UNIVERSIDADE DE SÃO PAULO

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Papel da Sirtuina 1 na ativação e diferenciação das células T CD4^{+} em um modelo murino de obesidade e transplante

Tese Doutoral

OMAR ALBERTO DOMÍNGUEZ AMOROCHO

SÃO PAULO / MADRID, 2022

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Area: Immunology

Director: Prof. Dr. Niels Olsen Saraiva Câmara.

Co-director: Prof. Dra. María Mittelbrunn Herrero

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CERTIFICADO

Certificamos que a proposta intitulada "Papel da Sirtuina 1 na ativação e diferenciação de células T CD4+ em animais obesos transplantados", protocolada sob o CEUA nº 9090200318, sob a responsabilidade de **Niels Olsen Saraiva Câmara** *e equipe; Omar Alberto Dominguez Amorocho; Paulo José Basso; Fernanda Fernandes Terra; Meire Ioshie Hiyane* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais do Instituto de Ciências Biomédicas (Universidade de São Paulo) (CEUA-ICB/USP) na reunião de 02/05/2018.

We certify that the proposal "Role of Sirtuin 1 in CD4+ T cell activation and differentiation in obese transplanted engrafted animals", utilizing 50 Heterogenics mice (50 males), 70 Genetically modified mice (GMO) (70 males), protocol number CEUA 9090200318, under the responsibility of **Niels Olsen Saraiva Câmara** *and team; Omar Alberto Dominguez Amorocho; Paulo José Basso; Fernanda Fernandes Terra; Meire Ioshie Hiyane* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Biomedical Sciences Institute (University of São Paulo) (CEUA-ICB/USP) in the meeting of 05/02/2018.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: 36 meses Depto/Setor: Imunologia

Origem:	Biotério de Experimentação do Departamento de Imunologia			
Espécie:	Camundongos heterogênicos	sexo: Machos	Idade ou peso:	8 a 12 semanas
Linhagem:	C57BL/6		**N amostral:**	50
Origem:	Biotério de Experimentação do Departamento de Imunologia			
Espécie:	Camundongo geneticamente modificado (OGM)	sexo: Machos	Idade ou peso:	8 a 10 semanas
Linhagem:	C57/BL6 SIRT1fl/fl-CD4-Cre		**N amostral:**	30
Origem:	Biotério de Experimentação do Departamento de Imunologia			
Espécie:	Camundongo geneticamente modificado (OGM)	sexo: Machos	Idade ou peso:	8 a 10 semanas
Linhagem:	C57BL/6-Tg(CAG-OVA)916Jen/J		**N amostral:**	10
Origem:	Biotério de Experimentação do Departamento de Imunologia			
Espécie:	Camundongo geneticamente modificado (OGM)	sexo: Machos	Idade ou peso:	8 a 10 semanas
Linhagem:	C57BL/6 (Sirt-/-) OT-II		**N amostral:**	30

São Paulo, 11 de junho de 2018

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Título da proposta: "Papel da Sirtuina 1 na ativação e diferenciação de células T CD4+ em animais obesos transplantados".

Parecer Consubstanciado da Comissão de Ética no Uso de Animais ICB (ID 000007)

A Comissão de Ética no Uso de Animais da Instituto de Ciências Biomédicas (Universidade de São Paulo), no cumprimento das suas atribuições, analisou e **APROVOU** a Emenda (versão de 30/maio/2018) da proposta acima referenciada.

Resumo apresentado pelo pesquisador: "Inclusão de linhagem prevista no texto da proposta mas não no campo específico do formulário.".

Comentário da CEUA: "".

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Instituição: .....

"*Try not to become a man of success, but rather try to become a man of value.*"

Albert Einstein

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RESUMO

As histonas desacetilases (HDACs) removem grupos acetil de resíduos de lisina em diferentes proteínas, incluindo histonas. Sirtuinas são membros da classe III das HDACs e sirtuina 1 (Sirt1) desempenham um papel importante no metabolismo celular e regulação da resposta imune. Na obesidade, a expressão de Sirt1 está reduzida na maioria dos tecidos com alta atividade metabólica. Nos últimos anos, tornou-se evidente a contribuição de células T CD4+ na obesidade. No entanto, a contribuição da Sirt1 em células T na obesidade não foi totalmente investigada. Nossa hipótese foi que a Sirt1 no contexto da obesidade teria um papel importante na polarização dos linfócitos T $CD4^+$, não somente por modificações epigenéticas, mas também por uma modulação metabólica da resposta imune, que culminaria em alterações no aceite de um transplante. Aqui, avaliamos o papel de Sirt1 na diferenciação e ativação de células T $CD4^+$ em um modelo experimental de obesidade. Animais $Sirt1^{+/+}$ e CD4-$Sirt1^{-/-}$ com oito semanas de idade foram submetidos à obesidade induzida por dieta (DIO) ou à dieta padrão por 12 semanas. Parâmetros morfológicos, bioquímicos, metabólicos, moleculares e de biologia celular foram avaliados durante e ao final do DIO. Após 12 semanas, os animais DIO tornaram-se obesos e inflamados e mostraram uma atividade reduzida de HDACs e uma expressão reduzida da expressão de Sirt1, Sirt3, CD36 e PGC-1α em células T $CD4^+$ em linfonodos. Foram observadas alterações na expressão desses genes também em momentos diferentes no sangue periférico. Nos linfonodos observou-se também aumento da frequência de Th1, Th17 e redução de células Treg. As células T $CD4^+$ também apresentaram um aumento nos marcadores relacionados à ativação ou exaustão, como KLRG-1 e PD-1 e uma mudança para um fenótipo de precursor de memória (MPEC). Observamos um aumento da captação de glicose nas células T

$CD4^+$ em animais DIO em comparação com os controles, especialmente em CD4-$Sirt1^{-/-}$. Além disso, um aumento da massa mitocondrial e produção de superóxido mitocondrial e um perfil respiratório mitocondrial alterado (em termos de vazamento de prótons, capacidade respiratória máxima e de reserva) foram observados em células T $CD4^+$ de animais obesos. A depleção condicional de Sirt1 em células T $CD4^+$ aumentou a frequência de células Th1 e Th17, no contexto da obesidade, em comparação com os resultados obtidos em animais $Sirt1^{+/+}$ em DIO. O perfil bioenergético das células T $CD4^+$ e a captação de glicose também mostraram respostas metabólicas e demanda de glicose aumentadas nos animais DIO em comparação com os controles. Em relação aos resultados do transplante, não foram observadas diferenças entre $Sirt1^{+/+}$ e CD4-$Sirt1^{-/-}$, mas uma taxa de rejeição acelerada foi observada nos animais em DIO. Em conclusão, a deleção de Sirt1 nas células T $CD4^+$ agrava o efeito da obesidade no perfil metabólico e funcional dessas células. Esses dados sugerem um papel protetor de Sirt1 nas células T $CD4^+$ no contexto de distúrbios metabólicos.

Palavras-chave: Sirtuin 1, plasticidade celular, linfócitos T $CD4^+$, obesidade induzida por dieta, imunometabolismo.

ABSTRACT

Histone deacetylases (HDACs) remove acetyl groups from lysine residues in different proteins, including histones. Sirtuins are members of class III HDACs and Sirtuin 1 (Sirt1) plays a role in cellular metabolism and immunological regulation. In obesity, the expression of Sirt1 is constitutively downregulated in most metabolic tissues. Recently, it has become evident the contribution of T cells to obesity. However, the importance of Sirt1 expression in T cells in the context of obesity has not been investigated. We hypothesized that Sirt1 in the context of obesity has an important role on $CD4^+$ T cell polarization, not just from an epigenetic point of view, but by a metabolic modulation of the immune response and these modifications could also be involved in the progression of transplant rejection.

Here, we evaluate the role of Sirt1 in the differentiation and activation of $CD4^+$ T cells in an experimental model of obesity. Eight weeks old $Sirt^{+/+}$ and CD4-$Sirt1^{-/-}$ animals were submitted to diet-induced obesity (DIO) or standard diet conditions for 12 weeks. Morphological, biochemical, metabolic, molecular and cell biology parameters were evaluated through and at the end of the DIO.

After 12 weeks, DIO animals became obese and inflamed and showed a reduced activity of HDACs and a reduced expression of Sirt1, Sirt3, CD36 and PGC-1α expression in $CD4^+$ T cells from lymph nodes, changes in the expression of these genes were observed also at different point times in peripheral blood. An increased frequency of Th1, Th17 and reduction of Treg cells in draining lymph nodes was also observed. $CD4^+$ T cells also presented an increase in markers related to activation or exhaustion, such as KLRG-1 and PD-1 and a shift to a memory precursor phenotype (MPEC). We observed an increased glucose uptake in DIO animals compared to controls, especially in CD4-$Sirt1^{-/-}$ supporting increased glucose

demand in T cells from DIO mice. In addition, an increased mitochondrial mass and mitochondrial superoxide production, an altered mitochondrial respiratory profile (in terms of proton leak, maximal and spare respiratory capacity) were observed in $CD4^+$ T cells from obese animals.

Conditional depletion of Sirt1 in $CD4^+$ T cells increased the frequency of Th1 and Th17 cells, in the context of obesity, compared with the obtained results in $Sirt1^{+/+}$ DIO animals. The bioenergetic profile of $CD4^+$ T cells and glucose uptake also showed increased metabolic responses and glucose demand in DIO animals compared to controls. Regarding transplantation outcomes, no differences were observed between $Sirt1^{+/+}$ and CD4-$Sirt1^{-/-}$ but an accelerated rejection rate was observed as a result of DIO.

In conclusion, deletion of Sirt1 in $CD4^+$ T cells aggravates the effect of obesity in the metabolic and functional profile of these cells. These data suggest a protective role of Sirt1 in $CD4^+$ T cells in the context of metabolic disorders.

All the procedures were evaluated and accepted by the ethical committees of the participant institutions and were performed according to the national and international regulations and guaranteeing the animal welfare. Ethics committee approval code: CEUA 9090200318

Keywords: Sirtuin 1, $CD4^+$ plasticity, diet induced obesity, immunometabolism.

RESUMEN

Las histonas desacetilasas (HDAC) eliminan los grupos acetilo de los residuos de lisina en diferentes proteínas. Las sirtuinas son miembros de las HDAC de clase III y la sirtuina 1 (Sirt1) juega un papel importante en el metabolismo celular y la regulación inmunológica. En la obesidad, la expresión de Sirt1 está constitutivamente regulada a la baja en la mayoría de los tejidos metabólicos. En los últimos años se ha hecho evidente la contribución de las células T a la obesidad. Sin embargo, no se ha investigado la contribución de Sirt1 expresada en células T al progreso de la obesidad. Nuestra hipótesis fue que Sirt1 tiene un papel importante en la polarización de las células T $CD4^+$, no únicamente desde un punto de vista epigenético sino participando de la regulación metabólica de la respuesta inmune y que esta modulación podría estar involucrada en el rechazo del trasplante.

Para ello, evaluamos el papel de Sirt1 en la diferenciación y activación de las células T $CD4^+$ en un modelo experimental de obesidad. Los animales $Sirt^{+/+}$ y CD4-$Sirt1^{-/-}$ de ocho semanas de edad se sometieron a obesidad inducida por la dieta (DIO) o condiciones de dieta estándar durante 12 semanas. Se evaluaron parámetros morfológicos, bioquímicos, metabólicos, moleculares y de biología celular a través y al final de la DIO.

Después de 12 semanas, los animales DIO se volvieron obesos e inflamados y mostraron una actividad reducida de las HDAC y una expresión reducida de la expresión de Sirt1, Sirt3, CD36 y PGC-1α en las células T $CD4^+$ de los ganglios linfáticos en diferentes momentos puntuales en la sangre periférica. También se observó una mayor frecuencia de Th1, Th17 y una reducción de las células Treg en el drenaje de los ganglios linfáticos. Los linfocitos T $CD4^+$ también presentaron un aumento de marcadores relacionados con la activación o el agotamiento, como

KLRG-1 y PD-1 y cambio a un fenotipo precursor de memoria (MPEC). Observamos una mayor expresión de la captación de glucosa en animales DIO en comparación con los controles, especialmente en CD4-Sirt1$^{-/-}$ apoyando una mayor demanda de glucosa en las células T de los ratones DIO. Además, se observó una mayor masa mitocondrial y producción de superóxido mitocondrial, un perfil respiratorio mitocondrial alterado (en términos de fuga de protones, capacidad respiratoria máxima y de reserva) en células T CD4^{+} de animales obesos.

La deleción condicional de Sirt1 en células T CD4^{+} aumentó la frecuencia de células Th1 y Th17, en el contexto de la obesidad, en comparación con los resultados obtenidos en animales Sirt1$^{+/+}$ DIO. El perfil bioenergético de las células T CD4^{+} y la absorción de glucosa también mostró un aumento de las respuestas metabólicas y la demanda de glucosa en los animales DIO en comparación con los controles. Con respecto a los resultados del trasplante, no se observaron diferencias entre Sirt1$^{+/+}$ y CD4-Sirt1$^{-/-}$ pero se observó una tasa de rechazo acelerada como resultado de la DIO.

En conclusión, la deleción de Sirt1 en las células T CD4^{+} agrava el efecto de la obesidad en el perfil metabólico y funcional de estas células. Estos datos sugieren un papel protector de Sirt1 en las células T CD4^{+} en el contexto de trastornos metabólicos.

Palabras clave: Sirtuina 1, plasticidad CD4^{+}, obesidad inducida por dieta, inmunometabolismo.

FIGURES

TABLES

ABBREVIATIONS

ADP - adenosine diphosphate

ATP – adenosine triphosphate

BMI - Body Mass Index

BSA – Bovine Serum Albumin

c-Myc – cellular myelocytomatosis oncogene

CCL-2 – C-C motif chemokine ligand 2

CCL-20 - C-C motif chemokine ligand 20

CD4 – cluster of differentiation 4

CD8 – cluster of differentiation 8

CEUA - Comitê de Ética em Uso de Animal

CTLA-4 – cytotoxic T-lymphocyte associated protein 4

DCs – dendritic cells

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

Drp-1 – dynamin-related protein 1

ECAR – extracellular acidification rate

EDTA – ethylenediamine tetraaceticacid

ER – endoplasmic reticulum

EX-527 - 6-chloro-2,3,4,9-tetrahydro-1H-Carbazole-1-carboxamide

FBS – fetal bovine serum

FOXO - Forkhead Family of Transcription Factors

Foxp3 – Forkhead Box P3

GTT – Glucose tolerance Test

HDACi - Histone Deacetylase inhibitors

HDACs - Histone Deacetylases

HIF-1a - hypoxia-inducible factor 1-alpha

ICAM-1 – intercellular adhesion molecule 1

IFNb-1a - Interferon-Beta-1a

IFNb-1b - Interferon-Beta-1b

IFN-γ – interferon gamma

IL – interleukin

IR - Insulin Resistance

ITT – Insulin Tolerance Test

MCP-1 – monocyte chemoattractant protein 1

MFI – mean fluorescence intensity

Mfn1 – mitofusin 1

Mfn2 – mitofusin 2

mTOR - Mammalian Target of Rapamycin

NAD – Nicotinamide adenine dinucleotide

NADPH – Nicotinamide adenine dinucleotide phosphate

NF-kB - nuclear factor kappa B

NLRP3- NACHT, LRR and PYD domains-containing protein 3

NO – nitric oxide

OCR – oxygen consumption rate

OXPHOS – oxidative phosphorylation

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PGC-1a – Peroxisome proliferator-activated receptor gamma coactivator-1 alpha

Rv – Resveratrol

ROS – Reactive oxygen species

RPMI – Roswell Park Memorial Institute Medium

RT-qPCR – reverse transcription quantitative polymerase chain reaction

SIRT1 – Sirtuin 1

STAT3 - Signal Transducers and Activators of Transcription

T2D – Type 2 Diabetes mellitus

T-bet - T-Box Transcription Factor TBX21

TBST - Tris-Buffered Saline and Tween 20

TGF-b – Transforming growth factor beta

Th1 – T-helper 1

Th17 – T helper 17

Treg – Regulatory T cell

WT – Wild Type

ΔΨm – Mitochondrial membrane potential

INDEX

1. INTRODUCTION

1.1. OBESITY

The World Health Organization (WHO) defines overweight and obesity as a condition in which excessive fat accumulation is related to several risks to health, being an important and growing problem for public health worldwide (Blüher, 2019; Capodaglio & Liuzzi, 2013; Fruh, 2017). Obesity is an important risk factor for development of noncommunicable diseases (NCDs), such as cardiovascular diseases, cancer and diabetes mellitus. NCDs represent over 70% of early deaths worldwide, and they are also considered the leading cause for premature disability (WHO, 2017; Al-Raddadi et al., 2019; Censin et al., 2019; Hingorani et al., 2019; Hruby et al., 2016). Increased body mass index (BMI) and high deposits of fat mass have been considered predictors for metabolic disorders (Goossens, 2017) and have been associated with a low-grade inflammation and metabolic dysfunction (Kittiskulnam et al., 2018) .

Obesity takes place when energy intake exceeds energy expenditure and nutrient reserves, resulting in an abnormal accumulation of fat (Wilding, 2012). Genetic predisposition, environmental conditioning (P.H. Wilding, 2001; Stunkard et al., 1986), diversity, amount and quality of food (energy density)(Al Kibria et al., 2019; Hruby et al., 2016), accessibility to diets with high caloric content (Cohen & Lesser, 2016; Holsten, 2009; Nguyen & El-Serag, 2010) and irregular physical exercise or physical inactiveness (Gokosmanoglu et al., 2019; Golshevsky et al., 2020; Narciso et al., 2019) are important intrinsic and extrinsic factors with relevant roles in weight gain.

1.2. MODELING OBESITY AND METABOLIC SYNDROME IN ANIMALS

Research strategies involving animal models that mimic or share human obesity characteristics and its comorbidities are crucial for a better comprehension of its physiopathology and for its prevention and treatment. Available obesity animal models can be organized into three main types: one, based on genetic mutations or manipulations; second, based on the exposition of genetically intact animals to obesogenic environments like high-fat diets; and third, a combination of both strategies (Kanasaki & Koya, 2011; Lutz & Woods, 2012).

Food composition and food intake are important aspects to be considered in animal models for obesity, since they can influence body adiposity and have important effects in biochemical and physiological signatures (Kleinert et al., 2018).

Animals on diet-induced obesity (DIO) resemble with more fidelity the main features of human obesity and may be the best choice for testing potential therapeutics in a close "real" context (de Moura e Dias et al., 2021). Likewise, transgenic or models with point mutations are commonly used to evaluate specific genes, molecules or components that could be useful to define therapeutic targets or to identify pathways with a potential role in obesity (Levin et al., 1997; Michel et al., 2004).

In terms of the DIO, animals become obese as a result of a high fat diet (HFD) or the human western diet (WD), also called "coffe shop" diet. HFD and WD-induced obesity induce hyperphagia which is partly compensated by increased energy expenditure (Pérez et al., 1999; Rogers & Blundell, 1984; Rothwell & Stock, 1979). Caloric density of HFD reduces the effect of insulin and leptin, most likely due to a post-receptors modification effect. HFD also seems to affect intracellular signaling

pathways in many cells types resulting in changes in cell fate (Banks et al., 2004; Benoit et al., 2009; Clegg et al., 2011; Hariri & Thibault, 2010; Woods et al., 2004).

1.3. OBESITY, INFLAMMATION AND THE IMMUNE SYSTEM

Obesity signature is a chronic, low-grade and systemically pervasive inflammation. The term "meta-inflammation" or metabolically-derived inflammation has been adopted to define this condition (Hotamisligil, 2006). In obesity, adipocytes expand their numbers and size resulting in cellular stress and stimulate cytokine secretion and infiltration of immune cells into different organs and tissues.

Metabolic changes and the meta-inflammation lead to immune cell activation, which play an important role in the appearance and perpetuation of NCD. In addition, they truly affect the immune response, as evidenced by vaccines failure and infection complications (Bremer et al., 2011; Sheridan et al., 2012), including COVID-19 (Gao et al., 2021; Petrova et al., 2020; Yates et al., 2021, Andersen et al., 2016; Feuerer et al., 2009; Grivennikov et al., 2010).

Some cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-1β are the main soluble inflammatory factors in obesity-induced inflammation, (Hotamisligil et al., 1993; Naugler & Karin, 2008; Rath et al., 2015; Stenlöf et al., 2003; Stienstra et al., 2012; Wang et al., 2005). TNF-α favors the activation of NF-κB, inhibits GLUT4 expression, enhances the blood levels of free fatty acids (FFA), activates TLR pathways (Baker et al., 2011; Chiang et al., 2009), increases the levels of leptin, and resistin, all leading to insulin resistance (Gómez-Hernández et al., 2016).

Different $CD4^+$ Th cell profiles have been observed in obesity, depicting increased numbers of Th1 cells, and stable number of Th2 cells in several tissues, mainly in

adipose tissue (S. Winer et al., 2009, McLaughlin et al., 2017; Rocha et al., 2008). Regarding the Th17 differentiation in obesity, data are still controversial. Some studies have shown that Th17 cell frequency is increased during obesity (Chehimi et al., 2017; Endo et al., 2015; Schindler et al., 2017). In fact, those observations have been endorsed to an accelerated progress of autoimmunity in murine models of experimental autoimmune encephalomyelitis (EAE) and colitis in DIO mice (Shawn Winer, Geoff Paltser, et al., 2009). Moreover, IL-17 secretion may affect several cell populations bearing IL-17 receptors, including adipocytes, neutrophils and endothelial cells (Cua & Tato, 2010; Zúñiga et al., 2010).

Finally, the chronic pro-inflammatory milieu in obesity has an important effect on vascular and endothelial dysfunction, increasing the levels of reactive oxygen species (ROS), promoting atherosclerosis and hypertension (Ellulu et al., 2017). Most of the aforementioned factors have been related with organ dysfunction and failure, in some cases, requiring organ transplantation as a therapeutic alternative (Grinyó, 2013).

1.4. ORGAN TRANSPLANTATION

According to the Global Observatory on Donation and Transplantation, the number of transplants of solid organs carried out in 2019 was 153.863 worldwide. Despite the rise of the number of transplants, the availability of donors does not reach the demand of individuals in the waiting list (http://www.transplant-observatory.org/). Kidney transplants lead as the most performed type of transplant worldwide (36%), followed by cardiac (21.3%), and liver (19%) (http://www.transplant-observatory.org/). According to the Brazilian Transplant Registry (RBT) and the Brazilian association for organ transplantation (ABTO), the

average number of solid organ transplants performed between January and March in 2021 was 1562, showing an important reduction compared with the previous year, mainly due to the COVID-19 pandemics (https://site.abto.org.br/publicacao/ano-24-numero-1/).

Even with the increase in the number of transplants performed, it is still necessary to explore how rejection develops and new approaches to alleviate the consequences of acute rejection in short-, medium- and long-term after the transplant. Even though more precise and effective immunosuppressive drugs have emerged in recent years (Arnol et al., 2020; Halloran, 2004), around 15-20% of transplanted patients still experience an acute rejection episode, being totally dependent on these drugs for the rest of their lives, despite their potential side effects.

1.5. SOLID ORGAN TRANSPLANTATION AND THE IMMUNE RESPONSE

Since transplantation techniques were introduced, many studies have been directed to understand the immune mechanisms in organ and tissue rejection. After many cases of failure and procedures without reproducibility, George Snell and Peter Gorer, with experiments made on animals, concluded that the rejection of some organ involved important phylogenetic factors (Nasr et al., 2016; Snell, 1948, 2014). Later, some of these factors, specially some of them present in leukocytes, were characterized as Human Leukocyte Antigens (HLA) and the Major Histocompatibility Complex (MHC), this last composed by the largest genomic loci, which when transcribed and translated, originate surface molecules that determine the compatibility among tissues at the molecular, cellular and tissue level (Dausset, 1958; Komlos et al., 1995; Snell, 2014).

In transplantation immunology, a critical step of the immune response is the presentation of donor-specific antigens to immune cells from the recipient and there are some known processes. In the direct presentation, T cells from the recipient are directly activated by recognition of alloantigens coupled to class I or II MHC molecules on the surface of donor APCs. Conversely, indirect presentation involves the capture and processing of donor antigens and presentation in class I and II MHC molecules by the recipient's APCs to the recipient's T cells. More recently, semi-indirect presentation was described, which is related to the T cells and APCs interaction, leading to MHC-peptides complexes exchange by cell-to-cell direct contact (Ingulli, 2010; Moreau et al., 2013; Nankivell & Alexander, 2010). Finally, in any type of antigenic presentation, the effector mechanisms that cause rejection are mostly commanded by CD4+ T lymphocyte subtypes (Ingulli, 2010; Marino et al., 2016; Siu et al., 2018).

Three types of rejection have been described: hyperacute, acute and chronic (Chalasani et al., 2004; Moreau et al., 2013). Hyperacute rejection is characterized by the preexistence of antibodies against donor antigens (IgG and IgM, usually previously formed), activating the complement system or innate immune system cells causing endothelial damage, leading to vascular thrombotic occlusion of the graft, within minutes or hours, soon after anastomosis between the graft vessels and the recipient's circulatory system (da Silva et al., 2017). Acute rejection, however, is due to effector mechanisms of $CD4^+$ and $CD8^+$ T lymphocytes against donor-specific antigen, causing damage to the graft parenchyma and blood vessels in weeks or months after transplantation. Similarly, chronic rejection is related to vascular occlusion caused by proliferation of intimal smooth muscle cells and collagen deposits in the parenchyma that result from the accumulation of cytokines and other

factors released by graft-reactive T cells and phagocytes (da Silva et al., 2017; Wood & Goto, 2012).

It is still necessary to carry out studies to delimitate the processes that trigger or exacerbate rejection, in the context of obesity. It is possible that low-grade and maintained inflammatory processes might influence the process of rejection.

1.6. THE IMPACT OF OBESITY IN TRANSPLANTATION

The graft survival of several transplanted organs is affected in the context of obesity. For example, a BMI greater than 30 is considered a relevant risk factor for delay of the surgical wound healing in recipients of kidney transplant (Humar et al., 2001; Kwan et al., 2016), and for the impairment in graft acceptance (Nagendran et al., 2016; Weiss et al., 2009).

Cardiovascular diseases are the leading causes of death in kidney transplant recipients, which are most often related to obesity (Costa et al., 2020; Rangaswami et al., 2019). In addition, heart and kidney transplant recipients with a BMI greater than 35 had worse long-term survival and higher rates of acute rejection, in addition to increased Type 2 Diabetes incidence (Armstrong et al., 2005; Ditonno et al., 2011; Russo et al., 2010). Patient survival after orthotopic liver transplantation is also compromised in recipients with BMI above 40 (Ayloo et al., 2015; Moctezuma-Velazquez et al., 2019; Shi et al., 2019). Interestingly, obesity acts as an independent risk factor in recipients who received lung grafts, contributing to up to 12% of deaths during the first year after transplantation (Lederer et al., 2009; Pienta et al., 2018; Upala et al., 2016). Given the inflammatory environment in obese people, it is possible that inflammation in obesity may drive immune response towards to transplant rejection.

1.7. T CELL METABOLISM AND IMMUNE RESPONSE

Immune cells are key factors in maintaining the homeostasis of biological systems and in tumor surveillance. During their different stages, immune cells are conditioned by signals from the microenvironment that modulate and regulate their state of differentiation, maturation, tissue tropism and effector or regulatory fate, influencing their role in the local or systemic immune response. Some of the signals originate from diet or metabolites derived from biological processes that diverge among cells and organs, thus influencing immune cells differentially.

A fine tune balance of metabolites, such as substrates for reducing/oxidizing reactions, reactive oxygen species (ROS) production, as well as the availability of growth factors and nutrients, directly influence which metabolic pathways will be prioritized (Buck et al., 2017; Domblides et al., 2018; Wang et al., 2019). The definition of energy metabolism and nutritional sensing suggest that, after food is digested and absorbed, adenosine triphosphate (ATP) can be directly metabolized from these absorbed nutrients or stored as alternative energy sources, such as proteins, glycogen, or lipids (Iyer et al., 2015). Specifically considering the function of immune cells, metabolic pathways alterations or dysfunctions have been associated with proliferation and acquisition of effector function by immune cells. In general, six metabolic pathways are generally considered: the glycolytic metabolic pathway (1); the pentose phosphate (PPP) pathway (2); the tricarboxylic acid (TCA) cycle (3); the oxidation of fatty acids (FAO) (4); or synthesis (5); and the amino acid metabolic pathway (6). The main cellular metabolic pathways are summarized by O'Neill et al (O'Neill et al., 2016) (Figure 1):

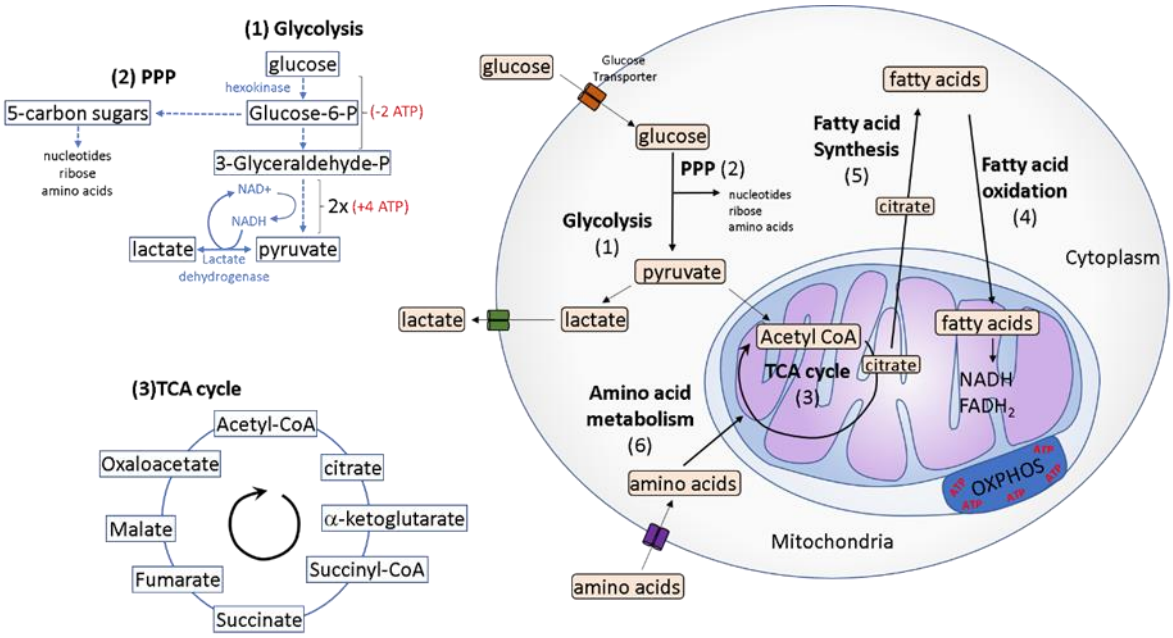

Figure 1. Six major metabolic pathways relevant to immune cell function.

Glycolysis (1) is a process that takes place in the cytoplasm and involves the conversion of glucose into pyruvate, which can enter the tricarboxylic acid (TCA) cycle (3) or be transformed into lactate and secreted. The pentose-phosphate pathway (2) parallels glycolysis and generates ribose for nucleotides, amino acids and nicotinamide adenine dinucleotide phosphate (NADPH), which is important for the synthesis of fatty acids and production of lipid ligands. Oxidation of fatty acids (4) is an aerobic mitochondrial-dependent process that consists of breaking down fatty acids into Acetyl-CoA units, generating NADH and FADH2, and leading to the production of ATP from the synthesis of E fatty acids. Fatty acid synthesis (5) is a complex cytoplasmic process. It is regulated by Acetyl-CoA, NADPH and fatty acid synthases to generate fatty acids. The metabolism of amino acids (6) is very diverse, also important for cell growth and protein biosynthesis, as a consequence of the large number of different amino acids, which can feed different carbon skeletons in pyruvate, acetyl CoA and citric acid cycle, which enter the TCA cycle. TCA: tricarboxylic acid; PPP: pentose phosphate pathway; OXPHOS: oxidative phosphorylation. Adapted from O'Neill, 2016.

Glycolytic pathway, also known as glycolysis, starts when the glucose is captured and transported from the extracellular space by specialized transporters (such as Glut1), to generate pyruvate and other products after a series of enzymatic

reactions. When glucose enters the cell, it is converted to glucose-6-phosphate (G6P) and degrades to glyceraldehyde-3-phosphate, which in turn is converted to pyruvate in the cytosol [6]. Pyruvate is internalized into the mitochondria and converted into acetyl-CoA, integrating the TCA cycle, which leads to the production of NADH and FADH2, co-factors in the electron transport chain (ETC), both molecules are important in ATP generation. Alternatively, in the cytosol, the enzyme lactate dehydrogenase can convert pyruvate to lactate, reoxidizing NADH to NAD^+ which is needed for glycolysis to continue (Palmer et al., 2015; Tanimine et al., 2019). In oxygen absence, glucose is preferentially converted to lactate rather than acetyl-CoA to enter the TCA cycle. The switch to glycolysis, even when oxygen is not a limitation, is seen in some processes known as aerobic glycolysis (fermentation) or the Warburg process, described by Otto Heinrich Warburg in which tumor cells tend to depend on glycolysis for ATP production than oxygen-dependent phosphorylation (L. Almeida et al., 2016; Patel & Powell, 2017).

Mitochondrial fatty acid oxidation (FAO) generates products such as acetyl-CoA, $NADH^+$ and FADH2 through two steps: "activation" and oxidation. First, the formation of an acyl-CoA fatty acid with the consumption of ATP and second, the β-oxidation for generation of acetyl-CoA, NADH and FADH2. These products enter the tricarboxylic acid cycle (TCA), where they can be used for the generation of ATP (O'Neill et al., 2016). In addition, cells need lipids to produce cell membranes and other necessary structures for cell growth and proliferation, in here, the fatty acid synthesis pathway (FAS) converts intermediate products of glycolysis and TCA into acetyl-coA, which it is used to generate lipids (Stincone et al., 2015). Inside the mitochondria, citrate is synthesized from acetyl-CoA and oxaloacetate, and exported to the cytosol where it is cleaved to produce acetyl-CoA and oxaloacetate, followed

by the transformation of cytosolic acetyl-CoA into palmitate. Palmitate or palmitic acid is the most common saturated fatty acid in the human body and important for the composition of membrane phospholipids, substrate for protein acylation, cholesterol and triacylglycerol synthesis in adipose tissue (L. Almeida et al., 2016; Palmer et al., 2015; Pearce & Pearce, 2013; Stincone et al., 2015; Weinberg et al., 2015).

In the context of transplantation, it has been described that blocking glycolysis and glutamine metabolism can prevent allograft rejection in skin and heart transplant models by suppressing effector T cells and generating allospecific Treg cells, suggesting that inhibition of metabolic pathways can exert cell selectivity related to metabolic processes (Lee et al., 2015). Another example of the complexity of the metabolic pathways is the fatty acid synthesis, which depends on the availability of intermediary products of glycolytic metabolism and TCA cycle and its relevant for the formation of cell membranes and other key lipid-based structures, necessary for proliferation (Inoki et al., 2003; Williams & O'Neill, 2018).

1.8. LIPIDS AS MODULATORS OF THE IMMUNE T CELL FUNCTIONS

Differentiation and effector functions of the adaptive immune system cells are linked to cellular metabolism. In this context, lipid metabolism controlling is crucial for the appropriate T cells functional differentiation and to the maintenance of immune tolerance.

Fatty acids (FA) are important dietary components which can act as intracellular and extracellular mediators, regulating physiological and pathological conditions (Pompeia et al., 2000). Cell and serum levels of FA are significantly increased under fasting conditions, hypoxia, obesity, exercise, type 1 and 2 diabetes and are

important energy sources, stored in the form of triglycerides. FA participates in the production of complex lipids such as cholesterol and incorporated into hormones and as cell signaling molecules (Howie et al., 2018). FA have been related to leukocyte function alterations, and depending on concentration and type, they can modulate both inflammatory and immune responses.

Functions of T cell subsets depend and are influenced by extra and intracellular FA content and changes on these parameters are related to memory establishment, polarization and survival. FA enter into T cells through two main mechanisms: first, entering to the cell by passive diffusion from the microenvironment and be incorporated into their membranes (Rossetti et al., 1997; Szamel et al., 1989), and second, being uptaken by controlled mechanisms mediated by transport proteins such as CD36, plasma membrane associated FABP, and FATPs. CD36 is a fatty acid translocase which imports long chain fatty acids (LCFAs) inside the cells and binds many ligands in addition to FAs including oxidized phospholipids (Podrez et al., 2002), oxidized low-density lipoprotein (LDL), native lipoproteins, and collagen (Calvo et al., 1998; Endemann et al., 1993; Nicholson et al., 1995; Tandon et al., 1989). Once inside the cell, FAs have different functions. In addition to acting as a fuel for mitochondrial respiration, they also act as molecular signals to control genic transcription to guarantee lipid homeostasis. Some molecules have been described as crucial for these processes, being the case of PPARs family members which are activated by lipophilic molecules and controlling genes mostly involved with lipid metabolism (Sonoda et al., 2008; Chawla et al., 2001; Zieleniak et al., 2008). Most FAs can act as activators or ligands for PPARs, but long-chain polyunsaturated fatty acyls (PUFAs) have been described as better ligands for PPARs (Echeverría et al., 2016).

PPAR family is composed of three subtypes; α, β/δ, and γ (Tyagi et al., 2011). All members are shown to play a role in T cell activation, differentiation into Th phenotypes and proliferation. Different studies demonstrated that PPARγ inhibits IL-2 production by T cells (Choi & Bothwell, 2012; Clark et al., 2000; Yang et al., 2000) and the induction of inflammation in models of colitis (Desreumaux et al., 2001; Su et al., 1999). In addition, PPARγ controls partially the Th17 or Treg differentiation (Barbi et al., 2013; Corral-Jara et al., 2021; Lee, 2018; Park & Pan, 2015).

Other important factors that are involved in lipid metabolism are the Sterol regulatory element-binding proteins (SREPB) which act as transcription factors that participate in the activation of necessary genes for FA synthesis and are also required for membrane synthesis during cell division (Horton et al., 2003). SREPB1 participates in *de novo* lipogenesis and SREBP2 acts as an activator of cholesterol synthesis and uptake (Jeon & Osborne, 2012).

1.9. EPIGENETIC REGULATORS AND IMMUNOMETABOLISM

Several epigenetic alterations in immune cells are frequently observed in metabolic-related conditions like obesity and type 2 diabetes mellitus, associated with phenotypic, functional, and migratory immune cell alterations. In these cells, the acquisition of effector or memory functions and proliferation capacity are accompanied by a dramatic metabolic change. Under homeostatic conditions, naive and quiescent T cells' metabolism depends mainly on FA for energy, and to a lesser extent on glucose. In contrast, activated and differentiated effector cells switch to aerobic glycolysis to fulfill their energy needs (L. Almeida et al., 2016). The nutrient availability also plays a crucial role in controlling the immune cell phenotype,

whereas malnutrition leads to an immunosuppressive state, and an a dietary or nutritional improvement restores immune functions (Alwarawrah et al., 2018; Gerriets et al., 2016; Hotamisligil et al., 1995; Mello et al., 2014; Wellen & Hotamisligil, 2005; Xu et al., 2017). The overabundance of substrates influences the phenotype of resident or infiltrating immune cells, in particular macrophages (Ganeshan & Chawla, 2014).

Emerging evidence has shown that preexisting epigenetic factors can respond to environmental stimuli by activating or repressing gene transcription (Tiffon, 2018). Epigenetic control of the immune response begins at the time of lineage compromise of hematopoietic stem cells, when naive immune cells are directed to differentiate through specific pathways by regulating lineage-specific gene repression or activation (Allis & Jenuwein, 2016; Busslinger & Tarakhovsky, 2014; Henning et al., 2018; Wang et al., 2017). DNA methylation, covalent modification of histones (acetylation and deacetylation) and expression of miRNA, are the most observed epigenetic signatures involved in the inflammatory processes.

1.10. HISTONE DEACETYLASES AS REGULATORS OF CELL METABOLISM AND IMMUNE RESPONSE

Histone deacetylases (HDACs) are biological molecules which remove the acetyl group from the lysine residues of histones resulting in chromatin compaction and transcription repression (Yuan et al., 2013). Sirtuins are, by definition, members of the Sir2 family, subdivided into seven sirtuins named 1–7 (Sirt1–7) and are characterized as a group of NAD^+ dependent HDACs (Kume & Maegawa, 2020; Kume et al., 2012).

Sirt1 is the most studied sirtuin, defined as a key molecule in cell metabolism and immune regulation. Sirtuins share a c75-central deacetylase domain, but their various N- and C-terminal domains specify their different physiological functions and subcellular localization (Michishita et al., 2005). Diverse studies have shown the role of Sirt1 in physiological and pathological conditions associated with aging, cancer, neurodegenerative diseases and metabolic diseases (Boutant & Cantó, 2013; H. C. Chang & L. Guarente, 2014; Herskovits & Guarente, 2014; Hubbard & Sinclair, 2014; Kane & Sinclair, 2018; Scisciola et al., 2020; Wan & Garg, 2021), however, its role as an immune system regulator has only been revealed recently.

1.10.1. Sirt1 in the general context of immunometabolism and inflammation

The nuclear sirtuins Sirt1, Sirt6 and the mitochondrial Sirt3 act as sensors of nutrient availability and changes in NAD^+ production or NAD/NADH ratio in macrophages and other tissue cells (Elibol & Kilic, 2018). Sirtuins act by reprogramming immunological, metabolic and bioenergetic pathways (Liu et al., 2015; Zhang et al., 2019). For instance, Sirt1 supports insulin secretion in pancreatic cells (Elibol & Kilic, 2018; Schug & Li, 2011), gluconeogenesis in hepatocytes, and FAO in macrophages (Gutierrez et al., 2009; Hernandez et al., 2014). Sirt1 also plays a role in the epigenetic reprogramming of immune cells altering histones and transcription factors such as NFκB and AP1 (Elibol & Kilic, 2018; Huang et al., 2017; Sung et al., 2021).

Several observations have been defined as relevant to the role of sirtuins in inflammation:

(1) The requirement of NAD^+ as a cofactor supports the role of sirtuins in detecting bioenergetic changes.

(2) Although sirtuin-dependent deacetylation activities have been mentioned as the main role of sirtuins in inflammation, other functions such as ADP-ribosylation by Sirt4 and the removal of succinyl, malonyl and glutamyl groups from lysine residues by Sirt5 may be also important factors in inflammation (Du et al., 2018; Kratz et al., 2021; Kumar & Lombard, 2018); and

(3) The effects of Sirt1 in inflammation can be a double-edged sword, in which reduction of Sirt1 levels increase the activity of NFκB RelA/p65 promoting the acute inflammation-related effects, and prolonged elevations in Sirt1 levels and activity during inflammation have been associated with immunosuppression and increased mortality (de Gregorio et al., 2020; Vachharajani et al., 2016; Yao et al., 2021).

Evidence shows that NAD^+ levels and Sirt1 transcription are reduced in specific tissues during chronic inflammation, such as the fat deposits in obesity, in the brain in Alzheimer's disease and in the vessels in atherosclerosis (Feng et al., 2013; Fujita & Yamashita, 2018; Serrano-Marco et al., 2012). Not unexpectedly, chronic inflammation is also accompanied by increased activation levels of the pro-inflammatory transcription factor NFkB RelA/p65 (Serrano-Marco et al., 2012). In this context, Sirt1 and Sirt6 deacetylate RelA/p65 and support its proteasomal degradation. A reduction in the levels or activity of Sirt1 increases the activity of NFkB RelA/p65 and amplifies the expression of pro-inflammatory molecules. Other evidence for Sirt1's role in chronic inflammation is that increasing NAD^+ levels (Imai & Guarente, 2016; Kane & Sinclair, 2018) or Sirt1 activation by pharmacological treatment with resveratrol reduces chronic inflammation and restore the balance between the metabolism and bioenergetic profile of immune cells (Iside et al., 2020).

Finally, some other observations regarding the role of Sirt1 in the control of the immune response have shown that the deleted in breast cancer-1 (DBC1) protein colocalizes and interacts with Sirt1, modulating its activity in multiple cell lines and tissues (Escande et al., 2010). Furthermore, DBC1-deficient mice were protected from HFD-induced liver steatosis and inflammation, despite the development of obesity (Escande et al., 2010).

1.10.2. **Sirt1 regulates T cell activation and differentiation**

Activation of T cells requires the cooperative interactions of several transcription factors, including AP-1, NF-κB and NFAT (Jinping Zhang et al., 2009). These transcription factors have been implicated in the regulation of transcription of genes relevant to differentiation of $CD4^+$ T cells. Murine activated T cells display higher levels of Sirt1 than naive T cells and these levels are related to activation state and differentiation (Gao et al., 2012; Gao et al., 2001). Sirt1-deficient $CD4^+$ T cells are hyperresponsive and can be activated by stimulation of T cell receptors (TCR) without CD28 co-activation. In mice immunized with OVA, Sirt1-deficient $CD4^+$ T cell proliferation as well as IL-2 production increased dramatically compared to naive T cells, suggesting that Sirt1 could act as a negative regulator of T cell activation (Kong et al., 2011). More recently, Sirt1 seems to negatively regulate the differentiation of IL-9-producing CD4+ T Cells (Wang et al., 2016).

Sirt1 may also modify other gene requirements for T cell activation and proliferation. For example, B-cell lymphoma-associated factor 1 (Bclaf1) identified primarily as an inducer of apoptosis (Haraguchi et al., 2004; Liu et al., 2007) has proven be a critical regulator in T cell activation (McPherson et al., 2009). The expression of Bclaf1 in Sirt1-deficient $CD4^+$ T cells after TCR/CD28 stimuli was

increased when compared to T cells from WT animals, suggesting Sirt1 as a negative regulator of Bclaf1 (Kong et al., 2011).

Upon TCR activation, $CD4^+$ T cells can differentiate into one of several Th cell lines, including Th1, Th2, Th9 and Th17 cells, as defined by the pattern of cytokine production and function (Butcher & Zhu, 2021; Kaiko et al., 2008; Zhu et al., 2010). Sirt1 may also have a potential role in Th differentiation modulation, as Sirt1 could suppress STAT3 activity by deacetylation (Hyung W. Lim et al., 2015; Wang & Green, 2012). Signaling through STAT3 triggered by IL-6 and IL-23 plays a crucial role in the development of Th17 cells (X. O. Yang et al., 2007).

Sirt1 also regulates $CD4^+$ T cell differentiation into Treg cells. The Foxp3 protein has a short half-life molecule and its acetylation prevents protein degradation and increases their levels. Sirt1, together with histone acetyltransferase p300, reciprocally regulates acetylation and, therefore, Foxp3 activity (van Loosdregt et al., 2011; van Loosdregt et al., 2010).

Moreover, inhibition of HDACs increases Foxp3 acetylation, which protects Foxp3 from protein degradation and increases its DNA binding and transcription activity (Beier et al., 2012; Liu et al., 2012; van Loosdregt et al., 2010; von Knethen et al., 2020). The specific deletion of Sirt1 in Treg cells or treatment with Sirt1 inhibitors promoted Foxp3 expression and increased the suppressor function of Treg cells, prolonging allograft survival (Beier et al., 2011).

1.11. ROLE OF SIRT1 IN THE CONTEXT OF TRANSPLANTATION

The importance of Treg cells in preventing allograft rejection provides a rationale for targeting Sirt1 in the context of transplantation (Miyajima et al., 2011). Kwon et al. (2012) reported a down-regulation of Treg activity by Sirt1 due to deacetylation

of three points in the foxP3 gene (Kwon et al., 2012). Strategies to inhibit Sirt1 can improve Treg activities and provide a therapeutic approach to treating allograft rejection. Sirt1 knockout mice resulted in increased FoxP3 expression, increased Treg suppressive activity and increased full mismatched MHC cardiac allograft survival, although effector T cell function was unaffected as determined by proliferation, activation or production of IL-2, IL-4, IL-17 or IFN-gamma (Kong et al., 2012; Tao & Hancock, 2008).

Although some studies have suggested that Sirt1 largely suppresses inflammation (Hu et al., 2013; Zwang & Leventhal, 2017), others demonstrate a clear pro-inflammatory role for Sirt1 in the context of Th17 effector cell differentiation. By deacetylating both RORγt and Foxp3, Sirt1 increases RORγt activity and reduces Foxp3 activity, promoting Th17 cell development at the expense of Tregs, and thus establishing favorable conditions for the development of autoimmunity and graft rejection (Levine et al., 2016; Rasha et al., 2020; van Loosdregt et al., 2010; J. Zhang et al., 2009). This suggests that Sirt1 inhibition may represent a potent therapeutic modality for the treatment of Th17 cell-mediated graft rejection (H. W. Lim et al., 2015).

Obesity has become a significant epidemic in recent decades. Regarding this topic, attempts have been made to explain the relationship among metabolic conditioning derived from obesity, $CD4^+$ T cell fate and function and the establishment and maintenance of meta-inflammation. Although an association between $CD4^+$ T cell immune activation and obesity has been described, the role of epigenetic regulators induced by Sirt1 on the alloimmune response and transplant rejection, under obesity conditions, remains unclear. Thus, we hypothesized that Sirt1, in the context of obesity, has an important role on $CD4^+$ Th cell polarization, not just from an

epigenetic point of view, but by a metabolic modulation of the immune response. These modifications could also be involved in the progression of transplant rejection.

2. OBJECTIVES

Main Objective:

To determine the role of Sirt1 in $CD4^+$ T cell activation and differentiation in a murine model of obesity and/or transplantation.

Specific objectives:

1. Evaluate the expression of Sirt1 and other interacting molecules related to $CD4^+$ T cell metabolism in the context of obesity and transplantation;
2. Characterize the metabolic parameters related to DIO in a murine model of conditional depletion of the Sirt1 in $CD4^+$ T cells;
3. Determine the role of Sirt1 in $CD4^+$ T cell activation and polarization under the metabolic conditioning derived from obesity and *in vitro* lipid conditioning; and
4. Identify key metabolic factors related to mitochondrial behavior in $CD4^+$ T cells in the context of obesity and transplantation in a murine model of $CD4^+$ T cells-depleted Sirt1.

3. METHODS

3.1. ANIMALS

All experimental procedures were performed according to Brazilian legislation approved by the Committee for Animal Use (CEUA IB/USP, number 9090200318). Experiments were performed on control male animals without the cre inserted: CD4cre$^{+/+}$ Sirt1 flox$^{+/+}$ (Sirt1$^{+/+}$), and in male animals carrying a Sirt1 conditional depletion in CD4 T cells: CD4cre$^{+/-}$ Sirt1 flox$^{-/-}$ (CD4-Sirt1$^{-/-}$), all on C57BL/6J background, and provided by the Institute vivarium at the University of São Paulo. Mice genotypes were confirmed according to the instructions provided by Jackson Laboratories. For transplantation, skin from male F1 animals (Balb/c x C57BL/6, H-2d/b) were used to evaluate the semi-allogeneic response. Mice were kept under a 12:12 light/dark cycle (800 – 1000 lux white LED light, ranging from 420 to 750 nm) at controlled temperature (22 ± 2°C) in SPF (specific pathogen free) conditions.

After completion of the obesity induction and transplantation procedures, animals were euthanized with overdose of anesthetics, and the death was assured by cervical dislocation. The organs, tissues and blood were harvested and immediately processed or stored at -80 ˚C as described below.

3.2. Diet-Induced Obesity (DIO)

To induce obesity in animals, 4 to 8 weeks old animals were subjected to a standard diet (20% lipids/kcal, SFD) or high-fat diet (60% lipids/kcal, HFD) *ad libitum*. HFD was prepared according to the following recipe: 3 g/Kg L-cystine, 2.5 g/Kg of choline bitartrate, 10 g/Kg of 10 g/Kg of vitamin mix, 35 g/Kg of mineral mix G, 100 g/Kg of sucrose, 50 g/Kg of microfine cellulose, 115.5 g/Kg of corn starch, 132 g/Kg of dextrinized starch, 200 g/Kg of casein (RhosterTM, São Paulo, Brazil), 315 g/Kg of

pork lard and 35 g/Kg of soybean oil. SFD and HFD were administered to animals for 12 weeks. After that, the establishment of obesity and metabolic syndrome was assessed by monitoring weight gain, as well as by performing GTT and ITT tests.

3.3. Mouse Weight and food Consumption

Mice weight values were expressed in grams. Food consumption was assessed by measuring the initial and every 3 to 4 days the weight of ration pellets and expressed in grams. All parameters were assessed every week at the same time of the day (from 10 to 11 a.m.).

3.4. Indirect calorimeter assessment

Lean and obese mice were normalized for body composition and acclimated for 24h before the 24-hour energy expenditure (EE) to be measured, which was obtained using an 8-chamber Oxymax FAST system (Columbus Instruments, Washington, USA) for the analysis of oxygen consumption (VO2), carbon dioxide production (VCO2) and the respiratory exchange rate (RER). The animals had the same amount of food (50 g), regardless of the type of food (HFD or SCD) and water ad libitum.

3.5. Blood withdrawal

Blood was obtained from the retro-orbital plexus with the use of heparinized micro-haematocrit tubes (Vitrex) in deep anesthesia (ketamine and xylazine). Blood was subsequently collected in tubes prefilled with EDTA (Kabe Labortechnik GmbH) for determination of differential blood counts or heparin (Sarstedt AG & Co) for determination of clinical chemistry parameters, respectively.

3.6. Hematological analyses of blood samples

Whole blood was immediately analyzed for complete blood count, i.e., red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), white blood cell (WBC) and platelets (PLT), using the fully automated BC-2800Vet Analyzer (Mindray, China). Briefly, 53 μL of blood were aspired into a needle, divided and distributed to the various chambers for sample analysis. Using the relationship between HCT HGB and erythrocyte count, it was possible to calculate the RBC indexes: mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC).

3.7. Biochemical tests

Serum levels of glucose, total cholesterol and triglycerides were measured via enzymatic colorimetric assay (Bioclin) following the manufacturer's instructions to verify the biochemical dynamics of animals after diet-induced obesity. Glucose and insulin tolerance tests were also performed. For the glucose tolerance test (GTT), we injected intraperitoneally, 1 g/Kg of glucose (diluted in sterile 1X PBS) according to the animal's body weight (Sigma-Aldrich®, Missouri, USA) after 12 hours of fasting. Blood glucose concentration was measured in animals at 0, 15, 30, 60, 90 and 120 minutes after glucose injection, using blood glucose strips (Accu-chek®, Basel, Switzerland). For the insulin resistance test (ITT), the animals received 1 U/Kg of regular human insulin (diluted in sterile PBS 1X) (Thermo Fisher ScientificTM, Massachusetts, USA) intraperitoneally, after 8 hours of fasting. The assessment of blood glucose was made in the same time intervals as for the GTT.

3.8. Nuclear magnetic resonance (NMR)

Lean and obese animals were scanned using the Bruker Minispec Live Mice Analyzer (model mq7.5, the "LF50") (Bruker Optics, Inc) to estimate the composition of total, fat and lean mass. On each test day, a check of internal tensions, temperature and quality control NMR parameters was performed using a standard provided by the manufacturer. Animals were placed in a clear plastic cylinder (50 mm in diameter) and held immobile by insertion of a plunger tight into the cylinder. The tube was then lowered into the instrument's sample chamber for approximately 2 minutes, the duration of the scanner.

3.9. Skin transplantation

F1 animals were used as skin transplant donors. The transplant was performed using an adaptation of the technique described by Larocca (Larocca et al., 2013) based on Markees (Markees et al., 1998). Donor mice were euthanized and, with the help of a scalpel, the skin on the back was removed. Removed skin was cut into 1 cm^2 pieces with the corners being curved. Recipient mice were anesthetized with 87.5 mg/Kg Ketamine and 12.5 mg/Kg Xylazine via i.p, and had their backs shaved and cleaned with 70 % ethanol. A "bed" for the dorsal graft was prepared by removing an area of the epidermis and dermis at the level of the intrinsic muscle, in the size of the graft. The grafts were placed on the "bed" covered with a dressing. Graft survival was monitored daily by visual touch assessment. The accepted graft was defined from the day the graft was incorporated into the mouse and the rejection day was defined when 100 % of the graft showed signs of necrosis.

3.10. Skin preparation and histological staining

After euthanasia, 1 cm^2 skin sample was removed from the transplanted animals. The material was preserved in 10 % formalin and fixed according to standard routine methods. The tissue was dehydrated through xylene and hydrated through ethanol series and transferred to xylene (3 × 15 minutes) and subsequently embedded in melted paraffin at 58 °C, for 24 hours at room temperature. Skin samples were allowed to harden in paraffin for two days and then cut into 8 μm sections on a rotary microtome. Serial sections from each specimen were obtained by microtomy and stained haematoxylin and eosin. Serial sections were qualitatively evaluated using a computer image acquisition system (Carl Zeiss microscope Axioscop 2 plus Axiocam camera).

3.11. Immunophenotyping

To assess the inflammatory infiltrate of the transplanted tissue and the inflammatory response, cells obtained from draining lymph nodes were stimulated with 50 ng/mL of phorbol myristate acetate (PMA) and 1.0 mg/mL of ionomycin (Sigma, St. Louis, MO, USA) in RPMI 1640 medium supplemented with 10 % fetal bovine serum and maintained at 37 °C with 5 % CO_2 for 1 hour and then cultured for a further 6 hours in the presence of 0.5 mg/mL brefeldin A (BFA, Sigma). After the stimulation process, cells were washed and labeled with the following fluorescent antibodies: CD3, CD4, CD25, FOXP3, IFNγ, IL-17 and IL-4, using BD fixation and permeabilization reagents and eBioscience following the indications manufacturers. Stimulated cells were collected and divided into two tubes. One tube was labeled with anti-CD3, anti-CD4, fixed and permeabilized with Cytofix/Cytoperm solution (Becton Dickinson, San Diego, USA), followed by labeling with anti-IL-17, anti-IFN-γ

and anti- IL-4 (Becton Dickinson, San Diego, USA). For Treg cell analysis, additional cells were labeled with permeabilized anti-CD3, anti-CD4, anti-CD25 mAb and fixed using the FoxP3 staining buffer set kit (eBioscience) according to the manufacturer's instructions. After permeabilization, cells were incubated with anti-FoxP3. A viability marker was included in the two marker panels to improve the definition of cell populations. Labeled cells were acquired on Facs Canto II® and LSR Fortessa® cytometers (Beckton Dickinson, California, USA). The acquired data were analyzed using FlowJo software (BD, USA).

3.12. Quantitative assay of HDAC activity

Histone acetylaces (HAT) and Histone deacetylaces (HDAC) activity was measured in $CD4^+$ T cells isolated from draining lymph nodes of lean and obese animals, using the Colorimetric Kit for determination of HDAC activity (BioVision, Mountain View, CA). Briefly, 8 days after skin transplantation, $CD4^+$ T cells isolated by sorting with magnetic particles (Miltenyi, USA) from draining lymph nodes were lysed using a RIPA buffer. Total protein was measured by the BCA Protein Assay Kit (Pierce, Rockford, IL) according to the manufacturer's instructions. All samples were incubated with HDAC substrate (Boc-Lys (Ac)-AMC). Deacetylation of the substrate sensitizes the substrate, and addition of lysine developer produces a signal which was measured using a plate reader. The standard curve was prepared according to the dilution range recommended by the kit protocol and the absolute HDAC activity was calculated based on the standard curve.

3.13. Analysis of metabolic molecular markers expression by qPCR

RNA extraction: (a) Analysis of gene transcript expression by real-time RT- PCR was initially performed via the processing of $CD4^+$ T cells in TRIzol (Invitrogen, USA). Briefly, RNA was extracted using incubation with chloroform and centrifugation at 1200 x g for 15 minutes in a refrigerated centrifuge (2-8 °C). Subsequently, RNA was precipitated by adding isopropanol following centrifugation at 12,000 x g for 10 minutes. The precipitated RNA was washed with a 75 % ethanol solution and centrifuged again at 10,500 x g. Subsequently, the precipitate was dried, resuspended in 50 µL of DEPC water and quantified in a Nanodrop device (Thermo-Scientific). RNA integrity was evaluated by placing total RNA in agarose gel to visualize the ribosomal RNA, particularly the 18 and 28S subunits. (b) cDNA synthesis: The mRNA was separated from the total RNA obtained through the use of Oligo-dT primers that annealed to the poly-A tail of the mRNA and the corresponding cDNA was obtained via reverse transcriptase reaction. (c) Real-time PCR: In order to amplify the gene transcripts, primers were used for: the housekeeping gene HPRT (hypoxanthine-guanine phosphoribosyltransferase) and for the molecules Sirtuinas 1 to 7 (Integrated DNA Technologies, USA). Each reaction was performed in triplicate and in each assay negative controls were used. The reaction was performed in a QuantStudio 12K Flex device (Applied Biosystem, UK). Primers used for the procedures are listed in table 1.

Table 1. Primer sequences used for measurement of the expression of metabolic-related factors by q-PCR.

Murine Primer	Sequence
Sirt1	F-5' AAACAGTGAGAAAATGCTGG
	R-5' GGTATTGATTACCCTCAAGC
Sirt2	F-5′ GAGCCGGACCGATTCAGAC
	R-5′ AGACGCTCCTTTTGGGAACC
Sirt3	F-5′ GGATTCGGATGGCGCTTGA
	R-5′ CACCTGTAACACTCCCGGAC
Sirt4	F-5′ GAGCATTCTTACTAGGGATTCCA
	R-5′ AACGGCTAAACAGTCGGGTT
Sirt5	F-5′ GCCACCGACAGATTCAGGTT
	R-5′ CCACAGGGCGGTTAAGAAGT
Sirt6	F-5′ CCAAATCGTCAGGTCAGGGA
	R-5′ CAGAGTGGGGTACAGGGATG
Sirt7	F-5′ CTAAGCGAAGCGGAGCCTAC
	R-5′ GTGGAGCCCATCACAGTTCT
Pgc1α	F-5' AAACTTGCTAGCGGTCCTCA

	R-5' TGGCTGGTGCCAGTAAGAG
Tfam	F-5' CCTTCGATTTTCCACAGAACA
	R-5' GCTCACAGCTTCTTTGTATGCTT
P21	F-5' CGAGAACGGTGGAACTTTGAC
	R-5' CAGGGCTCAGGTAGACCTTG
Glut4	F-5' AGAGTCTAAAGCGCCT
	R-5' CCGAGACCAACGTGAA
Pparγ	F-5’-TGTGGGGATAAAGCATCAGGC-3’
	R-5’-CCGGCAGTTAAGATCACACCTAT-3’
Hif1α	F-5’-ACCTTCATCGGAAACTCCAAAG-3’
	R-5’-CTGTTAGGCTGGGAAAAGTTAGG-3’
Srebp2	F-5' CCAAAGAAGGAGAGAGGCGG
	R-5' CGCCAGACTTGTGCATCTTG
Ucp2	F-5′ ACCAAGGGCTCAGAGCATGCA
	R-5′ TGGCTTTCAGGAGAGTATCTTTG
CD36	F-5' GATGTGGAACCCATAACTGGATTCAC

	R-5' GGTCCCAGTCTCATTTAGCCACAGTA

3.14. *In-vitro* Differentiation of Th and Treg cell populations

Cultivation and stimulation of cells for polarization to different Th cell phenotypes was performed using naive T cells from the spleen of wild C57BL/6j mice ($CD4^{+}CD44^{lo}CD62L^{hi}$) sorted in flow cytometry equipment "FACS ARIA II". In summary, these cells were subjected to activation by stimulation with anti-CD3 and anti-CD28 in the presence of Th1 (IL-2, IL-12), Th2 (IL-4), Th17 (TGF-β, IL-6,IL-23,) or Treg (IL-2, TGF-β), After 6 days in culture at 37 ˚C and 5 % CO_2, the cells were fixed, labeled with the respective antibodies described above for determination of lymphocyte polarization, including an anti-sirt1 monoclonal antibody (Clone 19A7AB4, Abcam, USA), and analyzed via flow cytometry.

3.15. Treatment of $CD4^{+}$ T cells with Sirt1 agonists and antagonists

The pharmacological compounds resveratrol (catalog: R5010-100MG, Sigma-Aldrich® Missouri, USA) and EX-527 (catalog: E7034-5MG, Sigma-Aldrich® Missouri, USA) were both diluted in DMSO and stored at -20 °C, as per manufacturer's instructions. Dose-response tests, varying time and concentration were performed to determine the dose that we would use in the work. After the previous tests, the concentration of 100 μM of resveratrol and the concentration of 20 μM were chosen for the other subsequent experiments, as they showed greater modulation of SIRT expression (increase with resveratrol and inhibition with EX-527) without harming cell viability.

3.16. Glucose Uptake Test

Cell populations obtained from draining lymph nodes of lean and obese animals were cultured in RPMI Medium without glucose, supplemented with 10% fetal bovine serum (FBS) and antibiotics. After 30 minutes of glucose restriction, cells were incubated with 150 μM of 2-NBDG in the presence of a viability marker and anti-CD3 and anti-CD4 fluorescent antibodies for 30 minutes. At the end of the incubation, cells were washed with PBS and acquired immediately using a FACS Canto II flow cytometer (BD Biosciences, USA).

3.17. Seahorse High Resolution Live Cell Respirometry

The oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) were recorded using a Seahorse XFe96 Analyzer (Agilent, USA). $CD4^+$ T cells from draining lymph nodes were freshly isolated using a magnetic separation technique (Myltenyi, USA) according to the instructions of the fabricant and resuspended in Agilent XF Assay Medium supplemented with 25 mM glucose, 1 mM sodium pyruvate, and 2 mM L-glutamine. Cells (0.4×10^6 cells/well) were then plated on Seahorse assay plates coated with 50μg/mL poly-D-lysine (Sigma Aldrich, USA) and let to rest at room temperature in the hood for 30 min. During the assay, cells were kept in the same medium and exposed to 1 μM olig9954omycin, 1.5 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), 100 nM rotenone and 1 μM antimycin A, purchased from Sigma-Aldrich, USA. Alternatively, a mix of phorbol myristate acetate (5 ng/mL, PMA) and ionomycin (1 μM), purchased from Sigma-Aldrich, USA, were used in the injections during the experiments.

3.18. Protein extraction and quantification

In order to extract the protein content of $CD4^+$ T cells from lean and obese mice, 500 µL of Radioimmunoprecipitation assay buffer (RIPA) (MerckTM, Darmstadt, Germany) with phosphatase and protease inhibitors (Roche®, Basel, Switzerland) was used for every $2x10^6$ cells. The RIPA buffer was composed of: 0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl, 2.5 %, deoxycholic acid, 10 % NP-40, 10 mM EDTA. After adding the solution to the pellet cells, they were incubated for 15 minutes on crushed ice (4 °C), for future quantification by the BCA kit following the manufacturer's instructions (Thermo Fischer ScientificTM, Massachusetts, USA) and storage in - 80 °C.

3.19. Statistical analysis

Body weight, food intake and flow cytometry were analyzed by Two-Way ANOVA followed by Bonferroni's post-test. For Seahorse data analysis, the area under the curve was calculated and the data were analyzed with One-Way ANOVA followed by Tukey for the comparison of more than two groups or with unpaired Student's t-test for two group comparisons. In all scenarios, $p<0.05$ was established to reject the null hypothesis. GraphPad Prism 7.0 was used for all statistical analyses (USA).

4. CONCLUSIONS

1. The experimental model of high-fat diet-induced obesity used in the project was effective in reproducing the metabolic profile described in obese patients with metabolic syndrome.
2. This obesity model accelerated graft rejection in an experimental model of allogeneic skin transplantation, however, Sirt1 depletion in $CD4^+$ T cells does not affect the transplant rejection.
3. Skin rejection in the context of obesity was accompanied by an inflammatory polarization in lymph nodes to Th1-Th17 profiles; these profiles were differentially expressed under Sirt1 depletion in $CD4^+$ T cells; and
4. Mitochondrial alterations in $CD4^+$ T cells, represented in Oxygen consumption ratios variations and increased superoxide production were observed under Sirt1 depletion and DIO.

5. CONCLUSIONES

El modelo experimental de obesidad inducida por dieta hiperlipídica usado en el Proyecto fue efectivo reproduciendo el perfil metabólico descrito en pacientes obesos con síndrome metabólico.

Este modelo de obesidad aceleró el rechazo del injerto en un modelo experimental de trasplante alogénico de piel, sin embargo, la depleción de Sirt1 en las células T $CD4^+$ no afectó este proceso de rechazo.

El rechazo del injerto de piel en el contexto de la obesidad estuvo acompañado por una polarización inflamatoria hacia perfiles Th1 y Th17 en los nódulos linfoides de los animales, esos perfiles presentaron una expresión diferencial como consecuencia de la depleción de Sirt1 en las células T $CD4^+$.

Alteraciones a nivel mitocondrial en las células T $CD4^+$, representadas en variaciones de consumo de oxígeno e incremento en la producción de superóxido fueron condiciones características de la depleción de Sirt1 en las células T $CD4^+$ y la obesidad inducida por la dieta.

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