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# IMMUNOMETABOLIC CHARACTERIZATION OF HUMAN EFFECTOR B CELL SUBSETS IN SYSTEMIC AUTOIMMUNITY

Dissertação no âmbito do Mestrado em Biologia Celular e Molecular orientada pela Doutora Maria Margarida Souto Carneiro e pelo Professor Doutor Rui de Albuquerque Carvalho e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

Setembro de 2019

Faculdade de Ciências e Tecnologia
da Universidade de Coimbra

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Setembro de 2019

*Be like a proton*
*and think positive*

The work presented here was performed at the AG Lorenz group, at the Otto-Meyherhof Zentrum, Rheumatology Department, Universitätsklinikum Heidelberg under the supervision of Dr. Margarida Souto-Carneiro (OMZ, UH) and Dr. Rui Carvalho (DCV, UC).

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## LIST OF ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| 2-DG | 2-deoxy-D-glucose |
| Ab | Antibody |
| ACPA | Anti–citrullinated Protein Antibody |
| AKT | Protein Kinase B |
| AMPK | AMP-activated Protein Kinase |
| anti-dsDNA | Anti-double-stranded DNA Antibodies |
| ANA | Antinuclear Antibodies |
| ATP | Adenosine Triphosphate |
| BAFF | B Lymphocyte Activating Factor |
| BCR | B-cell receptor |
| BLP | B cell-biased Lymphoid Progenitor |
| BM | Bone Marrow |
| BSA | Bovine Serum Albumin |
| BTK | Bruton's Tyrosine Kinase |
| CLP | Common Lymphoid Progenitor |
| EBV | Epstein Barr Virus |
| ECAR | Extracellular Acidification Rate |
| DMARD | Disease-Modifying Antirheumatic Drug |
| DNL | *De novo* lipogenesis |
| ETC | Electron Transport Chain |
| GC | Germinal Center |
| GSK3 | Glycogen Synthase Kinase 3 |
| GLUT1 | Glucose Transporter 1 |
| HIF-1α | Hypoxia-Inducible Factor 1-alpha |
| HK2 | Hexokinase 2 |
| IFN-α | Interferon alpha |
| Ig | Immunoglobulin |
| IgA | Immunoglobulin A |
| IgD | Immunoglobulin D |
| IgG | Immunoglobulin G |
| IGHV | Immunoglobulin Heavy-chain gene |
| IgM | Immunoglobulin M |
| IL-4 | Interleukin 4 |
| IL-7 | Interleukin 7 |
| IL-7R | Interleukin 7 Receptor |
| LDHA | Lactate Dehydrogenase A |
| LLPC | Long-Lived Plasma Cell |
| LPS | Lipopolysaccharide |
| mTOR | Mammalian Target of Rapamycin |
| mTORC1 | Mammalian Target of Rapamycin Complex 1 |
| MTX | Methotrexate |
| OCR | Oxygen Consumption Rate |
| OXPHOS | Oxidative Phosphorylation |
| PBMC | Peripheral Blood Mononuclear Cells |
| PDH | Pyruvate Dehydrogenase |
| PI3K | Phosphoinositide 3-Kinase |
| PKCβ | Protein Kinase Cβ |

| | |
|---|---|
| RA | Rheumatoid Arthritis |
| RF | Rheumatoid Factor |
| ROS | Reactive Oxygen Species |
| S6RP | S6 Ribosomal Protein |
| SLE | Systemic Lupus Erythematosus |
| SLPC | Short-Lived Plasma Cell |
| SS | Systemic Sclerosis |
| TCR | T-cell receptor |
| TGF-β | Transforming Growth Factor β |
| TLR4 | Toll-like Receptor 4 |
| TLR7 | Toll-like Receptor 7 |
| TLR9 | Toll-like Receptor 9 |
| TNF-α | Tumor Necrosis Factor Alpha |

## ABSTRACT

Immunometabolism is an exciting emerging field that explores the impact of metabolic processes in the regulation of immune cell function. Metabolic remodeling upon immune cell activation allows the production of energy and biosynthetic precursors necessary for normal differentiation and proliferation and has a key regulatory role in immune response and effector function.

B cells play a vital role in the development and maintenance of autoimmune diseases such as Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE). The metabolic configurations of T cells in autoimmunity are starting to be unraveled, however, there are no reports studying the metabolism of B cells in autoimmune context. Moreover, given the essential role of B cells in autoimmunity, it is of utterly importance to unravel the metabolic configurations of B cells in such maladies which, perhaps, could provide new targets for alternative therapies to current treatments. This is to the best of our knowledge the first study that attempt to characterize the metabolism of B cells from RA and SLE patients.

In our study we isolated naïve (CD27-) and memory (CD27+) B cells from the peripheral blood of RA and SLE patients. Besides stimulating the cells under hypoxia, we also attempted to inhibit some metabolic pathways in order to understand how it may impact cellular metabolism and effector functions. Therefore, we aimed to inhibit glycolysis, oxidative phosphorylation and the main metabolic regulator mTOR. To evaluate the effects of stimulation and inhibition we measured the expression of key metabolic enzymes, the glucose consumption and lactate production rates, the levels of immunoglobulin and cytokine production. We also evaluated the gene expression of antigen-specific B cell subsets isolated from the peripheral blood of RA and SLE patients.

The results we obtained showed that control B and RA cells exhibited a purely glycolytic profile upon stimulation. Oppositely, in B cells from SLE patients the levels of lactate production were reduced indicating a downregulation of glycolysis. The metabolic inhibition had scarcely any effect on enzymatic expression and intermediary metabolism. Nevertheless, we successfully downregulated antibody secretion in the SLE group, however this pattern was not so clear in cytokine secretion because we could simultaneously promote upregulation of pro-inflammatory and anti-inflammatory cytokines for both autoimmune subsets. The gene expression assay showed that plasmablasts SLE patients might exhibit a more glycolytic profile when compared to healthy controls.

Together these results suggest that the metabolism of B cells is altered in autoimmunity and understanding these changes could be essential for treating RA and SLE.

*Keywords: immunometabolism; autoimmunity; B cells; Rheumatoid Arhtitis; Systemic Lupus Erythematosus*

## RESUMO

O immunometabolismo é uma área que está a surgir e que explora o impacto dos processos metabólicos na regulação das funções imunitárias. A remodelação metabólica que ocorre após ativação das células imunitárias permite a produção energia e precursores de vias metabólicas, ambos necessários para sustentar os processos de proliferação e diferenciação. Esta remodelação metabólica também tem um papel chave na regulação da resposta e funções imunitárias.

Os linfócitos B desempenham funções vitais no desenvolvimento e progressão de doenças autoimunes como a Artrite Reumatoide (RA) e o Lupus Sistémico Eritematoso (SLE). As características metabólicas de linfócitos T na autoimunidade estão a ser revelados, no entanto, não existem ainda estudos sobre o metabolismo dos linfócitos B num contexto de autoimunidade. Dado o papel essencial dos linfócitos B em doenças autoimunes, o estudo do metabolismo destes linfócitos é de extrema importância podendo mesmo contribuir para a descoberta de novos alvos para novas terapias. Este é o primeiro estudo que visa caracterizar o metabolismo dos linfócitos B em pacientes com RA e SLE.

No nosso estudo foram isolados linfócitos B naïve ($CD27^-$) e de memória ($CD27^+$) do sangue periférico de pacientes com RA e SLE. Para além de estimular os linfócitos em condições de hipoxia, também era nosso objetivo tentar inibir algumas vias metabólicas e perceber como esta inibição podia afetar o metabolismo celular e as funções imunitárias. Deste modo, inibimos a glicólise, a oxidação fosforilativa e o regulador metabólico mTOR. De modo a avaliar os efeitos da estimulação e inibição usadas, foram verificados parâmetros como expressão de enzimas metabólicas, taxas de consumo de glucose e de produção de lactato, e os níveis de produção de imunoglobulinas e citocinas. Para além disso, verificámos a expressão génica de linfócitos B efetores do sangue periférico de pacientes com RA e SLE.

Os resultados obtidos mostraram que os linfócitos B controlo e RA quando estimulados exibiam um perfil puramente glicolítico. Por outro lado, os linfócitos B de pacientes com SLE apresentaram baixos níveis de lactato o que indica uma redução na utilização da via glicolítica. Os inibidores usados tiveram um efeito quase nulo na expressão enzimática e no metabolismo. No entanto, estes inibidores conseguiram suprimir a produção de anticorpos no grupo SLE. Este resultado não se verificou no caso das citocinas pois a inibição metabólica permitiu simultaneamente o aumento da secreção de citocinas pro-inflamatórias e anti-inflamatórias em ambos os grupos autoimunes. A expressão génica de células efetoras permitiu inferir que plasmoblastos de pacientes com SLE possuem um perfil mais glicolítico quando comparados a controlos saudáveis.

Deste modo, os resultados obtidos permitem concluir que o metabolismo dos linfócitos B está alterado na autoimunidade e a caracterização destas alterações pode ser essencial para o tratamento de RA e SLE.

*Palavras-chave:* imunometabolismo; autoimunidade; linfócitos B; Artrite Reumatoide; Lupus Sistémico Eritematoso

# 1. INTRODUCTION

## 1.1 THE IMMUNE SYSTEM

The immune system is, perhaps, one the most complex and less understood systems of the human body. The main function of the immune system is to protect the organism against pathogens and tumors while maintaining tissue homeostasis (Yatim and Lakkis, 2015). This protection is mediated by several types of cells that develop in the bone marrow (BM). Hematopoietic stem cells in the bone marrow differentiate into two lineages: myeloid and lymphoid. Natural killer cells, B lymphocytes and T lymphocytes develop from the lymphoid lineage, while monocytes, neutrophils, eosinophils, basophils and mast cells differentiate from a common myeloid progenitor (Chaplin, 2010). The immune system can be further subdivided in innate immunity and adaptive immunity which participate in distinct phases of response and have different specificity against infectious agents (Parkin and Cohen, 2001).

### 1.1.1 Innate Immunity

The innate immune system is the first line of defense providing immediate protection against pathogens and tissue perturbations. In fact, this arm of the immune system is designated innate because the mediated mechanisms of defense are inborn, i.e. embedded in our germline (Yatim and Lakkis, 2015). These mechanisms include cell receptors, proteins and small molecules (e.g. cytokines and chemokines) (Chaplin, 2010). Furthermore, the mechanisms of innate immunity are mediated by distinct cell types: monocytes, neutrophils, eosinophils, basophils, mast cells, macrophages, dendritic cells and natural killer cells. The cells from the innate system express receptors (e.g. Toll-like receptors, Dectin-1) that can recognize conserved molecular patterns that are present in pathogens allowing to discriminate between self and non self (Chaplin, 2010; Parkin and Cohen, 2001). Furthermore, the interaction between those foreign molecules and innate system cells will trigger a response that can result in phagocytosis of opsonized pathogens (Parkin and Cohen, 2001). The response mediated by the innate

system takes minutes to hours to develop. However, the unspecific character of this response may cause tissue damage and needs, therefore, to be tightly regulated by certain lymphocytes (Parkin and Cohen, 2001; Yatim and Lakkis, 2015).

### 1.1.2 The Adaptive side of the Immune System

Adaptive immunity is mediated by T lymphocytes and B lymphocytes that exhibit antigen specific receptors: T-cell receptor (TCR) and B-cell receptor (BCR), respectively (Parkin and Cohen, 2001). Unlike the innate system, lymphocyte receptors are the product of somatic gene rearrangements during lymphocyte development. This means that different subsets of lymphocytes will express several distinct receptors that will be able to target a broad range of pathogens (Yatim and Lakkis, 2015). However, cells from the adaptive system need to be exposed to a pathogen in order to be able to differentiate and exert effector functions. Interaction between a pathogen and a lymphocyte will lead to the activation of the latter that will proliferate and differentiate into effector cells that can mediate the immune response (Parkin and Cohen, 2001; Yatim and Lakkis, 2015). When the immune response ceases some of the lymphocytes will be converted into memory cells that can be quickly reactivated and act efficiently upon a second infection (Yatim and Lakkis, 2015). Therefore, adaptive immunity is much more specific and efficient than innate immunity, however the time to develop a response is much longer (7-12 days in a primary antigenic challenge, 3-4 days in a repeated infection) (Chaplin, 2010).

### 1.1.3 B cell development and function

The development of B cells starts in the BM from an undifferentiated common lymphoid progenitor (CLP) and proceeds to yield fully-differentiated cells with effector capacity in the spleen (Hoffman et al., 2016). Through this tightly regulated process, B cells assume several well-defined stages that occur in different organs of the human body. The maturation stages that occur in the BM are commonly defined as B lymphopoiesis where the CLP differentiates sequentially into pro-B cell, pre-B cell and

immature B cell (Signer et al., 2007). The rearrangements of the immunoglobulin heavy-chain genes (IGHV) responsible to yield several different receptors (BCRs) start during CLP and pro-B cell differentiation. The pre-B cell stage is characterized by the expression of the pre-BCR, while the expression of BCR happens when differentiation into immature B cell is completed (Herzog et al., 2009). Immature B cells home to the spleen where they will differentiate into naïve marginal or naïve follicular B cells (Pillai and Cariappa, 2009). The first group is retained in the spleen, while follicular B cells can circulate through the peripheral blood, the lymph, the lymph nodes and the spleen (Allman and Pillai, 2008). Naïve B cells that encounter and bind an antigen through the BCR get activated and engage the process of differentiation into memory B cells or plasma cells (Harwood and Batista, 2010). Memory B cells provide a lasting protection in case of a second infection strike, while plasma cells secrete antibodies and act as one of the main mediators of adaptive humoral immunity (Harwood and Batista, 2010; Hoffman et al., 2016).

### 1.1.4 Autoimmunity: Rheumatoid Arthritis and Systemic Lupus Erythematosus

The main hallmark of autoimmunity is the loss of self-tolerance, in other words, the immune system loses the capacity of distinguish between non-self and self-antigens leading to the appearance of self-reactive T cells and autoantibodies (Banchereau et al., 2017). Moreover, autoimmunity is thought to result from a combination of genetic predisposition and environmental factors (Rosenblum et al., 2015). The autoantibodies commonly present in the serum of autoimmunity patients are secreted by self-reactive B cells (Lleo et al., 2010). B cell development is a tightly regulated process and therefore the arise of autoreactive B cells results from a dysregulation of this process. The mechanisms of self-tolerance take place in the BM during B cell lymphopoiesis and later in the spleen. The gene rearrangements that take place during B cell development in order to yield the expression of BCR promote the generation of diverse receptors some of which can recognize self-antigens. B cells that express these self-reactive BCR must be eliminated or must go again through a rearrangement process (Hoffman et al., 2016).

Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE) are chronic systemic autoimmune diseases with high prevalence among population (Cooper and Stroehla, 2003). The main symptoms that RA patients experience are swelling, stiffness and pain in the joints. This inflammation is caused by leucocyte infiltration in the synovial membrane that will progressively promote joint and cartilage damage (Smolen et al., 2016). B cells participle actively in this inflammation process in the joint and are thought to contribute to disease initiation and progression. The most described role of B cells in RA is the production of autoantibodies that can be detected in patients serum and in the synovium (Chaiamnuay and Bridges Jr, 2005). The main produced antibodies are Rheumatoid Factor (RF) and Anti–citrullinated protein antibody (ACPA) and both have been described to have a pro-inflammatory role in disease context (Bugatti et al., 2018). On the other hand, SLE is a very complex disease that can affect several organs systems including the kidneys, skin, central nervous system, heart and joints. This heterogenicity causes difficulties in the diagnosis because SLE patients can exhibit a broad range of clinical manifestations that can be commonly mistaken with other pathologies (Kaul et al., 2016). The main feature common to all SLE patients is the production of autoantibodies that actively promote pathogenesis (Nashi et al., 2010). The most prevalent type of antibodies found in the serum of SLE patients are antinuclear antibodies (ANA) and anti-double-stranded DNA antibodies (anti-dsDNA) (Han et al., 2015; Nashi et al., 2010). Thus, B cells also play an essential role in the development and progression of SLE. B cells can have other roles in RA and SLE pathogenesis such as promoting the activation of T cells through antigen-presentation and producing pro-inflammatory cytokines (Chaiamnuay and Bridges Jr, 2005; Kaul et al., 2016). Thus, B cells are essential for autoimmunity development and progression.

## 1.2 IMMUNOMETABOLISM & DISEASE

For many years, immunology research overlooked the impact of cellular metabolism on immune cells. The pioneering studies that aimed to characterize the metabolic requirements of lymphocytes were carried out more than 40 years ago. Besides characterizing glycolysis and oxidative phosphorylation (OXPHOS) in lymphocytes, these studies also attempted to explore other metabolic pathways and study the impact of specific metabolites (e.g. glutamine) in lymphocyte function. It was concluded that lymphocytes depend on glycolysis, OXPHOS and lipid synthesis for proper proliferation (Chen et al., 1975; Roos and Loos, 1973). In addition, glutamine was considered to be essential for blastogenesis, plasma cell differentiation and antibody secretion (Ardawi and Newsholme, 1983; Crawford and Cohen, 1985; Schrek et al., 1973). Lymphocytes used glutaminolysis to yield glutamate and aspartate which were presumably used as precursors in protein synthesis for the increased proliferation (Ardawi and Newsholme, 1983). However, despite these promising early findings the number of studies exploring the impact of cellular metabolism on immune function remained quite incipient. In the past 10 years there has been a renewed interest in the field of immune metabolism with an increasing number of studies describing how, upon stimulation, leukocytes experience a metabolic remodeling that impacts their effector function. Therefore, the metabolic remodeling verified upon immune activation not only allows the production of energy but is also essential to yield biosynthetic precursors required for growth, proliferation and immune functions (e.g. through the production of effector molecules). Even though the bulk of these studies has focused on monocytes and T cells, the interest on B cell metabolism is slowly on the rise. Studies on human cells and mouse models are unveiling how metabolic remodeling is essential for B cell development, differentiation and immune function in health and disease.

### 1.2.1 B cell metabolism: from the bone marrow precursors to the final effectors

At steady state B cells are quiescent, however, upon stimulation they quickly mount an immune response, which demands a lot of cellular and molecular changes involving growth, proliferation, differentiation, migration, cytokines and antibodies production. These processes need a high amount of energy and biosynthetic precursors, which require particular metabolic remodeling (Boothby and Rickert, 2017). Additionally, during their development from the bone marrow precursors, B cells need to adapt their metabolic requirements to allow biosynthesis and differentiation in a challenging hypoxic environment (Taylor and Colgan, 2017). Overall, these changes require a tight control and coordination between metabolism, immune function and development.

#### 1.2.1.1 B cell lymphopoiesis

B cells develop from a common lymphoid progenitor in the BM and conclude their maturation in the spleen. After commitment of the CLP towards the B cell lineage, expression of Interleukin 7 receptor (IL-7R) and several transcription factors especially *PAX5*, *EBF1*, *FoxO1*, *E2A* and *IKAROS/IKFZ1* are essential for the B cell-biased lymphoid progenitor (BLP) to proceed through the subsequent phases of differentiation, by regulating genes associated not only with immunoglobulin recombination and repression of the T cell differentiation program, but also those associated with glucose metabolism (Grosschedl, 2014). Samples from pre-B-cell acute lymphoblastic leukemia patients with and without mutations in PAX5 and IKZF1 have presented evidence that these transcription factors and their downstream targets NR3C1 (a glucocorticoid receptor), TXNIP (a glucose-feedback sensor) and CNR2 (a cannabinoid receptor) are essential to reduce glucose uptake, expression of glycolytic enzymes and glycolysis, while at the same time control the mammalian target of rapamycin (mTOR) activity through phosphorylation of AMP-activated protein kinase (AMPK), thus ensuring the differentiation commitment towards the B cell lineage (Chan et al., 2017). A normal B cell differentiation is equally dependent on the overall metabolic integrity of the BM itself, since B-cell lymphopoiesis is compromised when high fat diet-induce BM adiposity reduces the expression of PAX5, IL-7 or EBF1 (Adler et al., 2014).

Differentiation of B cells in the BM comprises four well defined stages: pro-B cell, large pre-B cell, small pre-B cell and immature B cell (Herzog et al., 2009). The different cellular states and metabolic configurations of these four stages have been characterized in mouse and human cells. Pro-B cells, exhibit elevated levels of glucose uptake, increased mitochondrial mass and potential, suggesting that both glycolysis and OXPHOS are required to fulfil their metabolic needs. Large pre-B cells have the highest glucose uptake as well as augmented ROS levels and membrane mitochondrial potential, even though mitochondrial mass is reduced when compared to the other stages (Stein et al., 2017). This profile seems to be dependent on the upregulation of glycolytic targets of mammalian target of rapamycin complex 1 (mTORC1) and Myc signaling induced by IL-7 (Gobin et al., 2003). In mouse bone marrow cells, inhibition of glycolysis with 2-deoxy-D-glucose (2-DG) prevented the differentiation of early pre-B cells (or late pro-B) into pre-B cells (Kojima et al., 2010). Therefore, large pre-B cells seem to exhibit a metabolic profile that is more glycolysis dependent. These metabolic configurations are probably required to support the rapid proliferation and differentiation of pro and large pre-B cells. In mice BM cells when the small pre-B cell stage is reached, glucose uptake, glycolysis and mitochondrial potential are reduced, while mitochondrial mass is increased and metabolic genes such as *Glut1 and Pfkfb3 (*glycolysis), *Mdh and Atp5d* (OXPHOS) are less expressed (Stein et al., 2017). *In vivo* experiments also showed that pre-B cells uptake less glucose than pro and immature B cells in a hypoxia-inducible factor 1-alpha (HIF-1α) dependent manner (Kojima et al., 2010). Similarly, human BM CD25$^{+}$ pre-B cells present lower glycolytic and oxygen-consumption rates as pro-B cells (Zeng et al., 2018). Whether this metabolic remodeling resembling a quiescent state is required to guarantee the correct rearrangement of the IGHV and the assembly of the pre-BCR remains to be elucidated. Immature B cells from mouse bone marrow exhibit high glucose uptake levels and seem to be glycolysis dependent. This, however, contrasts with the human ones, which seem to stay metabolically quiescent exhibiting low 2-NBDG (glucose uptake) and EdU (DNA synthesis/ proliferation) incorporation, and reduced expression of genes linked to glycolysis, OXPHOS and one-carbon metabolism (Zeng et al., 2018). All these changes indicate that B cell differentiation in the BM is a tightly regulated process, where the glycolytic dependency varies through the diverse stages, with mTORC1 playing a central role in survival, development and metabolic regulation of B cells in the pro and B stages and a lesser role once the cells reach an immature and naïve state (Iwata et al., 2016).

### 1.2.1.2 Naïve B cells: a quiescent state

Immature B cells that successfully reach the follicles in the spleen will proceed the maturation process and differentiate either into follicular B cells or marginal zone B cells (Pillai and Cariappa, 2009). The data regarding the metabolic configurations of naïve B cells point out OXPHOS as the main source for energy and biosynthesis. In murine Peyer's Patches naïve B cells rely on Vitamin B1 to maintain OXPHOS activity, using this vitamin as a co-factor in the TCA (Kunisawa et al., 2015), whereas splenic naïve B cells from mice have a ratio of Oxygen Consumption Rate to Extracellular Acidification Rate (OCR:ECAR) close to 1, indicating that they rely both on OXPHOS and glycolysis to meet their metabolic needs (Caro-Maldonado et al., 2014; Mcfadden et al., 2016). Comparing to anti-IgM-stimulated or Epstein Barr Virus-infected (EBV) cells quiescent human B cells have less glucose uptake, lower glycolysis and higher spare respiratory capacity (Caro-Maldonado et al., 2014; Mcfadden et al., 2016). This is also true when quiescent B cells from healthy donors are compared to human B cell lymphoma cell lines (Vangapandu et al., 2017). In naïve B cells from mice the expression of glycogen synthase kinase 3 (Gsk3) is important to regulate metabolic remodeling, proliferation and survival (Jellusova et al., 2017). Since GSK3 acts as an AMPK-inhibitor, its activation by cytokines and CD40 permits naïve B cells to mature into antibody-producing cells, whereas in quiescent naïve B cells low expression of GSK3 allows AMPK to be expressed increasing catabolism and OXPHOS, thus maintaining a self-renewal state (Adams et al., 2016; Suzuki et al., 2013).

In the mouse, the dominant subset of B cells in the secondary lymphoid organs are the quiescent and long-lived B2 follicular cells expressing high cell surface levels of immunoglobulin D (IgD). B2 are mainly involved in T cell-dependent immune responses (Nutt et al., 2015). Both tissue localization and function distinguish B2 cells from B1 B cells, which abound in the peritoneal cavity and are able to quickly respond to pathogens independent of T cell-help (Cunningham et al., 2014). However, these are not the only differences between these two subsets. They also present distinct metabolic signatures. Namely, splenic follicular B2 B cells maintain their quiescent state by keeping a lower expression of genes encoding glycolytic enzymes (*Hk2*, *Slc2a1* and *Myc*), express less glucose transporter 1 (GLUT1), consume less glucose, have less cytoplasmatic neutral lipids and lower OXPHOS and glycolytic

rate than peritoneal B1 B cells (Clarke et al., 2018). In general, the quiescence of naïve follicular B2 and unstimulated B cells seem to be maintained by a state of metabolic paralysis, and yet it is still enough to fuel their low energetic and biosynthetic needs.

#### 1.2.1.3 B cell activation and germinal center differentiation

Mature naïve follicular B cells in the secondary lymphoid organs are activated upon antigen stimulation and move into the germinal centers (GC) where, upon contact with follicular T cells and follicular dendritic cells, they complete the maturation process by differentiating into antibody-secreting plasma cells and memory B cells (Mcheyzer-Williams et al., 2012). In the GC-microenvironment, activation of naïve B cells involves immunoglobulin class-switch recombination, clonal expansion and affinity maturation which are processes requiring plenty of energy and production of biosynthetic precursors (Mcheyzer-Williams et al., 2012).

A recent study that compared follicular B cells and GC B cells in mice concluded that GC B cells exbibit higher glucose uptake, enhanced mitochondrial biogenesis and higher protein content. The enhanced glycolysis observed in GC B cells is essential for proper growth and proliferation and was proved to be HIF-1α-driven due to the GC hypoxic environment. GSK3 also seems to be essential for the survival of GC B cells. During nutrient abundance this kinase is inhibited which allows cellular growth and proliferation, however, when there is nutrient restriction it is upregulated and limits cellular growth and proliferation in order to promote survival upon CD40L-IL-4 stimulation (Jellusova et al., 2017). The transcription factor c-MYC regulates the proliferation in mice GC mature and immature B cells and is essential for the formation, expansion and maintenance of GC (Calado et al., 2012; Dominguez-Sola et al., 2012). Moreover, given that c-MYC and mTORC1 have been intensely described as coordinators of cellular growth and metabolism, the high phosphorylation levels of these two factors in GC B cells suggest their role in coordinating the regulation of metabolic configurations (Dang et al., 2006; Jellusova et al., 2017; Saxton and Sabatini, 2017). Therefore, the metabolism of GC B cells seems to be a multiple regulated and firmly controlled process.

The first studies carried out in the immunometabolism field concluded that upon activation a naïve immune cell will go through a process of metabolic remodeling in order to support effector functions. In fact, more recent studies suggest that, as described earlier, mouse splenic B lymphocytes upon BCR crosslinking (with anti-IgG or anti-IgM), Lipopolysaccharide (LPS) or IL-4 stimulation increase glucose uptake, GLUT1 expression and glycolysis rates (Blair et al., 2012; Caro-Maldonado et al., 2014; Cho et al., 2011; Doughty et al., 2006; Dufort et al., 2007; Garcia-Manteiga et al., 2011). The expression of key metabolic enzymes such as *HK2* and *LDHA* is also upregulated and the activity of PDH is enhanced in mouse lymphocytes stimulated through BCR or Toll-like receptor 9 (TLR9) (Akkaya et al., 2018). In addition, IL-4 stimulated murine B cells exhibit an increase in *Glut1*, *PDH*, *Citrate Synthase*, and *ATP Synthase* gene expression (Cho et al., 2011). Besides increased glycolysis rates, lactate production is also augmented upon LPS or BCR stimulation, but is not changed upon IL-4 activation (Caro-Maldonado et al., 2014; Cho et al., 2011; Doughty et al., 2006; Garcia-Manteiga et al., 2011). Oppositely, IL-4 stimulated B cells increase glucose oxidation, OXPHOS and mitochondrial membrane potential (Cho et al., 2011). Mitochondrial mass, mitochondrial activity and ROS levels are also increased upon LPS-induced differentiation (Caro-Maldonado et al., 2014; Vene et al., 2010). Thus, this suggests that B lymphocytes upon stimulation first upregulate glycolysis and then gradually increase oxidative metabolism with the predominant substrates being glucose and glutamine (Caro-Maldonado et al., 2014; Price et al., 2018). However, it was also reported that mouse splenic B lymphocytes stimulated through BCR or TLR9 increase both glycolysis and oxidative phosphorylation right after stimulation and that oxidative phosphorylation is the predominant energy source in BCR-stimulated cells (Akkaya et al., 2018).

In addition to that, B cells also seem to require other metabolic pathways for proper proliferation and differentiation. After the initial glycolytic burst, IgM-stimulated B cells engage a metabolic profile that includes the pentose phosphate pathway (Doughty et al., 2006). *De novo* lipogenesis (*DNL*) is also upregulated with the main substrate being glucose. The main effector in this pathway is ACLY and its inhibition blocks glucose incorporation into fatty acids (Dufort et al., 2014). Oppositely, lipid oxidation is downregulated upon TLR4, BCR or IL-4 stimulation (Caro-Maldonado et al., 2014; Cho et al., 2011). Protein synthesis was also reported to be upregulated in IL-4 activated B cells (Cho et al., 2011). This might suggest that

glucose is not only used as a substrate to obtain energy but can also be used to yield biosynthetic precursors required for cellular proliferation (Vander Heiden et al., 2009). Importantly, glycolysis was also proved to be the metabolic pathway crucial for survival and proliferation in LPS-activated and BCR-crosslinking activated B cells (Caro-Maldonado et al., 2014; Doughty et al., 2006; Milasta et al., 2016).

Perhaps, the most interesting detail about this metabolic regulation process in B cells is the fact that each different stimulation will activate different pathways that will ultimately culminate in the same result which is glycolysis upregulation. In LPS-stimulated B cells, the metabolic reprogramming is regulated by both PI3K-Akt pathway and c-MYC (Caro-Maldonado et al., 2014). In another study, the anabolic state of LPS-stimulated B cells was associated with phosphoinositide 3-kinase (PI3K) and mTOR activation (Adams et al., 2016). Moreover, BCR-crosslinking with anti-IgM stimulation was also reported to require the combination of c-MYC and PI3K-Akt-pathway as promoters of glycolysis upregulation (Doughty et al., 2006). However, it was also reported that glycolysis upregulation upon anti-IgM BCR stimulation is controlled by Protein kinase Cβ (PKCβ) and the Bruton's tyrosine kinase (BTK) is also required (Blair et al., 2012). Interestingly, BTK and Akt are the two major PI3K downstream effectors (Baracho et al., 2011). In contrast, in IL-4 stimulated B cells the glycolytic configuration was proven to be Stat6-dependent and PI3K/Akt-independent (Dufort et al., 2007). It was also reported that IL-4-activated B cells reprogram their metabolism in an AMPK-dependent way (Cho et al., 2011), which is quite interesting because the PI3K/Akt pathway can indirectly activate mTORC1 while AMPK inhibits it (Jellusova and Rickert, 2016; Saxton and Sabatini, 2017). The different stimuli also affect the pathways that upregulate *DNL.* In LPS-stimulated cells this process was suggested to be PI3K-independent, while in B cells simultaneously stimulated with IL-4 and anti-IgM, lipid synthesis was suggested to be PI3K-dependent (Dufort et al., 2014).

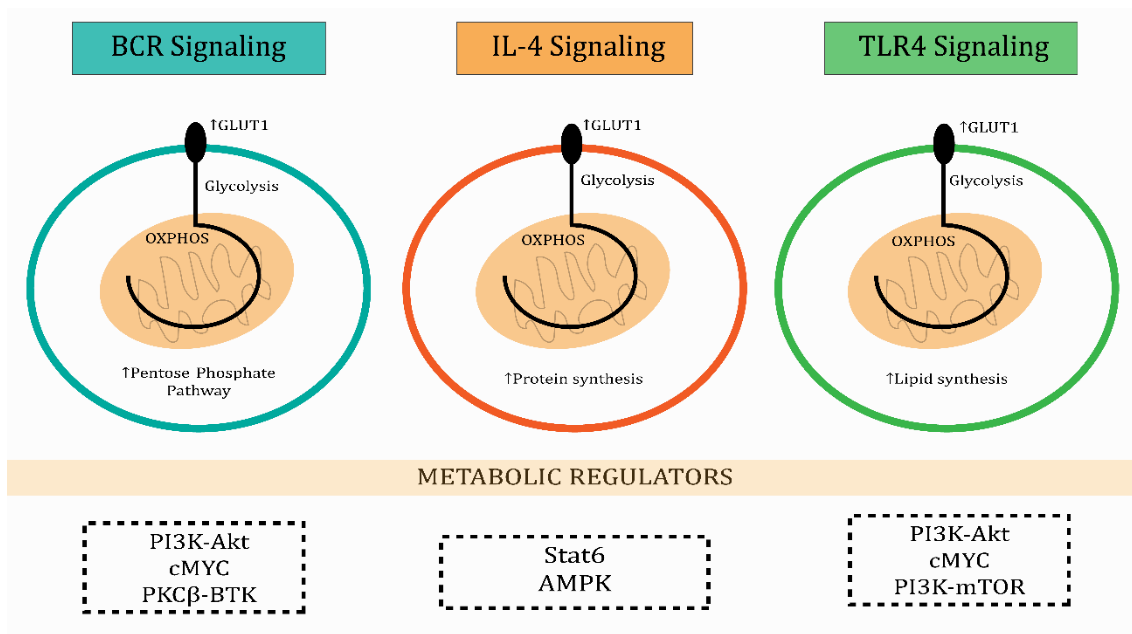


**Figure 1 -** B cell stimulation and activation are regulated through several pathways that include both adaptive and innate immunity. These different pathways include BCR-stimulation through antigen-binding, T-cell-dependent activation through CD40L and IL4 or microbial-dependent activation through TLR receptors. In general, upon stimulation B cells engage a metabolic profile where both glycolysis and oxidative phosphorylation are upregulated, however there are differences concerning the use of other metabolic pathways between different signaling pathways. Interestingly, the three stimulation signals lead to the activation of different pathways that will tightly regulate the cellular metabolism.

#### 1.2.1.4 Memory B cells and Plasma cells: the fully differentiated effectors

Antigen stimulation promotes the activation of naïve B cells which will differentiate into either memory B cells or antibody-secreting plasma cells (Mcheyzer-Williams et al., 2012). This maturation process is also tightly regulated and the cellular metabolic configurations impact directly the differentiation into each one of the subsets. For instance, the functional state of mitochondria seems to be of utterly importance for B cell differentiation into class-switched or plasma cells. Activated mice splenic B cells that exhibit high ROS levels as a result of elevated mitochondrial mass and potential will engage the process of class-switch recombination, while activated B cells with lower ROS levels due to decreased mitochondrial potential and mitochondrial mass will commit to differentiate into plasma cells (Jang et al., 2015). Moreover, activation of human B cells with two different stimuli leads to different cellular metabolic configurations which seem to be fundamental for differentiation into

different populations. Stimulation of human unswitched memory B cells with CpG/TLR9 and IFN-α resulted in augmented mTORC1 expression and upregulated glycolysis, leading to their differentiation into plasmablasts, whilst activation with only CpG enhanced the activity of AMPK1 resulting in a more catabolic and oxidative metabolism and leading to their differentiation into $CD27^{-}IgD^{-}$ memory B cells (Torigoe et al., 2017). In line with this, mTORC1 was also proven to be essential for plasma cell differentiation and proliferation in mouse models (Jones et al., 2016). Moreover, when mitochondrial fission and autophagy are inhibited leading to accumulation of high ROS levels, LPS-activated B cells differentiate into plasmablasts. On the other hand, inhibition of glycolysis with 2DG or galactose maintained the cells in a self-renewal state repressing B cell differentiation (Adams et al., 2016).

Therefore, one might hypothesize that differentiation into memory B cells is a process that requires high OXPHOS levels and mitochondrial activity, while plasma cell differentiation seems to be a more glycolytic-dependent process which is supported by the evidence that glycolysis is essential for antibody production since *in vivo* experiments reported that GLUT1 deletion impaired antibody production (Caro-Maldonado et al., 2014). However, it has also been reported that OXPHOS is progressively upregulated during differentiation into plasmablasts and that plasma cells in mice upregulate both glycolysis and OXPHOS in order to support antibody production and secretion (Kunisawa et al., 2015; Price et al., 2018). Moreover, in mouse activated B cells when Ig secretion is maximal, glutamine consumption with glutamate and alanine production augment (Garcia-Manteiga et al., 2011).

The metabolic configurations of the two types of plasma cells also seem to differ. Glucose uptake and mitochondrial respiratory capacity are higher in long-lived plasma cells (LLPCs) than in short-lived plasma cells (SLPCs). The imported glucose in LLPCs is mainly used to support antibody glycosylation but is also the major fuel source of pyruvate for mitochondrial respiration. Moreover, long-chain fatty acids are used during basal respiration of LLPCs (Lam et al., 2016). In another study it was reported that mTORC1 kinase is not required for bone marrow LLPCs survival but is essential for antibody synthesis by these cells (Jones et al., 2016).

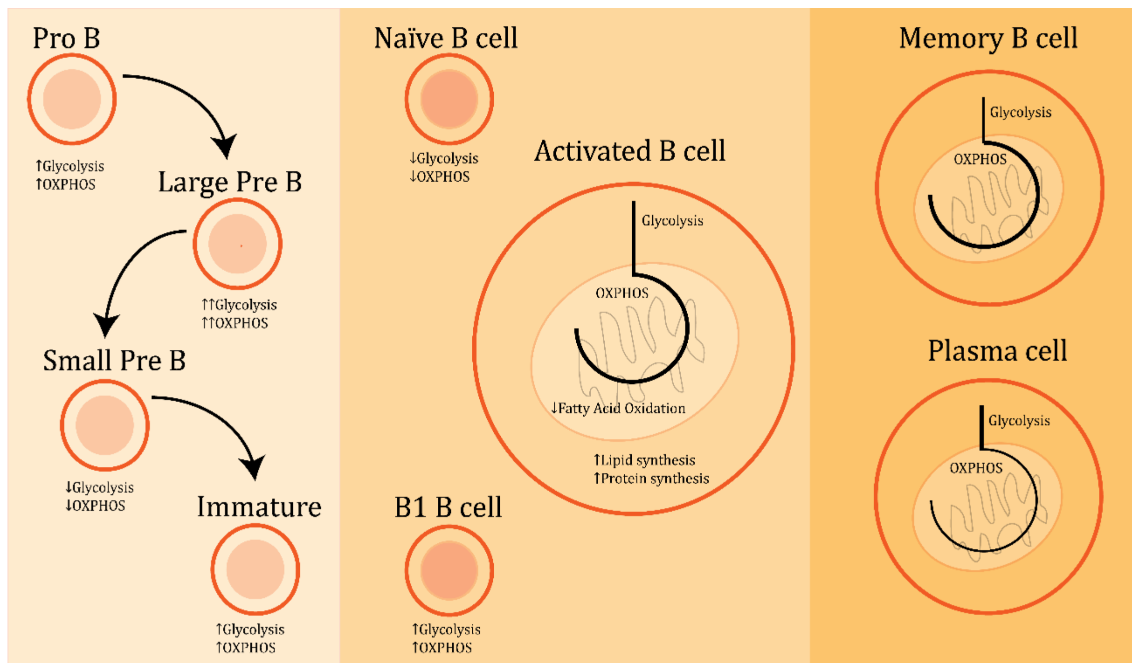


**Figure 2 -** The development of B cells is a tightly regulated process that starts in the Bone Marrow from an undifferentiated lymphoid precursor cell and ends in the Germinal Centers with a fully differentiated and effector immune cell. These differentiation stages have been metabolically characterized and exhibit several differences. Stages that involve cellular alterations, proliferation and effector function exertion enhance the use of metabolic pathways, whereas stages in which the cells are resting exhibit a rather quiescent metabolic state.

### 1.2.2 B cell metabolism in autoimmunity

B cells have a key regulatory role in the development and maintenance of autoimmune diseases. The role of B cells in autoimmunity comprise events such as activation of T cells and secretion of proinflammatory cytokines, however, the most explored role for B cells in autoimmunity is the production of autoantibodies (Mandik-Nayak et al., 2008; Musette and Bouaziz, 2018). Some of the examples include the secretion of anti-dsDNA antibodies in Systemic Lupus Erythematosus, RF and ACPA in Rheumatoid Arthritis or anti–Scl-70 in Systemic Sclerosis (SS) (Suurmond and Diamond, 2015).

B lymphocyte activating factor (BAFF) is a cytokine essential to promote B cell survival and maturation. Overexpression of BAFF in mouse animals leads to the development of autoimmune-like symptoms (Mackay and Browning, 2002). The serum levels of BAFF cytokine are increased in SLE, RA and Sjögren's syndrome (Moisini and Davidson, 2009). Therefore, besides the already established genetically modified autoimmunity mouse models, another

approach to study autoimmunity is the overexpression of BAFF in animals and chronic stimulation of BAFF in cells for an *in vitro* approach.

The few available studies regarding B cell metabolism in autoimmunity suggest that metabolic configurations of autoreactive B cells are altered. Indeed, BAFF-chronically stimulated cells engage a metabolic remodeling program in order to shift to an aerobic glycolytic profile (Caro-Maldonado et al., 2014). However, it has also been reported that B cells stimulated through the BAFF receptor not only upregulate the transcription of glycolytic genes but also increase the mitochondrial potential. These metabolic configurations verified upon stimulation happen in a PKCβ-Akt-dependent manner (Patke et al., 2006). Moreover, BAFF stimulated B splenic cells increase the glucose and amino acid uptake along with the glycolytic rate upon activation. However, blastogenesis is not promoted through this stimulus and instead requires the PI3K-Akt-mTOR pathway (Woodland et al., 2008). Healthy human B cells were reported to exhibit a positive correlation between the expression of CD24 and glucose consumption along with lactate production upon stimulation with BAFF, $CD40^+$ and $CpG^+$. Since, it has been reported that $IgD^+$ naïve cells from Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) patients have higher $CD24^+$ expression and enhanced frequency, one could hypothesize that the metabolic processes in these cells are also dysregulated. Memory B cells ($IgD^-CD27^+$) were also proved to have higher mitochondrial mass when compared to naive B cells in these patients. Moreover, $CD24^+$ memory B cells were also reported to have higher expression of AMPK. Nevertheless, a more precise and deep study should be carried out in order to properly characterize ME/CFS B cells metabolic requirements (Mensah et al., 2018).

HIF-1α-deficient mice were reported to have autoimmunity symptoms, i.e. increased IgM, IgG, RF autoantibodies serum levels, deposits of IgG and IgM in the kidneys, and proteinuria along with abnormal B cell development in the bone marrow (Kojima et al., 2002). As mentioned before, HIF-1α-regulated glycolysis is essential for B cell maturation in the BM, therefore, the levels of HIF-1α protein in the BM of autoimmunity models should be assessed in order to understand how it affects B cell development in autoimmune diseases.

Another possible main metabolic regulator of B cells in autoimmunity is the mTOR kinase. B cells from a RA mouse model (K/BxN) have higher mTORC1 activation levels when compared to B cells from a control mice (Abboud et al., 2018). It has also been reported that

inhibition of mTOR activity with rapamycin inhibits cellular proliferation and survival in BAFF-stimulated primary mouse splenic B cells (Zeng et al., 2015). Inhibition of mTOR kinase with rapamycin has been also reported in several SLE mouse models. Indeed, rapamycin treatment reduced serum anti-dsDNA antibodies to pre-disease numbers in MRL/l mice (Warner et al., 1994). Treatment with rapamycin in the NZB/W model resulted in the decrease of serum IgG antibodies due to the reduction of immature plasma cell frequencies in the spleen, bone marrow and GC B cells depletion (Jones et al., 2016). However, no metabolic parameters were evaluated in these studies and therefore one can't conclude that mTOR is interfering with cellular metabolism since this kinase is a key regulator for other cellular functions. mTORC1 was found to be overexpressed in $CD19^+$ B cells from SLE patients. As mentioned before, mTORC1 has a key regulatory role in the formation of plasmablasts. Moreover, the levels of p-mTOR in $CD19^+$ B cells from patients were positively correlated with plasmablasts frequency, serum levels of anti-dsDNA antibodies and disease index score (Torigoe et al., 2017). However, it was also reported that mTOR phosphorylation is downregulated in CpG-stimulated B cells from SS patients (Forestier et al., 2018).

In a rheumatoid arthritis mouse model (K/BxN), resting B cells were shown to have higher glycolysis, OCR and mTORC1 activation levels when compared to B cells from a control mice (Abboud et al., 2018). In line with this, more recent studies attempted to treat and prevent SLE and RA in mouse models using metabolic inhibitors. Treatment of a rheumatoid arthritis mouse model (K/BxN) with 2DG, a glycolysis inhibitor, improved disease symptoms by causing reduction of joint thickness and reducing the clinical score. However, the levels of arthrogenic antibodies weren't reduced and the disease wasn't completely abrogated. The 2DG treatment was able to reduce the frequency of splenic germinal center B cells, splenic plasma cells and GC B cells in the joint draining lymph node. The researchers that carried out the study concluded that the ineffectiveness in preventing the disease was probably due to insufficient dose or possibly because glycolysis is not the only mechanism driving disease and therefore this treatment should be combined with others currently available (Abboud et al., 2018). A combined therapy of 2-DG and metformin, a mitochondrial complex 1 inhibitor, was tested in several SLE disease models. A three-month carried out treatment of TC mice with this combined therapy resulted in lower production of serum anti-dsDNA IgG, ANAs and reduction of germinal center B cells. Moreover, one month of treatment was enough to

reduce the same parameters in the NZB/W model (Yin et al., 2015). Treatment of B6.*lpr* model with this combined therapy resulted in decreased percentages of GC B cells and plasma cells. In terms of disease activity, the combined therapy allowed a significant serum anti-dsDNA IgG and ANA reduction (Yin et al., 2016).

Thus, targeting the metabolism of immune cells could be a new approach to treat and prevent autoimmunity, either in combination or as an alternative to current therapies. The current therapeutic approaches include glucocorticoids, methotrexate and anti-TNF inhibitors (Li et al., 2017). It has been reported that glucocorticoids are potential metabolic deregulators and TNF-α can cause metabolic syndrome in arthritis patients which means that anti-TNF inhibitors might affect cellular metabolism (Maruotti and Cantatore, 2014; Vegiopoulos and Herzig, 2007). Therefore, it is important to understand the metabolic configurations of B cells in autoimmunity, but also to understand whether if the current therapies are inhibiting or promoting these metabolic settings. A possible approach could the use of metabolic inhibitors that are currently being tested or already approved for cancer treatment, since tumor cells are also metabolically very active (Luengo et al., 2017; Sabnis et al., 2017).

Another emerging topic that requires further investigation is the role of epigenetics in autoimmunity-driven metabolic alterations. The epigenetic configuration of cellular DNA can directly affect the metabolic state through the expression of metabolic enzymes (Yu et al., 2018). However, the metabolic configurations of the cell can also affect the regulation of the epigenetic modifications processes (Keating and El-Osta, 2015). In fact, epigenetic alterations in key metabolic genes have been verified in tumors. Cancer cells generally adopt an aerobic glycolytic metabolism which has been considered to be driven by the alteration in methylation patterns and consequent overexpression of glycolysis rate-limiting enzyme HK2 and by the glycolytic shift-responsible enzyme PKM2 (Lee et al., 2016; Liu et al., 2017). Recently it was reported that naïve B cells from SLE patients exhibit epigenetic differences when compared to healthy controls which promote their differentiation into antibody-secreting cells (Scharer et al., 2019). Thus, it is important to understand if the environment of the BM and lymph nodes is altered in autoimmunity and how this could be influencing the epigenetic program of B cells concerning their differentiation and metabolic alterations.

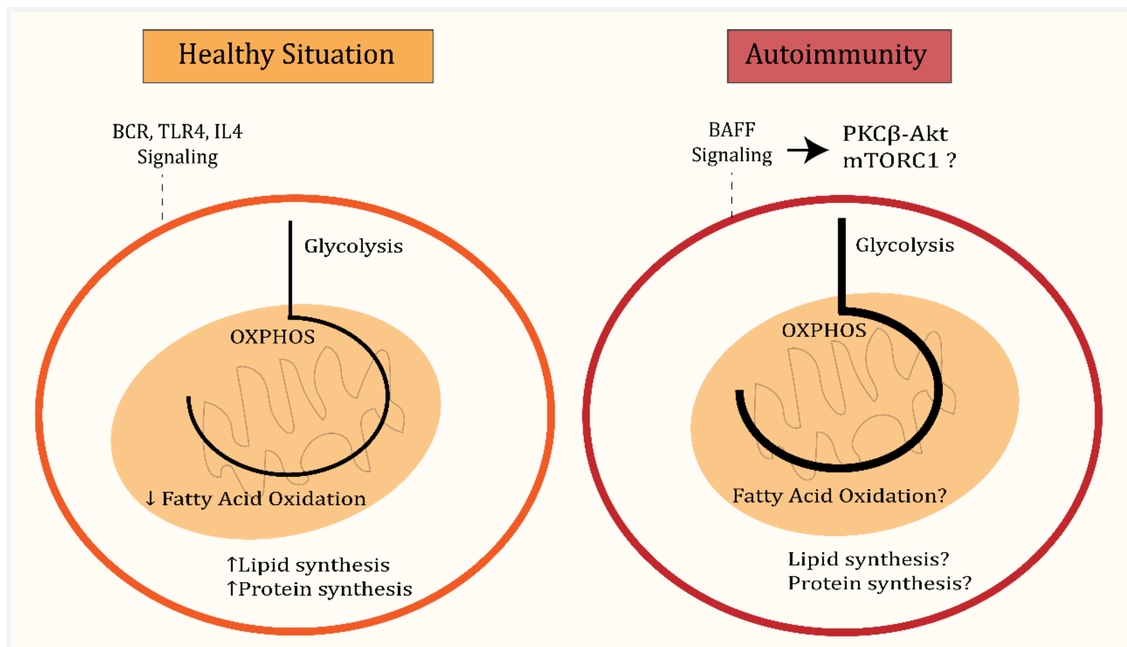


**Figure 3 -** Comparison of the metabolic requirements of an activated B cell between a healthy and autoimmune situation. Upon activation B cells upregulate the use of glycolysis and OXPHOS, however, this enhancement seems to be greater in BAFF chronically stimulated B cells (mimicking an autoimmunity state). Other pathways that concern lipid and protein metabolism or which signaling pathways are regulating these processes are still to be explored.

## 1.3 OBJECTIVES

The metabolic requirements of B cells throughout their lifespan is starting to be revealed and comprehended, however there is still a wide path to explore especially in terms of metabolic impact in autoimmunity. Nevertheless, there is a lack of studies in human cells concerning the B cell metabolism of healthy population and in autoimmunity. Thus, our main objective will be to characterize the metabolism of two B cell subsets, naïve and memory, in healthy controls and in autoimmunity patients, i.e. RA and SLE patients. This will allow us to further elucidate the metabolic requirements of healthy B cells and understand if there are changes for different subsets. Moreover, it will allow us to characterize the metabolic requirements of B cells in RA and SLE upon stimulation and under hypoxic conditions, thus mimicking the environment of chronical inflammation sites. This study will also allow us to understand if the metabolism of B cells is altered in autoimmunity. Finally, this study is of utterly importance since, to the best of our knowledge, we are the first group that will describe the metabolic requirements of B cells in autoimmunity patients.

# 2. MATERIALS AND METHODS

## 2.1 REAGENTS AND MATERIALS

**Table 1 -** Reagents list.

| Reagent | Company | Headquarters |
|---|---|---|
| [1,6-$^{13}C_2$]Glucose | Sigma-Aldrich | St. Louis, Missouri, USA |
| [U-$^{13}C_2$]Glutamine | Sigma-Aldrich | St. Louis, Missouri, USA |
| AffiniPure F(ab')$_2$ | Jackson ImmunoResearch | Cambridgeshire, UK |
| APC/Fire™ 750 anti-human CD20 Antibody (Clone 2H7) | BioLegend | San Diego, Califórnia, USA |
| BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit | Becton Dickinson & Company | Franklin Lakes, New Jersey, USA |
| Biocoll | Biochrom | Berlin, Germany |
| BODIPY™ 493/503 | Thermo Fisher Scientific | Waltham, Massachusetts, USA |
| Bovine Serum Albumin | Thermo Fisher Scientific | Waltham, Massachusetts, USA |
| Brilliant Violet 421™ anti-human CD19 Antibody | BioLegend | San Diego, Califórnia, USA |
| Brilliant Violet 510™ anti-human CD38 Antibody | BioLegend | San Diego, Califórnia, USA |
| CD40L | R&D Systems | Minneapolis, Minnesota, USA |
| CellROX® Deep Red Reagent | Thermo Fisher Scientific | Waltham, Massachusetts, USA |
| Deuterium oxide | Sigma-Aldrich | St. Louis, Missouri, USA |
| Dulbecco's Phosphate Buffered Saline (DPBS) | Sigma-Aldrich | St. Louis, Missouri, USA |
| FX11 | Sigma-Aldrich | St. Louis, Missouri, USA |
| Gibco™ Fetal Bovine Serum | Thermo Fisher Scientific | Waltham, Massachusetts, USA |
| Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) | Abcam | Cambridge, UK |
| High Pure RNA Isolation Kit | Roche | Basel, Switzerland |
| Human Glut1 APC-conjugated Antibody | R&D Systems | Minneapolis, Minnesota, USA |
| Human/Mouse HIF-1 alpha APC-conjugated Antibody | R&D Systems | Minneapolis, Minnesota, USA |
| Imiquimod | InVivoGen | San Diego, California, USA |
| Invitrogen™ EDTA | Thermo Fisher Scientific | Waltham, Massachusetts, USA |
| LDHA (C4B5) Rabbit mAb | Cell Signaling Technology | Danvers, Massachusetts, USA |
| LEGENDplex™ Human Immunoglobulin Isotyping Panel (6-plex) with V-bottom Plate | BioLegend | San Diego, California, USA |
| Memory B Cell Isolation Kit (Human) | Miltenyi Biotec | Gladbach, Germany |
| Pan B Cell Isolation Kit (Human) | Miltenyi Biotec | Gladbach, Germany |
| PE/Cy7 anti-human IgD Antibody (Clone IA6-2) | BioLegend | San Diego, California, USA |
| Penicillin-Streptomycin (Pen-Strep) | Thermo Fisher Scientific | Waltham, Massachusetts, USA |
| PerCP/Cyanine5.5 anti-human CD71 Antibody (Clone CY1G4) | BioLegend | San Diego, California, USA |
| QuantiTect® Reverse Transcription Kit | Qiagen | Hilden, Germany |

| | | |
|---|---|---|
| Rapamycin | Sigma-Aldrich | St. Louis, Missouri, USA |
| riL-21 | R&D Systems | Minneapolis, Minnesota, USA |
| Rotenone | Sigma-Aldrich | St. Louis, Missouri, USA |
| RPMI Medium 1640 | Biological Industries | Beit-Haemek, Israel |
| Sodium phosphate dibasic | Sigma-Aldrich | St. Louis, Missouri, USA |
| Sodium phosphate monobasic monohydrate | Sigma-Aldrich | St. Louis, Missouri, USA |
| Sodium Fumarate dibasic | Sigma-Aldrich | St. Louis, Missouri, USA |
| PE anti-human CD27 Antibody | BioLegend | San Diego, California, USA |
| Phospho-mTOR (Ser2448) Monoclonal Antibody (MRRBY), PE, eBioscience™ | Thermo Fisher Scientific | Waltham, Massachusetts, USA |
| Phospho-S6 Ribosomal Protein (Ser235/236) (D57.2.2E) XP® Rabbit mAb (Alexa Fluor® 488 Conjugate) #4803 | R&D Systems | Minneapolis, Minnesota, USA |
| PowerUp™ SYBR™ Green Master Mix | Applied Biosystems | Foster City, California, USA |

**Table 2 -** Materials list.

| Material | Company | Headquarters |
|---|---|---|
| 15 mL CELLSTAR® Polypropylene Tube | Greiner Bio-One | Kremsmünster, Austria |
| 50 mL CELLSTAR® Polypropylene Tube | Greiner Bio-One | Kremsmünster, Austria |
| 50 ml Leucosep™ Tubes | Greiner Bio-One | Kremsmünster, Austria |
| BD FACSCelesta™ Flow Cytometer | Becton Dickinson & Company | Franklin Lakes, New Jersey, USA |
| BD FACSAria™ II Cell Sorter | Becton Dickinson & Company | Franklin Lakes, New Jersey, USA |
| BD™ LSR II Flow Cytometer | Becton Dickinson & Company | Franklin Lakes, New Jersey, USA |
| Bruker AVANCE™ 600 MHz Spectrometer | Bruker | Billerica, Massachusetts, USA |
| CELLSTAR® 96W Microplate | Greiner Bio-One | Kremsmünster, Austria |
| Heracell 150i Incubator | Thermo Fisher Scientific | Waltham, Massachusetts, USA |
| Heraeus™ Biofuge Pico™ Microcentrifuge | Thermo Fisher Scientific | Waltham, Massachusetts, USA |
| MACS Multistand | Miltenyi Biotec | Gladbach, Germany |
| MidiMACS Separator | Miltenyi Biotec | Gladbach, Germany |
| PCR 8-tube Strips with attached caps | Greiner Bio-One | Kremsmünster, Austria |
| Rotofix 32 A | Andreas Hettich | Tuttlingen, Germany |
| StepOnePlus™ Real-Time PCR System | Applied Biosystems | Foster City, California, USA |
| T3000 Thermocycler | Biometra | Göttingen, Germany |
| NMR Tubes | Bruker | Billerica, Massachusetts, USA |

## 2.2 PATIENTS

Peripheral blood samples were obtained from 29 Rheumatoid Arthritis patients, 15 Systemic Lupus Erythematosus patients and 13 Healthy Controls. The patients were recruited at the University Hospital of Heidelberg. The clinical and demographic data of each group is exhibited in **Table 3**.

**Table 3 – Clinical and demographic data of healthy controls, RA and SLE B patients.**

| | **RA (n=29)** | **SLE (n=15)** | **CNT (n=13)** |
|---|---|---|---|
| **Female:Male** | 23:6 | 11:4 | 6:7 |
| **Age (years)**<br>**(minimum-maximum in years)** | 62.8<br>(35-81) | 51.9<br>(23-70) | 42.9<br>(23-57) |
| **Disease Duration**<br>**(minimum-maximum in years)** | 11.6<br>(1-37) | 18.7<br>(2-33) | - |
| **% active DAS28 (>3.2)** | 31 % | - | - |
| **% remission DAS28 (<2.6)** | 62 % | - | - |
| **CRP (± SEM) (mg/L)** | 4.87 (±0.76) | 4.41 (±1.27) | - |
| **RF positive:negative** | 25:4 | - | - |
| **Anti-CCP positive:negative** | 25:4 | - | - |
| **Anti-dsDNA positive:negative** | - | 11:3 | - |
| **ANA positive:negative** | - | 14:0 | - |
| **MTX (± SEM) (mg)**<br>**% from Total Patients** | 9.88 (±1.71)<br>55.2 % | -<br>- | -<br>- |
| **Glucocorticoids (± SEM) (mg)**<br>**% from total patients** | 4.27 (±0.37)<br>51.7 % | 3.50 (±0.77)<br>33 % | -<br>- |
| **Leflunomide (± SEM) (mg)**<br>**% from Total Patients** | 1.57 (±0.20)<br>24.1 % | -<br>- | -<br>- |

## 2.3 B CELL ISOLATION, CULTURE, STIMULATION AND METABOLIC INHIBITION

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from the obtained samples through centrifugation (1000g, 15 minutes, Room Temperature, brakes off) using a density separating solution (Biocoll). The supernatants were transferred to new tubes and PBMCs were centrifuged twice with DPBS for 10 minutes at 550g. Total B cells destined to be sorted were isolated from PBMCs using a Pan B Cell Isolation Kit through magnetic separation following the protocol provided with the kit (available at *www.miltenyibiotec.com*), while total B cells destined to be cultured were isolated using a Memory B Cell Isolation Kit. This kit involved two isolation steps through magnetic separation, first we isolated a fraction of total B cells and second we separated two fractions: naïve B cells ($CD27^-$) and memory B cells ($CD27^+$) according to the protocol provided with the kit (available at *www.miltenyibiotec.com*). These two subsets were then cultured in non-stimulated or stimulated medium. The non-stimulated medium was composed by: RPMI Medium 1640 (without glucose or glutamine) with 5mM [1,6-$^{13}C_2$]glucose, 2mM [U-$^{13}C$]glutamine, 10% FCS and 1% Pen-Strep. The stimulated medium had the exact same composition as the non-stimulated one plus a cocktail mix of: 1.5 µg/mL Imiquimod (anti-TLR7), 156 ng/mL riL-21, 100 ng/mL CD40L and 6 µg/mL $F(ab')_2$ (anti-IgG). Isolated-naïve and memory B cells were cultured under 5 different conditions: non-stimulated, stimulated, FX11 inhibition, rapamycin inhibition and rotenone inhibition. A total of 250000 cells was distributed per each condition/well in the naïve subset, however this number was always inferior in the memory subset given the low number of cells that we obtained through magnetic isolation. Naïve and memory B cells were cultured under hypoxic conditions with 3% $O_2$ at 37° for 7 days. The metabolic inhibitors were added to the cell cultures 24h after incubation with the following concentrations: 20 µM/well of FX11, 2 µM/well of rapamycin and 2.5 µM/well of rotenone.

## 2.4 TOTAL B CELL STAINING AND SORTING

Isolated-total B cells were centrifuged for 5 minutes at 550g and the pellet was resuspended in MACS Buffer (DPBS, 4% EDTA, 2% BSA). Total B cells were incubated for 30 minutes at room temperature in the dark with a mix solution of CD19, CD71, IgD, CD38 and CD20 antibodies diluted in MACS buffer (dilution of 4:150 for each Ab). The cells were washed twice with MACS buffer and resuspended at a final concentration of $5x10^6$ cells/mL MACS. Total B cells were sorted into two populations using a BD FACSAria II cell sorter: $CD19^+IgD^-CD71^+CD38^+CD20^-$ and $CD19^+IgD^-CD71^+CD38^{dim}CD20^+$ **(Figure 4)**.

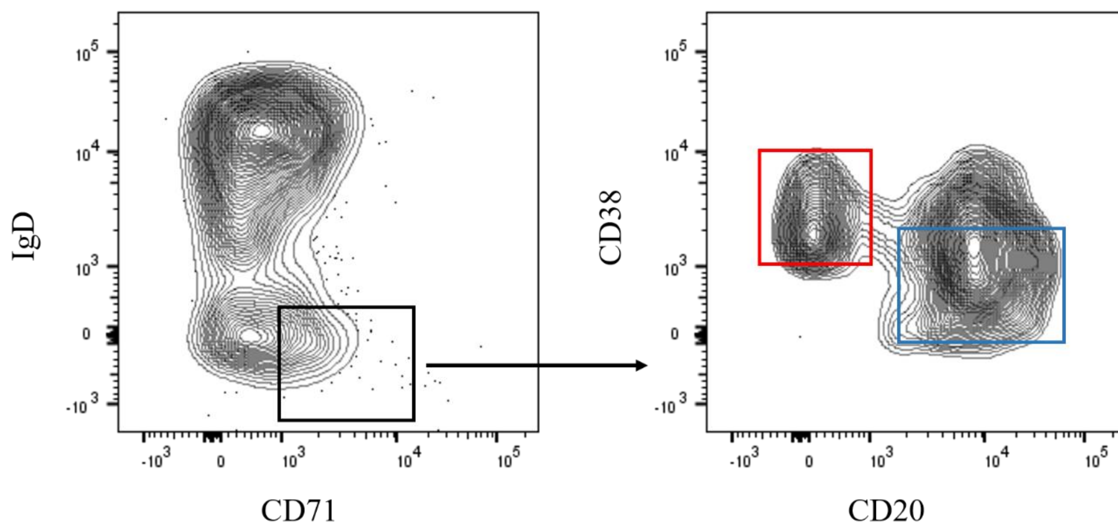


**Figure 4 -** Flow cytometry of $CD19^+$ PBMCs isolated from a healthy control with the gates used to identify and sort the two populations. $CD19^+IgD^-CD71^+CD38^+CD20^-$ (red square) and $CD19^+IgD^-CD71^+CD38^{dim}CD20^+$ (blue square).

## 2.5 RNA ISOLATION, DNA SYNTHESIS AND GENE EXPRESSION

The RNA from the two sorted populations ($CD38^+CD20^-$ and $CD38^{dim}CD20^+$) was extracted using the High Pure RNA Isolation Kit and following the provided protocol (available at *www.lifescience.roche.com*). QuantiTect® Reverse Transcription Kit and T3000 Thermocycler were used to convert the isolated RNA into cDNA and we followed the kit

associated protocol (available at *www.qiagen.com*). Next, we evaluated the gene expression of *RPLP0*, *HK2*, *PKM2*, *LDHA*, *TSC2*, *SREBP1*, *PRKAA1*, *MYC, HIF1A* and *CPT1A* with the StepOnePlus™ Real-Time PCR System and StepOne™ Software. The obtained cDNA was amplified following the protocol provided with the PowerUp™ SYBR™ Green Master Mix. The primer sequences for each assessed gene are detailed in **Table 4**.

**Table 4** – Forward and reverse primer sequences.

| Gene | Forward Primer | Reverse Primer |
|---|---|---|
| RPLP0 | TGGTCATCCAGCAGGTGTTCGA | ACAGACACTGGCAACATTGCGG |
| HK2 | GAGTTTGACCTGGATGTGGTTGC | CCTCCATGTAGCAGGCATTGCT |
| PKM2 | ATGGCTGACACATTCCTGGAGC | CCTTCAACGTCTCCACTGATCG |
| LDHA | GGATCTCCAACATGGCAGCCTT | AGACGGCTTTCTCCCTCTTGCT |
| TSC2 | GCACCTCTACAGGAACTTTGCC | GCACCTGATGAACCACATGGCT |
| SREBF1 | ACTTCTGGAGGCATCGCAAGCA | AGGTTCCAGAGGAGGCTACAAG |
| PRKAA1 | AGGAAGAATCCTGTGACAAGCAC | CCGATCTCTGTGGAGTAGCAGT |
| MYC | CCTGGTGCTCCATGAGGAGAC | CAGACTCTGACCTTTTGCCAGG |
| HIF1A | TATGAGCCAGAAGAACTTTTAGGC | CACCTCTTTTGGCAAGCATCCTG |
| CPT1A | GATCCTGGACAATACCTCGGAG | CTCCACAGCATCAAGAGACTGC |

## 2.6 PROTEIN EXPRESSION, OXIDATIVE STRESS AND LIPID LEVELS QUANTIFICATION

Naïve and memory B cells were collected after 7 days and for that we centrifuged the culturing plates at 550g for 10 minutes. The supernatant was collected and stored for further experiments and the pelleted cells were resuspended in DPBS. Naïve and memory B cells were incubated with CD19, CD71, IgD, CD38, CD20, CD27 antibodies diluted in DPBS (dilution 2:100) for 45 min at room temperature in the dark. Then, the cells were incubated with Bodipy (dilution 1:1250) and CellRox (dilution 2:1250) diluted in DPBS for 15 min at 37°C in the dark. After that, we fixed and permeabilized the cells for 20 min at room temperature in the dark with BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit. Naïve and memory B cells were incubated with LDHA, HIF-1α, pS6RP, pmTOR and GLUT1 antibodies for 45 min at room temperature in the dark. The secondary antibody Alexa Fluor 488 diluted in DBPS (dilution 1:500) was added and allowed to incubate for 30 min at room temperature in the dark. Finally, the cells were resuspended in DPBS and FACS analysis were performed using BD™ LSR II flow

cytometer and the provided software BD FACSDiva™. The obtained data was processed and analyzed using FlowJo® v10 software (Becton Dickinson & Company, Franklin Lakes, New Jersey, USA).

## 2.7 GLUCOSE CONSUMPTION AND LACTATE PRODUCTION

The supernatant from centrifuged naïve and memory B cells cultures (i.e. cell culture media) was collected to measure the amount of [1,6-$^{13}C_2$]glucose consumed and the amount of [3-$^{13}C$]lactate produced in the 5 different conditions using Proton Nuclear Magnetic Resonance ($^{1}H$ NMR) Spectroscopy. The prepared samples consisted of 100 µL of cell culture media, 50 µL of $D_2O$ (99.9%) and 35 µL of 10 mM sodium fumarate and phosphate buffer dissolved in $D_2O$ (99.9%), the latter was used for internal reference. The spectra were acquired in Bruker AVANCE$^{TM}$ 600 MHz Spectrometer equipped with a 5mm broadband indirect detection probe. The applied pulse program for $^{1}H$ NMR spectra acquisition consisted of a 30 ° radiofrequency observation pulse, a 3 s acquisition time which provided a total of 65k point defining a spectral width of 10 Hz, and an interpulse delay of 10 s between each scan to warrant quantitative spectra. Each $^{1}H$ NMR spectra was processed and quantitatively analyzed using NUTSpro™NMR software (Acorn NMR Inc., Livermore, CA, USA).

## 2.8 IMMUNOGLOBULIN AND CYTOKINE PROFILING

The supernatant from centrifuged naïve and memory B cells cultures (i.e. cell culture media) was also used to identify and quantify the immunoglobulins (Igs) and cytokines produced in the 5 different conditions. The first assessment was performed using LEGENDplex™ Human Immunoglobulin Isotyping Panel (6-plex) to evaluate the secretion of IgG1, IgG2, IgG3, IgG4, IgM and IgA. The second assessment was performed using a LEGENDplex™ Human Essential Immune Response Mix and Match Subpanel to measure the expression of the following cytokines: IL-4, IL-2, TNF-α, IL-6, IL-10, TGF-β1. Both kits provided

an associated protocol (available at *www.biolegend.com*) that we carefully followed to perform the assay. Data acquisition was performed using BD FACSCelesta™ Flow Cytometer and the provided software BD FACSDiva™. The obtained data was processed and analyzed using LEGENDplex™ Data Analysis Software v8.0 (Becton Dickinson & Company, Franklin Lakes, New Jersey, USA).

### 2.9 STATISTICAL ANALYSIS

All the obtained data was analysed using GraphPad Prism 7.04 software (GraphPad Software, Inc., San Diego, California, USA). All results were represented as scatter plots with the corresponding median and outliers were tested using the ROUT method (Q=1%). The data was tested for normal distribution with the Shapiro-Wilk normality test. The analysed data that assumed a normal distribution was then tested with One-Way ANOVA followed by Uncorrected Fisher's LSD, while the statistical significance of the data that did not assume a normal distribution was further determined with Kruskal-Wallis test followed by Uncorrected Dunn's test. The only exception were the results of gene expression because these results were represented as columns with the corresponding mean and the statistical significance was determined using the Holm-Sidak method.

# 3. RESULTS

## 3.1 PRELIMINARY RESULTS: B CELL GENE EXPRESSION PROFILING IN RHEUMATOID ARTHRITIS AND SYSTEMIC LUPUS ERYTHEMATOSUS PATIENTS VERSUS HEALTHY INDIVIDUALS

Published data regarding metabolic configurations in B cells from autoimmunity patients is almost non-existent. However, a gene expression profiling in B cells from SLE and RA patients has been published. In this study, Lauwerys and his colleagues assessed the differential expression of more than 6000 genes through microarray analysis of B cells sorted from total peripheral blood mononuclear cells (available at *https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE4588*). The first step of our work was to use this data published on Gene Expression Omnibus database and analyze the expression of metabolic genes generating heatmaps to compare between SLE patients, RA patients and healthy controls (**Figure 5**).

A brief overview of the results showed that total B cells from RA patients had highest expression of key glycolytic genes such as *LDHA*, *PKM* and *HK2* and the highest gene expression of the metabolic regulator *MYC*. SLE patients also had higher expression of the mentioned genes when compared to healthy controls, however these genes were expressed to a less extent when compared to RA patients. The gene of the metabolic regulator *mTOR* was found to be more expressed in SLE patients, while RA and CNT seemed to have similar values. Looking into OXPHOS, the gene expression of *PDHA*, responsible to convert pyruvate into acetyl-CoA, was highest in SLE patients. The expression of *CPT1A* a gene essential for fatty acid oxidation was higher in RA and SLE patients. In opposition to that, healthy controls and SLE patients had higher expression of the lipid synthesis regulator *SREBF1*.

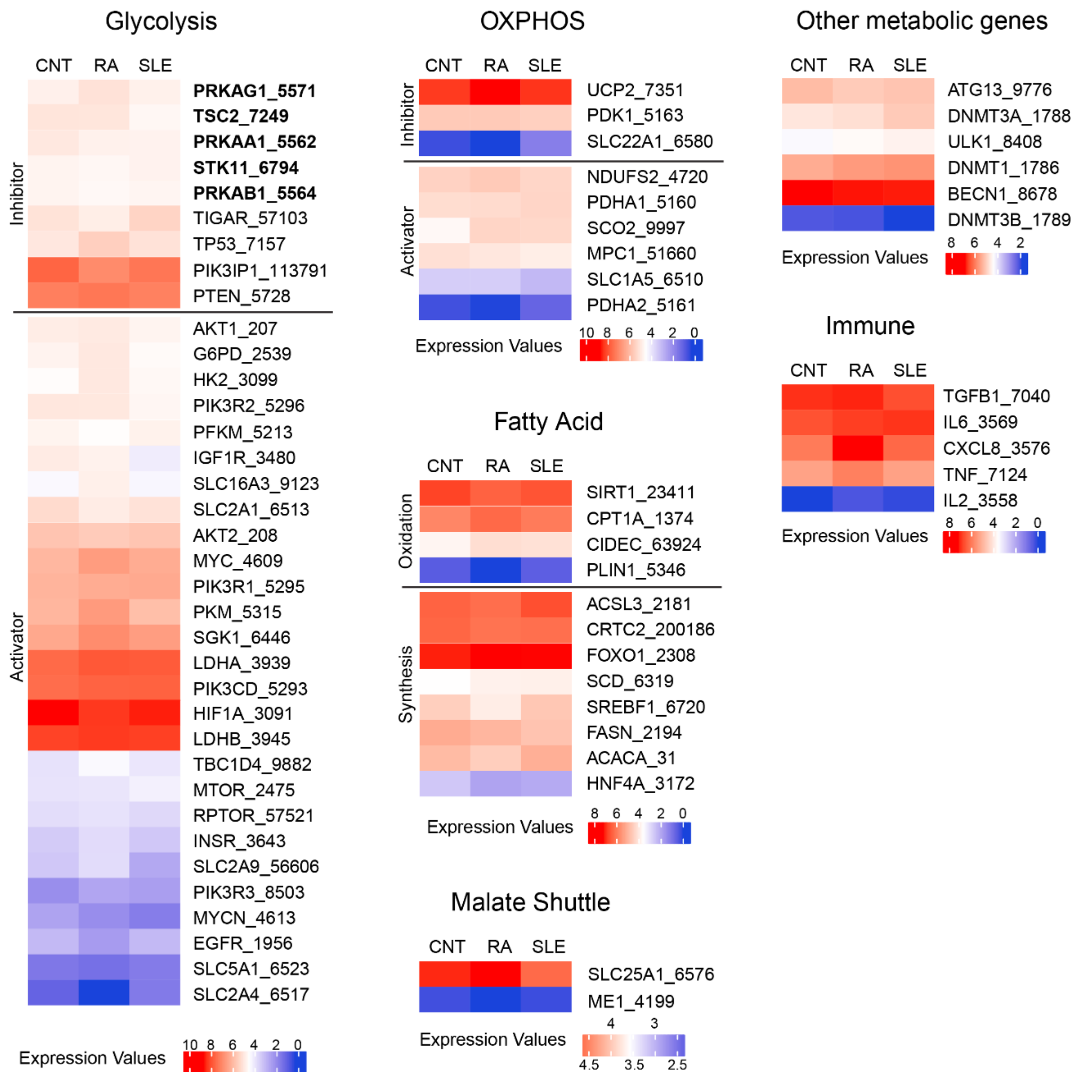


**Figure 5 - There is a clear differential expression of glycolytic and oxidative genes between patients and controls with these genes being upregulated in the first group.** Heatmap generated using microarrays analysis of metabolic genes in B cells from 7 RA patients, 7 SLE patients and 9 controls (CNT).

### 3.2 RHEUMATOID ARTHRITIS AND SYSTEMIC LUPUS ERYTHEMATOSUS METABOLIC GENE EXPRESSION IN ANTIGEN-SPECIFIC B CELL SUBSETS

Growing evidence suggests that the frequency of plasmablasts and memory B cells ($IgD^-CD27^+$) is increased in the peripheral blood of RA (Arroyo-Villa et al., 2014; Souto-Carneiro et al., 2009) and SLE patients (Anolik et al., 2004; Odendahl et al., 2000) which presumably is linked to the role of these two subsets as disease mediators in autoimmunity. Antigen-interactions that result from infections or vaccination stimulate naïve B cells to differentiate into two different subsets: plasmablasts and memory B cells (Tangye and Tarlinton, 2009). Recently, two subsets were identified among antigen-specific proliferating B cells ($CD71^+$) after infection or vaccination. These subsets were defined as plasmablasts (pBs, $CD19^+IgD^-CD71^+CD38^+CD20^-$) and activated B cells that were identified to be memory B cell precursors (mBs, $CD19^+IgD^-CD71^+CD38^{dim}CD20^+$) (Ellebedy et al., 2016).

Since it has been described that antigen-specific B cells levels are altered in both RA and SLE and since there is a clear differential gene expression in RA and SLE total B cells, we sought to characterize the expression of key metabolic genes in pBs and mBs. We attempted to characterize the main metabolic pathways by assessing the expression of the following genes: *HK2* and *PKM2* – these enzymes catalyze two irreversible steps in glycolysis (Allen and Locasale, 2018; Tanner et al., 2018) and have been linked to promote aerobic glycolysis (Christofk et al., 2008; Lee et al., 2016)*; LDHA* – catalyzes pyruvate-lactate conversion (Valvona et al., 2016)*; CPT1A* – key regulator of fatty acid oxidation (Gobin et al., 2003)*; SREBF1* – key activator of lipid synthesis (Shao and Espenshade, 2012); *MYC* and *HIF1A* – glycolysis positive-regulators (Dang et al., 2006; Majmundar et al., 2010); *PRKAA1* and *TSC2* – mTORC1 inhibitors (Saxton and Sabatini, 2017). The expression of these key metabolic genes was assessed by quantitative PCR and the obtained results showed explicit differences between a healthy situation and autoimmunity.

Results are represented as the fold change of the mean ΔCt of each group relatively to the mean ΔCt of the CNT group for each gene **(Figure 6)**. In the mB subset, no differences were found between control and autoimmune groups. This also applied to pBs from the RA group, however in SLE pBs significant changes were observed relatively to the CNT group. Indeed, in SLE pBs the expressions of *PKM2*, *LDHA*, *HIF1A* and *TSC2* were upregulated when compared to the CNT group.

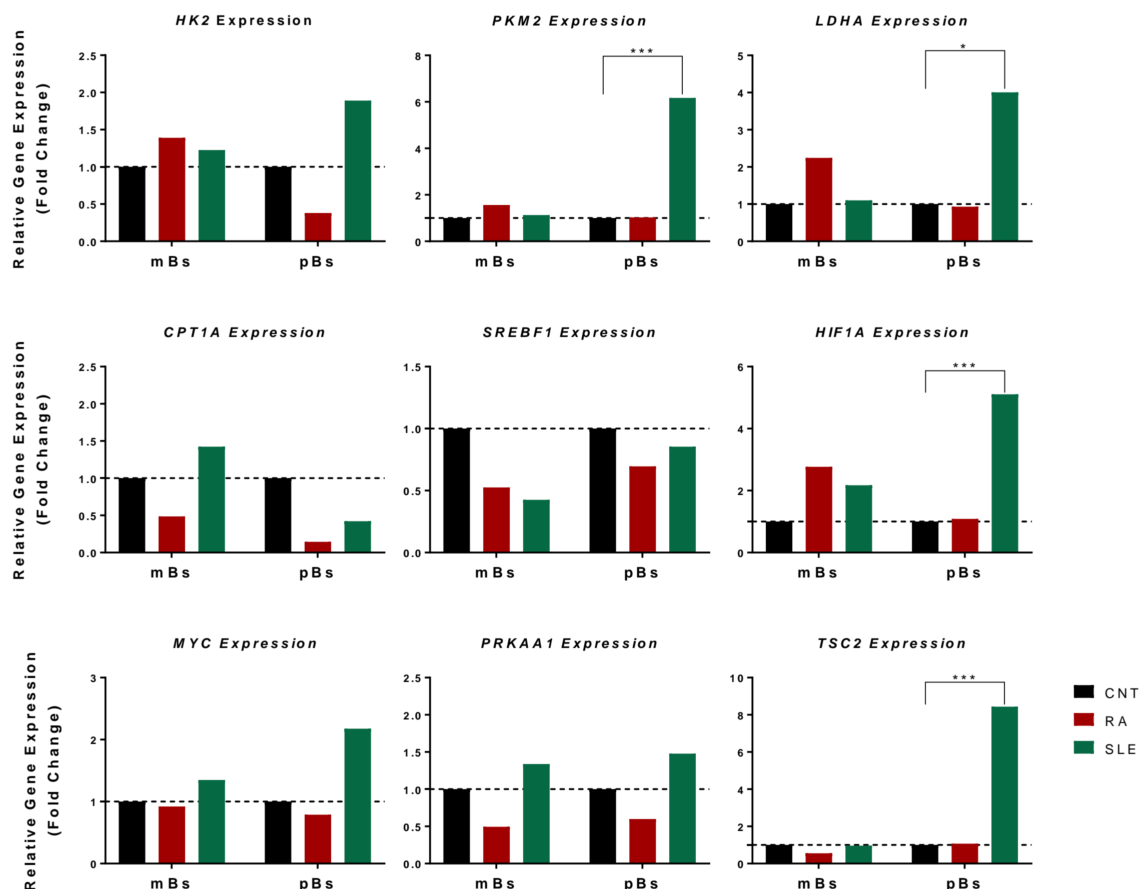


**Figure 6 - The expression of key metabolic genes is upregulated in RA and SLE B cell subsets when compared to healthy controls (CNT).** The expression values were assessed by qPCR and the results are represented using the fold change of the mean ΔCt of each gene for each group (CNT, RA or SLE) relatively to the mean ΔCt of the CNT group and the number of replicates is 5-6. For each subset (mBs or pBs) the p-values and statistical significance were determined using the Holm-Sidak method (α=0.05). *p<0.05; ***p<0.001.

### 3.3 METABOLIC PROFILING OF NAÏVE AND EFFECTOR B CELLS IN RHEUMATOID ARTHRITIS AND SYSTEMIC LUPUS ERYTHEMATOSUS

After finding differences regarding the gene expression of key metabolic enzymes in SLE pBs, we sought to characterize more in depth the metabolic profile of B cells in both diseases and understand how the modulation of the cellular metabolism can impact the exertion of effector functions. The study was designed to comprise 4 groups: Healthy controls (CNT), RA patients with conventional treatment (RADMARD), RA patients with Anti-TNF treatment (RATNF) and SLE patients. The standard drugs used for the treatment of Rheumatoid Arthritis are designated as disease-modifying antirheumatic drugs (DMARDs). These drugs are mainly used to control the disease by preventing inflammation, pain and joint damage and can be further divided in two groups: conventional or non-biologic DMARDs and biologic DMARDs (Bijlsma et al., 2006; Guo et al., 2018). Non-biologic or conventional DMARDs act through several broader mechanisms, while biologic DMARDs target specific molecules or pathways. One example is the targeting and binding inhibition of the pro-inflammatory cytokine TNF-α to the correspondent receptors (Abbasi et al., 2019; Guo et al., 2018). The first-line option recommended for RA treatment is Methotrexate (MTX) and this conventional DMARD is the most widely-used (Emery et al., 2013). If conventional DMARDs fail to control the disease, then biologic DMARDs are recommended, being the TNF inhibitor the predominant choice (Kumar and Banik, 2013). Therefore, for our study we decided to divide the RA patients into these two groups: RADMARD where the main treatment received was non-biologic DMARDs and predominantly MTX or the RATNF group with anti-TNF treatment.

First, we isolated naïve ($CD27^-$) and memory ($CD27^+$) from the peripheral blood of RA and SLE patients. Both populations were maintained under five different conditions: non-stimulated (NStim); stimulated (Stim); stimulated + FX11 (FX11) – an LDHA specific inhibitor (Gao et al., 2016); stimulated + Rapamycin (RAPA) – mTORC1 inhibitor (Saxton and Sabatini, 2017); and stimulated + Rotenone (ROT) – natural compound that inhibits the complex I of the electron transport chain (ETC) (Ozay et al., 2018). For both naïve and memory B cell subset we started by evaluating the expression of key metabolic enzymes: GLUT1 – glucose transporter (Galochkina et al., 2019); LDHA - catalyzes pyruvate-lactate conversion (Valvona et al., 2016); HIF-1α – glycolysis promoting factor (Majmundar et al., 2010); mTOR and S6RP –

main metabolic regulator and its downstream effector, respectively (Saxton and Sabatini, 2017). Besides, the amount of neutral lipids (Bodipy) and the cellular oxidative stress through the production of reactive oxygen species (ROS) (CellRox) were also measured. The metabolic profile of both subsets was further characterized through the quantification of the consumed glucose and produced lactate, with this we sought to unravel more in depth which main metabolic pathways were being used by both subsets. Finally, in order to characterize how the effector function differs in a disease context and how it is affected by metabolic inhibition, we verified the immunoglobulin and cytokine production in the five different conditions.

### 3.3.1 Metabolic enzyme expression of naïve and differentiated switched-memory B cells in autoimmunity

First, naïve B cells that were positive for the CD19 surface marker were gated for IgD, a marker that allows to characterize and distinguish between naïve or memory B cells, and for CD38, a typical plasma cells marker (Hamblin, 2003; Shi et al., 2003). Therefore, we were able to define three populations: naïve B cells ($IgD^{+}CD38^{-}$), switched-memory B cells ($IgD^{-}CD38^{-}$) and plasma cells ($IgD^{-}CD38^{+}$) **(Figure 7)**. The percentage of naïve B cells was significantly higher in all non-stimulated (Nstim) conditions when compared to the respective stimulated (Stim) condition. For the switched-memory B cells the opposite happened, with this population being higher in a stimulated condition. The percentage of plasma cells seemed to be constant throughout all the conditions, however there was a significant increase in the percentage of these cells in the SLE group upon stimulation. Still regarding the percentage of plasma cells, naïve and switched-memory B cells, no differences were found for NStim or Stim conditions when comparing the RA or SLE groups with the CNT group.

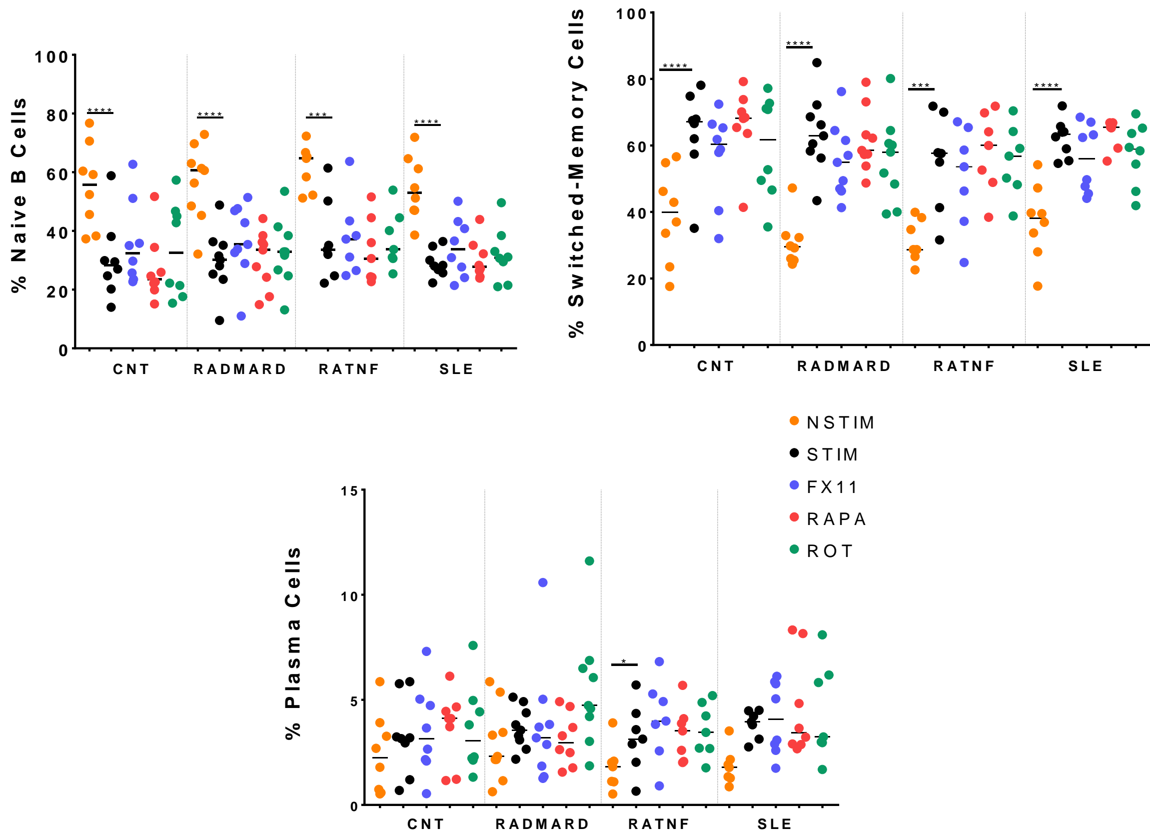


**Figure 7 -** **Selected CD19⁺ B cells were gated for IgD and CD38 which allowed to define 3 populations: Naïve B cells (IgD⁺CD38⁻), Switched-Memory B cells (IgD⁻CD38⁻) and Plasma Cells (IgD⁻CD38⁺).** The results are represented as the percentage of each population relatively to the total CD19⁺ B cells, each dot represents an individual donor (n=7-9) and the median bar of the data is also presented. The p-values and statistical significance were determined using One-Way ANOVA and Uncorrected Fisher's LSD (α=0.05). *p<0.05;***p<0.001; ****p<0.0001.

In **Figure 8** are represented the Mean Fluorescence Intensities (MFI) relative to each group for target enzymes, intracellular lipids and ROS levels in naïve B cells non-stimulated and stimulated. Results show that the CNT group exhibited higher expression levels of GLUT1 in non-stimulated conditions and higher LDHA levels upon stimulation when compared to RAs and SLE groups. Besides the observed decrease in LDHA expression there was a significant decrease of HIF-1α expression in the CNT group upon stimulation. In the RADMARD group no changes were verified upon stimulation. GLUT1 suffered a downregulation when B cells were stimulated in the SLE group. The main changes regarding the RATNF group are verified in the levels of neutral lipids and phosphorylation of S6RP which significantly increased when B cells were stimulated.

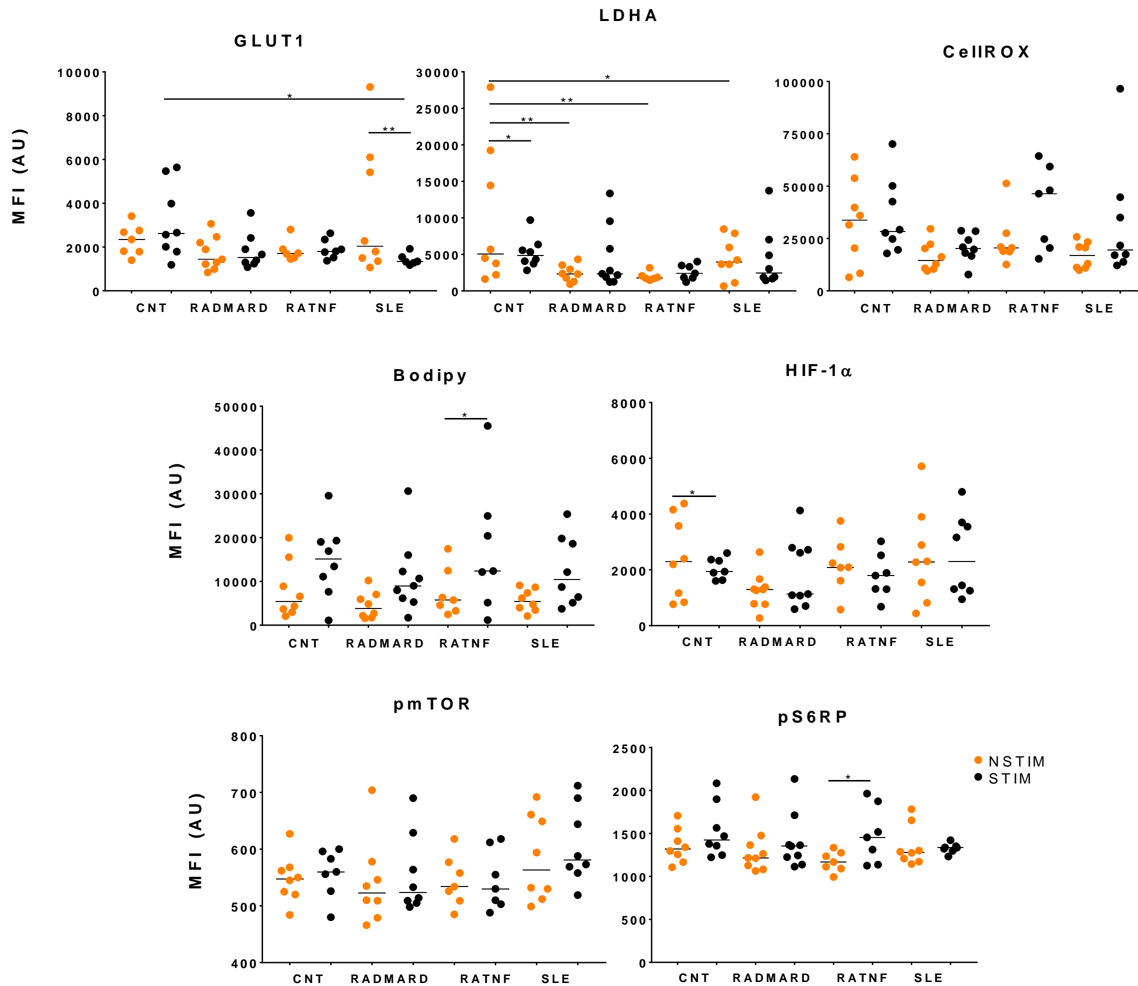


**Figure 8 - The expression of GLUT1 and LDHA is downregulated in naïve B cells in autoimmunity.** Levels of intracellular lipids, ROS and enzyme expression were assessed by flow cytometry and the results are expressed as the Mean Fluorescence Intensity (MFI). Each dot represents an individual donor (n=7-9) and the median bar of the data is also presented. One-way ANOVA and Uncorrected Fisher's LSD (α=0.05) were used to compare NStim and Stim conditions for each group and NStim and Stim conditions from the CNT group with the respective correspondents in RA and SLE groups. *p<0.05; **p<0.01.

We also evaluated the expression of these key enzymes and metabolic parameters in the three conditions treated with inhibitors, the results are represented as the fold change of the MFI of each condition (Stim, FX11, RAPA or ROT) relatively to the respective non-stimulated condition **(Figure 9)**. The results obtained for the CNT, RATNF and SLE groups showed that treatment with rotenone promoted a significant augmentation in mTOR and S6RP phosphorylation. In the RADMARD group it was verified an augmentation in mTOR phosphorylation and HIF-1α expression with rotenone treatment. Moreover, the levels of

intracellular lipids decreased drastically in all cells treated with the three inhibitors when compared to the stimulated condition of the RATNF group.

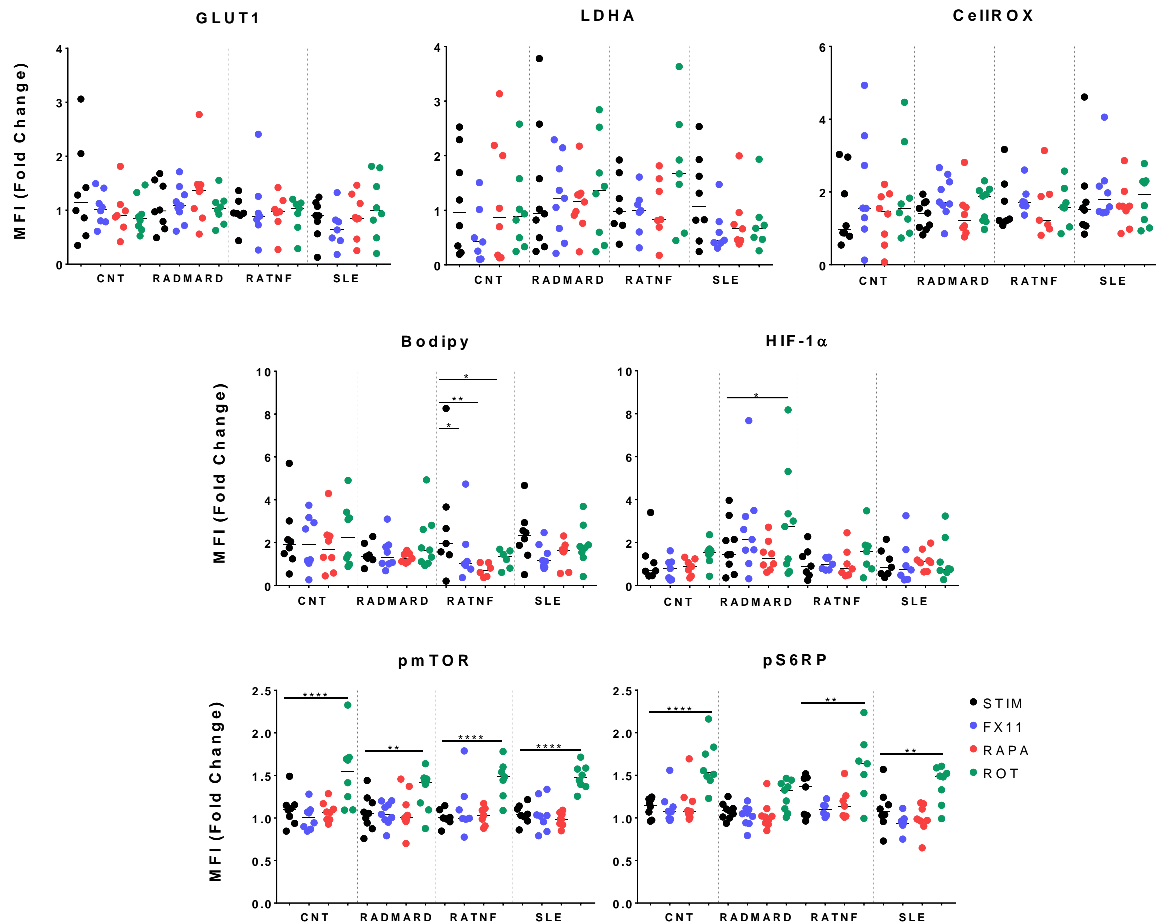


**Figure 9 - Rotenone can impact the phosphorylation levels of mTOR and its downstream effector in RA and SLE activated naïve B cells.** Levels of enzyme expression, intracellular lipids and ROS were assessed by Flow cytometry and the results are expressed as the Fold Change of the MFI relatively to the NStim condition of each group. Each dot represents an individual donor (n=6-9) and the median bar of the data is also presented. One-way ANOVA and Uncorrected Fisher's LSD (α=0.05) were used to compare Stim, FX11, RAPA and ROT conditions for each group. *p<0.05; **p<0.01; ****p<0.0001.

**Figure 10** presents results obtained for the switched-memory subset (IgD$^-$CD38$^-$), where NStim and Stim conditions were compared. Interestingly, the main differences were found between the control and autoimmune groups. The levels of LDHA under non-stimulating conditions were found to be higher in the CNT group when compared to RA groups. The levels of GLUT1 upon stimulation were found to be higher in the CNT group when

compared to the RADMARD group. The measurements for ROS levels were also found to be higher for the CNT group in non-stimulated conditions when compared to the SLE group, while upon activation the SLE group had significantly higher levels of mTOR phosphorylation.

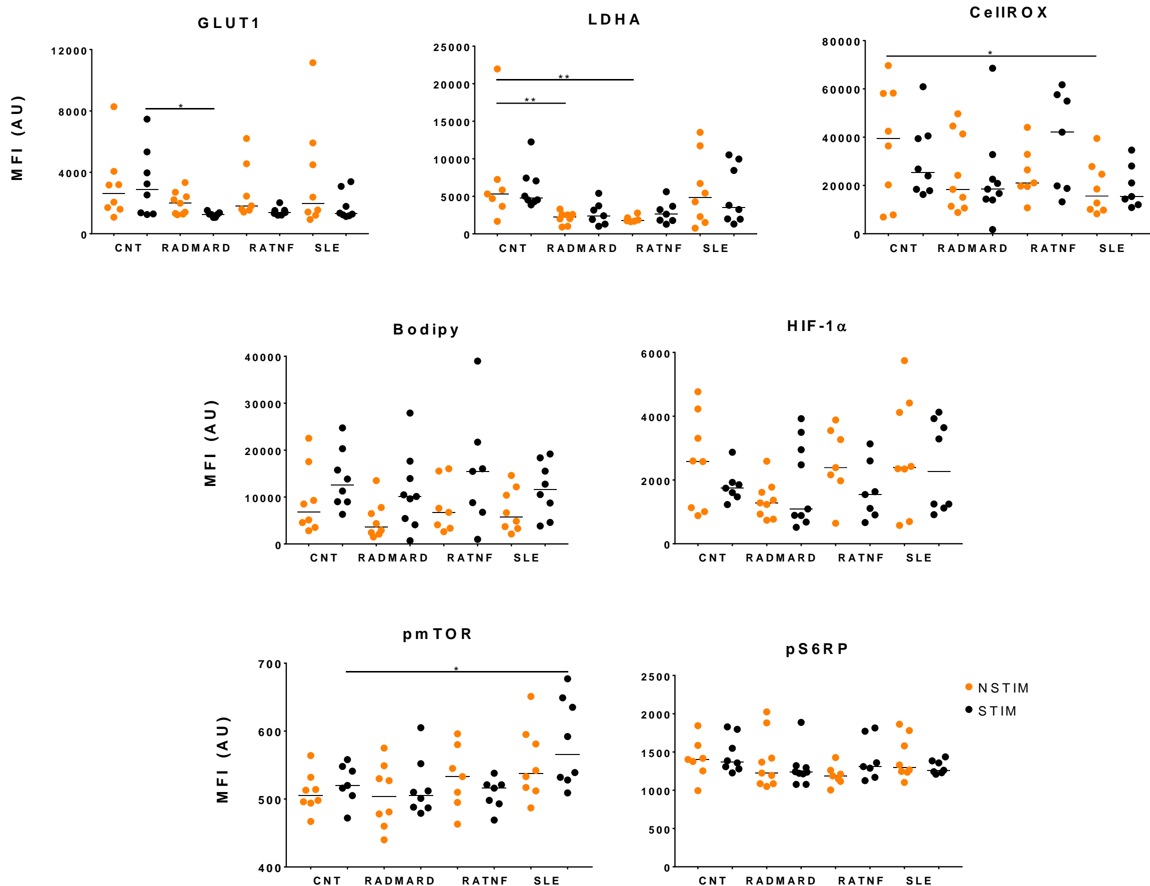


**Figure 10 - Switched-memory B cells in autoimmunity exhibit a similar profile under non-stimulated and stimulated conditions.** Levels of intracellular lipids, ROS and enzyme expression were assessed by Flow cytometry and the results are expressed as the MFI.Each dot represents an individual donor (n=6-9) and the median bar of the data is also presented. One-way ANOVA and Uncorrected Fisher's LSD (α=0.05) were used to compare NStim and Stim conditions for each group and NStim and Stim conditions from the CNT group with the respective correspondents in RA and SLE groups. *p<0.05; **p<0.01.

The treatment with the FX11 or rotenone promoted a downregulation of GLUT1 expression in the CNT group, however, no effect was verified in the autoimmune counterparts. Like what was observed for naïve B cells from the CNT group, rotenone-mediated inhibition promoted increase mTOR and S6RP phosphorylation. In the RADMARD the levels of

intracellular lipids decreased with FX11-mediated inhibition, while rotenone promoted augmented phosphorylation of mTOR. Similarly, in the RATNF group the treatment with rotenone produced the most pronounced effects where the levels of HIF-1α expression, and mTOR phosphorylation were significantly increased relatively to the stimulated condition. Moreover, the levels of intracellular neutral lipids decreased with rapamycin treatment. In the SLE group it was also verified an increase in the levels of mTOR phosphorylation upon rotenone-mediated inhibition **(Figure 11)**.

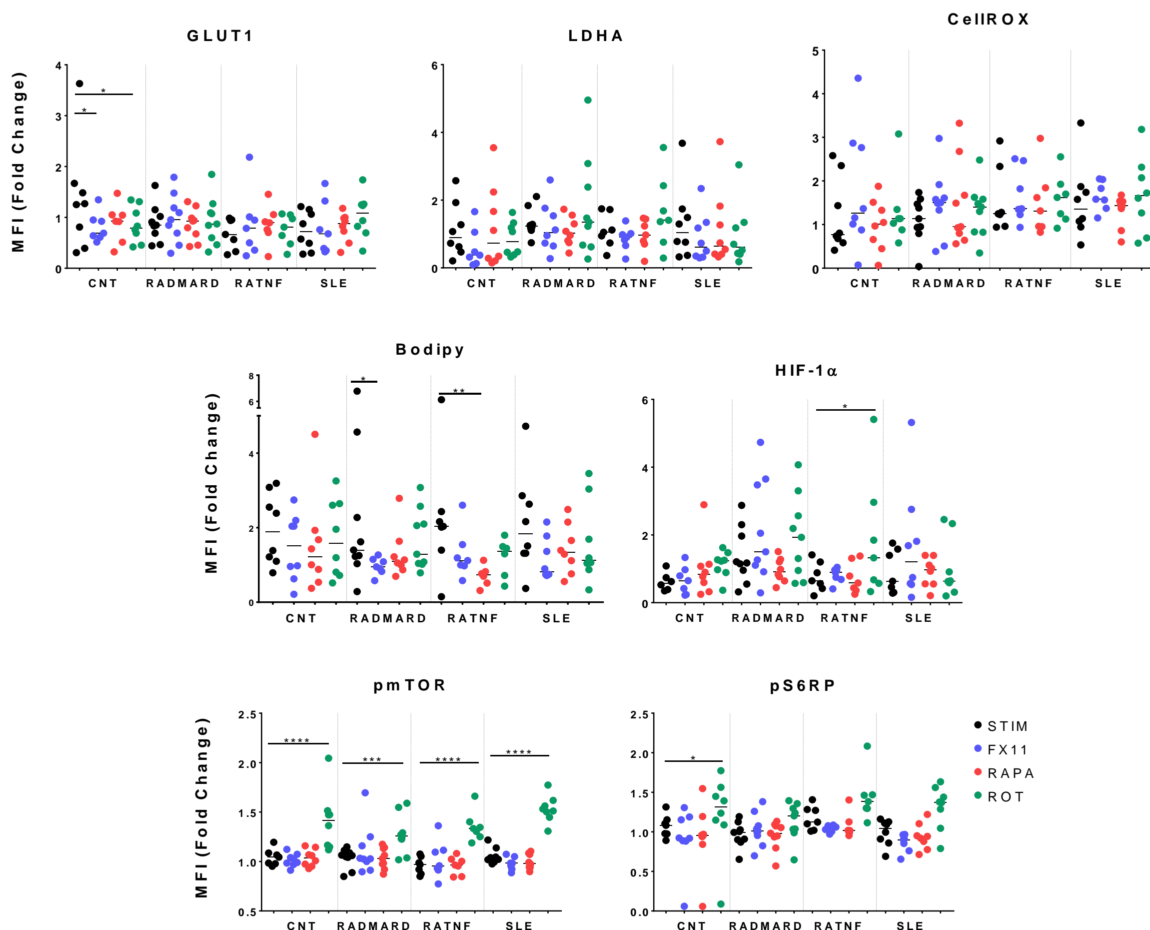


**Figure 11 - Switched-memory B cells treated with rotenone increase the levels of mTOR phosphorylation.** Levels of enzyme expression, intracellular lipids and ROS were assessed by Flow cytometry and the results are expressed as the Fold Change of the MFI relatively to the NStim condition of each group. Each dot represents an individual donor (n=6-9) and the median bar of the data is also presented. One-way ANOVA and Uncorrected Fisher's LSD (α=0.05) were used to compare Stim, FX11, RAPA and ROT conditions for each group. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

In **Figure 12** we compare the expression of the analyzed metabolic enzymes and the levels of ROS and intracellular lipids upon stimulation between naïve and switched-memory B cells of each group. Overall, the expression pattern is similar between the two subsets.

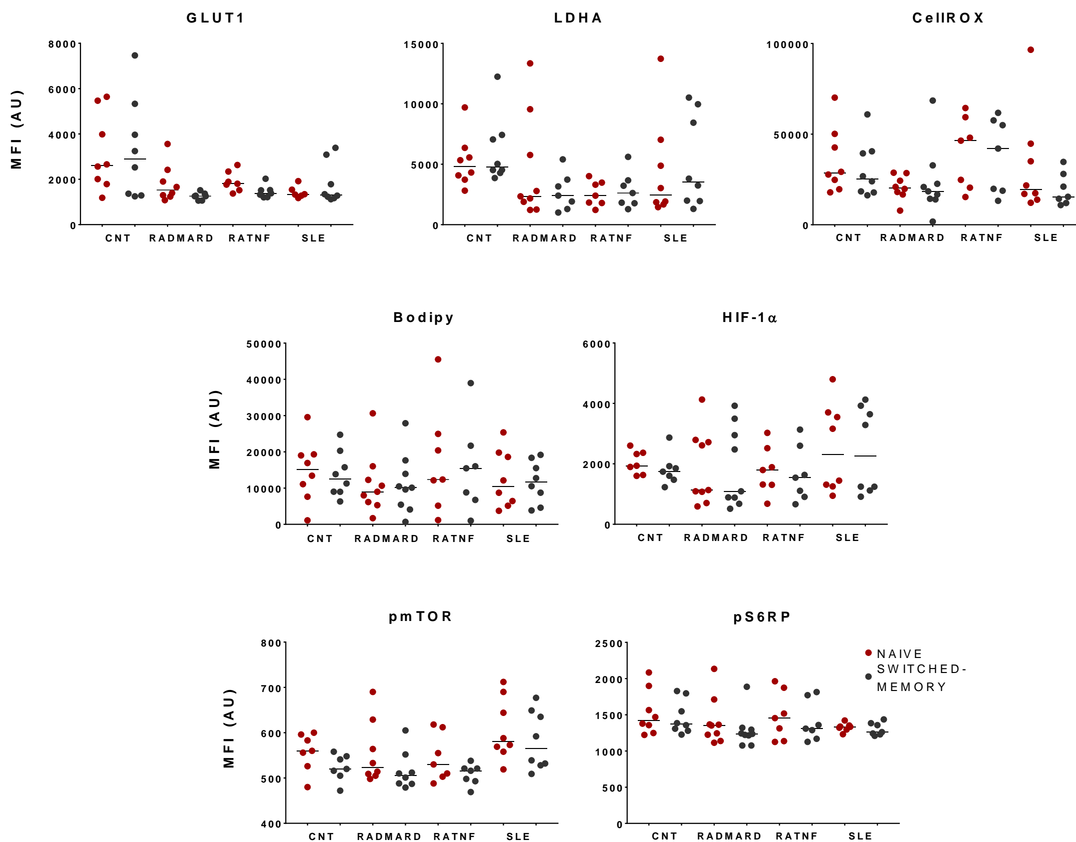


**Figure 12 - Levels of enzyme expression, intracellular lipids and ROS are similar when comparing stimulated naïve and switched-memory B cells.** Each dot represents an individual donor (n=6-9) and the median bar of the data is also presented. One-way ANOVA and Uncorrected Fisher's LSD (α=0.05) were used to compare the two subsets for each group. No significant differences were found.

The levels for enzyme expression, intracellular lipids and ROS of non-stimulated and stimulated memory B cells ($CD27^+$) isolated from the peripheral blood are represented in **Figure 13**. The CNT group was also observed to have higher mTOR phosphorylation levels under non-stimulated conditions when compared to the RATNF group, however upon stimulation the amount of mTOR phosphorylation was found to be higher in the SLE group. Moreover, the downstream target of mTORC1 was also observed to have higher

phosphorylation levels in the CNT group when compared to RATNF group in stimulated conditions. No significant changes were found for both RA groups. The most accentuated change observed for the SLE group upon stimulation was the augmentation of mTOR phosphorylation.

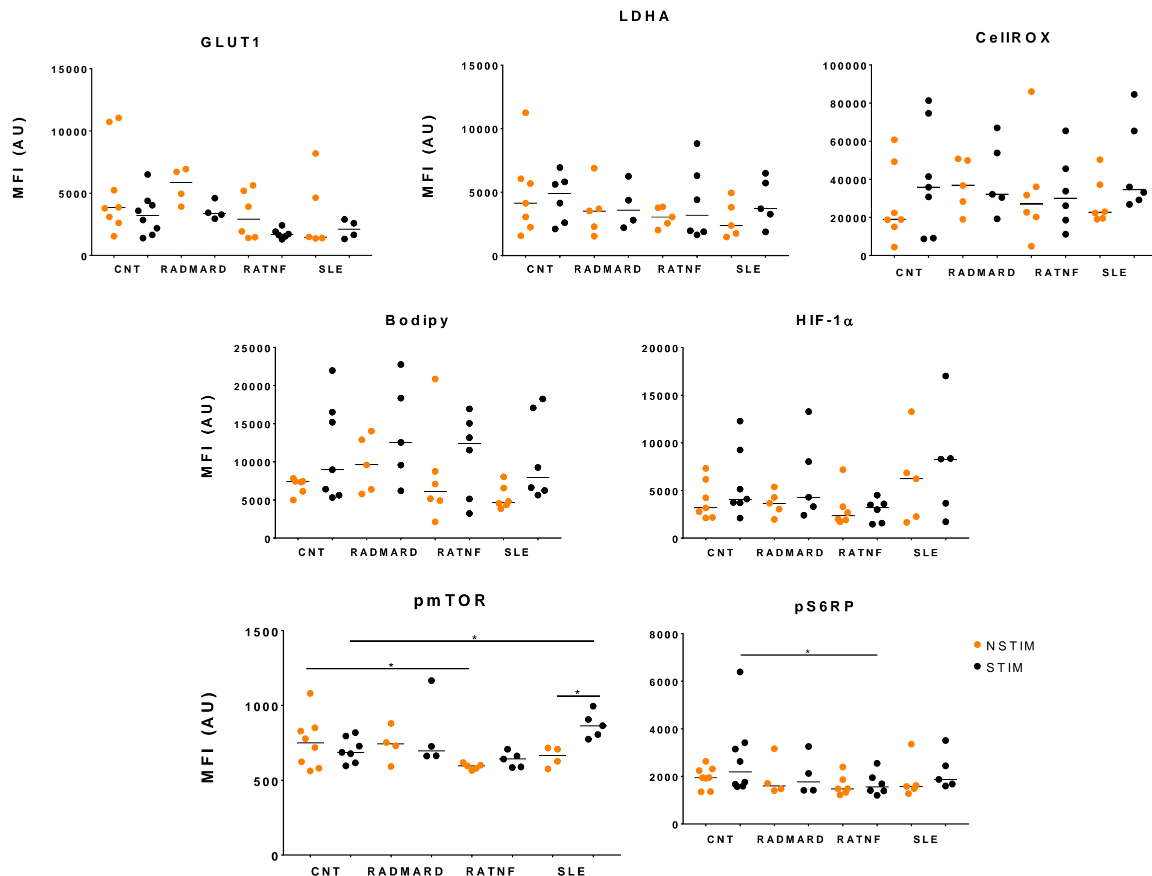


**Figure 13 - CD27⁺ Memory B cells in autoimmunity don't exhibit marked changes upon stimulation.** Levels of intracellular lipids, ROS and enzyme expression were assessed by Flow cytometry and the results are expressed as the MFI. Each dot represents an individual donor (n=4-8) and the median bar of the data is also presented. One-way ANOVA and Uncorrected Fisher's LSD (α=0.05) were used to compare NStim and Stim conditions for each group and NStim and Stim conditions from the CNT group with the respective correspondents in RA and SLE groups. *p<0.05.

Next, we evaluated the impact of FX11, rapamycin and rotenone in enzymatic expression, intracellular lipids and ROS levels in memory B cells **(Figure 14)**. Inhibition with rotenone resulted in increased phosphorylation of mTOR in the CNT group, while rapamycin promoted a decrease in the phosphorylation of mTOR target S6RP. The main change observed

for the RADMARD group was a decrease in the levels of LDHA when either one of the inhibitors was used. In the RATNF group the levels of mTOR phosphorylation were also shown to be upregulated upon inhibition with rotenone. Treatment with rotenone promoted an upregulation of GLUT1 and mTOR phosphorylation levels in the SLE group.

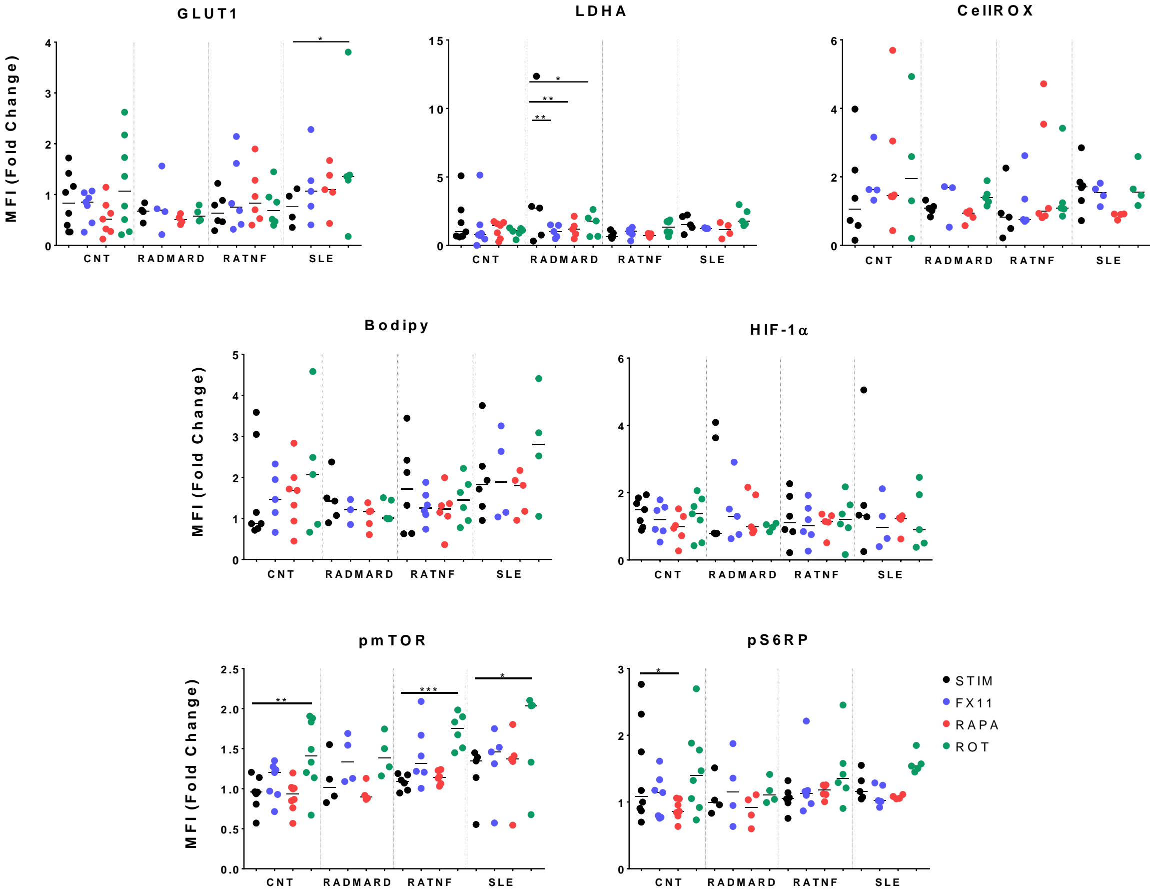


**Figure 14 - Treatment with rotenone promotes changes in the profile of memory B cells from RA and SLE patients.** Levels of enzyme expression, intracellular lipids and ROS were assessed by Flow cytometry and the results are expressed as the Fold Change of the MFI relatively to the NStim condition of each group. Each dot represents an individual donor (n=4-6) and the median bar of the data is also presented. One-way ANOVA and Uncorrected Fisher's LSD (α=0.05) were used to compare Stim, FX11, RAPA and ROT conditions for each group. *p<0.05; **p<0.01; ***p<0.001.

We also compared these metabolic and enzymatic parameters between naïve B cells ($CD19^{+}IgD^{+}CD38^{-}$) and memory B cells ($CD27^{+}$) under stimulating conditions **(Figure 15)**. The results obtained show that for the CNT group the levels of HIF-1α expression, mTOR and S6RP phosphorylation were increased in memory B cells. In the RADMARD group, memory B cells were also shown to upregulate the expression of GLUT1 and HIF-1α and the mTOR phosphorylation when compared to their naïve counterparts. No changes were observed in the RATNF group between memory cells and naïve B cells. The levels of HIF-1α expression, and mTOR phosphorylation were shown to be higher in memory B cells when compared to naïve B cells of SLE patients.

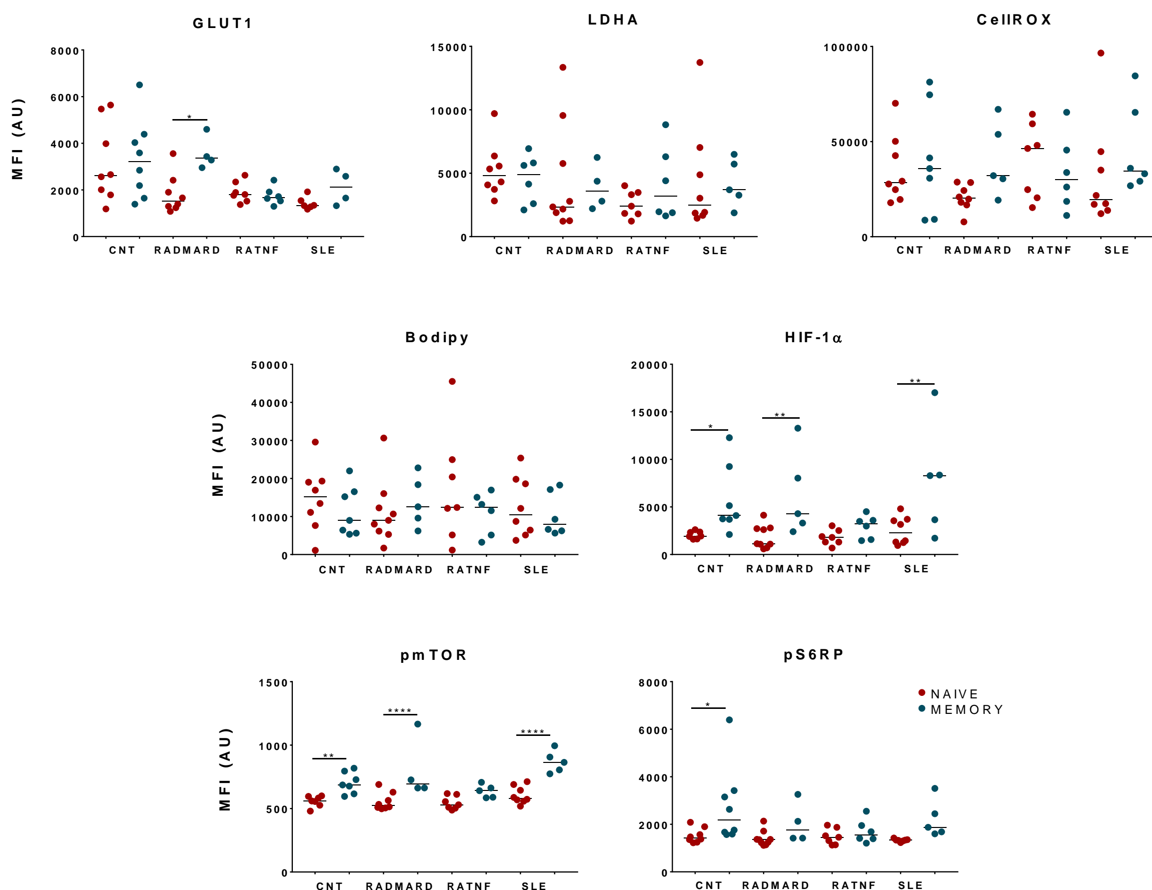


**Figure 15 - Stimulated naïve ($CD19^{+}IgD^{+}CD38^{-}$) and memory ($CD27^{+}$) B cells exhibit profound differences in healthy controls and autoimmune situations.** Each dot represents an individual donor (n=5-9) and the median bar of the data is also presented. One-way ANOVA and Uncorrected Fisher's LSD (α=0.05) were used to compare the two subsets for each group. *p<0.05; **p<0.01; ****p<0.0001.

The expression of key metabolic enzymes and parameters analyzed through flow cytometry was also assessed for the total naïve B cells ($CD19^{+}$) and the results are presented in the Supplementary Data section **(Figure S1 and S2)**. The results obtained showed that the expression of LDHA in the CNT group under non-stimulated conditions was found to be significantly higher than in RAs or SLE groups. In the RADMARD and RATNF groups there were no differences between non-stimulated and stimulated conditions. For the SLE group upon stimulation there was a downregulation of GLUT1. When metabolic inhibition was tested in the CNT group the levels of mTOR and S6RP phosphorylation were significantly increased when rotenone was used. Treatment with FX11 promoted an increase in HIF-1α expression in RADMARD group whereas rotenone-mediated inhibition promoted the upregulation of mTOR phosphorylation. In the RATNF group, treatment with rotenone also promoted upregulation of mTOR and S6RP phosphorylation. Likewise, in SLE rotenone-mediated inhibition also promoted increased mTOR and S6RP phosphorylation.

### 3.3.2 Metabolic profiling of naïve and memory B cells in autoimmunity

The next step was to characterize the metabolic profile of naïve and memory B cells. Our aim was to understand if cellular metabolism was altered in autoimmunity and if its modulation was possible. Therefore, we quantified the amount of [1,6-$^{13}C_2$]glucose consumed and the amount of [3-$^{13}$C]lactate produced per condition. In **Figure 16** are represented the obtained results for naïve B cells (CD27$^-$) isolated from the peripheral blood of each subject. The amount of consumed glucose upon stimulation was observed to be higher in the SLE group when compared to the CNT group. Overall, the effect of the inhibitors did not promote marked differences in the consumption of glucose. Stimulation of naïve B cells was shown to have no impact in the production of lactate in CNT and RA groups. Interestingly, the levels of lactate production were found to be downregulated in the SLE group and significantly different from the values found for the CNT group under non-stimulated or stimulated conditions. Similarly to what was observed regarding glucose consumption, the levels of lactate production were not affected by the treatments with the three metabolic inhibitors.

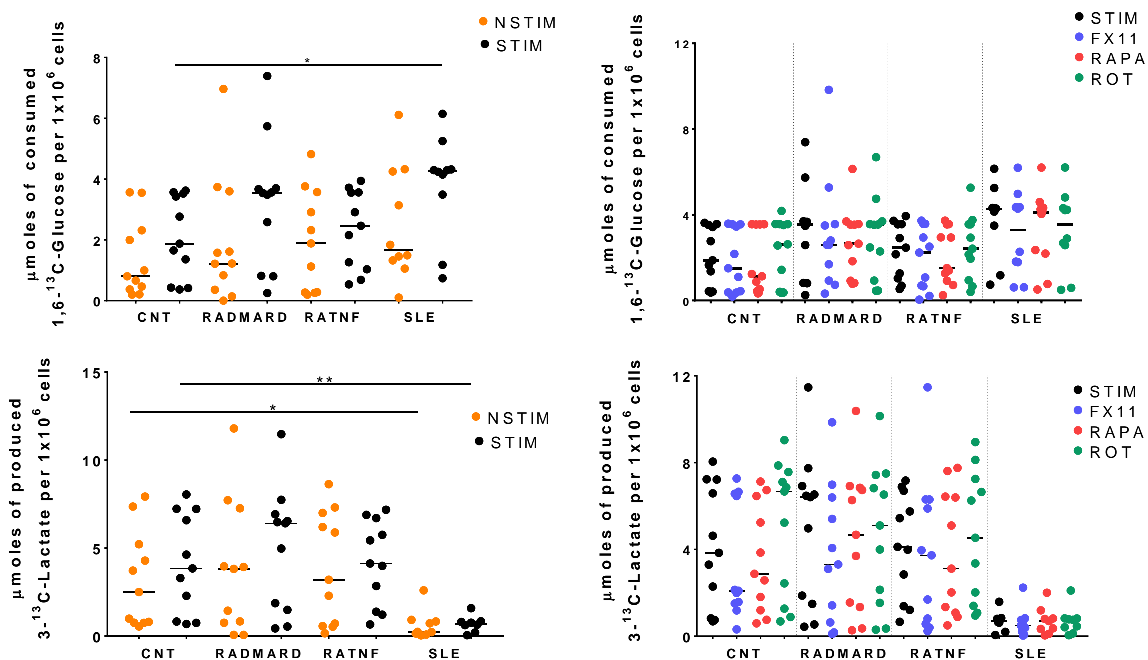


**Figure 16 - The amount of consumed glucose is augmented in SLE activated total naïve B cells.** The quantification of consumed [1,6-$^{13}C_2$]glucose and [3-$^{13}$C]lactate was assessed using $^1$H NMR and the results are expressed per million of cells. Each dot represents an individual donor (n=8-11) and the median bar of the data is also presented. One-way ANOVA and Uncorrected Fisher's LSD (α=0.05) were used to compare NStim and Stim or Stim, FX11, RAPA and ROT conditions for each group. NStim and Stim conditions from the CNT group were also compared with the respective correspondents in RA and SLE groups. *p<0.05; **p<0.01.

The results obtained for memory B cells show that these cells increased the rates of glucose consumption upon stimulation in the three autoimmune groups. Similarly to what was observed in total naïve B cells, metabolic inhibition did not impact in the amount of consumed glucose. The rates of produced lactate were showed to augment in the CNT group upon stimulation, however no changes were verified in the autoimmune groups. Interestingly, metabolic inhibition had the highest effect in the CNT group where the three inhibitors successfully decreased the amount of produced lactate. No marked effects were observed in the RATNF, RADMARD or SLE groups **(Figure 17)**.

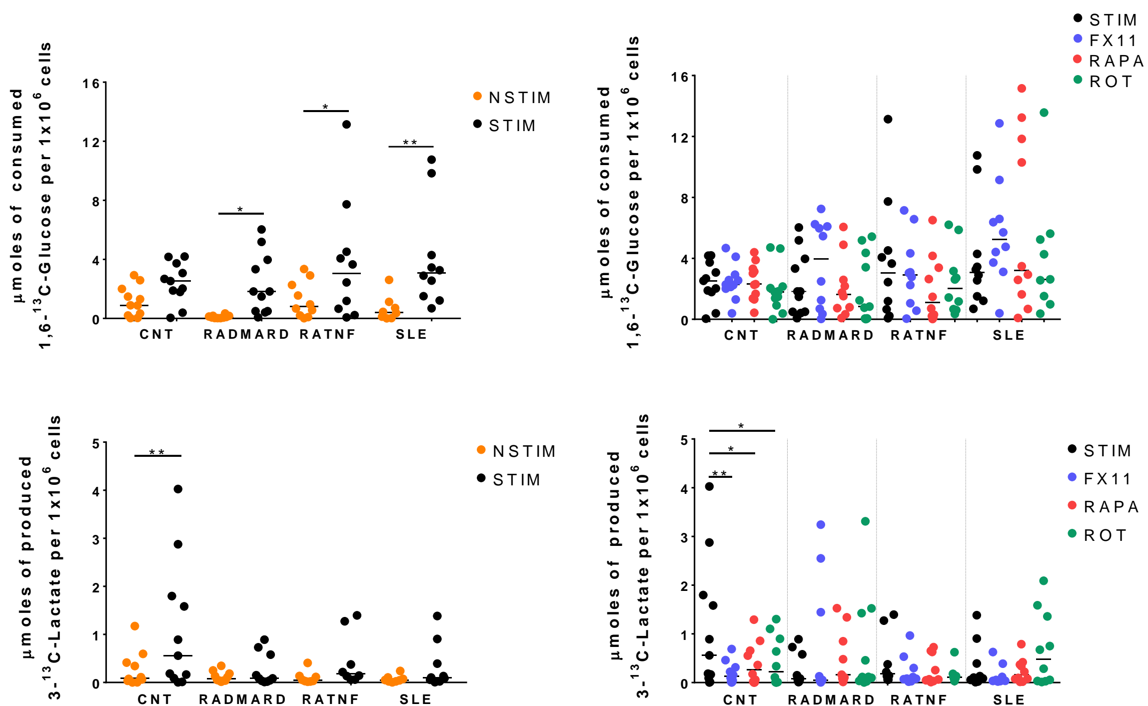


**Figure 17 - The levels of consumed glucose augment upon stimulation of autoimmune memory B cells.** The quantification of consumed [1,6-$^{13}C_2$]glucose and [3-$^{13}$C]lactate was assessed using $^1$H NMR and the results are expressed per million of cells. Each dot represents an individual donor (n=8-11) and the median bar of the data is also presented. One-way ANOVA and Uncorrected Dunn's test (α=0.05) were used to compare glucose measurements and Kruskal-Wallis test (α=0.05) were used to compare lactate measurements between NStim and Stim or Stim, FX11, RAPA and ROT conditions for each group. NStim and Stim conditions from the CNT group were also compared with the respective correspondents in RA and SLE groups *p<0.05; **p<0.01.

We also compared the amount of glucose consumed and lactate produced under stimulated conditions between naïve and memory B cells **(Figure 18)**. No changes were found between total naïve and memory B cells regarding glucose consumption in all groups. In terms of lactate production, there was a drastic decrease of these values in memory B cells from both RA groups and in the CNT group. Interestingly, no differences were observed for lactate production in the SLE group in naïve B cells between naïve and memory B cells.

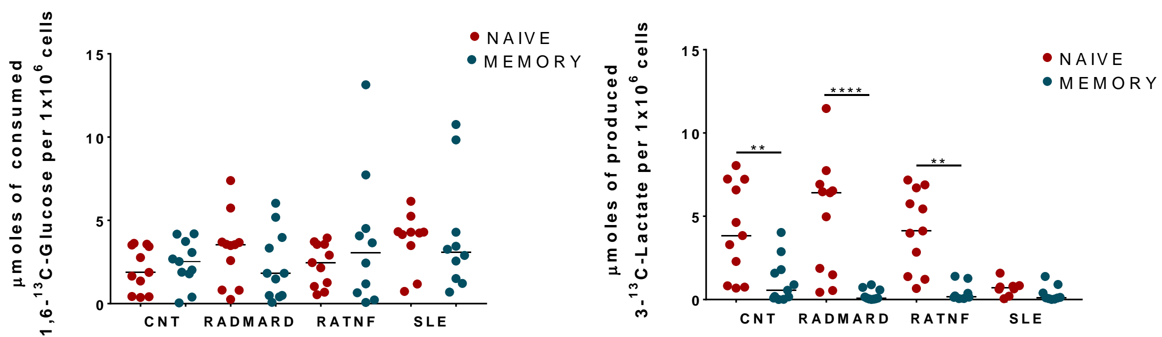


**Figure 18 -** Naïve B cells exhibit higher levels of glucose consumption and lactate production when compared to their memory counterparts. Each dot represents an individual donor (n=8-11) and the median bar of the data is also presented. One-way ANOVA (α=0.05) were used to compare glucose consumption between naïve and memory subsets, while Kruskal-Wallis test and Uncorrected Dunn's test (α=0.05) were used to compare the lactate production between these two subsets. **p<0.01; ****p<0.0001

### 3.3.3 Metabolic modulation of effector functions in activated naïve and memory B cells in autoimmunity

Since one of the main roles of B cells in RA and SLE is the production of autoantibodies and cytokines that will actively contribute to the development and progression of the disease, we also decided to characterize the profile of secreted immunoglobulins and cytokines in autoimmunity. In both RA and SLE the main antibodies classes secreted are IgG and IgM, while the main proinflammatory cytokines produced are IL-6, IL-10 and TNF-α (Bugatti et al., 2014; Nashi et al., 2010). Therefore, we aimed to test a panel of secreted immunoglobulins (Igs) - IgG1, IgG2, IgG3, IgG4, IgM and IgA – and a panel of secreted cytokines – IL-2, IL-4, IL-6, IL-10, TNF-α and TGF-β.

In **Figure 19** are represented the results obtained for immunoglobulin isotyping in naïve B cells ($CD27^-$). The CNT group was found to secret widely every immunoglobulin analyzed and in some cases in higher amounts than the autoimmune groups for the stimulated condition, however the inhibitors did not have a marked effect when used. The CNT group had higher IgG1 and IgG4 levels when compared to the SLE group, the secretion of IgG2 was also higher when compared to the RADMARD value, while the levels of IgG3 were observed to be lower than the values for the RATNF group. IgG1, IgG3 and IgG4 were observed to be the IgG subclasses more secreted by the RADMARD group. In the RATNF group all the four subclasses of IgG were secreted by naïve B cells. Metabolic inhibition did not seem to affect immunoglobulin production in both RA groups. In the SLE group, IgG3 was found to be the predominant secreted IgG subclass followed by IgG4 and IgG2. Treatment with FX11 and rotenone promoted a downregulation of IgG3 secretion in the SLE group. Finally, IgM and IgA were found to be secreted in the SLE group and inhibition with rotenone or FX11 promoted a reduction in IgM levels.

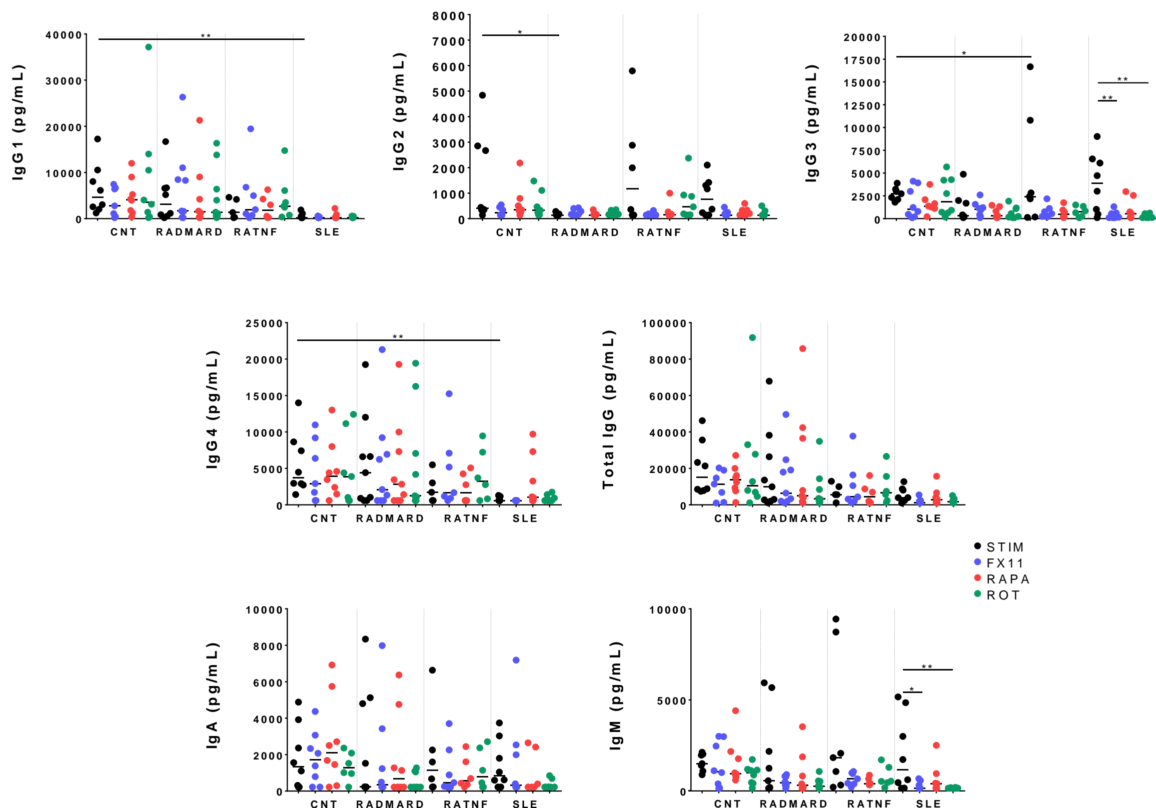


**Figure 19 - Metabolic inhibition can impact the secretion of immunoglobulins in SLE total naïve B cells.** Levels of Igs secretion were assessed by Flow cytometry and the results are expressed in pg/mL. Each dot represents an individual donor (n=5-9) and the median bar of the data is also presented. Kruskal-Wallis test (α=0.05) were used to compare Stim, FX11, RAPA and ROT conditions for each group; Stim conditions from CNT group with RAs and SLE group. *p<0.05; **p<0.01.

Next, we measured the amount of cytokines produced by stimulated and metabolic inhibited total naïve B cells **(Figure 20)**. IL-4 secretion by the CNT group upon stimulation was significantly higher when compared to the RADMARD and RATNF group. The treatment with either one of the three inhibitors promoted increased secretion in levels of TNF-α in the CNT group. IL-4 was the cytokine secreted in higher quantity by the RADMARD group, however along with IL-10 these values were significantly lower than in the CNT group. Inhibition with FX11 leaded to increased TNF-α and TGF-β secretion in the RADMARD total naïve B cells. Moreover, in this group IL-10 secretion increased upon rapamycin mediated inhibition. In the RATNF group the cytokines found to be more secreted were IL-6 and IL-2. Similarly to what was observed in the RADMARD group, the levels of secreted TNF-α augmented upon treatment with FX11 and the levels of TGF-β1 also suffered a significant increase with FX11 or rotenone treatment. Finally, the SLE group was found to secret predominantly IL-4, IL-2 and IL-6. FX11-mediated inhibition leaded to increased secretion levels of TNF-α and TGF-β1. IL-10 secretion was inhibited when the cells were treated with either FX11 or rotenone.

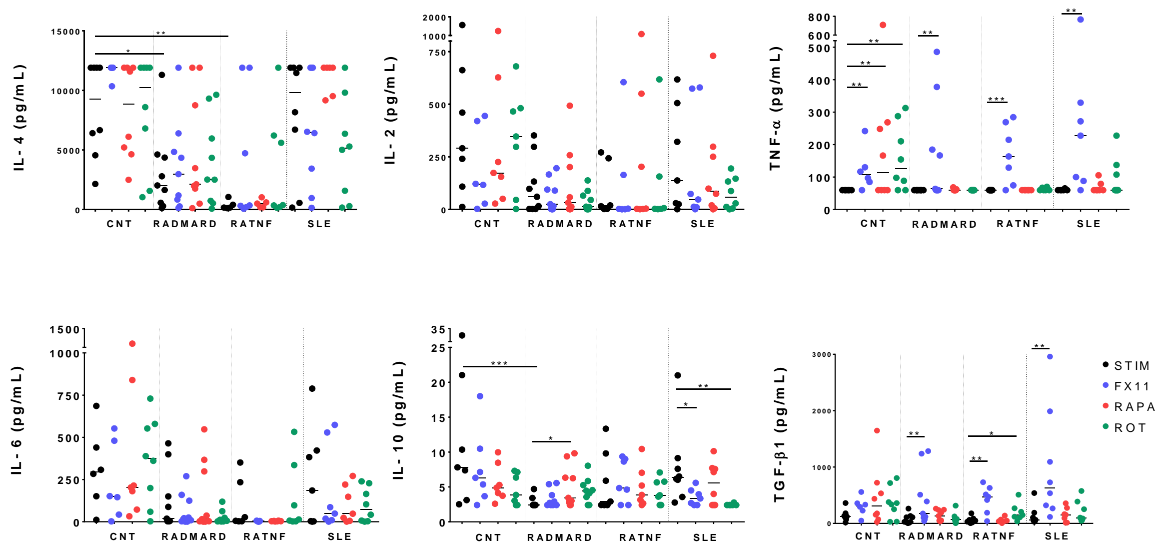


**Figure 20 - Metabolic inhibition can impact the cytokine secretion in autoimmune total naïve B cells.** Levels of cytokine secretion were assessed by flow cytometry and the results are expressed in pg/mL. Each dot represents an individual donor (n=5-9) and the median bar of the data is also presented. Kruskal-Wallis test and Uncorrected Dunn's test (α=0.05) were used to compare Stim, FX11, RAPA and ROT conditions for each group; Stim conditions from CNT group with RAs and SLE groups. *p<0.05; **p<0.01, ***p<0.001.

In **Figures 21 and 22** are represented the quantifications of secreted Igs and cytokines by memory B cells ($CD27^{+}$). The levels of IgG2 and IgG3 are not represented in **Figure 21** because these two subclasses were found to be secreted in very low quantities and in the same range of quantity for each condition of each group. Overall, the stimulated condition from the CNT group was observed to have the highest amount of secreted Igs. Metabolic inhibition had only effect in the Ig secretion levels of the CNT group. Indeed, inhibition with rapamycin decreased significantly the levels of IgG1, while the three treatments were found to be effective in reducing the levels IgG4. Moreover, the total IgG levels were significantly reduced with FX11 and rotenone. The levels of IgA secretion were also successfully reduced with FX11 and rotenone, while in the IgM group only FX11 was able to promote this reduction. In the RADMARD group IgG1 was the most secreted, while the values for the RATNF and SLE groups were found to be very close to the detection limit and with limited variance.

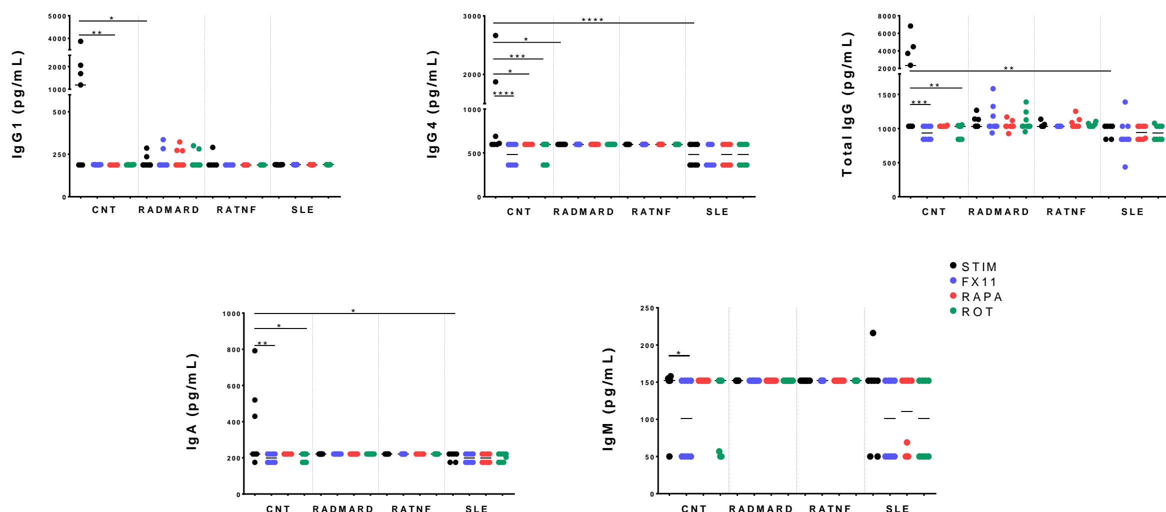


**Figure 21 - Immunoglobulin production is higher in stimulated memory B cells from the control group.** Levels of Igs secretion were assessed by Flow cytometry and the results are expressed in pg/mL. Each dot represents an individual donor (n=5-8) and the median bar of the data is also presented. Kruskal-Wallis test and Uncorrected Dunn's test (α=0.05) were used to compare Stim, FX11, RAPA and ROT conditions for each group; Stim conditions from CNT group with RAs and SLE group. *p<0.05; **p<0.01.

Similarly to what happened in immunoglobulin quantification, the levels of TNF-α and IL-10 are not represented in **Figure 22** because no variance was found between each value in each condition and group since these values were on the lower detection limit. Overall, inhibited conditions in the CNT, RADMARD, RATNF and SLE groups did not seem to have any effect on cytokine secretion. The only detected alteration was regarding TGF-β1 secretion, where treatment with FX11 or rotenone promoted increased secretion levels in the RADMARD group, while a reduction in TGF-β1 levels was observed in the RATNF group for rapamycin condition. Finally, the levels of IL-6 in the CNT group were found to be significantly higher when compared to the RADMARD levels, while the levels of TGF-β1 were observed to be higher in the RATNF group.

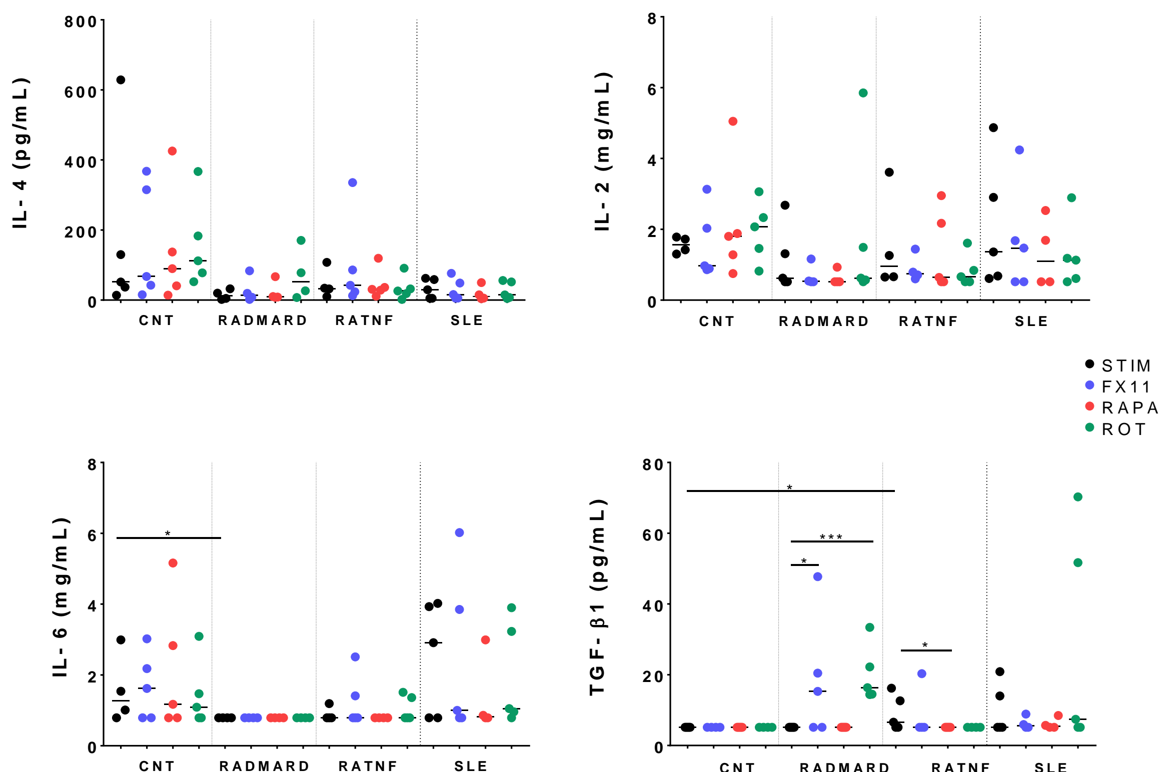


**Figure 22 - Immunoglobulin production is higher in stimulated memory B cells from the control group.** Levels of Igs secretion were assessed by Flow cytometry and the results are expressed in pg/mL. Each dot represents an individual donor (n=4-6) and the median bar of the data is also presented. Kruskal-Wallis test and Uncorrected Dunn's test (α=0.05) were used to compare Stim, FX11, RAPA and ROT conditions for each group; Stim conditions from CNT group with RAs and SLE group. *p<0.1; **p<0.01.

We also compared the levels of Ig and cytokine secretion between naïve and memory stimulated B cells (**Figures 23 and 24**). Overall, naïve B cells had significant higher secretion levels of both Igs and cytokines. The data obtained for TNF-α levels is not represented in **Figure 24** because there was no variance between the obtained values.

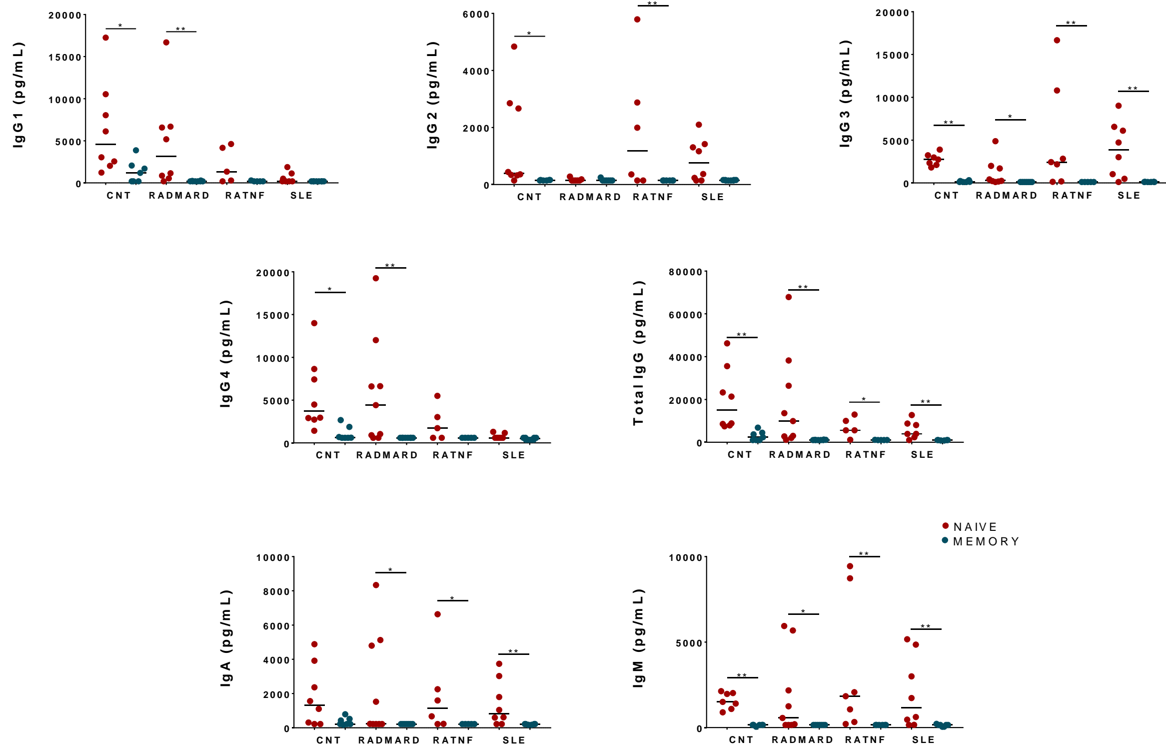


**Figure 23 -** Immunoglobulin secretion is higher in stimulated naïve B cells (CD27-). Each dot represents an individual donor (n=6-8) and the median bar of the data is also presented. Kruskal-Wallis test and Uncorrected Dunn's test (α=0.05) were used to compare naïve and memory B cells for each group. *p<0.1; **p<0.01.

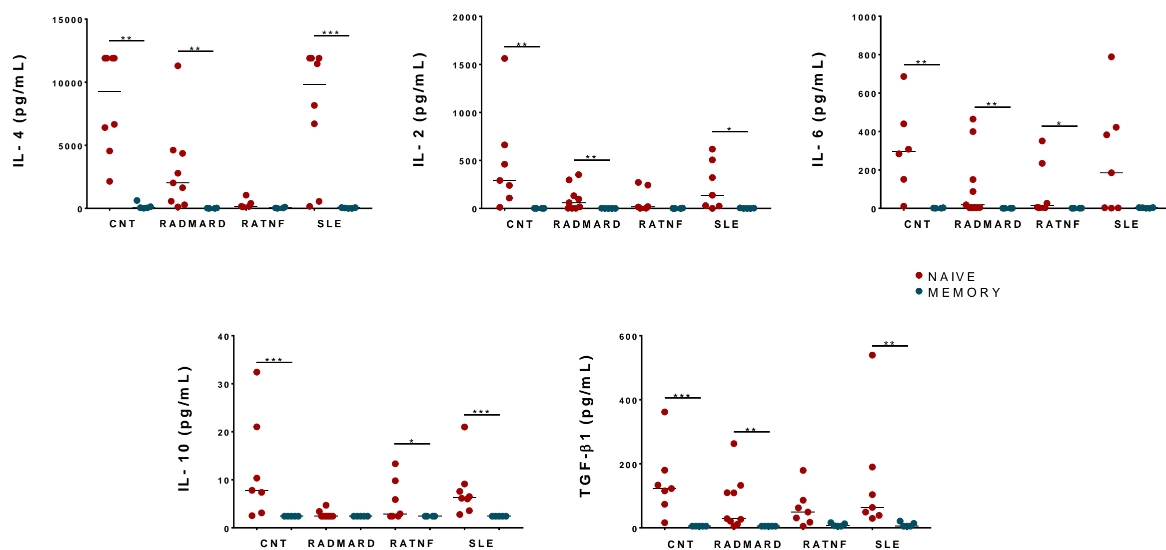


**Figure 24 -** Cytokine secretion is higher in stimulated naïve B cells (CD27-). Each dot represents an individual donor (n=4-8) and the median bar of the data is also presented. Kruskal-Wallis test and Uncorrected Dunn's test (α=0.05) was used to compare naïve and memory B cells for each group. *p<0.1; **p<0.01.

# 4. DISCUSSION

The preliminary results obtained by our group regarding the microarray analysis of metabolic genes in total B cells from RA and SLE patients showed some differences when compared to healthy individuals. These results suggested that B cells from both RA and SLE patients had highest expression of key glycolytic genes (*LDHA,PKM, HK2, MYC*), while *PDHA* (key OXPHOS gene) was found to be overexpressed only in SLE cells. Total B cells from SLE patients were also found to overexpress the genes of metabolic regulator mTOR. Indeed Torigoe and his colleagues also reported that phosphorylation of mTORC1 was upregulated in $CD19^+$ B cells from SLE patients (Torigoe et al., 2017). Regarding lipid metabolism we found that the fatty acid oxidation gene *CPT1A* was upregulated in RA and SLE B cells, while CNT B cells had higher expression of the lipid synthesis regulator *SREBF1*. However, this array was performed in peripheral total B cells and since these cells have multiple roles in promoting RA and SLE pathogenesis not all subsets might have a role and contribute equally for the development and maintenance of autoimmunity. Indeed, in RA it has been described that, besides producing autoantibodies, B cells can act as antigen-presenting cells thereby activating autoreactive T cells, while in SLE B lymphocytes act essentially as autoantibodies producers (Karrar and Graham, 2018; Silverman and Carson, 2003). Therefore, one could conclude that switched memory B cells and plasma cells might play a more predominant role in autoimmunity. Nevertheless, this preliminary analysis allowed us to conclude that there were pronounced differences between patients and healthy controls regarding metabolic gene expression.

Given that specific B cell subsets might have different roles or impact in the development and maintenance and that their frequencies are altered in RA and SLE, we characterized the gene expression of two antigen-specific subsets in these two diseases. The results showed that gene expression in RA and SLE mBs did not differ from the CNT group. The results for pBs showed the same pattern for the RA groups, however significant changes were found in the SLE group. In fact, SLE pBS exhibited higher expression of *LDHA*, *PKM2* and *HIF1A*, which indicated that glycolysis was upregulated in this subset. Moreover, the expression of mTOR inhibitor *TSC2* was increased in SLE pBs when compared to the control subset. This suggested that mTORC1 was less expressed in SLE pBS, which does relate with a previous study

where the proportion of plasmablasts in the peripheral blood of SLE patients was correlated with high levels of mTORC1 phosphorylation (Torigoe et al., 2017). In conclusion, while mBs from both RA and SLE patients and RA pBs exhibited a gene expression that was not altered when compared to the CNT group, results from SLE pBs suggested that glycolysis was upregulated and mTORC1 inhibited.

Since the results from both microarray analysis and gene expression profiling showed marked differences between healthy controls and autoimmune patients, we sought to characterize the metabolic requirements of B cells more in depth. Therefore, we assessed the expression of metabolic enzymes, characterized the intermediary metabolism and the effector functions of the two main B cells populations: total naïve ($CD27^-$) and memory ($CD27^+$). The obtained results provided new insights not only regarding the unexplored field of immunometabolism of B cells in autoimmunity, but also concerning the growing field of general B cell metabolism. First, we determined and compared the percentage of naïve B cells ($IgD^+CD38^-$), switched-memory B cells ($IgD^-CD38^-$) and plasma cells ($IgD^-CD38^+$) for stimulated and non-stimulated conditions in total naïve cells ($CD27^-$) for each group. The significantly increased percentage of naïve cells under non-stimulated conditions and significant augmentation of memory B cells under stimulated conditions revealed that the used stimuli was working, and the cells were responding properly. In contrast, the percentage of plasma cells was only verified to be altered between non-stimulated and stimulated conditions of the SLE group, which is very interesting given the predominant role of plasma cells in antibody production in SLE (Malkiel et al., 2018).

Contrarily to what has been described before, in our experiments naïve B cells from healthy controls did not upregulate GLUT1 or LHDA expression upon stimulation, in fact, the LDHA expression is downregulated upon stimulation. However, in the previous studies these measurements were always assessed six hours to two days after stimulation, a very short period when compared to our seven-day protocol which might explain why our results were different (Blair et al., 2012; Caro-Maldonado et al., 2014; Doughty et al., 2006; Dufort et al., 2007; Price et al., 2018). In our experiments, HIF-1α expression was also found to be downregulated upon CNT naïve B cell activation and since this transcription factor under hypoxic conditions upregulates glycolysis by enhancing the expression of glucose transports and LDHA, this destabilization could be the reason why GLUT1 and LDHA expression was not

being upregulated (Majmundar et al., 2010). In addition to that, even though this HIF-1α downregulation under hypoxia might seem like a paradox, it has been reported before that cancer cells destabilize HIF-1α when exposed to chronic hypoxic conditions (i.e. for more than 2 days) (Lin et al., 2011). Nonetheless, GLUT1 expression in stimulated conditions was higher in the CNT group when compared to the SLE group. In non-stimulated conditions the CNT group exhibited higher LDHA expression when compared to both RA and SLE groups. Moreover, upon stimulation, the expression levels of GLUT1 were reduced in SLE naïve B cells which suggested that glucose uptake was decreased. There are only a few reports regarding the expression of these enzymes in RA or SLE, however none of these studies was carried in B cells. The activity of LDH was found to be increased in the synovial tissue of RA patients when compared to healthy controls (Lindy et al., 1971). LDH was also found to be overexpressed in the serum of patients with lupus-derived renal severe pathology when compared to only mild affected patients, while memory effector $CD4^+$ T cells from SLE patients had higher GLUT1 expression levels when compared to healthy control cells (Inoue et al., 1986; Koga et al., 2019). Naïve B cells from the RATNF group augmented the levels of S6RP phosphorylation upon stimulation. Since S6RP is a downstream target of mTOR Complex 1 (a complex formed by mTOR, Raptor protein and mLST8 protein), this protein can be used as an activation marker of mTORC1 and mTOR pathway (Pallis et al., 2016). Therefore, this suggests that mTORC1 pathway was upregulated in the RATNF group. Indeed, Abboud and his colleagues reported that B cells from a murine RA model exhibited higher mTORC1 activation levels than B cells from control mice (Abboud et al., 2018). The naïve B cells from the RATNF group also increased the levels of intracellular lipids upon stimulation which indicated that lipid synthesis was upregulated. Indeed, previous studies reported that upon B cell activation lipid oxidation is inhibited and *de novo lipogenesis* is enhanced, however this was reported in healthy B cells (Caro-Maldonado et al., 2014; Cho et al., 2011; Dufort et al., 2007). When the naïve B cells from the RATNF group were treated with the FX11, rapamycin or rotenone the levels of intracellular lipids decreased. Rotenone inhibits the ETC and under hypoxic conditions can prevent HIF-1α stabilization in cancer cells (Agani et al., 2000; Giaccia et al., 2004). A functional mitochondrial ETC is required under hypoxic conditions to promote HIF-1α stabilization (Chua et al., 2010). In hypoxic conditions, HIF-1α is essential to glucose uptake and glycolysis upregulation (Ziello et al., 2007). FX11 inhibits LDHA and will consequently reduce the rates of glycolysis and glucose uptake, while rapamycin inhibits mTORC1. Moreover, mTORC1 is also

required for HIF-1α regulation under hypoxia (Heberle et al., 2015). Lipid synthesis is upregulated by HIF-1α under hypoxic conditions and B cells have been described to fuel lipid synthesis with glucose upon stimulation (Dufort et al., 2014). Therefore, these results suggest that HIF-1α might be a critical regulator of *de novo* lipogenesis which might be fueled by glucose in the RATNF naïve B cells. Inhibition with rotenone promoted an increase in the levels of mTOR and S6RP phosphorylation in the CNT, RATNF and SLE groups, while only increased mTOR phosphorylation was observed in RADMARD naïve B cells. Therefore, these results suggest that mTOR was activated and the pathway was functional. There is no information concerning the impact of rotenone on these proteins in B cells. On one hand, as previously mentioned, rotenone prevents HIF-1α stabilization and, on the other hand, HIF-1α expression requires a functional mTORC1 (Heberle et al., 2015). Thus, the possible HIF-1α destabilization would explain why upon inhibition with rotenone there was an activation of the mTORC1-pathway. It is important to notice that we did not observe any downregulation when we assessed HIF-1α expression in CNT, RATNF or SLE groups which could mean that mTORC1 was working in promoting its stabilization. However, the expression of HIF-1α augmented upon treatment with rotenone in the RADMARD group which could be a result of the observed increased phosphorylation of mTOR. Another hypothesis could also rely on rotenone mediated ETC inhibition. Since mTORC1 promotes a shift in cellular metabolism from oxidative phosphorylation to glycolysis, this could explain why mTORC1 pathway was activated upon treatment of the naïve B cells with rotenone (Saxton and Sabatini, 2017).

Overall, the results for switched-memory B cells showed no difference between non-stimulated or stimulated conditions for all the groups but rather showed a difference between the CNT and autoimmune groups. Indeed, the CNT group had higher LDHA expression levels compared to both RA groups for non-stimulated conditions. A reduction of ROS levels under non-stimulated conditions was verified when we compared the SLE post-switched memory subset to the CNT counterparts, while upon stimulation the levels of mTOR phosphorylation were observed to be increased in the SLE group. This could suggest a higher activity of mTORC1 in SLE switched-memory B cells when compared to healthy ones, an outcome that was reported in peripheral B cells from SLE patients (Torigoe et al., 2017). When the three inhibitors were tested in the switched-memory B cell CNT group, GLUT1 expression was decreased with FX11. Since FX11 inhibits LDHA consequently it will inhibit glycolysis and

glucose transporters, an outcome that has been previously reported in cancer cells (An et al., 2017; Le et al., 2010). Switched-memory B cells from the RATNF group that were treated with rapamycin reduced the levels of lipid droplets, the same effect was observed for switched-memory B cells from the RADMARD group incubated with FX11. This suggests that similarly to what was observed in the naïve counterparts, lipid synthesis was probably fueled with glucose and upregulated in a mTORC1-HIF-1α-dependent way. In the CNT group, inhibition with rotenone upregulated mTOR and S6RP phosphorylation while inhibited GLUT1 expression. Since HIF-1α upregulates the expression of GLUT1 under hypoxic conditions, this could suggest that rotenone was downregulating GLUT1 through HIF-1α destabilization (Sadlecki et al., 2014). The levels HIF-1α expression were not altered which could indicate a mTORC1-mediated stabilization, however, this did not seem to be enough to revert the inhibition effect caused by rotenone on GLUT1 expression. Inhibition with rotenone promoted mTOR increased phosphorylation, while no effect was observed on its target, S6RP, in switched-memory B cells from the RAs and SLE group. Moreover, the treatment with rotenone increased the expression of HIF-1α in the RADMARD group which could be a result of the increased mTOR phosphorylation. Interestingly, no differences were observed for enzyme expression, ROS or intracellular lipid levels when naïve and switched-memory B cells from all the groups were compared.

The results for memory B cells (CD27$^{+}$) showed no differences between stimulated and non-stimulated conditions for the CNT and both RA groups. The main change verified in these groups was the downregulation of mTOR phosphorylation in the RATNF group when compared to the CNT group under non-stimulated conditions, while upon stimulation this downregulation in the RATNF group was verified for the phosphorylation of S6RP. SLE memory B cells upon stimulation augmented the levels of mTOR phosphorylation and these levels were significantly higher when compared to the CNT group. Therefore, this suggests a higher activity of mTORC1 in the SLE subset which was also verified in the switched-memory group and in the peripheral B cells from SLE patients (Torigoe et al., 2017). Moreover, rotenone-mediated inhibition promoted again increased levels of mTOR phosphorylation in the CNT, RATNF and SLE groups. Moreover, in the SLE group the treatment with rotenone also promoted an increased upregulation of GLUT1 which could suggest a successful HIF1α stabilization or a shift to glycolytic metabolism. The levels of S6RP phosphorylation decreased

with rapamycin treatment in the CNT group, which was expected since this compound inhibits mTORC1 (Saxton and Sabatini, 2017). Moreover, treatment with either one of the inhibitors caused a reduction of LDHA expression levels in the RADMARD group which was expected since glycolysis is regulated in a mTORC1/HIF-1α-dependent way under hypoxic conditions. We could explain the observed LDHA downregulation with rotenone if we assume that this molecule prevents HIF-1α stabilization and since this treatment did not promote mTOR phosphorylation in the RADMARD group. Furthermore, when memory B cells and naïve stimulated subsets were compared some profound differences were found. The levels of HIF-1α expression, mTOR and S6RP phosphorylation were augmented significantly in memory B cells from the CNT group, while no significant changes were observed for the SLE subset. In the memory B cells of RADMARD group the expression of GLUT1, HIF-1α and mTOR phosphorylation were found to be upregulated when compared to the naïve subset, while only HIF-1α was found to be upregulated in memory B cells from the RATNF group. Nevertheless, this could suggest that mTOR could have a predominant role in coordinating cellular events upon stimulation in memory B cells in the CNT and RADMARD groups.

The intermediary metabolism monitoring of total naïve B cell ($CD27^-$) subset reflects the metabolic rates of naïve B cells, switched-memory B cells and plasma cells. Results showed that upon stimulation CNT total naïve B cells did not change glucose consumption or lactate production rates, however, it was also showed that 1 mole of glucose yielded about 2 moles of lactate. This ratio was also verified for non-stimulated B cells and this indicated that there was a total conversion of glucose into lactate (Semenza, 2008). However, these results did not correlate with previous reports where glucose uptake and the rates of glycolysis and OXPHOS were increased upon stimulation of healthy B cells (Blair et al., 2012; Caro-Maldonado et al., 2014; Doughty et al., 2006; Dufort et al., 2007; Price et al., 2018). Furthermore, glycolysis seemed to be the main pathway used by non-stimulated and stimulated CNT total naïve B cells which was expected since these cells were under hypoxic conditions. Similarly, total naïve B cells from both RA groups did not change the rates of glucose consumption or lactate production upon stimulation. No differences were also found between both RA groups and the CNT group for either glucose consumption or lactate production. In fact, upon stimulation, each mole of glucose yielded approximately 1.5 moles of lactate in the RADMARD group and 1.8 moles of lactate in the RATNF group which suggested that these cells were using anaerobic

glycolysis almost exclusively. Under non-stimulated conditions, these ratios were approximately 1 mole of glucose to 2 moles of lactate for both groups. Therefore, the results from intermediary metabolism don't correlate with previous reports where resting B cells from a RA murine model exhibited higher rates of glycolysis when compared to B cells from control animals or where BAFF-chronically stimulated B cells shifted their metabolism to a glycolytic profile (Abboud et al., 2018; Caro-Maldonado et al., 2014). These results suggested that non-stimulated and stimulated B cells from both RA groups were using only glycolysis as the main energetic source. However, the amount of intracellular lipids augmented when the RATNF naïve B cells were stimulated which suggested that these cells shifted their metabolism to include other pathways such as lipid synthesis. One possible explanation why we were obtaining different results for the CNT and both RA groups might be again the time-lapse of our experiences since all these measurements were performed only hours to two days after stimulation or culturing in contrast with our seven days duration. Another possible explanation why we were not observing differences between stimulated and non-stimulated conditions for the intermediary metabolism is the fact that these cells were cultured under hypoxia and glycolysis is the predominantly used pathway in this condition. Upon stimulation, total naïve SLE B cells did not alter the rates of glucose consumption, however, these rates were significantly higher when compared to the CNT group stimulated condition. On the other hand, lactate levels were significantly lower in the SLE group under non-stimulated or stimulated conditions when compared to the CNT group counterparts. Stimulation of SLE total naïve B cells showed that 5 moles of consumed glucose were required to yield 1 mole of produced lactate. For non-stimulated conditions, this ratio was 3.9 moles of consumed glucose to 1 mole of produced lactate. Therefore, these cells were not using only glycolysis to meet their energetic demands. We also did not observe an increase in the amount of intracellular lipids, thereby *de novo* lipogenesis was not upregulated with stimulation. These cells were cultured under hypoxic conditions which inhibit OXPHOS, but this inhibition is not complete. Thus, SLE total naïve B cells were presumably using OXHPOS as the main metabolic pathway. There are no studies concerning the metabolism of B cells in SLE, however, studies on T cells reported that $CD4T^{+}$ cells from SLE patients and mouse models exhibited elevated glycolysis and OXPHOS upon stimulation. Inhibition of glycolysis and oxidative phosphorylation with 2DG and metformin ameliorated disease symptoms in SLE murine models, while inhibition of lactate production did not cause the same effect suggesting that

aerobic glycolysis was not essential for disease progression (Yin et al., 2015, 2016). Finally, the treatment with the FX11, rapamycin or rotenone did not affect lactate or glucose levels in the CNT, RAs or SLE groups.

The results for memory B cells ($CD27^{+}$) showed that glucose consumption rates did not change between the non-stimulated and stimulated conditions of the CNT group. On the other hand, the lactate production rates augmented upon stimulation and 2 moles of consumed glucose were required to yield 1 mole of produced lactate, while under non-stimulated conditions this ratio was 4:1. Glucose consumption increased upon stimulation in both RA groups and this result was never observed for memory B cells in healthy situations or autoimmunity, however, it was observed for healthy activated-naïve B cells (Caro-Maldonado et al., 2014; Cho et al., 2011; Doughty et al., 2006; Dufort et al., 2007). The increase in glucose uptake upon stimulation did not correlate with the lactate production levels, because upon stimulation 8.7 moles of glucose were required to yield 1 mole of produced lactate in the RADMARD group, and this ratio was 8.2:1 in the RATNF group. Under non-stimulated conditions, this ratio was 1:1 in the RADMARD group and 12:1 in the RATNF group. The amount of consumed glucose was also increased upon stimulation of memory SLE B cells, but this change was not verified in lactate production. Stimulated memory B cells required 11.8 moles of consumed glucose to yield 1 mole of lactate and under non-stimulated conditions this ratio was 18:1. Thus, this indicated that memory B cells were not using glycolysis as the main metabolic pathway and might be using other pathways. $CD8^{+}$ Memory T cells have been reported to increase the levels of OXPHOS to meet their metabolic demands (O'Sullivan et al., 2014; van der Windt et al., 2012). Thus, memory B cells could be using OXPHOS as the main source to obtain energy. However, which other metabolic pathways could be required for memory B cells needs is still an open issue and needs further investigation. Inhibition with FX11, rapamycin and rotenone did not impact the consumption of glucose, but the three inhibitors affected negatively the production of lactate in memory B cells from the CNT group. Since FX11 inhibits lactate dehydrogenase the consequent lactate production downregulation is not surprising. However, the result for rotenone treatment was surprising, since the inhibition of mitochondrial ETC should promote a shift in the metabolism to a glycolytic profile mediated by mTOR. Another hypothesis is the HIF-1α destabilization caused by rotenone along with the mTORC1 inhibition caused by rapamycin, since mTORC1 promotes HIF-1α

stabilization under hypoxia and the latter upregulates glycolysis. Treatment with the three inhibitors did not promote any effect on glucose uptake or lactate production in both RA and SLE groups. This result did not correlate at all with the observed LDHA decreased expression upon treatment of the RADMARD group with the three inhibitors. Therefore, either the inhibitors were not enough to suppress the enzyme activity or the low amounts of detected lactate could be affecting the results. Indeed, the amount of detected lactate for the memory B cells was extremely low and this could be affecting the observed results for the 5 conditions of the CNT, RAs and SLE groups. We also compared the rates of glucose consumption and lactate production between total naïve and memory B cells. In the CNT and RAs groups there was no difference between total naïve and memory B cells in the glucose consumption rate. On the other hand, the rates of lactate production were significantly higher in the total naïve B cell subsets. Thus, total naïve B cells did presumably have a more glycolytic profile than memory B cells in the CNT and both RA groups. Oppositely, no differences were observed for the glucose consumption or the lactate production rates between SLE memory and total naïve B cells which suggests that there is no difference between the metabolic profile of these two subsets.

When we assessed the immunoglobulin secretion profile, we found that IgG1, IgG3, IgG4, IgA and IgM were highly secreted by RADMARD total naïve B cells, while IgG2 expression was lower than in the CNT group. The RATNF group secreted widely the four IgG subclasses, IgA and IgM. Moreover, the most common Ig isotypes detected in the peripheral blood of RA patients are IgM for RF and IgG for ACPA and these isotypes were found to be highly secreted by our both RA groups (Sieghart et al., 2018). The profile of secreted immunoglobulins by the SLE total naïve B cells was composed by IgG3, IgG2, IgA and IgM. Even though all the immunoglobulins were secreted by the SLE group, the amount of total IgG was lower than in the CNT group. The class of immunoglobulins predominant in autoantibodies (e.g. anti-dsDNA Ab, ANA) in the peripheral blood of SLE patients is the IgG class (Egner, 2000; Villalta et al., 2013). Moreover, IgG1 and IgG3 subclasses are the most prevalent subclasses in anti-DNA antibodies in the serum of SLE patients and in our results the most secreted immunoglobulin in this group was IgG3 (Liu et al., 2004). In general, treatments with inhibitors did not affect the secretion of immunoglobulins except for the SLE group. Indeed, treatment with FX11 or rotenone promoted a reduction in the levels of IgG3 and IgM secretion. This suggested that

glycolysis was critical for antibody secretion and it could be presumably mediated by HIF-1α. In fact, it has been reported that glycolysis was required for proper antibody production in plasma cells (Caro-Maldonado et al., 2014; Jones et al., 2016). Importantly, we successfully decreased the production of IgG3 in total naïve SLE B cells with FX11 and rotenone which could be of extreme importance since this Ig subclass is one of the most prevalent in the serum of SLE patients, as previously mentioned.

The results obtained from produced cytokine profiling showed that IL-4, IL-2 and IL-6 were the predominantly secreted cytokines in the SLE group. The levels of pro-inflammatory cytokines TNF-α, IL-2, IL-6, and anti-inflammatory cytokine IL-10 were all found to be increased in the serum of SLE patients (Lourenço and La Cava, 2009). Since we observed high expression of IL-6 and IL-2 we can partially correlate our results with the reports from SLE patients' serum. The RADMARD group secreted IL-4, IL-2, IL-6, IL-10 and TGF-β1, while in the RATNF group IL-2, IL-6, IL-10 and TGF-β1 were secreted. However, none of these cytokines was found to be oversecreted when compared to the CNT group. Several analyses of cytokine profile in the synovial fluid of RA patients concluded that IL-1 and TNF-α were the pro-inflammatory cytokines found in higher quantities (Feldmann et al., 1996). IL-6 is another proinflammatory cytokine that has been detected in the synovial fluid of RA patients and is one important mediator of pathogenesis in RA, while IL-2 is barely detected in the synovial fluid and its role is still uncertain (Magyari et al., 2014). Nevertheless, it is interesting how the pro-inflammatory cytokines IL-6 and IL-2 were secreted in higher amount by RATNF total naïve B cells, while in the RADMARD group this happened for IL-4. Moreover, IL-10, TGF-β1 and IL-4 are all anti-inflammatory cytokines, while IL-10 and TGF-β1 were found to be widely expressed in the synovium in RA, the IL-4 levels were not detectable (Chen et al., 2019; Feldmann et al., 1996). Thus, it is interesting how both RA groups secret cytokines that mediate pathogenesis, but at the same time produce cytokines that can control disease progression (e.g. IL-10, TGF-β1). Treatment with the three inhibitors increased expression of TNF-α in total naïve B cells from the CNT group. Contrarily to our results, FX11 and rotenone affected negatively TNF-α expression in macrophages (Chandel et al., 2000; Kaushik et al., 2019). TNF-α has been reported to upregulate HIF-1α expression in myeloid cells (Lin and Simon, 2016). Treatment with FX11 inhibits LDHA and consequently inhibits glycolysis which is regulated by HIF-1α under hypoxic conditions. Treatment with rotenone and rapamycin

could be promoting a HIF-1α destabilization. Therefore, TNF-α augmented secretion could be an attempt of B cells restoring HIF-1α and glycolysis levels. In the RADMARD group the use of metabolic inhibitors caused very different effects because FX11 promoted the upregulation TNF-α secretion but also increased the secretion of anti-inflammatory cytokine TGF-β1. Rapamycin also promoted IL-10 secretion which suggested that an anti-inflammatory profile can be achieved by inhibiting mTORC1. In the RATNF group, FX11 caused increased secretion of both TNF-α and TGF-β1, while treatment with rotenone also promoted increased secretion of TGF-β1. These observations suggested that glycolysis inhibition could promote the upregulation of the proinflammatory cytokine TNF-α which has a predominant role in RA pathogenesis (Feldmann et al., 1996). However, glycolysis and mTORC1/HIF-1α inhibition could also promote the increased secretion of anti-inflammatory cytokines (IL-10 and TGF-β1) which have been reported to limit disease damage (Feldmann et al., 1996). In SLE total naïve B cells, treatment with FX11 promoted increased levels of TNF-α and TGF-β1, while IL-10 secretion was observed to be reduced. The secretion of IL-10 was also successfully reduced when cells were treated with rotenone. Therefore, inhibition of glycolysis and presumably HIF-1α promoted the reduction of anti-inflammatory disease mediator cytokine IL-10. Inhibition of glycolysis promoted the increased secretion of pro-inflammatory TNF-α which actively contributes to SLE pathogenesis, but also promoted increased production of TGF-β1 which has a protective role in this disease. Thus, metabolic inhibition could be considered as a new therapy to limit pathogenic cytokines in RA and SLE, however, this theme needs to be more tightly explored.

Finally, the results obtained for the secretion of immunoglobins in the memory B cell subset showed very small differences and the levels of these molecules were very low and close to the detection limit. Indeed, IgG1 was highly secreted by the RADMARD group, while in RATNF the secretion values were very low for every analyzed Ig. The levels of IgG1 and IgG2 secretion by SLE memory B cells were significantly reduced when comparing to the control group. Treatment with the three inhibitors did not impact the secretion of Igs in both RA and SLE group, while the opposite was verified for the CNT group. Indeed, the three inhibitors decreased the levels of IgG4 secretion, while only FX11 and rotenone could produce the same effect on IgA. Moreover, FX11 promoted successfully decreased levels of IgM and rapamycin inhibited IgG1 secretion. These results might indicate that immunoglobulin secretion was a

glycolysis or HIF-1α-dependent process, since both were presumably inhibited with these treatments, and in fact, glycolysis was already proved to be essential for antibody production in B cells (Caro-Maldonado et al., 2014). The evaluation of cytokine secretion profiles did not show differences between stimulated and inhibited conditions in the CNT and SLE groups. The most secreted cytokines by the SLE group were IL-4, IL-2, IL-6 and TGF-β1. The results obtained also showed that IL-4 and IL-2 were secreted by the RADMARD group, while in the RATNF group this was verified for IL-4, IL-2 and TGF-β1. Indeed, the levels of TGF-β1 secretion were higher in the RATNF group when compared to the CNT group which is interesting given the anti-inflammatory and protective role of this cytokine. In the RADMARD group, treatment with rotenone or FX11 promoted increased levels of TGF-β1, oppositely the treatment with rapamycin reduced the secretion of this anti-inflammatory cytokine in the RATNF memory B cell subset. Therefore, glycolysis and presumably HIF-1α inhibition could promote the increase secretion of protective cytokine TGF-β1, while mTORC1 inhibition had the opposite effect in RA groups. However, the very low amount of detected Igs and cytokines in memory B cells could be affecting our results.

# 5. CONCLUSION

In conclusion, our results for the naïve B cell subset in healthy controls showed that GLUT1 and LDHA expression did not change upon stimulation and a lack of effect was also verified regarding the rates of glucose uptake and lactate production. However, the ratio of consumed glucose to produced lactate suggests that total naïve B cells were using glycolysis as the main energy source which was predictable given that the cells were cultured under hypoxic conditions.

The results for both RA groups showed that total naïve B cells also had a metabolic profile that was almost dependent on glycolysis, however, lipid synthesis was also suggested to be upregulated upon stimulation in the RATNF group. Unlike previous results, the levels of GLUT1, LDHA or glycolysis were not upregulated upon stimulation. Moreover, the RATNF naïve B cells also showed a strong mTORC1-pathway activation. The metabolic inhibitors had barely any effect on enzyme expression or on glucose and lactate metabolism. However, the metabolic inhibitors had a significant impact on effector molecules production since we could successfully promote the augmentation of cytokine secretion.

The metabolic results from total naïve B cells in the SLE group were of most interest since it seemed that a total downregulation of lactate production occurred when compared to CNT B cells under non-stimulated or stimulated conditions. This was supported by the downregulation of LDHA expression levels when compared to CNT naïve B cells under non-stimulated conditions. Upon stimulation, SLE B naïve cells drastically reduced GLUT1 expression, but the amount of glucose consumption did not change with stimulation and was significantly higher than in the CNT group. Therefore, SLE total naïve B cells must be using another metabolic pathway to obtain energy. The amount of intracellular lipids did not change upon stimulation which suggests that lipid synthesis was not upregulated. This suggests that OXPHOS was the main energy source used by SLE total naïve B cells, however, the question of which pathway is used to metabolize the consumed glucose remains to be elucidated. Upon stimulation, switched-memory and memory B cells had higher mTOR phosphorylation which suggests an upregulation of its pathway. The metabolic inhibitors did not have a great impact on enzymatic expression or glucose and lactate metabolism. However, we could successfully decrease the quantity of secreted antibodies with FX11 or rotenone. The effect on cytokines

was very mixed because we could promote the increase of pro-inflammatory and anti-inflammatory cytokines with FX11 and rotenone.

The intermediary metabolism results for memory B cells from the CNT, RADMARD, RATNF and SLE groups showed that these cells were not using glycolysis as the principal metabolic pathway to obtain energy and OXPHOS could be considered as the main alternative. However, the low levels of lactate that we detected in these cells could be affecting our results.

The results from naïve, switched-memory and memory B from the CNT, RADMARD, RATNF and SLE groups exhibit one common feature: the upregulation of mTOR upon inhibition with rotenone which could presumably be an attempt to prevent HIF-1α destabilization. However, it is important to highlight that we did not observe any change in HIF-1α expression upon treatment with rotenone, so another possible mechanism could be responsible for mTOR/mTORC1 activation.

Furthermore, our study presents some limitations that could be affecting the results. For instance, we were not able to support previous results concerning the upregulation of GLUT1 and glycolysis upon stimulation. However, as pointed before, the other reports assessed these parameters only from six hours to two days after stimulation, while we were measuring them seven days after stimulation. Therefore, this could be a reason why we were not able to support previous results. Another reason could be the hypoxic culturing conditions since hypoxia and HIF-1α can drastically modulate cellular metabolism. Another limitation that we found concerns the use of the three metabolic inhibitors: FX11, Rapamycin and Rotenone. These inhibitors had barely any effect on glucose or lactate metabolism or enzymatic expression and only showed some effect on antibody or cytokine secretion. Moreover, the failure of the inhibitors could be also explained by insufficient dose or with the necessity of administrating the inhibitor more than once.

Thus, the metabolism of B cells of both RA and SLE patients' needs to be characterized more in-depth. One possible approach could be studying cells under normoxic conditions to understand what is happening in the peripheral circulating cells and how it may differ from the synovium cells. However, our study provides already some basic knowledge about how metabolism is regulated in SLE and RA B cells from patients.

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## SUPPLEMENTARY DATA

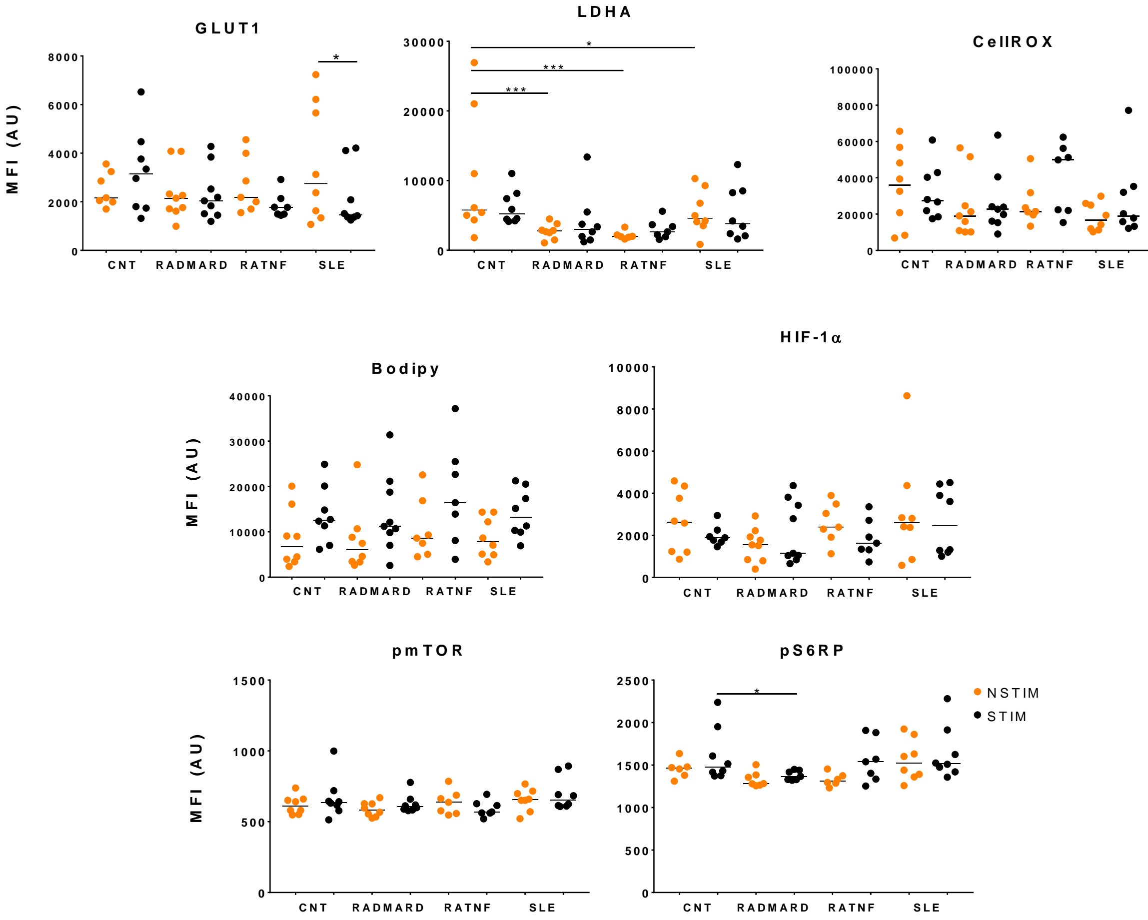


**Figure S1 - The expression of metabolic enzymes isn't altered upon activation of B cells in autoimmunity.** Levels of intracellular lipids, ROS and enzyme expression were assessed by flow cytometry and the results are expressed as the Mean Fluorescence Intensity (MFI). Each dot represents an individual donor (n=6-9) and the median bar of the data is also presented. One-way ANOVA and Uncorrected Fisher's LSD (α=0.05) was used to compare NStim and Stim conditions for each group and NStim and Stim conditions from the CNT group with the respective correspondents in RA and SLE groups. *p<0.05; **p<0.01.

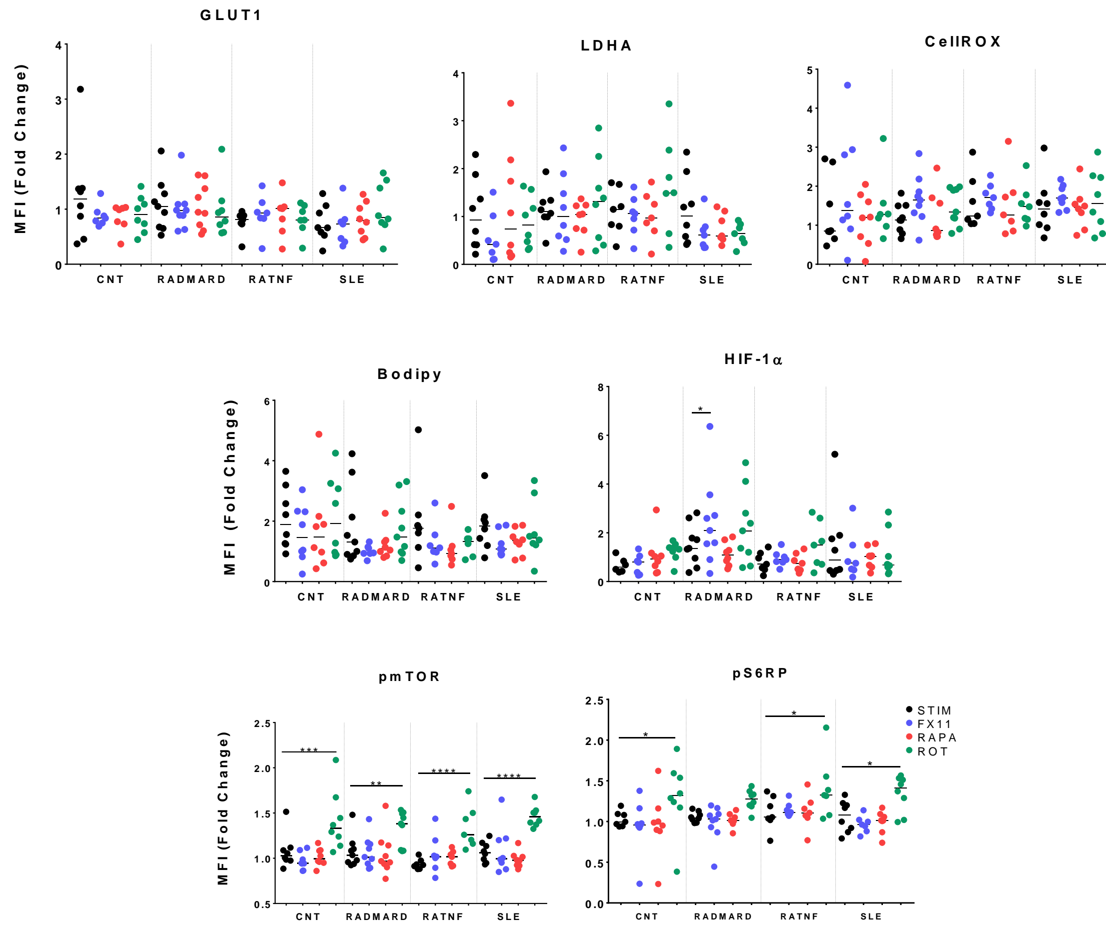


**Figure S2 - The levels of mTOR and its downstream effector S6RP are affected when stimulated B cells are treated with rotenone**. Levels of enzyme expression, intracellular lipids and ROS and were assessed by Flow cytometry and the results are expressed as the Fold Change of the MFI relatively to the NStim condition of each group. Each dot represents an individual donor (n=6-9) and the median bar of the data is also presented. One-way ANOVA and Uncorrected Fisher's LSD (α=0.05) was used to compare Stim, FX11, RAPA and ROT conditions for each group. *p<0.05; **p<0.01, ****p<0.0001.