and respective temperatures of annealing used for RT-PCR analyses.
activities were normalized to the initial cell number and to cellular protein content.

Quantitative RT-PCR

Total RNA was isolated by Absolutely RNA miniprep (Stratagene, La Jolla, CA) following the manufacturer's instructions. The first strand cDNA was synthetized by 1.0 µg of total RNA was reverse-transcribed with 300 ng of Random Hexamers primers using (Invitrogen, Carlsbad, CA) and AccuScript High Fidelity Reverse Transcriptase (Stratagene). Quantitative real-time PCR reactions were performed with the thermocycler MX3000P (Stratagene) with 1.5 µl of cDNA, 300 nM primers (Table 2) and Brilliant SYBR Green QPCR Master Mix (Stratagene) in a reaction volume of 25 µl. The reaction program included preincubation at 95°C for 3 min and 40 cycles consisting of denaturation (95°C for 30 s), annealing (at the temperatures indicated in Table 2 for 30 s), and elongation (72°C for 15 s). The β-actin gene amplification was used as a reference standard to target the signal using the 2ΔΔCt method. Analysis of the melting curves and agarose gel electrophoresis were performed to confirm the specificity of the amplified products.

Western blotting

Two×10^6 PBMC were washed twice with cold PSB and lysed in RIPA buffer (62.5 mmol/L Tris-Cl, pH 6.8, 2% (w/v) SDS, 5 mmol/L dithiotreitol). Cell lysate was centrifuged at 13,000 rpm for 10 min at 4°C, the resulting supernatant collected and assayed for protein by the Bradford method. Forty µg protein of cell lysate were separated by SDS-PAGE on 12.5% polyacrylamide and transferred by electro-blotting on PVDF membrane (polyvinylidene fluoride, Bio-Rad) for 1 h at 100 mV at 4°C using as transferring buffer 20 mM Tris base, 150 mM glycine, 20% v/v methanol and then treated with blocking buffer (10% FBS in TTBS) for 1 h at room temperature. The membrane was incubated with the primary Ab for the protein of interest for 16 h at 4°C after then the TTBS-washed membrane was incubated for 1 h at room temperature with 1:8000 horseradish peroxidase-conjugated suited secondary antibody (Santa Cruz) and analysed/visualized by chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific) with the VersaDoc Imaging System (Bio-Rad, Quantity One software). Densitometric analysis of the digitalized images was carried out with the public domain software ImageJ (rsweb.nih.gov) by a dedicated tool. The Abs used and the respective dilutions were the following: rabbit anti-β2-AR (1:300, Santa Cruz), mouse anti-STAT3 and rabbit anti-P-STAT3-Ser727 (1:1000, Cell Signaling), mouse anti-β-actin (1:10000, Sigma).

Statistical analysis

Two tailed Student’s t test was applied to evaluate the significance (i.e. P<0.05) of differences measured throughout the data sets reported. The data from a given measurement are represented as box-and-whiskers plot with the bottom and top of the box indicating the first and third quartile respectively, the band inside the box is the median and the ends of the whiskers are one standard deviation above and below the mean of the data.

Results

A cohort of 11 patients affected by psoriasis was enrolled in this study. The PASI index ranged from 5 to 35 (Table 1). Nine healthy subjects were used as control. PBMC were isolated from patients and controls and the activation state of T-lymphocyte subsets was assessed evaluating the transcript levels of interferon gamma (IFN-γ) and interleukin 10 (IL-10) specifically expressed by Th1 and Th2/Treg respectively [18,19]. The box plot in Figure 1 shows that the transcript level of IFN-γ resulted in a significant three-fold increase, on an average basis, in patients’ PBMC as compared with controls. The transcript level of IL-10 in patients’ PBMC was also increased albeit not-reaching statistical significance vs. control cells. These data indicated the occurrence in psoriatic patients of an altered immunologic homeostasis resulting in a Th1-mediated pro-inflammatory setting.

Isolated PBMC were subjected to respirometric analysis by high-resolution oxymetry. Figure 2A details the experimental protocol used and is representative of a comparative measurement. It is shown that patients’ cells exhibited a significant three fold enhanced rate of oxygen consumption (OCR) under resting respiration relaying on endogenous substrates (Figure 2B). Addition of the F0F1-ATP synthase inhibitor oligomycin resulted in a significant depression of the OCR indicating that a significant part of the mitochondrial respiration was coupled to ATP synthesis (Figure 2C). Conversely, in control PBMC, oligomycin caused only a slight decrease of the OCR. Consequently, the respiratory control ratio (RCR), attained dividing the resting OCR by that in the presence of oligomycin, was higher in patients’ cells indicating a more efficient OxPhos therein (Figure 2D). Collapse of the mitochondrial transmembrane potential (∆Ψm) by addition of the K+-ionophore valinomycin restored the OCR to the levels of the resting respiration in both patients’ and control cells. These activities were almost fully inhibited by KCN and therefore attributable to the mitochondrial respiratory chain. In keeping that PBMC represent a heterogeneous population of mononucleate cells isolation of CD3+T cells by cell sorting was attempted. However, the yield obtained from patients’ samples was insufficient for the respirometric tests to be performed. Moreover, pilot experiments with sufficient amount of sorted control samples resulted to be time-consuming and detrimental to the functional properties of mitochondria (i.e. very low or absent respiratory control ratio). In addition, the utilization of the selecting antibody introduced a further
variable to be considered. Therefore, our conclusions rely on the notion that metabolically active lymphocytes represent the largest cell population (≈75%) in freshly prepared PBMC.

To get deeper insights into the cause of the observed enhanced mitochondrial respiration in patients' PBMC, the activities of the respiratory chain complexes I (NADH:ubiquinone oxidoreductase) and IV (cytochrome c oxidase) were assessed on cell lysates by specific spectrophotometric assays. Figures 3A and 3B show that the activity of complex I was significantly two-fold increased whereas that of complex IV was practically unchanged in patients' cells as compared with controls.

The activity of complex I is controlled post-translationally by covalent modifications including PKA-mediated phosphorylations [20]. Upstream of PKA the β2-adrenergic receptor (β2-AR) has been reported to play a role in T-lymphocyte subsets activation [21-23] and more specifically in the development of psoriasis [24-28]. The expression of the β2-AR is reported in Figures 4A and 4B. It is shown that both at the mRNA and protein level the β2-AR did not change significantly in patients' PBMC as compared with controls. Moreover, when the activity of complex I was assessed in PBMC preincubated with the β2-AR agonist isoproterenol plus the phosphodiesterase pan-inhibitor IBMX no changes were observed both in patients' and control PBMC as compared with untreated samples (Figure 3A). All

**Figure 2:** Measurement of mitochondrial respiratory activities in control and psoriatic intact PBMC. (A) Representative oxymetric traces of mitochondrial respiration in PMBC from one control ((C), control #8), black lines) and one psoriatic ((P), patient #6), red lines), respectively. The measurements were carried out simultaneously in 2 ml tween chambers as detailed in Materials and Methods. Where indicated the following was added: 4×10^6 PBMC, 8 µg/ml oligomycin (olig), 2 µg/ml valinomycin (val), 3 mM KCN. The continuous and dashed lines represent the O₂ concentration (left axis) and the oxygen consumption rate (right axis, OCR normalized to cell number) measured simultaneously every 0.1 s throughout the time-course of the assay. (B) Box-plot representation of the normalized and KCN-insensitive-corrected oxygen consumption rates measured under resting conditions (RR), in the presence of oligomycin (OL) and in the presence of valinomycin (VAL) (see panel A). The plots were constructed from the average values of 2-3 independent measurements for each control or psoriatic PBMC sample. (C) ATP-dependent O₂ consumption measured as absolute difference between that obtained in the absence and that in the presence of oligomycin (RR-OL) or normalized to the basal respiration ((RR-OL)/RR). (D) Respiratory control ratios obtained dividing the oxygen consumption rates measured under resting conditions by that in the presence of oligomycin (RR/OL). White boxes, control; red boxes, psoriatic. The statistical significance between related pairs of measurements in control and psoriatic samples is shown as P.

**Figure 3:** Activity of complex I and complex IV in control and psoriatic PBMC. PBMC from control and psoriatic patients were lysed and assayed spectrophotometrically as described in Materials and Methods for complex I and complex IV activity (A and B respectively). Where indicated in (A) PBMC were pre-treated with 100 µM isoproterenol plus 100 µM 3-isobutyl-1-methylxanthine (IMBX) for 15 min at 37°C in a CO₂-incubator. The box-plots were constructed from the average values of 2-3 independent measurements for each control or psoriatic PBMC sample. White boxes, control; red boxes, psoriatic. Statistically significant differences are shown when P<0.05; NS, non-statistically significant (i.e. P>0.05).

**Figure 4:** Expression of the adrenergic receptor β2-AR in control and psoriatic PBMC. (A) Quantitative RT-PCR was performed on total mRNA extract from control and psoriatic PBMC, as detailed in Materials and Methods; each sample was assayed in duplicate and the averaged values used for the box-plot representation. (B) Upper panel: representative Western blotting on total cell protein extract (40 µg/lane) from three control and three psoriatic PBMC. Lower panel: box-plot representation of the densitometric analysis of the immunodetected β2-AR band normalized to that of β-actin. White boxes, control; red boxes, psoriatic. NS, non-statistically significant (i.e. P>0.05).
together the presented results show that the observed enhanced activity of complex I in patients PBMC was unrelated to the β2-AR/cAMP/PKA signalling pathway.

To ascertain if the enhanced activity of complex I was caused by up-regulation of its transcription we assessed by quantitative RT-PCR the expression levels of the main transcription factors involved in the biogenesis of the mitochondrial respiratory chain. The results of this analysis showed that only the expression of the nuclear respiratory factor 1 (NRF1), nuclear respiratory factor 2 (NRF2) and mitochondrial transcription factor A (TFAM) did not change significantly as compared with control samples. The transcript level of both the nuclear and mitochondrial coded structural subunits of complex I, NDUFS8 and ND5 respectively, resulted to be unaffected in patients' PBMC. As TFAM is also known to regulate the mitochondrial genome copy number its unchanged expression in psoriatic PBMC would indirectly indicate no significant variations in their mitochondrial mass.

GRIM19 (Genes associated with Retinoid-IFN-induced Mortality) is a nuclear encoded protein found to be associated with the mitochondrial complex I and to be necessary for the assembly and activation of the complex. Consistent with this notion, patients' PBMC exhibited a significant up-regulation of the transcript level of GRIM19. A function recently disclosed for GRIM19 is its role in the import of the signal transducer and activator of transcription 3 (STAT3) into the mitochondria where it interacts with complex I and regulates its activity. Prompted by this finding we investigated by immunoblotting the total content of STAT3 by a polyclonal specific antibody. Figure 6A shows the results for a representative subset of control and psoriatic samples in which a large inter-individual variability is observed. To note, in the 70-100 KDa range, two immunoreacting bands were observed. This is consistent with the notion that STAT3 is also expressed as a splice-variant with lower molecular weight. Most notably, psoriatic PBMC exhibited a shift in the relative amount of the two STAT3 isoforms resulting in a large significant increase of the STAT3β/STAT3α ratio. To interact with GRIM19, STAT3α needs to be phosphorylated at serine 727 with this residue lacking in the STAT3β isoform. Immunoblotting with an antibody specific for the p-(S727)-STAT3α resulted in a larger staining in most of the patients' samples. When referred to the total amount of STAT3α the densitometric analysis confirmed a statistically significant increase of STAT3α.
the \( p\)-STAT3/STAT3 ratio in psoriatic samples with respect to controls (Figure 6D).

**Discussion**

A recently conducted meta-analysis of genome-wide association studies has increased to 36 the number of susceptibility loci associated with psoriasis in European individuals [37]. The newly identified loci shared with other autoimmune diseases include candidate genes with roles in regulating T-cell function. Notably, they included candidate genes whose products are involved in innate host defence. These results are consistent with the current change in the perspective of psoriasis from a localized autoimmune skin disease to a systemic inflammatory disease with autoimmune features and severe comorbid conditions [38].

As central players of the adaptive immune response T lymphocytes have evolved a metabolic machinery that is finely regulated to match the energy needs of the different functional states of the cell. In the naive state T cells rely largely on fatty acid oxidation [15]. Once activated T lymphocytes begin to proliferate, the cells engage distinct transcriptional programs that drive them into functional subsets depending on the context (cytokines and other extracellular signals) in which they were activated [39,40]. After the clearance of pathogens, most clonally expanded and differentiated T cells undergo apoptosis in an abrupt contraction phase. The remaining antigen-specific T cells (memory T cells) are responsible for enhanced immunity after re-exposure to the pathogen [41]. Of these various T cell subsets, the induced regulatory T cells (iTreg) cells and memory T cells rely on lipid oxidation as a major source of energy, whereas cytotoxic T lymphocytes and effector T cells sustain high glycolytic activity and glutaminolytic activity [42-45]. Importantly the metabolic reprogramming accompanying the T lymphocyte subsets transitions is now emerging not simply as a marker but as a determinant of the cell fate. Consistently, the occurrence of “metabolic checkpoints”, interconnecting the availability of specific metabolites and signal transduction pathways affecting T lymphocyte responses, has been proposed [46].

However, if the above-described scenario applies to T lymphocytes acutely stimulated by invading pathogens some differences appear to characterize chronically activated T cells. In autoimmune diseases such as psoriasis, autoreactive lymphocytes are persistently stimulated by self-antigen and acquire a chronically activated phenotype characterized by sustained proliferation and effector functions [47]. Although the metabolic pathways used by chronically activated lymphocytes have not been studied thoroughly, several lines of evidence suggest that pathogenic lymphocytes in diseases like lupus, rheumatoid arthritis, psoriasis, and graft-versus-host disease preferentially rely on mitochondrial metabolism [48-51]. In lupus autoreactive splenocytes the characterization of metabolic pathway unveiled more reliance on mitochondrial oxidative phosphorylation than on aerobic glycolysis; moreover, in vitro repetitive stimulation of T cells through the T-cell receptor resulted in mimicking of the chronically activated phenotype and dependence on oxidative metabolism [52].

Therapeutic strategies aimed to interfere with lymphocyte metabolism are emerging in the treatment of immune-related diseases. Dimethyl fumarate (DMF) is an effective oral antipsoriatic drug reported to suppress T cells by a mechanism of action that though to be fully understood involves inhibition of the NFκB nuclear translocation, possibly by interference of the intracellular redox balance, leading to inhibition of pro-inflammatory cytokines production, dendritic cell differentiation and induction of apoptosis [53,54]. However, it is worth noting that DMF is a potential inhibitor of the mitochondrial respiratory complex II succinate dehydrogenase whose reaction product is fumarate. Thus, in addition to or perhaps causally linked to the abovementioned effects modulation of the mitochondria-related terminal oxidative metabolism may represent an unappreciated pharmacological action of DMF worthy of further investigations.

The straight and novel finding in the present study is that PBMC isolated from patients affected by psoriasis exhibit a large increase in their mitochondria-related respiratory activity (Figure 2). This activity is largely depressed by the ATP-synthase specific inhibitor oligomycin indicating that under resting condition the oxygen consumption is largely utilized to synthesize ATP by the mitochondrial OxPhos system. Conversely, PBMC from healthy control subjects displayed much lower oxygen consumption, which, in addition, was almost insensitive to oligomycin. This result would support the occurrence of a transition from a glycolytic to an oxidative metabolism in psoriatic PBMC. Importantly, in control PBMC the resting respiration matched that attained under uncoupled conditions (that defines the maximal respiratory capacity) thereby implying the absence of a kinetic reserve under coupled phosphorylation-linked respiration. As a consequence enhancement of the respiratory chain activity can be achieved either by an "activating mechanism" or by up-regulating the expression of the enzymatic rate limiting step, or by a combination of both.

The gatekeeping of the overall electron transfer through the respiratory chain is provided by complex I and/or complex IV activities depending on the prevailing conditions [55]. Herein, we found that complex I activity was specifically enhanced in patients’ PBMC whereas that of complex IV was comparable with that of control PBMC (Figure 3).

Complex I is constituted by 45 subunits in mammals [31] with seven of them encoded by the mitochondrial genome and the others by nuclear genes. Thus, the biogenesis of complex I requires the orchestrated activities of two expression/translation systems. However, we did not find evidence for a significant change in the expression both of the main transcription factors controlling the mitochondrial respiratory chain biogenesis and of nuclear and mitochondrial genes of complex I subunits in patients’ PBMC (Figure 5). This observation would rule out that the increased activity of complex I shown in the PBMC of psoriatic patients was due to substantial up-regulation of its expression rather suggesting the occurrence of a post translational regulatory process.

PKA-mediated phosphorylation of complex I proved to be a reversible covalent modification enhancing electron transfer activity of complex I by a not yet defined mechanism [20]. Changes in the \( \beta2\)-AR and in cAMP have been reported in keratinocytes and in T-cell subsets of psoriatic patients and proposed to be involved in the development of the disease [27,56,57]. In this study we did not find evidence of up-regulation of the \( \beta2\)-AR in psoriatic PBMC neither of stimulation of complex I activity both in control and patient samples following treatment with \( \beta2\)-AR and phosphodiesterase inhibitor. Possibly, the control of mitochondrial respiration by the cAMP/PKA axis is not functioning in normal PBMC as proved in serum-starved preconditioned fibroblasts [20]. In any case the results provided would rule out a major contribution of the \( \beta2\)-AR-mediated signalling pathway in the modulation of complex I activity in psoriatic PBMC (Figure 3A). In addition to covalent modification, the complex I activity was found to be controlled by assembly factors and interacting proteins. GRIM-19, initially identified as an apoptosis-related gene in human cancer cell lines [29] proved to be a nuclear-encoded subunit of...
mitochondrial complex I [30,58,59]. Strikingly, deletion of GRIM-19 alone totally destroys the assembly and enzymatic activity of the 43-subunit mammalian complex I. Knockout of GRIM-19 in mice causes early embryonic lethality [58]. A GRIM19-mediated linkage between the mitochondrial respiratory chain and the innate immunity has been recently reported by a paper showing that heterogeneous mice (GRIM-19−/−) are prone to spontaneous infection [60]. Macrophages from these mice have a compromised complex I activity and defective production of pro-inflammatory cytokines such as interleukins and IFN-γ. Consistent with this data, we provide in the present study the evidence that transcription of GRIM19 is significantly up-regulated in PBMC from psoriatic patients (Figure 5B). Although the mechanistic function of GRIM19 in stabilizing the multimeric complex I remains to be clarified recent reports disclosed its role as chaperone in STAT3 localization in mitochondria [33,34]. STAT3 is a member of a family of transcription factors and downstream product of cytokine and growth factor pathways playing a central role in the cell biology of immunocytes, controlling the expression of genes involved in the innate and acquired immunity and in the pro-inflammatory response [61-64]. Once phosphorylated by receptor associated kinases STAT3 moves into the nucleus where it binds to multiple genes involved in Th17 cell differentiation, cell activation, proliferation, and survival, regulating both expression and epigenetic modifications. Alterations in STAT3 have been linked to immune diseases including psoriasis [65,66] as well as in cancer [63].

Two distinct STAT3 isoforms originating from alternative splicing have been described. STAT3α (92 kDa) is 770 amino acids in length, whereas STAT3β (84 kDa) is identical in sequence with the exception of 55 amino acids at the C-terminal tail that are replaced with a unique seven-amino-acid sequence [35,67]. As a consequence, the transactivation domain of STAT3β is truncated relative to this domain in STAT3α. This has led to suggestions of impaired transcriptional activity and a role as a dominant-negative regulator of STAT3β [35]. However, accumulating data do not support STAT3β’s role as a classical dominant-negative regulator of STAT3α but rather as a transcriptional regulator associated with its own specific set of target genes [68-71]. Nevertheless, the occurrence of cross-talking between the two STAT3 isoforms has been revealed by co-expression experiments showing that STAT3β enhanced and prolonged the phosphorylation and nuclear retention of STAT3α [71].

The involvement of STAT3 in the development of psoriasis is supported by the observation that STAT3 is consistently activated in epidermal keratinocytes both in disease-animal model and human psoriatic lesions [65,72] and by recent genotyping studies [37,73]. This has led to the development of STAT3 inhibitors to be used for the topical therapeutic treatment of psoriasis [74,75]. However, given the notion of a crosstalk between keratinocytes and leukocytes creating the proinflammatory cytokines-mediated vicious circle of chronic skin inflammation seen in psoriasis, its optimal treatment needs to target pathogenic pathways in both leukocytes and keratinocytes [76].

The observations here reported, for the first time, unveiled in psoriatic PBMC the occurrence of an alteration in the STAT3 homeostasis resulting in a shift toward the preferential expression of the STAT3β isoform (Figures 6A-6C) even though the relative activation of STAT3α via phosphorylation of S727 was confirmed as reported in stimulated keratinocytes (Figure 6D) [66]. The impact of this finding in the understanding of the pathogenesis of psoriasis warrant further investigations.

In addition to its canonical functions, STAT3 enters mitochondria and regulates OxPhos though complex I activation in a transcriptional-unrelated manner [32,33,77-79]. Phosphorylation of STAT3 at S727 appears to be essential for its import. The mechanism by which STAT3 controls complex I is not clear since its amount in mitochondria is largely substechiometric with respect to the respiratory chain components and therefore not relying on direct interaction [80]. One possibility is that complex I subunits exist in the mitochondrial membrane as inactive or partially active sub-complexes [81] and that GRIM-19/STAT3 catalyse their fully functional assembly. Alternatively they might inhibit complex I degradation. The consequence in either cases would be the up-regulation of the complex I driving enhanced mitochondrial respiration and OxPhos without the need to change the bigenomic expression profile of the constituting complex I subunits.

A side-effect of the electron transfer through the mitochondrial respiratory chain is the production of reactive oxygen species (ROS) with complex I being the major source [82,83]. ROS are now recognized as intermediates in redox signalling controlling fundamental aspect of cell biology [46,84-87]. Importantly, cellular signaling pathways such as mitogen-activated protein kinase/activator protein 1, nuclear factor kB, and Janus kinase–signal transducers and activators of transcription are known to be redox sensitive and proven to be involved in the progress of psoriasis whereby setting the rationale for the beneficial utilization of antioxidant as therapeutic strategy [51]. Most notably, it has been reported that mitochondrial ROS contribute significantly to the dominant Th effector phenotype in autoimmunity in addition to the cytokine milieu [88]. Moreover, in a recent study it has been shown that mitochondrial derived hydrogen peroxide selectively enhances T cell receptor-initiated signal transduction through JNK/cJun [89].
Intriguingly, ROS have been also implicated in the activation of the JAK-STAT pathway [90,91].

A further described relevant role of mitochondria is linked to the calcium dependent T-cell activation. It has been shown that following formation of immunological synapse mitochondria localize at the subplasmalemmal level of the T-cell and function as calcium buffer preventing a large local calcium increase, which inactivates the plasma membrane Ca²⁺ channel ORAI [92,93]. This nano-scale distribution maximizes the efficiency of calcium influx through ORAI channels, resulting in a more sustained NFAT activity and subsequent activation of T cells. To note, calcium entry into mitochondria is mediated by a Ca²⁺-unipporter and electrophoretically driven by the ΔΨm, which in turn depends on the electron transfer-driven protonotive activity of the respiratory chain.

In keeping the abovementioned notions it is tempting to speculate that the enhanced activity of the mitochondrial respiration featuring the psoriatic PBMC, as described in the present study, is functional either/both to the development of disease-related lymphocyte subsets (i.e. by redox signalling) and to the maintenance of their chronically activated state (i.e. by calcium signalling); see the scheme in Figure 7. The causes of the observed up-regulation of the respiratory activity and in particular of the complex I warrant further investigations. The clue here provided is the cytoxynes-related up-regulation of GRIM19/STAT3β and possibly of other related respiratory chain assembly factors.

Finally, the observations reported in this study provide hints to explore the use of available specific inhibitors of complex I [94] to reduce cytokine production in the treatment of psoriasis as well as other auto-immune related diseases.

Acknowledgement

The authors thank the assistance of Dr. Maria Piccolo for performing respiratory assays on frozen-thawed PBMC and Dr. Caterina Ceccarini for helping in the selection of psoriatic patients and isolation of PBMC.

Financial Support

Local research grants from the University of Foggia to N.C. and M.M.

References


