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Cold Adaptation in Mice and Its Impact on Immunomodulatory Properties of Mesenchymal
Stem Cells

Chladová Adaptace u Myši a Její Vliv na Imunomodulační Mechanismy Mesenchymálních
Kmenových Buněk

Diploma thesis

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Declaration

I hereby declare that my thesis represents my original research work. Wherever the contribution of others is involved, every effort is made to indicate this clearly, including reference to the literature. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma.

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Prohlášení

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Abstract

Adaptation to cold ambient temperatures is accompanied by local changes in the adipose tissue microenvironment. Besides impacting stromal cells within the adipose tissue, cold shapes the adipose tissue immune compartment. Previous research showed that acute cold exposure is associated with stress response, whereas prolonged exposure is associated with an anti-inflammatory setting, contributing to adaptation to the cold environment. We believe that cold-induced changes in the adipose tissue immune system might impact the properties of adipose tissue-derived mesenchymal stem cells (MSCs). Understanding these alterations could provide a novel strategy for preconditioning MSCs to improve their immunomodulatory potential. Thus, we focused on alterations in the metabolism and immunomodulatory function of MSCs isolated from cold-exposed mice. We observed that cold exposure resulted in a different MSC metabolic state and the use of glycolytic and oxidative metabolism. We noticed a significant upregulation of nitric oxide production in MSCs isolated from mice adapted to cold temperatures, pointing to an augmented immunomodulatory potential of those cells. The immunomodulatory capacity was, however, not confirmed in co-culture experiments with splenocytes.

Another aim of this project was to examine the effect of culturing MSCs at reduced temperature, as alterations in the oxidative stress and metabolism in MSCs cultured at reduced temperature were reported. We showed that MSCs cultured at reduced temperature exhibit increased metabolic parameters compared to MSCs cultured at standard conditions. Reduced temperature did not change their immunomodulatory potential.

Overall, we showed that MSCs respond to cold-induced changes in the adipose tissue microenvironment and *in vitro* culture at low temperatures. These conditions influenced MSC metabolism, but not their immunomodulatory function, at least in our setting.

Key words: cold adaptation, adipose tissue, mesenchymal stem cells, metabolism, immunomodulation

Abstrakt

Adaptace na nízké okolní teploty je doprovázena lokálními změnami v mikroprostředí tukové tkáně. Kromě vlivu na stromální buňky ovlivňuje chlad i imunitní buňky tukové tkáně. Předchozí výzkumy ukázaly, že akutní vystavení chladu je spojeno se stresovou reakcí, zatímco dlouhodobé vystavení chladu je spojeno s protizánětlivým nastavením, což přispívá k adaptaci na chladné prostředí. Domníváme se, že změny v imunitním systému tukové tkáně vyvolané chladem mohou mít vliv na vlastnosti mezenchymálních kmenových buněk (MSC) izolovaných z tukové tkáně. Pochopení těchto změn by mohlo poskytnout nové strategie pro stimulaci MSC s cílem zlepšit jejich imunomodulační potenciál. Zaměřili jsme se na změny v metabolismu a imunomodulační funkci MSC izolovaných z myši vystavených chladu. Pozorovali jsme, že vystavení chladu způsobilo změny v metabolismu MSC a rozdílnému využívání glykolytického a oxidačního metabolismu. U MSC izolovaných z myši adaptovaných na nízké teploty jsme zaznamenali výraznou produkci oxidu dusnatého, což poukazuje na zvýšený imunomodulační potenciál těchto buněk. Imunomodulační schopnost však nebyla potvrzena v kokultivačních experimentech se splenocyty.

Dalším cílem tohoto projektu bylo prozkoumat účinek kultivace MSC při snížené teplotě, jelikož dřívější publikace zaznamenaly změny v oxidačním stresu a metabolismu u MSC kultivovaných při snížené teplotě. Ukázali jsme, že MSC kultivované při snížené teplotě vykazují zvýšené metabolické parametry ve srovnání s MSC kultivovanými za standardních podmínek. Snížená teplota však nezměnila jejich imunomodulační potenciál.

Celkově jsme prokázali, že MSC reagují na změny v mikroprostředí tukové tkáně vyvolané chladem a na kultivaci *in vitro* při nízkých teplotách. Tyto podmínky ovlivnily metabolismus MSC, nikoliv však jejich imunomodulační funkci, alespoň při našem nastavení.

Klíčová slova: chladová adaptace, tuková tkáň, mezenchymální kmenové buňky, metabolismus, imunomodulace

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List of abbreviations

ASC	adipose tissue-derived mesenchymal stem cell
asWAT	anterior subcutaneous white adipose tissue
BAT	brown adipose tissue
CCL	C-C motif chemokine ligand
CD	cluster of differentiation
CXCL/R	C-X-C motif chemokine ligand/receptor
DC	dendritic cell
DMEM	Dulbecco's modified eagle's medium
ECAR	extracellular acidification rate
ETC	electron transport chain
EV	extracellular vesicles
FGF21	fibroblast growth factor 21
FOXP3	forkhead box protein 3
GLUT	glucose transporter
GvHD	graft versus host disease
HLA	human leukocyte antigen
HIF1 α	hypoxia induced factor 1- α
ICAM1	intercellular adhesion molecule 1
IDO	indolamine-2,3-dioxygenase
IL	interleukin
ILC2	innate lymphoid cells group 2
IFN	interferon
iNKT	invariant natural killer T cells
iNOS	inducible nitric oxide synthase
iWAT	inguinal white adipose tissue
LPS	lipopolysaccharide
M1 macrophages	classically activated macrophages
M2 macrophages	alternatively activated macrophages
MHCII	major histocompatibility complex class II

MSC	mesenchymal stem/stromal cells
mTOR	mammalian target of rapamycin
NE	norepinephrine
NK	natural killer cells
NO	nitric oxide
NST	non-shivering thermogenesis
OCR	oxygen consumption rate
PBS	phosphate saline buffer
pWAT	perigonadal white adipose tissue
PGE ₂	prostaglandin E2
RPMI	Roswell Memorial Park Institute 1640 medium
ROS	reactive oxygen species
ROR γ t	retinoic acid-related orphan receptor c
RT	room temperature
STAT	signal transducer and activator of transcription
sWAT	subcutaneous white adipose tissue
TCA cycle	tricarboxylic acid cycle
TGF β	transforming growth factor β
Th	helper T cells
TLR	Toll-like receptor
TNF	tumor necrosis factor
Treg	regulatory T cell
TRP (M8)	transient potential receptor (melastatin 8)
TSG-6	tumor necrosis factor-stimulated gene 6
UCP	uncoupling protein
VEGF	vascular endothelial growth factor
vWAT	visceral white adipose tissue
WAT	white adipose tissue
WST	water-soluble formazan

1. Introduction

Exposure to cold ambient temperatures triggers physiological changes in endothermic animals to maintain the core body temperature. In small endothermic animals, such as mice, local changes in the adipose tissue are of great importance. Cold-induced adrenergic signalization leads to the activation of thermogenic adipocytes, which upregulate uncoupling protein 1 (UCP1) expression. Through the action of this protein, they uncouple the proton gradient from ATP production and generate heat. Recently, it was suggested that immune cells, both resident and infiltrating into the adipose tissue upon cold exposure, are largely involved in regulating heat generation by thermogenic adipocytes and, therefore, in adaptation to cold environments. Immune cells, mainly associated with type 2 immune response and unconventional lymphocytes, can regulate adipocytes, neurons, and other immune cell populations by producing soluble factors.

These cold-induced changes in adipose tissue composition and function raise important questions about how they might affect other cell populations within this tissue. Mesenchymal stem cells (MSC) represent one such population. MSCs are multipotent cells that maintain tissue homeostasis and regeneration, including in the adipose tissue. Beyond their regenerative capacity, MSCs possess potent immunomodulatory properties. They can suppress inflammatory immune cells and promote anti-inflammatory responses by producing various immunomodulatory molecules, extracellular vesicles (EV), or through direct cell-to-cell contact. These properties have made MSCs attractive candidates for stem-cell-based therapies targeting tissue injuries and inflammatory or autoimmune diseases. However, their clinical application is still limited, mainly due to low survival and functionality after transplantation, arising from different conditions during MSC cultivation and the *in vivo* microenvironment. Several preconditioning strategies were developed to support the survival of MSCs after transplantation and boost their immunomodulatory activity.

Due to its accessibility, MSCs are often isolated from adipose tissue for research and transplantation purposes. No studies have addressed the potential impact of cold-induced changes in adipose tissue on adipose tissue-derived MSCs. The cold-induced changes in the cellular composition and soluble factor production within the adipose tissue microenvironment likely affect resident MSCs. Mimicking the conditions in adipose tissue during cold acclimation might represent a potential preconditioning strategy to improve MSC cellular and immunomodulatory properties.

2. Literature overview

2.1. Cold exposure

Exposure to cold ambient temperatures triggers a complex physiological response to maintain a stable core body temperature in endothermic organisms. The cold stimulus is sensed through thermoreceptors expressed in the periphery, such as the transient receptor potential (TRP) melastatin 8 (M8) channel or the recently discovered glutamate receptor of the kainate family GluK2 (Bautista et al., 2007; Cai et al., 2024). Afferent neurons transmit the information to the preoptic area of the hypothalamus, leading to the activation of a physiological response to cold. The main purpose of this circuit is to trigger cold-avoidance behavior, reduce heat loss, and increase heat production, aiming to prevent a drop in core body temperature. Mechanisms preventing excessive heat loss include cutaneous vasoconstriction, which reduces blood flow to the skin and minimizes heat transfer to the environment. Heat generation is achieved through thermogenesis. We discriminate between two types of thermogenesis – shivering and non-shivering. Activation of shivering thermogenesis leads to increased muscle tone and frequency of muscle contractions that generate heat. Non-shivering thermogenesis (NST) is based on the activation of brown adipose tissue (BAT), composed of thermogenic adipocytes able to generate heat through the expression of uncoupling protein 1 (UCP1). UCP1 then acts by uncoupling the electron transport chain (ETC) from ATP production, releasing energy as heat instead of ATP generation (reviewed in Mota-Rojas et al., 2021).

It is important to note that mice and humans have significantly different mechanisms of heat generation. While humans rely mainly on shivering thermogenesis, mice employ NST as their primary thermoregulatory strategy. There is a negative correlation between the surface-to-volume ratio and NST activity, explaining the species-specific differences in metabolic activity and adaptive mechanisms to cold exposure. Due to their higher surface-to-volume ratio, mice experience higher heat dissipation and consequently require more robust thermogenic mechanisms than humans (reviewed in Bastías-Pérez et al., 2020).

2.1.1. Adipose tissue and non-shivering thermogenesis

Adipose tissue is composed of adipocytes, among other cells. We distinguish between 3 types of adipocytes based on their morphology and function (see Fig.1). White adipocytes contain one large lipid droplet, have lower mitochondrial content, and their primary function is storing energy in the form of triglycerides. Brown adipocytes contain large numbers of small lipid droplets, have high mitochondrial content with elevated expression of UCP1, and are involved in the adaptive mechanisms to cold temperatures. White adipocytes constitute white adipose tissue (WAT) depots located either under the skin, subcutaneous WAT (sWAT), or inside the body cavities, visceral WAT (vWAT). Brown adipocytes, on the other hand, compose BAT, which is in rodents located mainly in the interscapular region (reviewed in Cohen & Kajimura, 2021). These two adipocyte subtypes also arise from different precursors (Seale et al., 2008; Shamsi et al., 2021). Beige adipocytes represent an intermediate phenotype. Their origin is still

debatable; it is believed that beige adipocytes can arise from progenitors mobilized upon cold exposure or transdifferentiate from white adipocytes. Following stimulation with cold temperature, white adipocytes are transformed into beige phenotype in a process termed “beiging”, upregulate UCP1 content, and contribute to NST (reviewed in Cohen & Kajimura, 2021). Beige adipocytes can be found within WAT, and it was suggested that sWAT has a higher potential for the generation of thermogenic adipocytes than vWAT (Wu et al., 2012).

The induction of NST depends mainly on the activation of the sympathetic nervous system following cold exposure. Sympathetic neurons innervating BAT release norepinephrine (NE), activating β 3-adrenergic receptors expressed on adipocytes and triggering adenylate cyclase and protein kinase A signaling pathway. Activation of this pathway causes the mobilization of fatty acids from adipocyte lipid droplets, which then activate UCP1. UCP1 transports protons generated by the activity of the ETC from the intermembrane space to the mitochondrial matrix, thereby bypassing ATP synthase and dissipating the energy in the form of heat (reviewed in Cannon & Nedergaard, 2004) (see Fig.1).

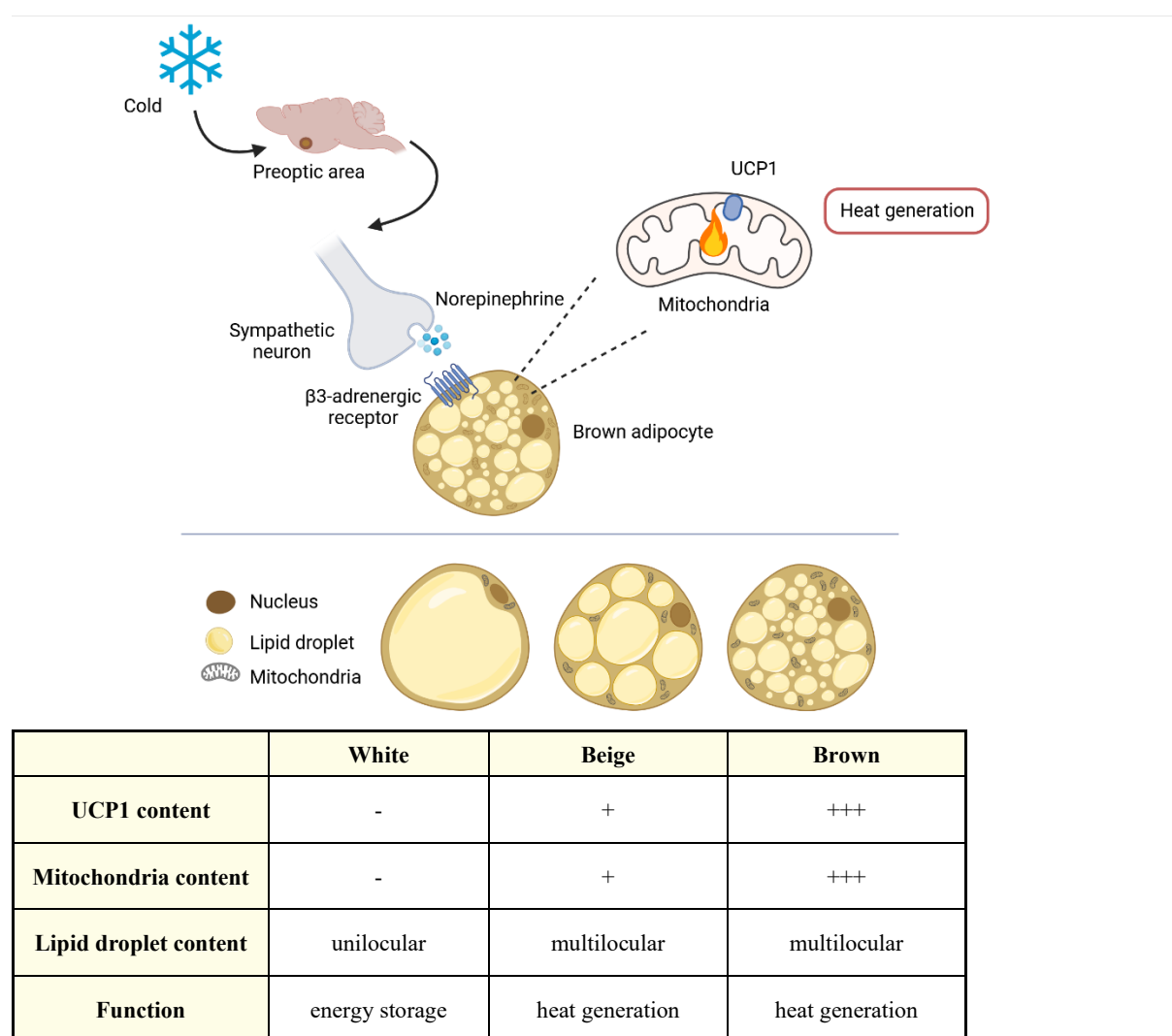


Figure 1. The noradrenergic stimulation of NST and different types of adipocytes with their principal characteristics. Cold stimulus is transduced into the preoptic area of the hypothalamus and through the sympathetic neurons into the thermogenic adipose tissue. Norepinephrine released by sympathetic neurons, activates β 3-adrenergic receptors and subsequent signalization increases UCP1 expression and function, thus generating heat. UCP1...uncoupling protein 1. Taken and adapted from Cohen and Kajimura (review by Cohen & Kajimura, 2021). Created in Biorender.com.

Cold exposure and NST have recently become of great interest due to their impact on the metabolic state in both mice and humans. Research is focused on developing strategies for targeting thermogenesis to ameliorate obesity and its associated metabolic syndrome and type 2 diabetes (reviewed in Bastías-Pérez et al., 2020). Pharmacological approaches aiming to activate thermogenesis, expand thermogenic adipose tissue, and increase energy expenditure have shown efficacy in mouse models but also in humans. For example, glucagon-like peptide 1 analogs induced interleukin (IL) 6-mediated UCP1 expression in adipose tissue and increased core body temperature during cold challenge (Gutierrez et al., 2022). Likewise, treatment with mirabegron, a β 3-adrenergic receptor agonist, increased BAT activity (Cypess et al., 2015) and glucose tolerance in obese individuals (Finlin et al., 2020).

2.1.2. Cold exposure and the immune system

There is little evidence regarding the impact of cold exposure on the immune system. Prolonged or repeated cold exposure has been suggested to benefit the immune system. In mice, it was shown that long-term cold exposure limits the expression of major histocompatibility complex class II (MHCII) on monocytes and the production of inflammatory effector cytokines by T cells during experimental autoimmune encephalomyelitis (Spiljar et al., 2021). Rats exposed 10 days to cold temperature exhibited reduced production of type 1 and type 2 immune response-related cytokines in serum, suggesting a systemic immunomodulatory effect (Marvanova et al., 2023). Previous research in our laboratory also showed that cold-acclimated rats exhibit an elevated abundance of $\gamma\delta$ T cells in several tissues. A similar trend was observed in humans practicing regular swimming in cold water compared to the unacclimated group (Vasek et al., 2024). Mice housed at sub-optimal temperature, meaning temperature below their thermoneutral zone of around 30°C, caused hypothermia upon infection with *Escherichia coli*, associated with improved infection tolerance and lower mortality rate (Ganeshan et al., 2019).

Conversely, housing mice suffering from asthma at sub-optimal temperatures increased inflammatory cell infiltration in the lungs and skewed T cells toward type 2 helper T cells (Th2) (Liao et al., 2017). Apart from allergic diseases, cold temperatures also compromised the anti-viral response of nasal epithelial cells by reducing their production of EVs (D. Huang et al., 2023).

Cold exposure triggers physiological responses, enabling endothermic animals to deal with altered environmental conditions. These responses include alterations in the immune profile, changing the dynamic and intensity of immune responses. Moreover, cold exposure and acclimation to the cold environment significantly affect the local immune system, particularly in the adipose tissue, the site of adaptive NST. The following section is devoted to the cold-induced changes in the adipose-tissue immune compartment.

2.1.3. Cold exposure shapes the adipose tissue immune system

Adipose tissue undergoes significant morphological and physiological changes following exposure to cold temperatures, as illustrated above. Literature suggests that cold exposure changes the abundance, infiltration, function, and regulation of immune cells. Moreover, these cells can directly or indirectly contribute to acclimation to the cold environment by producing a wide range of effector molecules (reviewed in Agueda-Oyarzabal & Emanuelli, 2022). These changes are unique for specific adipose tissue depots, as different adipose tissue depots exhibit different response dynamics after cold stimulation (Chang et al., 2019; Kohlgruber et al., 2018; Vasek et al., 2024; Ye et al., 2024).

Generally, the immune system in thermogenic adipose tissue after prolonged cold exposure is skewed toward an anti-inflammatory and type 2 immune response. Eosinophils, monocytes, macrophages, regulatory T cells (Treg), $\gamma\delta$ T cells, invariant natural killer T cells (iNKT), or type 2 innate lymphoid cells (ILC2) were proposed to play a role in the process of NST (reviewed in Agueda-Oyarzabal & Emanuelli, 2022).

Eosinophils infiltrate sWAT upon cold exposure and contribute to the elevated levels of Th2 cytokines (IL-4, IL-13), thereby regulating other type 2 immune cells within the adipose tissue and contributing to beiging of sWAT (Qiu et al., 2014). Fibroblast growth factor 21 (FGF21) and C-C motif chemokine ligand (CCL) 11 derived from adipocytes, CCL22 secreted by M2 macrophages, and IL-5 secreted by ILC2s participate in the recruitment of eosinophils into the adipose tissue (Z. Huang et al., 2017; Molofsky et al., 2013; Yuan et al., 2024). Eosinophil-secreted IL-4 and IL-13 drive the alternative polarization of macrophages after cold exposure (Qiu et al., 2014). Alternatively activated macrophages (M2) were shown to upregulate the expression of tyrosine hydroxylase, leading to increased production of catecholamines, specifically NE, which in turn induces beiging of sWAT (Qiu et al., 2014). This view was later challenged by Fisher et al., who did not observe any attenuation of thermogenesis when the expression of tyrosine hydroxylase, an enzyme of catecholamine synthesis, was depleted in hematopoietic cells, including macrophages. Moreover, IL-4-stimulated macrophages did not induce a thermogenic program in primary adipocytes, challenging the importance of IL-4 and M2 macrophages in the adaptation to cold (Fischer et al., 2017). Conversely, a population of sympathetic neuron-associated macrophages in BAT can uptake released NE from sympathetic neurons and metabolize it, resulting in impaired thermogenesis and ability to adapt to a cold environment (Pirzgalska et al., 2017).

Macrophages modulate the innervation of thermogenic adipose depots besides NE regulation (Wolf et al., 2017; Yadav et al., 2024). For example, M2 macrophages secrete Slit3 cytokine, which increases sWAT sympathetic innervation (Y. N. Wang et al., 2021). A specific population of macrophages termed cholinergic adipose macrophages secrete acetylcholine, which participates in the beiging of sWAT (Knights et al., 2021). Apart from the impact on tissue innervation, macrophages modulate the infiltration of other effector cells (Yuan et al., 2024) or may directly contribute to the induction of thermogenesis (D. Lee et al., 2021). Similarly, in humans exposed to cold, adipose tissue is enriched for M2 macrophages expressing cluster of differentiation (CD) 163 or CD206. CD163⁺ macrophages were even shown to express UCP1, but the authors of this study did not identify any direct effect of macrophages on thermogenesis induction (Finlin et al., 2021).

To sustain the macrophage population after cold exposure, monocyte infiltration into adipose tissue is enhanced (Qiu et al., 2014; Ye et al., 2024). Monocytes also promote the expansion of BAT directly (Gallerand et al., 2021).

ILC2s serve as central regulators of thermogenic activation. By secreting IL-5 and IL-13, they regulate eosinophil and M2 macrophage recruitment into adipose tissue (Brestoff et al., 2015; Molofsky et al., 2013). The concerted action of eosinophils and ILC2s results in the proliferation of adipocyte progenitors and their commitment to beige phenotype (M. W. Lee et al., 2015). Further, eosinophils activated in response to ILC2-derived IL-5 produce nerve growth factor, thus supporting sympathetic innervation of WAT (Meng et al., 2022). It was suggested that ILC2s also regulate thermogenesis independently of eosinophils and M2 macrophages via producing methionine-enkephalin peptide, which directly elevates the expression of UCP1 in adipocytes (Brestoff et al., 2015).

iNKT cells promote the cold adaptation process by enhancing the expression of FGF21 in adipocytes and subsequently driving UCP1 expression (Lynch et al., 2016). Another unconventional lymphocytes involved in the induction of thermogenic response are $\gamma\delta$ T cells. After cold exposure, they exhibit increased abundance in adipose tissue and produce IL-17A, which appears to be a key factor for upregulating adipocyte thermogenic gene expression (Kohlgruber et al., 2018). IL-17F, also produced by $\gamma\delta$ T cells, acts on BAT adipocytes to induce transforming growth factor (TGF) β expression, leading to enhanced sympathetic innervation of the tissue, thereby contributing to thermogenesis (Hu et al., 2020).

Finally, conventional T cells are crucial contributors to the regulation of thermogenic activity. BAT of cold-exposed mice contains a transcriptionally unique population of Tregs, which restrain the inflammation within BAT and support BAT activation after cold stimulation (Medrikova et al., 2015). Adoptive transfer of Tregs promotes beiging of sWAT by enhancing the expression of thermogenic genes in adipocytes and supporting M2 macrophage population (Fang et al., 2020). A role for signal transducer and activator of transcription (STAT) 6 signaling was proposed to maintain the Treg population in BAT and sWAT (Kälin et al., 2017). iNKT and $\gamma\delta$ T cells were demonstrated to regulate Treg population in thermogenic adipose tissue by producing IL-2 and IL-17A, respectively (Kohlgruber et al., 2018; Lynch et al., 2014). Th2 cells

might be involved in cold adaptation, as increased infiltration of Th2 cells was observed in a model of accelerated thermogenic response (Kikuchi et al., 2019). Furthermore, the increased abundance of naïve T cells in BAT supports the role of T cells during the response to a cold environment (Ye et al., 2024).

2.1.4. Cold-induced changes in the secretion profile of adipose tissue

From the earlier stated observations, it is evident that soluble factors produced by immune and stromal cells play an essential role in mediating adipose tissue thermogenesis. Cytokines associated with type 2 immune response (IL-4, IL-5, and IL-13) appear indispensable for activating the thermogenic activity of sWAT (Molofsky et al., 2013; Qiu et al., 2014). Other cytokines involved in the induction of UCP1 expression and thermogenesis activation are cytokines of the IL-17 family, particularly IL-17A and IL-17F (Hu et al., 2020; Kohlgruber et al., 2018). IL-33 produced by stromal cells maintains Treg cells (Kohlgruber et al., 2018) and ILC2s within adipose tissue (Molofsky et al., 2013). IL-27 has been reported to drive UCP1 expression in white adipocytes, alleviating metabolic disease associated with obesity (Q. Wang et al., 2021). IL-6 is involved in regulating body temperature during cold exposure, and a role for IL-6 in regulating UCP1 expression and beiging was proposed (Knudsen et al., 2014; Kristóf et al., 2019). In contrast, IL-10 serves as a negative regulator of NST, as IL-10-deficient mice exhibited a marked increase in thermogenic gene expression (Rajbhandari et al., 2017).

The production of soluble factors depends on the specific adipose tissue, as well as the duration of cold exposure. Previous research in our laboratory, performed on rats, suggested that short-term (1 day) cold exposure triggers a stress response in the adipose tissue associated with increased levels of pro-inflammatory (IL-1 β , IL-6, IL-17, tumor necrosis factor- α (TNF α)), Th2-related (IL-4), and anti-inflammatory (IL-10) cytokines, while long-term cold, leading to acclimation (14 days), results in cytokine levels similar to control. This effect was more pronounced in WAT than in BAT, where the cytokine levels were higher overall (Vasek et al., 2024). One exception to this observation was the levels of IL-17A and IL-17F in BAT, which significantly increased after 14 days of cold exposure, confirming their role in BAT thermogenesis (Vasek et al., 2024), as suggested by others (Hu et al., 2020; Kohlgruber et al., 2018).

Growth factors were suggested to be involved in the induction of WAT beiging and BAT activation. FGF21, as mentioned earlier, is produced after cold exposure by adipocytes in sWAT and BAT, which appears essential for thermogenic activation (Z. Huang et al., 2017). Moreover, iNKT cell-derived FGF21 was proposed to induce sWAT beiging (Lynch et al., 2016). TGF β production by brown adipocytes is induced by $\gamma\delta$ T cell-derived IL-17F and plays an important role in the innervation of BAT (Hu et al., 2020). To the superfamily of TGF β also belongs the growth and differentiation factor 15, which is elevated after cold challenge and modulates classically activated macrophages (M1) (Campderrós et al., 2019). Cold also upregulated the expression of vascular endothelial growth factor (VEGF), corresponding with increased vascularization of BAT and sWAT and expression of thermogenic genes in cold-exposed mice

(Xue et al., 2009). Moreover, levels of insulin-like growth factor 1 within BAT are raised during the first days of cold exposure, and the lack of its receptor results in impaired thermogenic activation (Duchamp et al., 1997; Viana-Huete et al., 2016).

Apart from cytokines, adipokines produced by adipocytes in WAT and BAT regulate the thermogenic process. BAT-secreted C-X-C motif chemokine ligand (CXCL) 14 supports the infiltration of M2 macrophages and BAT activation (Cereijo et al., 2018). Another factor secreted by adipocytes upon exposure to cold is adiponectin. Adiponectin expression is upregulated in chronic cold and supports beiging of sWAT by promoting the proliferation of M2 macrophages through interaction with T cadherin on the surface of macrophages (Hui et al., 2015). However, the role of adiponectin in regulating BAT thermogenesis is still debated (Hui et al., 2015; Qiao et al., 2014). After cold exposure, adipocytes secrete a lot of other factors, including meteorin-like hormone (Rao et al., 2014), 12.13-dihydroxy-9Z-octadecenoic acid (Lynes et al., 2017), or prostaglandin E2 (PGE₂) (García-Alonso et al., 2013), all of which are involved in mediating thermogenic activation of adipocytes.

The thermogenic capacity of adipose tissue is heavily dependent on its sympathetic innervation (Hu et al., 2020; Jiang et al., 2017). Therefore, the production of neural modulators is critical for cold adaptation. Cells in the adipose tissue secrete many factors facilitating sympathetic innervation after prolonged cold challenge, such as neuregulin 4 (Henriques et al., 2020), neurotrophic factor 3 (Cui et al., 2021), TGFβ (Hu et al., 2020), or brain-derived neurotrophic factor (Blaszkiewicz et al., 2020).

This section demonstrated that cold exposure triggers substantial changes in adipose tissue, significantly influencing stromal, neural, and immune cells beyond the adipose tissue.

2.2. Mesenchymal stem cells

Mesenchymal stem cells (MSC), also called mesenchymal stromal cells, are multipotent cells with high migratory, proliferative, and differentiation potential. They can be found in almost every tissue, where they maintain tissue homeostasis and regeneration. Thanks to their properties, MSCs became of great interest in transplantation research. They can be isolated from many tissues, most easily from bone marrow, adipose tissue, dental tissue, synovial fluid, or umbilical cord. Generally, to define human MSCs, three minimal criteria must be met. The first criterion is adherence to plastic when cultured *in vitro*. The second is the expression of specific surface markers, such as CD73, CD105, or CD90, as well as the lack of expression of markers of cells that are most likely to contaminate MSC cultures, including CD45, CD34, CD11b, CD19, or human leukocyte antigen (HLA) DR. The last characteristic of MSCs is their differentiation potential into adipocytes, chondrocytes, and osteocytes (Dominici et al., 2006). However, the criteria listed above are not universally applicable, as MSCs isolated from different tissues may possess distinct characteristics, and the same holds for MSCs of different donor species (Dominici et al., 2006; Sung et al., 2008; Xu et al., 2017). MSCs isolated from diverse tissues may also differ in their functional potential. Adipose tissue-derived MSCs (ASC) exhibit higher immunomodulatory activity than bone marrow-derived MSCs (Melief et al., 2013). On the other hand, ASC-derived exosomes show decreased immunosuppressive potential but increased angiogenic potential compared to bone marrow-derived MSC exosomes (Y. Liu et al., 2024). Umbilical cord-derived MSCs have an increased content of proteins involved in tissue remodeling (Z. Gang Wang et al., 2020).

MSCs play essential roles in wound healing, tissue regeneration, angiogenesis, and immunomodulation. Their mode of action is mainly through the paracrine secretion of cytokines, chemokines, or growth factors. Another possibility is direct cell-to-cell contact through the expression of inhibitory and adhesive molecules (reviewed in G. Yang et al., 2023). MSCs can influence many cellular processes by transferring mitochondria to other cells (reviewed in Malekpour et al., 2023), as also shown in our laboratory (Porubska et al., 2021). Lastly, MSCs produce exosomes packed with immunomodulatory factors, membrane-bound molecules, and regulatory microRNA (reviewed in Hassanzadeh et al., 2021).

MSCs have demonstrated efficacy in promoting wound healing and tissue regeneration in many animal models of tissue damage. They promote the wound-healing process by secretion of paracrine factors involved in immune recruitment and polarization (Whelan et al., 2020), regulation of the inflammatory immune response (L. Liu et al., 2014), re-epithelialization (R. Li et al., 2025; Ramos-Gonzalez et al., 2020), angiogenesis (Cao et al., 2024), or reduction of scar formation (R. Li et al., 2025; Millán-Rivero et al., 2019). MSCs are involved in neovascularization following tissue injury. ASC-derived exosomes enhance the proliferation of human umbilical vein endothelial cells and tube formation *in vitro*. (Heo & Kim, 2022). Also, MSCs overexpressing VEGF showed effective blood flow restoration in a mouse model of limb ischemia (Beegle et al., 2016). Another feature of MSCs is their anti-fibrotic effect (Chu et al., 2023; C. Y. Yang et al., 2021). The application of MSCs, or MSC-derived exosomes,

mitigates liver fibrosis by inhibiting the activation of hepatic stellate cells (Zheng et al., 2023). MSCs can indirectly modulate liver fibrosis by re-polarizing pro-fibrotic macrophages (Y. hui Li et al., 2021).

MSCs promote tissue repair thanks to their potential to differentiate into multiple cell types. MSCs can differentiate into hepatocyte-like cells (Saito et al., 2021; Sgodda et al., 2007), cells of neuronal lineage (Bueno et al., 2021; Choi et al., 2021), osteogenic lineage (Ma et al., 2024), cardiomyocytes (Z. hua Li et al., 2020), or chondrocytes (J. Huang et al., 2021); therefore, they represent a promising tool for regenerative medicine. However, MSCs support tissue regeneration mostly through their paracrine activity, supporting the proliferation and differentiation of tissue-resident cells and modulating the immune system rather than through direct differentiation.

The capacity to regulate the immune response is essential to MSC function. The next chapter will be devoted to the mechanisms MSCs employ to modulate individual immune cell types, the interconnection of the immunomodulatory capacity of MSCs and their metabolic state, and ways to enhance their immunomodulatory potential.

2.2.1. Immunomodulatory potential of mesenchymal stem cells

MSCs are now extensively studied for their capabilities to modulate immune responses. Thanks to their self-renewal, migratory potential, and low immunogenicity, they have been proposed as a promising therapeutic tool in treating numerous immune-mediated disorders, such as inflammatory, autoimmune, neurodegenerative disorders, or graft versus host disease (GvHD). Several stem-cell-based therapies using MSCs are in different phases of clinical trials or have proven effective in murine experimental disease models (reviewed in G. Yang et al., 2023).

However, the immunomodulatory activity is not intrinsic; MSCs must first be activated. MSCs are licensed by inflammatory signals, which will be discussed further, hypoxic environment (Martinez et al., 2017; H. Ren et al., 2023), or culture conditions, for example, 3D cultures (Bartosh et al., 2010; Thakur et al., 2022).

The target cells of MSC immunomodulatory activity are both adaptive and innate immune cells. MSCs can alter the polarization of macrophages, suppress antigen presentation by dendritic cells (DC), induce regulatory immune cells, limit antibody production, inhibit natural killer (NK) cell cytotoxicity, or regulate differentiation of Th17 lymphocytes. MSC immunomodulatory activity toward various immune cells is summarized in Table 1 and Fig.2. A special section will be devoted to the immunomodulatory effect of MSCs on T cells.

Table 1. The immunomodulatory effects of MSCs on specific immune cell types. MSC...mesenchymal stem cell, IL...interleukin, ROS...reactive oxygen species, ALI...acute lung injury, TGFβ...transforming growth factor-β, PGE₂...prostaglandin E₂, PCA...passive cutaneous anaphylaxis, CA...chronic urticaria, DC...dendritic cell, FLT3L...Fms-related tyrosine kinase ligand 3, MHCII...major histocompatibility complex class II, HLA-DR...human leukocyte antigen DR, CD...cluster of differentiation, SLE...systemic lupus erythematosus, pDC...plasmacytoid dendritic cell, IFN...interferon, TSG-6...tumor necrosis factor-stimulated gene 6, iNOS...inducible nitric oxide synthase, TNFα...tumor necrosis factor-α, MerTK...Mer tyrosine kinase, M1...classically activated macrophages, M2...alternatively activated macrophages, NO...nitric oxide, NADH...nicotinamide adenine dinucleotide, WAT...white adipose tissue, IDO...indolamine-2,3-dioxygenase, NK...natural killer, Breg...regulatory B cell, GC...germinal center, LN...lymph node, iNKT...invariant natural killer T cell.

Cell type affected	Proposed mechanisms	Immunomodulatory effect	In vivo model	Reference
Neutrophils	IL-6	↑ viability, phagocytic activity, oxidative burst ↓ extracellular trap formation, ROS production	ALI - ↑ apoptosis, limit tissue injury ALI - ↓ inflammatory phenotype	(Brandau et al., 2014; Feng et al., 2023; D. Jiang et al., 2016; Raffaghello et al., 2008; V. Y. F. Su et al., 2019; Taghavi-Farahabadi et al., 2021)
Mast cells	TGFβ, PGE ₂	↓ degranulation, chemotaxis, inflammatory cytokine production	PCA - ↓ degranulation, CA - ↓ disease severity	(Brown et al., 2011; Hyun et al., 2023; H. S. Kim et al., 2015)
DCs	IL-6, TGFβ, galectin 3, PGE ₂ , FLT3L, Notch	tolerogenic DC induction, ↓ MHCII/HLA-DR, CD40, CD86, inflammatory cytokine production (IL-12) upon activation, ↓ T cell proliferation	SLE - ↑ tolerogenic DCs colitis - DC suppression	(Djouad et al., 2007; X. Li et al., 2020; Nikolic et al., 2018; Spaggiari et al., 2009; X. Yuan et al., 2019)
pDCs	CD39/CD73/adenosine	↓ production of IFNα		(M. Chen et al., 2019; Ríos-Ríos et al., 2024)
Macrophages	TSG-6, PGE ₂ , exosomes	↓ M1 polarization, ↓ iNOS, MHCII/HLA-DR, CD86, inflammatory cytokine production (IL-12, TNFα) ↑ M2 polarization, ↑ arginase 1, CD206, MerTK, IL-10 production metabolic switch toward oxidative phosphorylation ↑ microbicidal activity (↑ phagocytosis, NADH expression, NO and superoxide production)	wound healing - ↑ M2, IL-33 secretion obesity - ↑ M2, WAT beiging colitis - ↓ M1, inflammatory gene expression ALI - ↓ pyroptosis of alveolar macrophages	(D. I. Cho et al., 2014; H. Choi et al., 2011; Y. Li et al., 2019; Z. H. Li et al., 2023; P. Liu et al., 2024; Rabani et al., 2018; Vasandan et al., 2016; Y. Wang et al., 2025; Y. Yuan et al., 2022; Zhao et al., 2018)

NK cells	IDO, PGE ₂	↓ proliferation of IL-2- or IL-15-activated NK cells ↓ cytotoxic response, ↓ expression of activating receptors ↑ cytotoxicity toward MSCs, some studies suggest ↑ perforin and granzyme release, ↑ IFN γ production		(Blanco et al., 2016; R. Cui et al., 2016; Najar, Fayyad-Kazan, et al., 2019; Spaggiari et al., 2008; Yigitbilek et al., 2022)
B cells	IL-35, PGE ₂ , exosomes indirectly through T cell and DC modulation	↓ proliferation upon activation, differentiation, antibody production induction of Bregs, ↑ IL-10 production	ALI - ↓ chemokine and immunoglobulin gene expression SLE - ↓ GC B cells, autoantibody production	(X. Chen et al., 2019; K. A. Cho et al., 2017; Corcione et al., 2006; Feng et al., 2020; Ma et al., 2013; Park et al., 2015; Rosado et al., 2014; Tabera et al., 2008)
$\gamma\delta$ T cells	IDO, PGE ₂	↓ IL-17 production	psoriasis-like dermatitis - ↓ abundance in LN and IL-17 production	(Y. Chen et al., 2022; Fechter et al., 2017; Prigione et al., 2009)
iNKT cells		↓ proliferation, IFN γ production		(Prigione et al., 2009)

2.2.1.1. The immunomodulatory effect on T cells

MSCs modulate the proliferation of T cells, their differentiation into effector subsets, cytokine production, and cytotoxicity. Close contact is important for MSC immunosuppressive effects. MSCs produce several chemokines, for instance, ligands of the T-cell-specific chemokine receptor CXCR3, which recruit T cells to their proximity (G. Ren et al., 2008). MSCs also upregulate the expression of adhesion molecules, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 to sustain close contact with T cells (G. Ren et al., 2010).

Nitric oxide (NO) is a critical molecule in murine MSC-mediated T-cell suppression (Sato et al., 2007; Yu et al., 2024). While indolamine-2,3-dioxygenase (IDO) expression is the main immunosuppressive factor in humans or monkeys, the expression of inducible nitric oxide synthase (iNOS) and NO production is a key in rodent MSC immunomodulation (Su et al., 2013). NO dampens stimulation-induced proliferation of T cells *in vitro*. The addition of iNOS inhibitors or iNOS deficiency resulted in impaired ability of MSCs to suppress T-cell proliferation (G. Ren et al., 2008; Sato et al., 2007). It was reported that NO suppresses the phosphorylation of STAT5, which is involved in cytokine signaling necessary for T cell expansion (Sato et al., 2007).

Human MSCs, on the other hand, employ the IDO enzyme as the major mechanism of T-cell suppression (H. J. Lee et al., 2021; Meisel et al., 2004; Tipnis et al., 2010). IDO is involved in the degradation of tryptophan into kynurenine, depriving activated T cells of the necessary substrate for expansion. The addition of tryptophan to the co-culture of MSCs with T cells

restored T-cell proliferation (Meisel et al., 2004). Conversely, the inhibition of IDO activity abrogated the suppressive effects of MSCs (H. J. Lee et al., 2021; Tipnis et al., 2010). The importance of IDO expression in MSCs was also shown in a model of acute lung injury, where MSCs deficient in IDO exhibited impaired ability to alleviate the disease (G. Wang et al., 2017). Evidence suggests that products of tryptophan degradation may also impact T-cell proliferation. L-kynurenine suppresses T-cell proliferation, and kynurenic acid positively regulates the expression of TNF-stimulated gene (TSG)-6, another immunosuppressive molecule employed by MSCs (Frumento et al., 2002; G. Wang et al., 2017).

MSCs modulate the polarization of activated Th cells, as well. Generally, MSCs suppress the response of inflammatory Th cells. They reduced the relative abundance of IFN γ -producing Th1 cells in the spleen of mice suffering from inflammatory bowel disease or chronic urticaria, which at least partially contributed to the amelioration of these diseases (Hyun et al., 2023; X. Li et al., 2019). TGF β and additional factors are involved in the Th1 response suppression (Hyun et al., 2023). The activation and proliferation of Th1 cells were blocked in a co-culture with MSCs *in vitro* (Luz-Crawford et al., 2013). Furthermore, the transfer of mitochondria from MSCs to Th cells, in concerted action with secreted PGE $_2$, impacted Th1 differentiation, IFN γ production, and expression of the transcription factor T-box expressed in T cells (Akhter et al., 2023). TSG-6 was shown to suppress Th1 responses either directly or indirectly by inducing regulatory antigen-presenting cells (Kota et al., 2013).

Th17 and Treg cells serve opposing functions during immune responses, yet their differentiation is closely interconnected. TGF β drives the polarization of both subsets, but in an inflammatory microenvironment, it favors Th17 development. In the absence of inflammation, the Treg cell master regulator Forkhead box protein 3 (FOXP3) suppresses retinoic acid-related orphan receptor c (ROR γ t) expression, the key Th17 transcription factor, shifting differentiation toward Treg cells (Ichiyama et al., 2008). A balance between Th17 and Treg cells must be carefully maintained, and MSCs proved effective in regulating this balance. Several mechanisms were proposed to be involved, including the production of PGE $_2$ (English et al., 2009; Ghannam et al., 2010), TGF β (English et al., 2009), or programmed death-ligand 1 and 2 (Azevedo et al., 2020; J. Y. Kim et al., 2018). Additionally, the production of soluble HLA-G5 or adenosine by MSCs contributes to the induction of Treg cells (M. Chen et al., 2013; Selmani et al., 2008). An *in vitro* study suggested that MSC-mediated Treg expansion results from effector Th cell transdifferentiation rather than proliferation of the existing Treg cells, as MSCs did not stimulate the proliferation of Treg cells in co-culture experiments (Azevedo et al., 2020). Another report showed that co-cultivation of naïve T cells with MSCs diminished ROR γ t expression while increasing FOXP3 expression (Ghannam et al., 2010). The *in vitro* findings on MSC modulation of Th17/Treg balance were corroborated by *in vivo* studies, for example, by using arthritis, urticaria, or inflammatory bowel syndrome models (M. Chen et al., 2013; Hyun et al., 2023; X. Li, Wang, et al., 2019).

Although most literature refers to MSC immunosuppressive activity toward T cells, there are reports of MSC immunostimulatory capacity. MSCs co-cultured with activated T cells enhance the expression of ROR γ t, leading to enhanced differentiation of Th17 cells (Najar et al., 2019). MSCs isolated from lymph nodes support the survival and proliferation of activated T cells through the production of CCL2. However, when isolated from mice with ongoing inflammation, these lymph node-derived MSCs lost their supportive properties and instead exhibited immunosuppressive activity (He et al., 2022). Different outcomes of MSC application were also observed when MSCs were injected at the early stages of a disease versus later during the progression (Hoseinzadeh et al., 2023). Considering that, the immunomodulatory activity of MSCs is highly plastic and vary depending on many factors, such as the source tissue for MSC isolation (Y. Liu et al., 2024), the ratio of MSCs to T cells in co-culture experiments (W. Li et al., 2012; Najar et al., 2019; Porubska et al., 2021), and inflammatory conditions for the proper stimulation of MSC immunosuppressive potential (He et al., 2022; Hoseinzadeh et al., 2023; W. Li et al., 2012). The necessity of inflammatory stimulation of MSCs will be discussed in the next chapter.

2.2.1.2. Preconditioning of MSCs with inflammatory cytokines

A great deal of literature reporting the immunosuppressive capacity of MSCs, both *in vitro* and *in vivo*, underscores their potential for treating various inflammatory or autoimmune disorders. On the other hand, some studies show variable outcomes of MSC administration on disease progression (Constantin et al., 2009; Hoseinzadeh et al., 2023; Sudres et al., 2006). The plasticity of MSC immunomodulation was reviewed extensively by Wang and colleagues (reviewed in Y. Wang et al., 2014).

It is now well-established that MSCs require adequate inflammatory stimulation to acquire immunosuppressive function (Chan et al., 2006; W. Li et al., 2012; G. Ren et al., 2008). IFN γ is a very potent cytokine for MSC priming. IFN γ -pretreated MSCs upregulate IDO and iNOS expression in humans and mice, respectively, and exhibit enhanced suppressive abilities in co-culture assays and in the model of GvHD (D. S. Kim et al., 2018; G. Ren et al., 2008). The signaling pathways triggered by IFN γ leading to IDO/iNOS expression induction include Janus-kinase-STAT1 and nuclear factor- κ B pathway (Gu et al., 2018; D. S. Kim et al., 2018). Conversely, anti-inflammatory signals, such as TGF β or steroid therapy, limit the immunosuppressive function of MSCs by targeting IFN γ -induced signaling pathways, for example, STAT1 phosphorylation (X. Chen et al., 2014; Yu et al., 2024).

IFN γ can also elicit immunosuppressive properties of MSCs in combination with other pro-inflammatory cytokines, such as TNF α or IL-1 β , which act synergistically with IFN γ (G. Ren et al., 2008). Apart from IFN γ preconditioning, MSCs can be primed by stimulation with IL-17, which increases PGE₂ production by MSCs, thus improving their potential to alleviate renal injury (Bai et al., 2018). IL-17 also improves the immunoregulatory function of MSCs triggered by pretreatment with IFN γ and TNF α via NO upregulation (Han et al., 2014).

Pretreatment of MSCs with pro-inflammatory cytokines increases their potency in alleviating the disease progression in several models. IFN γ -priming alleviated renal fibrosis by upregulating IDO and PGE₂ production (Kanai et al., 2021; Kurawaki et al., 2024), improved survival after GvHD (D. S. Kim et al., 2018), and exosomes derived from IFN γ -primed MSCs exhibited enhanced therapeutic effect in experimental colitis (R. Yang et al., 2020). IFN γ , TNF α , and L-1 β pretreatment further improved the immunomodulatory effect of MSC-EVs in a GvHD model (Hackel A et al., 2023).

In addition to cytokine-mediated priming of MSCs, danger-associated molecules can potentiate MSC-immunomodulatory capacity. MSCs express several types of Toll-like receptors (TLR). A study by Waterman et al. showed that MSC immunomodulatory potential varies based on ligation of specific TLRs. TLR3 ligation with polyinosinic:polycytidylic acid leads to enhanced suppressive activity, whereas TLR4 ligation by lipopolysaccharide (LPS) leads to anti-inflammatory polarization of MSCs with decreased ability to suppress T-cell proliferation (Waterman et al., 2010). This finding was later challenged by the observation that prolonged LPS stimulation can also enhance the immunomodulatory properties of MSCs. In fact, the deletion of TLR4 mitigated the beneficial effect of MSC infusion in experimental autoimmune encephalomyelitis (Kurte et al., 2020).

2.2.2. Metabolic regulation of MSC immunomodulatory activity

Preconditioning of MSCs is essential for their immunomodulatory function. It was recently demonstrated that MSCs alter their metabolism following inflammatory stimulation, driving their immunoregulatory functions. Soon, it was suggested that metabolic reprogramming is an essential determinant of MSC immunomodulatory properties (reviewed by H. Li et al., 2023). This chapter will briefly summarize current knowledge regarding the interconnection between the metabolic state and immunomodulatory capacity of MSCs. Fig.2 summarizes MSC immunomodulatory effects and metabolic reprogramming.

Glycolytic metabolism is essential for ensuring sufficient energy supplies and substrates for biosynthesis. Glucose is taken up by the cell through glucose transporters (GLUT) and then transformed into pyruvate by a series of enzymatic reactions. Depending on oxygen availability, pyruvate can be further broken down into lactate or acetyl coenzyme A. Several signaling pathways regulate glycolysis. The mammalian target of rapamycin (mTOR) is one of the pivotal sensors of energy availability and modulators of cellular metabolism. Its upstream regulators, such as phosphoinositide 3-kinase, Akt, or AMP-activated protein kinase, also play an essential role in energy sensing. The downstream targets of mTOR pathway are transcription factors hypoxia-inducible factor 1 α (HIF-1 α) and c-Myc, which regulate the transcription of key parts of the glycolytic pathway. Activation of these glycolytic regulators is a key determinant for metabolic reprogramming in highly proliferating cells (e.g., stem cells or cancer cells) (reviewed by Szwed et al., 2021).

MSCs naturally rely on glycolysis for ATP production in their native environment. This metabolic preference reduces oxidative damage, which is crucial for maintaining their stemness properties (Estrada et al., 2011). Upregulating glycolysis while inhibiting oxidative phosphorylation also leads to enhanced immunosuppressive activity of MSCs. MSCs treated with oligomycin, an inhibitor of ATP synthase, exhibited an increased rate of glycolytic metabolism as demonstrated by increased extracellular acidification rate (ECAR, proxy for glycolytic activity), GLUT1 expression, and lactate efflux. This metabolic reprogramming was associated with enhanced suppression of Th1 and Th17 cell proliferation. The inhibition of glucose uptake by MSCs had the opposite effect (Contreras-Lopez et al., 2021).

A similar effect was accomplished when MSCs were pretreated with inflammatory cytokines. TNF α and IFN γ preconditioning increased glucose uptake, ECAR, and genes involved in glycolysis, highlighting the metabolic switch toward glycolysis (Contreras-Lopez et al., 2021; Y. Liu et al., 2018). It also enhanced the expression of HIF-1 α , which appears essential for T cell suppression (Contreras-Lopez et al., 2020). Oligomycin-primed MSCs showed improved therapeutic potential in a delayed-type hypersensitivity model, while HIF-1 α silencing aggravated the disease (Contreras-Lopez et al., 2020, 2021). Moreover, IFN γ -primed MSCs exhibited enhanced Akt and S6K, mTOR effector protein, phosphorylation. Conversely, blocking the mTOR activity by rapamycin decreased IDO and PGE₂ production by MSCs, underlying the importance of this signaling pathway in MSC suppressive activity (Y. Liu et al., 2018). STAT1 phosphorylation is an important mediator of MSC immunosuppressive activity after inflammatory stimulation. Glycolysis inhibition mitigated the increase in STAT1 phosphorylation and IDO expression after inflammatory licensing (Jitschin et al., 2019). Additionally, the inhibition of phosphoinositide 3-kinase diminished IFN- γ -induced IDO upregulation, also through STAT1 modification (Mounayar et al., 2015). These findings improved the understanding of the connection between glycolysis and signaling pathways involved in immunomodulation (Jitschin et al., 2019; Mounayar et al., 2015).

The above-mentioned studies demonstrated an elevation of lactate efflux associated with metabolic switch after inflammatory MSC preconditioning. In addition, it was suggested that exported lactate partially mediates the suppressive capacity of MSCs (Pradenas et al., 2023).

IFN γ licensing led to increased production of both mitochondrial and total reactive oxygen species (ROS). Higher ROS production appears to be associated with MSC immunosuppression, as ROS inhibition with antioxidant agents resulted in decreased IDO production and impaired ability to moderate T-cell proliferation (Contreras-Lopez et al., 2020; Y. Liu et al., 2018).

When oxygen supplies are sufficient, pyruvate generated in the glycolytic pathway is metabolized in the tricarboxylic acid cycle (TCA) and oxidative phosphorylation. In MSCs, stimulation with inflammatory cytokines caused either unchanged or slightly decreased oxygen consumption rate (OCR, a proxy for mitochondrial metabolism) based on available data (Contreras-Lopez et al., 2021; Y. Liu et al., 2018). Diminished activity of complex I of the ETC was reported following inflammatory stimulation (Y. Liu et al., 2018). Proteomic analysis

showed that MSCs primed with the combination of IFN γ and hypoxia have decreased expression of proteins involved in the TCA cycle, ETC, and fatty acid oxidation (Wobma et al., 2018). Moreover, rotenone and antimycin A, inhibitors of complex I and complex III, respectively, further strengthened IFN γ -induced glycolysis upregulation and immunosuppression (Y. Liu et al., 2018).

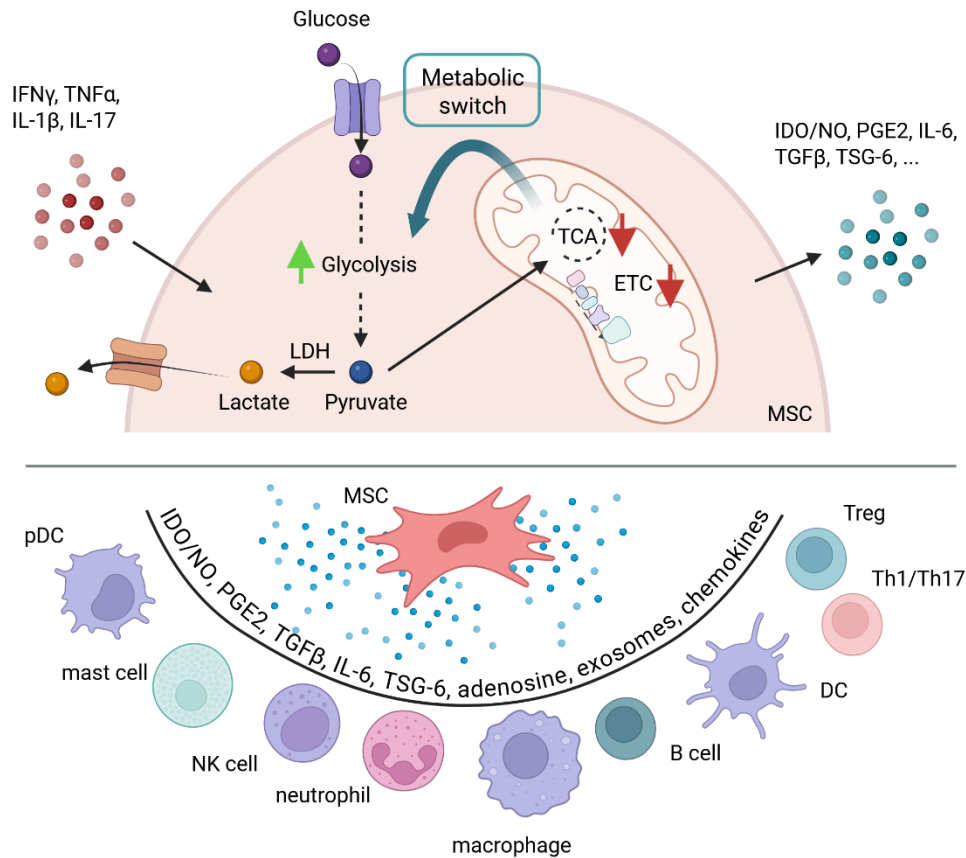


Figure 2. Immunomodulatory potential of MSCs. The upper picture demonstrates the inflammatory stimulation of MSC immunomodulatory function, which is dependent on a metabolic switch, reducing the use of mitochondrial metabolism to preferentially use glycolytic metabolism. The lower picture demonstrates the immune cell types affected and soluble factors involved in MSC-mediated immunosuppression. IFN γ ...interferon- γ , TNF α ...tumor necrosis factor- α , IL...interleukin, LDH...lactate dehydrogenase, TCA...tricarboxylic acid cycle, ETC...electron-transport chain, IDO...indolamine-2,3-dioxygenase, PGE2...prostaglandin E2, TGF β ...transforming growth factor- β , TSG-6...tumor necrosis factor-stimulated gene 6, MSC...mesenchymal stem cell, pDC...plasmacytoid dendritic cell, NK...natural killer, DC...dendritic cell, Treg...regulatory T cell, Th...helper T cell. Taken and adapted from Li et al. and Yang et al. (review by H. Li et al., 2023; G. Yang et al., 2023). Created in Biorender.com.

These findings highlight the importance of MSC preconditioning and metabolic reprogramming toward glycolysis with enhanced immunosuppressive capacity of MSCs. They provide foundations for the development of new modification strategies to improve MSC therapeutic potential.

2.2.3. The effect of reduced culture temperature on MSCs

MSCs are affected not only by soluble factors in their surroundings and interactions with other cells but also by physical parameters. As mentioned earlier, the physical conditions of MSC culture, e.g., hypoxia or culture in aggregates, might affect MSC immunomodulatory capacity (Bartosh et al., 2010; H. Ren et al., 2023). Temperature represents another factor affecting several aspects of MSC biology, including their proliferation, viability, differentiation, and metabolic properties. However, the literature concerning the impact of reduced temperature on MSCs is scarce. Culture of rat MSCs under reduced temperature (32-35°C) leads to a decrease in ROS production compared to culture under a standard temperature of 37°C. (Stolzing et al., 2006; Tirza et al., 2020). Moreover, MSCs isolated from 6-week-old rats exhibited decreased NO production while increased expression of heat shock proteins and activity of superoxide dismutase and glutathione peroxidase after cultivating at reduced temperature, suggesting a beneficial effect of reduced culture temperature on the oxidative state of MSCs (Stolzing et al., 2006). The apoptotic rate of MSCs is lower in MSCs cultured at 35°C, moreover, the expression of inflammatory factors, such as IL-1, chemokines, or matrix metalloproteinases, is reduced (Tirza et al., 2020). Similar observations of decreased ROS production, apoptotic rate, and inflammatory gene expression were also reported for human ASCs cultured at reduced temperatures (Tirza et al., 2020). The differentiation potential of MSCs is affected by temperature, as well. Treatment with menthol, an agonist of TRPM8, promoted the osteogenic differentiation of human MSCs (Henao et al., 2021). Myogenic differentiation of murine MSCs was enhanced by a short cooling period at 17°C (Takagi et al., 2023). Adipogenic differentiation of MSCs at reduced temperature was enhanced (Tirza et al., 2020; Velickovic et al., 2018). Interestingly, adipogenic differentiation under reduced temperature resulted in the generation of adipocytes containing a higher number of small lipid droplets, higher UCP1 content, metabolic activity, and thermogenic gene expression, indicating enhanced differentiation of thermogenic adipocytes at reduced temperature (Velickovic et al., 2018).

MSCs can sense cold temperatures thanks to the expression of TRP channels. TRP channels are mainly expressed by neural cells, where they are essential for perceiving changes in ambient temperature. Several TRP channels were implicated in regulating the properties of stem cells (reviewed in Guo et al., 2022); however, a connection with temperature sensing was not demonstrated. Nevertheless, the expression of the TRPM8 was shown in MSCs at the transcriptomic and protein levels, and using immunofluorescent microscopy (Henao et al., 2021). It was demonstrated to be activated by cooling (Takagi et al., 2023).

Even though the literature regarding the impact of low temperatures on MSCs is limited, the reports stated above suggest that MSCs respond to changes in ambient temperature and modify their cellular and metabolic properties to adapt to new conditions. Whether reduced temperatures prime MSC immunomodulatory function is not known.

3. Thesis aims

Cold exposure triggers substantial changes in the morphology and functionality of adipose tissue. Besides impacting adipocytes, cold affects the immune compartment and secretory profile of adipose tissue. The reciprocal regulation between stromal and immune cells after cold exposure was recently described. However, the impact of cold on MSCs residing within adipose tissue remains to be explored. MSCs possess great immunomodulatory potential, which is activated by inflammatory stimulation. The complex alterations in adipose tissue after cold exposure might lead to the activation of MSC immunomodulatory capacity.

The main objective of this project is to evaluate the impact of cold-induced changes in adipose tissue on the characteristics and functional qualities of MSCs isolated from the adipose tissue of cold-exposed mice. We hypothesize that cold-induced changes, including changes in the production of soluble factors and intercellular interactions, might activate the immunomodulatory activity of MSCs, which could then be utilized as a strategy for improving MSC immunomodulatory potential in stem cell therapies for inflammatory or autoimmune disorders. This project aims to:

- evaluate the immune-related changes in different tissues following short-term and long-term cold exposure in mice
- characterize MSCs isolated from cold-exposed mice
- elucidate the impact of cold-induced changes in adipose tissue on the properties of adipose tissue-derived MSCs, namely concerning their metabolic properties and immunomodulatory activity

The second part of this project is devoted to the individual impact of reduced temperature on the properties of MSCs. Specifically, this project will:

- determine the effect of reduced culture temperature on the metabolic properties of MSCs
- determine the effect of reduced culture temperature on the immunomodulatory activity of MSCs

4. Methods

4.1. Animals

Inbred mice of BALB/c strain (AnLab s.r.o., Prague, Czech Republic) were used in all experiments. The mice were 6-8 weeks old, both sexes.

In the first part of the project, mice exposed to room temperature (RT) or cold temperature (8°C) were used. Mice were divided into three experimental groups: control (C) housed for 14 days at RT (25°C), mice housed 1 day at 8°C (1D-cold), or 14 days at 8°C (14D-cold), see Fig.3. Mice were housed in pairs in well-bedded cages to minimize environmental and social stress. In each independent experiment, 4-6 mice were used per group.

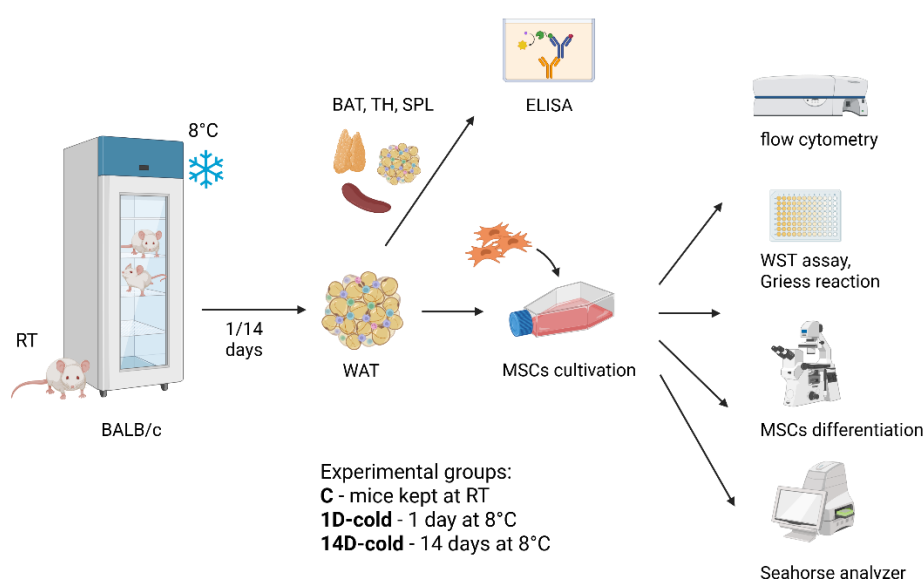


Figure 3. Experimental setup of the first part of the diploma project. RT...room temperature, WAT...white adipose tissue, BAT...brown adipose tissue, TH...thymus, SPL...spleen, MSCs...mesenchymal stem cells, WST...water-soluble formazan. Created with Biorender.com.

4.2. Media

MSCs were cultivated in Dulbecco's Modified Eagle's Medium (DMEM; Biosera, Cholet, France, catalog number: LM-D1111/500) supplemented with 10% Fetal Bovine Serum (FBS; ThermoFisher, Waltham, Massachusetts, USA; catalog no: A5256701), 10 mM HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Carl Roth, Karlsruhe, Germany, catalog no: R6763.2), and antibiotics (penicillin – 100 U/ml, Merck and Co., St. Luis, Missouri, USA, catalog no: P8721 and streptomycin – 100 mg/ml, Merck, catalog no: S6501).

Co-cultivation experiments of MSCs with splenocytes were performed using Roswell Memorial Park Institute 1640 medium (RPMI; Merck, catalog no: R8758) supplemented with 10% FBS, 10 mM HEPES buffer, 0.05 mM β -mercaptoethanol (Merck, catalog no: M3148), and antibiotics (penicillin – 100 U/ml, Merck and streptomycin – 100 mg/ml, Merck).

For the evaluation of glucose uptake, MSCs were cultivated in a glucose-free RPMI medium (ThermoFisher, catalog no: 11879020) supplemented with 200 mM L-glutamine (ThermoFisher, catalog no: 25030081), 10% FBS, 10 mM HEPES buffer, 0.05 mM β -mercaptoethanol, and antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin).

Evaluation of MSCs metabolism using Agilent Seahorse XF Analyzer was performed using Seahorse XF DMEM medium (pH 7.4; Agilent Technologies, Inc., Santa Carla, California, USA, catalog no: 103575-100) supplemented with 1 M Glucose Solution (Agilent Technologies, catalog no: 103577-100), 100 mM Pyruvate Solution (Agilent Technologies, catalog no: 103578-100), and 200 mM Glutamine Solution (Agilent Technologies, catalog no: 103579-100).

4.3. Solutions

1x phosphate saline buffer (PBS): pH 7.2-7.4

- 10 mM phosphate
- 2.3 mM KCl
- 37 mM NaCl

ELISA:

Washing solution:

- 1.625 ml Tween-20 (0.05%, Merck, catalog no: P1379)
- 125 ml 20x PBS
- 2.5 l TK water

Coating solution: pH 8.2

- 1.68 g 0.1M NaHCO₃ (Merck, catalog no: S5761)
- 200 ml distilled water

Blocking solution:

- 15 ml bovine serum albumin (BSA, 7.5%; Merck, catalog no: A2153)
- 200 ml PBS

Reagent Diluent:

- 1% BSA in PBS

Substrate for ELISA BD Pharmingen: pH 4.35

- 7.68 g anhydrous citric acid (Merck, catalog no: C0759)
- 120 mg 2,2'-azino-bis(3-ethylbezothiazoline-6-sulfonic acid) (Merck, catalog no: A1888)
- 11 μ L 30% H₂O₂ (Merck, catalog no: H1009)
- 400 ml TK water

Griess reaction:

Solution A:

- 0.5 g sulphanilamide (Merck, catalog no: S9251)
- 1.5 ml H₃PO₄
- 48.5 ml water

Solution B:

- 0.15 g N-(1-naphtyl)ethylenediamine dihydrochloride (Merck, catalog no: 222488)
- 1.5 ml H₃PO₄
- 48.5 ml water

BCA method:

Reagent A – pH 11.25

- 6.84 g anhydrous sodium carbonate
- 1.6 g sodium tartrate
- 100 ml distilled water

Reagent B

- 4 g bicinchoninic acid
- 100 ml distilled water

Reagent C

- 0.4 g 5x hydrated copper sulfate
- 10 ml distilled water

4.4. Technical equipment

Agilent Seahorse XF Analyzer (Agilent Technologies)

Centrifuge Hettich Universal 32R (DJB Labcare, Buckinghamshire, England)

Centrifuge 5430 (Eppendorf, Hamburg, Germany)

Centrifuge MiniSpin (Eppendorf, Hamburg, Germany)

Flow cytometer LSR II (BD, Franklin Lakes, New Jersey, USA)

Incubator (Sanyo, Osaka, Japan)

Laminar Flow Box CleanAir (Schoeller Instruments s.r.o., Prague, Czech Republic)

Laminar Flow Box SafeFAST premium (DASIT group, Cornaredo, Italy)

LightCycler 480 II (Roche, Rotkreuz, Switzerland)

Light microscope Leica Dmi1 (Leica Microsystems, Wetzlar, Germany)

MyCyclerThermal Cycler (Bio-Rad, Hercules, CA, USA)

Spectrophotometer microplate reader ELx800 (Biotek, Winooski, VT)

Spectrophotometer NanoDrop 1000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA)

4.5. Software

Agilent Seahorse Analytics (Agilent Technologies)

FlowJo 10.10 (BD Biosciences, Franklin Lakes, New Jersey, USA)

FACSDiva Software (BD, Franklin Lakes, New Jersey, USA)

GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA)

LightCycler 480 (Roche, Rotkreuz, Switzerland)

Operating software for Nanodrop ND-1000 (Coleman Technologies, Orlando, FL)

Operating software for spectrophotometer Revelation Quicklink (Dynex Technologies, Chantilly, VA)

4.6. Isolation of MSCs

MSCs were isolated from the iWAT, which was washed in PBS, cut into small pieces, and digested by adding a 10% solution of collagenase I (Merck, catalog no: C2674) and incubating for 60 min at 37°C with one agitation over the incubation period. The cell suspension was washed with PBS and centrifuged (8 min, 8°C, 170 g). The pellet was resuspended in PBS, and the washing step was repeated three times. After the last washing step, the pellet was resuspended in DMEM and placed onto tissue culture flasks (75 cm², ThermoScientific BioLite). The cells were cultured in an incubator with 5% CO₂ and 37°C. After 48 hours, non-adherent cells were washed away. The adherent cells were cultured for several weeks with regular passages after reaching 80-100% confluency. After 4-6 passages, the cells were used for experiments.

For the second part of this project, MSCs were isolated from iWAT of control BALB/c mice of both sexes according to the above-mentioned protocol and cultivated for 4-6 passages. To assess the impact of cultivation in reduced temperature, MSCs were divided into 3 experimental groups: MSCs cultured in a standard culture temperature of 37°C (control, C), MSCs cultured

for 1 day in a reduced temperature of 34°C (1 day, 1D), and MSCs cultured for 3 days in a reduced temperature of 34°C (3 days, 3D), see Fig.4. To evaluate the impact of inflammatory stimulation on MSCs cultured at reduced temperature, MSCs were stimulated with LPS (1.25 µg/ml; Sigma, catalog no: L2880) and IFN γ (20 ng/ml; Baria, catalog no: 315-05) for 3 or 24 hours. For flow cytometric analysis, MSCs were kept at 34/37 °C during the stimulation period until the cells were prepared for the analysis. For Seahorse analysis, MSCs were kept at 34/37°C during the stimulation period, but all MSC samples were transferred to 37°C for the actual measurement.

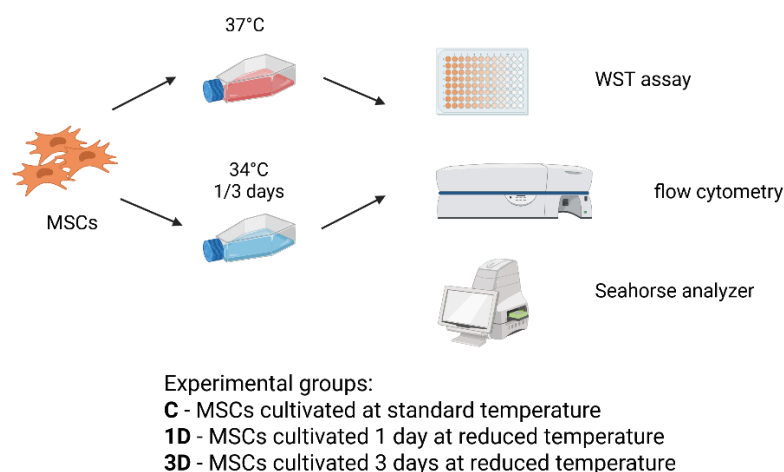


Figure 4. *Experimental setup of the second part of the diploma project concerning the impact of reduced culture temperature on MSCs. MSCs...mesenchymal stem cells, WST...water-soluble formazan.*

4.7. Isolation of splenocytes

The spleen was harvested, washed in 10% RPMI, and cut into small pieces. The tissue was then transferred through nylon gauze into a centrifuge tube and centrifuged (8 min, 8°C, 170 g). The pellet was resuspended, and splenocyte counts were determined using the Bürker chamber.

4.8. Tissue homogenates

Perigonadal WAT (pWAT), anterior subcutaneous WAT (asWAT), iWAT, interscapular BAT, spleen, and thymus were harvested, weighed, and washed in PBS. Fig.5 shows the location of isolated adipose tissue depots. All tissues were cut into small pieces with scissors and stored in a -20°C freezer. Three cycles of thawing and freezing were repeated to allow cell membrane rupture. The homogenates were centrifuged (6-8°C, 5 min, 5 000 g), and clear supernatants were collected.

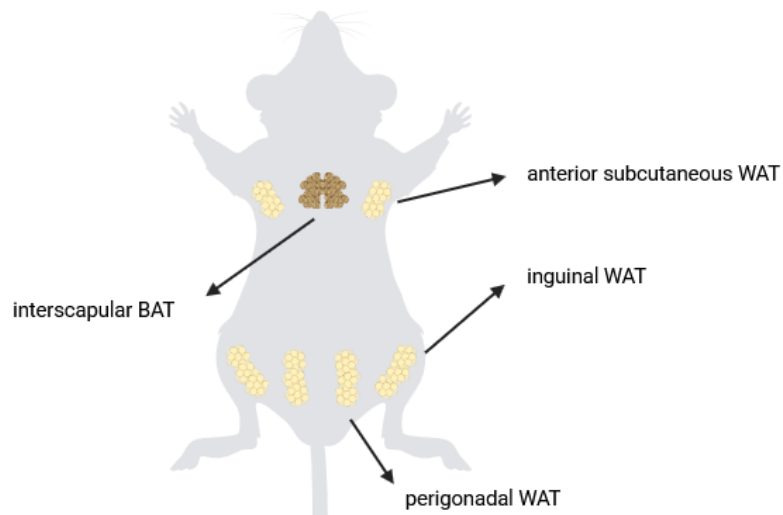


Figure 5. Isolated adipose tissue depots. WAT...white adipose tissue, BAT...brown adipose tissue. Created in Biorender.com.

4.9. ELISA

ELISA was used to determine the levels of cytokines IL-1 β , IL-2, IL-4, IL-6, IL-7, IL-10, IL-17, TNF α , and VEGF in tissue homogenates of BAT, iWAT, asWAT, pWAT, spleen, and thymus. ELISAs were performed using 96-well plates with flat bottoms (Baria, catalog no: 3590).

4.9.1. ELISA DuoSet Development R&D Systems

This ELISA kit was used to determine the levels of IL-1 β , IL-4, IL-7, IL-10, IL-17, TNF α , and VEGF in tissue homogenates according to this protocol:

- Plates were coated with 100 μ l of capture antibody diluted in PBS (see Table 2) and incubated overnight at RT.
- Plates were washed 3x with washing solution
- Plates were incubated with 200 μ l of Reagent Diluent for 1 hour at RT
- Plates were washed 3x with washing solution
- Plates were incubated with 100 μ l of blank (PBS), standards, and samples for 3 hours at RT
- Plates were washed 3x with washing solution
- Plates were incubated with 100 μ l of Streptavidin-horse-radish peroxidase diluted in Reagent Diluent for 20 min in the dark at RT
- Plates were washed 3x with washing solution
- Plates were incubated with 100 μ l of a substrate (Reagent A+Reagent B, 1:1; R&D Systems, catalog no: DY999) in the dark at RT until the reaction was visible (4-20 min)
- 50 μ l of 2N H₂SO₄ was added to stop the reaction

- The measurement was performed using the spectrophotometer ELx800 (Biotek) at wavelength 450 nm and reference wavelength 570 nm
- The final concentration of cytokines in a sample was calculated according to calibration curves for each cytokine. Representative calibration curves are shown in Fig.6.

Table 2. List of ELISA kits.

	Catalog number	Capture Antibody [c]	Detection Antibody [c]
IL-1β	DY401-05, R&D Systems	4 μ g/ml	250 ng/ml
IL-4	DY404, R&D Systems	4 μ g/ml	150 ng/ml
IL-7	DY407, R&D Systems	1.6 μ g/ml	400 ng/ml
IL-10	DY417, R&D Systems	4 μ g/ml	300 ng/ml
IL-17	DY421, R&D Systems	2 μ g/ml	400 ng/ml
TNFα	DY410, R&D Systems	0.8 μ g/ml	75 ng/ml
VEGF	DY493, R&D Systems	4 μ g/ml	250 ng/ml

4.9.2. ELISA BD Pharmingen

- Plates were coated with 50 μ l of capture antibody diluted in a coating solution (see Table 3) and incubated 24 h in a refrigerator.
- Plates were washed 3x with washing solution
- Plates were incubated with 200 μ l of a blocking solution for 2 hours at RT
- Plates were washed 4x with washing solution
- Plates were incubated with 100 μ l of blank (PBS), standards, and samples overnight in a refrigerator
- Plates were washed 4x with washing solution
- Plates were incubated with 100 μ l of detection antibody diluted in a blocking solution and incubated for 1 h at RT
- Plates were washed 6x with washing solution
- Plates were incubated with 100 μ l of avidin peroxidase (2.5 μ g/ml; Merck, catalog no: A3151) diluted in a blocking solution for 1 h at RT
- Plates were washed 8x with washing solution
- Plates were incubated with 100 μ l of substrate in the dark until the reaction was visible (2-5 min)
- The measurement was performed using the spectrophotometer ELx800 (Biotek) at wavelength 405 nm and at reference wavelength 630 nm
- The final concentration of cytokines in a sample was calculated according to calibration curves for each cytokine. Representative calibration curves are shown in Fig.6.

Table 3. List of antibodies used for IL-6 and IL-2 detection.

	Capture antibody		Detection antibody	
	Catalog number	Concentration	Catalog number	Concentration
IL-2	554424, BD Pharmingen	0.5 mg/ml	554426, BD Pharmingen	0.5 mg/ml
IL-6	554400, BD Pharmingen	0.5 mg/ml	554402, BD Pharmingen	0.5 mg/ml

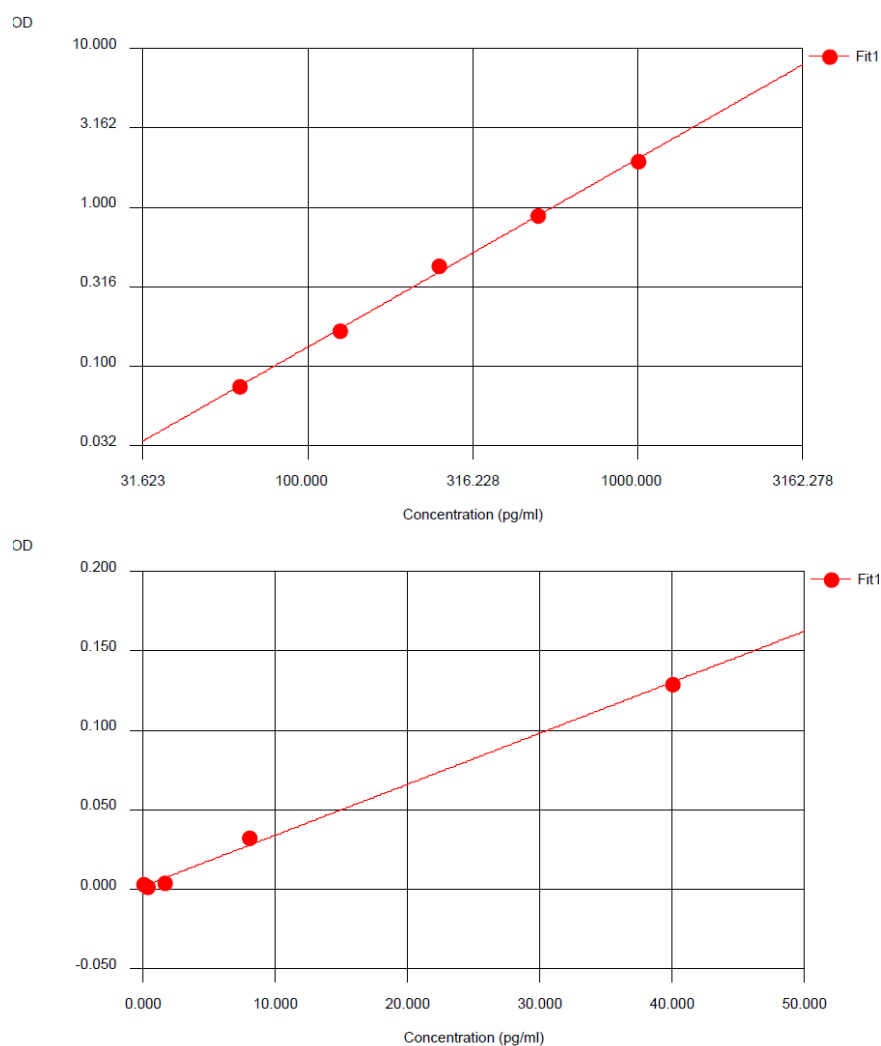


Figure 6. Representative calibration curves for ELISA DuoSet Development R&D Systems and ELISA BD Pharmingen. Representative calibration curves are shown for IL-17A in the upper graph (ELISA DuoSet Development R&D Systems) and IL-6 in the lower graph (ELISA BD Pharmingen). The X-axis shows cytokine concentration in pg/ml, and the Y-axis shows the optical density of the colored product. OD...optical density, Fit1 ...curve fitting.

4.10. Differentiation of MSCs

MSCs were differentiated into adipogenic, chondrogenic, and osteogenic lineages to characterize MSCs isolated from cold-exposed animals. Before the induction of differentiation, the cells were washed with PBS, trypsinized, centrifuged (8°C, 8 min, 170 g), counted, and seeded into 12-well plates (5×10^4 cells per ml; Nunc, Roskilde, Denmark). After reaching 80-100% confluency, the differentiation was induced by cultivating MSCs in differentiation media.

Adipogenic differentiation was induced by StemPro Adipogenesis Differentiation Kit (ThermoFisher, catalog no: A1007001). MSCs were cultivated in this medium for 10 days (+/- 2 days) until adipocytes were visible, changing the medium every 2-3 days. To visualize differentiated adipocytes, Oil Red O staining was used following this protocol:

- Cells were washed 2x with PBS
- Cells were fixed with 4% paraformaldehyde (Sigma, catalog no: P6148) for 15 min
- Cells were washed with PBS for 5 min
- The washing step was repeated 3x
- Cells were washed with 60% isopropanol (IBiotech, catalog no: 39559.02)
- The Oil Red O solution (Merck, catalog no: 102419) was added for 20 min
- Cells were washed with 60% isopropanol several times
- Cells were washed with PBS and observed under a microscope (Oil Red O detects neutral lipids present in lipid droplets of adipocytes)

Chondrogenic differentiation was induced by StemPro Chondrogenesis Differentiation Kit (ThermoFisher, catalog no: A1007101). MSCs were cultured in this medium for 10 days, changing the medium every 2-3 days. Differentiated chondrocytes were visualized with Alcian blue staining following this protocol:

- Cells were washed 2x with PBS
- Cells were fixed with 4% paraformaldehyde for 15 min
- Cells were washed with PBS for 5 min
- The washing step was repeated 3x
- Cells were washed with 0.1M HCl
- Alcian Blue solution (Sigma, catalog no: B8438) was added and incubated for 30 min
- Cells were washed several times with HCl and then with PBS and observed under a microscope (Alcian Blue binds to glycosaminoglycans produced by chondrocytes)

Osteogenic differentiation was induced by StemPro Osteogenesis Differentiation Kit (ThermoFisher, catalog no: A1007201). MSCs were cultured for 21 days. Differentiated osteocytes were visualized with Alizarin Red S staining following this protocol:

- Cells were washed 2x with PBS
- Cells were fixed with 4% paraformaldehyde for 15 min
- Cells were washed with PBS for 5 min
- The washing step was repeated 3x
- Cells were incubated for 20 min in 2% Alizarin Red S stain diluted in distilled water (Sigma, catalog no: A5533)
- Cells were washed several times with distilled water and then with PBS and observed under a microscope (Alizarin Red S detects calcium depots in bone tissue)

All pictures were taken with optical microscope Leica Dmi1 (Leica Microsystems, Wetzlar, Germany).

4.11. FACS analysis

FACS analysis was used for MSC phenotyping, evaluation of MSC metabolic properties, and analysis of splenocytes from co-culture experiments. Single-cell suspensions were kept on ice throughout the method, except when the cells were fixed. Hoechst 33258 (1:40 000, Merck, catalog no: 94403) was used to exclude dead cells in the case of surface staining and Live/Dead Fixable Violet Dead Cell Stain Kit (Live/Dead) (Invitrogen, catalog no: L34955) in the case of intracellular staining.

All samples were measured using LSR II (BD Biosciences). Flow cytometry data were analyzed using FlowJo 10.10 software (BD Biosciences).

4.11.1. Phenotypic characterization of MSCs

FACS staining was used to phenotype MSCs isolated from cold-exposed mice. The expression of markers CD90, CD73, CD105, CD44, CD34, CD31, CD29, CD45, CD11b was determined. Single-cell suspension of MSCs was stained according to this protocol:

- Cells were scraped from tissue culture plates, washed in PBS, and centrifuged (8 min, 8°C, 170 g)
- The pellet was resuspended in PBS and transferred into a 96-well plate with a round bottom (Nunc, Roskilde, Denmark)
- Cells were centrifuged (3 min, 8°C, 170 g), the supernatant was discarded, and the plate vortexed to resuspend the pellet
- Cells were stained with 10 µl antibodies (see Table 4) diluted in PBS, vortexed gently, and incubated for 30 min at 4°C
- Cells were washed 2x with PBS, centrifuged (3 min, 8°C, 170 g)
- 200 µl PBS was added
- Cells were stained with 20 µl Hoechst 33258

4.11.2. Analysis of metabolic properties of MSCs

To describe the metabolic properties of MSCs, ROS production, mitochondrial membrane potential, and glucose uptake were measured using FACS staining.

All samples were prepared according to the following protocol:

- Cells were scraped from tissue culture plates, washed in PBS, and centrifuged (8 min, 8°C, 170 g)
- The pellet was resuspended in PBS and transferred into a 96-well plate with a round bottom
- Cells were centrifuged (3 min, 8°C, 170 g), the supernatant was discarded, and the plate vortexed to resuspend the pellet
- 200 µl PBS was added
- Cells were stained with 20 µl Hoechst 33258 and measured

4.11.2.1. Glucose uptake

To evaluate the glucose uptake, cells were washed with PBS, incubated for 5 min with trypsin (Media and Washing facility, IMG, Prague), scraped, transferred into centrifuge tubes, and centrifuged (8 min, 8°C, 170 g). Cells were counted using the Bürker chamber, seeded in a concentration of 1.5×10^5 cells/ml into a 12-well plate, and cultured in a glucose-free RPMI 24 hours before the measurement to deprive the cells of glucose. After 24 h, fluorescently labeled glucose analog 2-NBDG (196 µg/ml; 2-(N(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose, excitation/emission maximum: 465/540 nm; ThermoFisher, catalog no: N13195) was added to the culture for 30 min and incubated in the dark. Cells were then prepared for FACS analysis.

4.11.2.2. ROS production and mitochondrial potential

To assess mitochondrial potential and ROS production, MSCs were washed with PBS, trypsinized, scraped, centrifuged (8 min, 8°C, 170 g), and counted. Cells were then seeded in a concentration of 1.5×10^4 cells/ml into a 12-well plate, and DMEM medium was added. The cells were cultured for 1 hour to let them adhere. Then, the cells were stained with MitoTracker Red CMXRos (100 nM; excitation/emission maximum: 579/599 nm; Invitrogen, catalog no: M7512) to visualize functional mitochondria and DCFDA (15 µM; 2',7'-dichlorofluorescein diacetate, excitation/emission maximum: 492/527 nm; Invitrogen, catalog no: C369) to visualize ROS and incubated for 30 min. After the incubation, cells were prepared for FACS analysis.

4.11.3. Intracellular NO expression

The production of NO in MSCs cultured at reduced temperature (C, 1D, 3D) was evaluated intracellularly by FACS staining. MSCs were washed with PBS, trypsinized, scraped, centrifuged (8°C, 8 min, 170 g), and counted. MSCs were seeded (1.5×10^5 cells/ml) into a 12-well plate and cultured in DMEM. To evaluate the effect of inflammation on NO production, MSCs were also stimulated with the combination of LPS (1.25 µg/ml) and IFN γ (20 ng/ml) for 3 or 24 h. After the given incubation period, the cells were stained with DAF-2 DA (2.5 µM; 4,5-diaminofluorescein diacetate dimethylsuloxide solution, excitation/emission maximum: 485/538 nm; Biotech, catalog no: ab146631) and incubated for 30 min in the dark. Then, the cells were prepared for FACS analysis following the protocol from 4.11.2.

4.11.4. Analysis of splenocytes in co-culture with MSCs

We employed co-cultivation experiments with splenocytes to assess the immunosuppressive function of MSCs. Splenocytes were isolated as described in Chapter 4.7. MSCs were washed with PBS, trypsinized, scraped, centrifuged (8°C, 8 min, 170 g), and counted. MSCs were seeded (10^5 cells per 0.5 ml DMEM) into 12-well plates and left to adhere for a few minutes. Splenocytes (10^6 per 0.5 ml DMEM) were added to MSCs. The ratio between MSCs and splenocytes was 1:10 for the experiments with MSCs isolated from cold-exposed mice and 1:20 (5×10^4 MSCs to 10^6 splenocytes in 1 ml of DMEM) for the experiments with MSCs cultured at reduced temperature (the protocol was otherwise the same for both experiments). Co-cultures were then activated by the addition of Concanavalin A (1.25 µg/ml, Merck, catalog no: C2010). MSCs were co-cultured with activated splenocytes for 24 h to evaluate activation markers, 48 h for the measurement of cytokine production by splenocytes, and 72 h to measure the proliferation rate and expression of transcription factors.

4.11.4.1. Activation marker expression

The following protocol was employed to evaluate the activation of splenocytes co-cultured with MSCs based on the expression of activation markers CD25 and CD69.

- Cells were scraped from tissue culture plates, washed in PBS, and centrifuged (8 min, 8°C, 170 g)
- The pellet was resuspended in PBS and transferred into a 96-well plate with a round bottom
- Cells were centrifuged (3 min, 8°C, 170 g), the supernatant was discarded, and the plate vortexed to resuspend the pellet
- Cells were incubated with antibodies diluted in PBS (see Table 4) at a 10 µl volume for 30 min at 4°C
- Cells were washed 2x with PBS and centrifuged (8°C, 3 min, 170 g)
- 200 µl PBS was added

- Cells were stained with 20 µl Hoechst 33258 and measured

4.11.4.2. Cytokine production

The production of cytokines IL-2, IL-10, and IL-17 by splenocytes co-cultured with MSCs was assessed. The co-cultures were restimulated with PMA (phorbol 12-myristate 13-acetate, 20 ng/ml; Merck, catalog no: P8139), Brefeldin A (5 µg/ml; Invitrogen, catalog no: 00-4506-51) and Ionomycin (500 ng/ml; Merck, catalog no: I0634) 4-5 hours before the preparation for FACS analysis with following this protocol:

- Cells were scraped from tissue culture plates, washed in PBS, and centrifuged (8 min, 8°C, 170 g)
- The pellet was resuspended in PBS and transferred into a 96-well plate with a round bottom
- Cells were centrifuged (3 min, 8°C, 170 g), the supernatant was discarded, and the plate vortexed to resuspend the pellet
- Cells were incubated with surface antibodies diluted in PBS (Table 4) and Live/Dead stain in a 10 µl volume for 30 min at 4°C
- Cells were washed 2x with PBS and centrifuged (8°C, 3 min, 170 g)
- Cells were incubated with 100 µl of fixation buffer (eBioscience Intracellular Fixation and Permeabilization Buffer Set; Invitrogen; catalog no.: 88-8824-00) for 20 min in the dark at 4°C and 10 min in the dark at RT
- 100 µl of permeabilization buffer (10x diluted in PBS, eBioscience Intracellular Fixation and Permeabilization Buffer Set; Invitrogen; catalog no.: 88-8824-00) was added, and the plate was centrifuged (23°C, 4 min, 300 g)
- 200 µl of permeabilization buffer was added, and the plate was centrifuged again (23°C, 4 min, 300 g)
- Cells were incubated with intracellular antibodies (Table 4) diluted in permeabilization buffer at a 10 µl volume for 30 min in the dark at RT
- Cells were then washed 2x with permeabilization buffer and centrifuged (23°C, 4 min, 300 g)
- 200 µl of permeabilization buffer was added, and cells were measured

4.11.4.3. Proliferation and expression of transcription factors

To assess the proliferation rate of splenocytes in the co-culture with MSCs, we employed staining for the proliferation marker Ki67. We also evaluated the expression of transcription factors FOXP3 and RORγt to distinguish Th1 and Th17 lymphocytes. The protocol used for the intracellular staining of single-cell suspensions is the same as in Chapter 4.11.4.2, with one exception. For the fixation of cells, a Fixation Concentrate diluted in Fixation Diluent was used (3x diluted, eBioscience Foxp3/Transcription Factor Staining Buffer Set; Invitrogen; catalog no: 00-5523-00) to allow the staining of nuclear proteins.

Table 4. List of monoclonal antibodies used for flow cytometry analysis. Green color designates surface markers, and blue designates intracellular markers. FITC...fluorescein isothiocyanate, APC...allophycocyanin, PE...phycoerythrin, AF...Alexa Fluor, SB...Super Bright.

Marker	Fluorochrome	Dilution	Clone	Manufacturer	Catalog no:
CD4	FITC	1:150	GK1.5	BioLegend	103128
CD4	APC	1:150	GK1.5	BioLegend	100412
CD8a	FITC	1:160	53-6.7	BioLegend	100705
CD11b	APC	1:100	M1-70	BioLegend	101212
CD19	FITC	1:90	6D5	BioLegend	115505
CD25	APC	1:200	PC61.5	eBioscience	17-0251-82
CD29	PE	1:100	eBioHMB1-1	Invitrogen	12-0291-82
CD31	PE	1:40	390	BioLegend	102407
CD34	PE	1:100	HM34	BioLegend	128609
CD44	FITC	1:40	IM7	BioLegend	103005
CD45	AF700	1:150	30-F11	Invitrogen	56-0451-82
CD45	SB780	1:150	30F-11	Invitrogen	11-0451-82
CD69	PE	1:120	H1.2F3	BioLegend	104507
CD73	PE	1:120	TY/11.8	Invitrogen	12-0731-82
CD90.2	APC	1:100	30-H12	SONY	1126560
CD105	AF488	1:40	MJ7/18	BioLegend	120406
F4/80	FITC	1:50	BM8.1	SONY	35-4801
FOXP3	APC	1:80	FJK-16S	Invitrogen	17-5773-82
ROR γ t	PE	1:80	AFKJS-9	Invitrogen	12-6988-82
Ki67	FITC	1:150	SolA15	eBioscience	11-5698-82
Ki67	PE	1:150	16A8	BioLegend	652404
IL-2	APC	1:100	JES6-5H4	Invitrogen	17-7021-82
IL-10	PE	1:80	JES5-16E3	BioLegend	505010
IL-17A	PE	1:70	eBio17B7	Invitrogen	12-7177-81

4.12. WST assay

The water-soluble tetrazolium salt (WST) assay is used for spectrophotometric evaluation of cell viability, proliferation, and metabolic activity. It is based on reducing WST-1 salts by mitochondrial succinate dehydrogenase into a formazan dye, which can be quantified by spectrophotometry. The cells were prepared for this assay using the following protocol: cells were washed with PBS, trypsinized, scraped, centrifuged (8°C, 8 min, 170 g), and counted using a Bürker chamber. Cells were seeded into a 96-well plate (1.5×10^4 cells in a volume of 200 μ l). To assess the response to inflammation, cells were stimulated with LPS (1.25 μ g/ml) and IFN γ (20 ng/ml) for 1 and 24 hours. After the incubation period, WST-1 was added (1:10, Roche; catalog no: 5015944001) and incubated for 2 hours at 37°C. Optic density was measured using an ELx800 spectrophotometer at 450 nm. DMEM was used as a blank for the measurement.

4.13. Extracellular NO production

Extracellular NO production was evaluated using the Griess reaction. MSCs were prepared using the following protocol: cells were washed with PBS, trypsinized, scraped, centrifuged (8°C, 8 min, 170 g), and counted using a Bürker chamber. Cells were seeded into a 12-well plate (2.5×10^5 cells per ml). Cells were left unstimulated or stimulated with LPS (1.25 µg/ml) and IFN γ (20 ng/ml) for 48 hours. After incubation, 100 µl of supernatant and standards (standard curve of NaNO $_3$ solution) in doublets were transferred into a 96-well plate. The detection solution (Solution A and Solution B, 1:1, see Chapter 4.3) was added in a 100 µl volume. Optic density was measured using spectrophotometer ELx800 at 570 nm. DMEM was used as a blank for the measurement. Representative calibration curve for NO detection is shown in Fig.7.

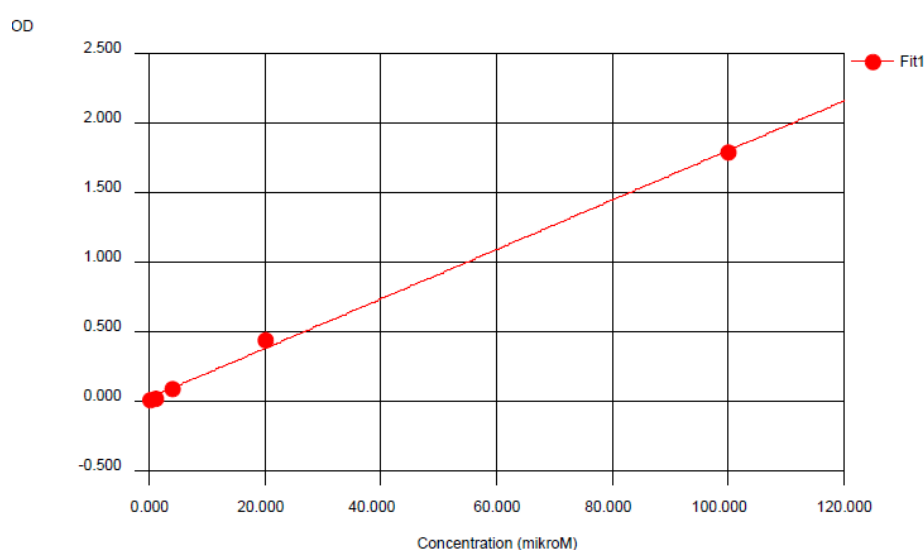


Figure 7. Calibration curve for extracellular NO detection. The X-axis shows NO concentration in µM, and the Y-axis shows the optical density of the colored product. OD...optic density, Fit1...curve fitting.

4.14. Real-Time qPCR

The real-time quantitative polymerase chain reaction (RT qPCR) was employed to measure the expression of genes involved in metabolism and immunomodulation in MSCs isolated from cold-exposed mice.

4.14.1. RNA isolation

MSCs were washed with PBS, trypsinized, scraped, centrifuged (8°C, 8 min, 170 g), and counted using the Bürker chamber. Cells were seeded into a 12-well plate in a concentration of 1.5×10^5 cells/ml. Cells were left unstimulated or stimulated with LPS (1.25 µg/ml) and IFN γ (20 ng/ml) for 24 and 48 hours. The RNA was then isolated as follows:

- Cells were centrifuged (8°C, 8 min, 170 g) and the supernatant discarded
- Cells were resuspended in 300 µl of TRI reagent (Molecular Research Center, Inc., Cincinnati, Ohio, USA; catalog no.: TR118) and transferred into DNA/RNA-free microtubes
- 60 µl of chloroform was added and microtubes were left to rest for 5 min at RT
- Cells were centrifuged (8°C, 15 min, 14 000 g)
- Surface phase was transferred into a new DNA/RNA-free microtube, and the same volume of 60% isopropanol was added
- Microtubes were left to rest for 5 min at RT and then centrifuged again (8°C, 8 min, 14 000 g)
- Supernatant was discarded
- 50 µl of ice-cold 70% ethanol was added
- The ethanol was discarded, and open microtubes were placed into a thermoblock set at 40°C to evaporate the rest of the ethanol
- 25 µl of RNase-free water was added, and microtubes were placed into a thermoblock set at 65°C for 5 min to dissolve RNA
- Microtubes were then put on ice
- The quality and concentration of isolated RNA were measured using a Spectrophotometer Nanodrop (Thermo-Fisher Scientific). RNA was stored at -80°C

4.14.2. Reverse transcription

Reverse transcription (RT) was performed using a kit SuperScript IV RT (Invitrogen, catalog no: 18090200) following the manufacturer's instructions.

- 1 µl of random hexamer primers (Thermo Scientific; catalog no: SO142) and 1 µl of deoxynucleotides (Thermo Scientific, catalog no: R0192) were added to 1 µg of RNA, and RNase-free water was added to a 13 µl volume
- Microtubes were placed in a thermocycler for 5 min at 65°C and immediately put on ice
- In the meantime, a premix was prepared (for 1 µg RNA): 4 µl 5x SuperScript IV RT buffer, 1 µl 0.1M DTT (dithiothreitol), 1 µl SuperScript IV reverse transcriptase, and 0.5 µl RNaseOUT Recombinant Ribonuclease Inhibitor (Thermo Scientific, catalog no: 10777019)
- The premix was added to the microtubes, mixed, and placed in a thermocycler for a cycle of 5 min at 25°C, 60 min at 50°C, 15 min at 75°C, and a final cooldown at 4°C
- Prepared cDNA was stored at -20°C

4.14.3. RT qPCR reaction

The qPCR reaction was performed using LightCycler 480 II (Roche). All used primers are listed in Table 5. The relative expression of genes was evaluated using genes for actin B (*Actb*), β 2-microglobulin (*B2m*), and ribosomal protein L13a (*Rpl13a*) as a housekeeping control.

- Every sample was prepared by mixing 1 μ l cDNA, 1 μ l forward primer, 1 μ l reverse primer, 1.5-2 μ l 5x HOT FIREPol EvaGreen qPCR supermix (Solid BioDyne, Tartu, Estonia; catalog no: 08-36-00020), and 6 μ l RNase-free water
- The samples were put into a 384-well plate (BIOplastics, Landgraaf, Netherlands) in triplicates
- Samples were pre-incubated for 12 min at 95°C, and 50 cycles of 17 s at 95°C, 22s at 62°C, and 20 s at 72°C, were repeated followed by a final cooldown to 40°C

Table 5. Primers used for qPCR reaction.

Gene	Gene ID	Protein	Forward sequence	Reverse sequence
<i>Actb</i>	11461	Actb	CGGAACCGCTCATTGCC	ACCCACACTGTGCCCATCTA
<i>Rpl13a</i>	22121	Rpl13a	AGGGGCAGGTTCTGGTATTG	TGTTGATGCCTTCACAGCGT
<i>B2m</i>	12010	B2m	CATGGCTCGCTCGGTGAC	CAGTTCAGTATGTTCTGGCTTCC
<i>Hk1</i>	15275	Hk1	CGGAATGGGGAGCCTTTGG	GCCTTCCTTATCCGTTTCAATGG
<i>Hk2</i>	15277	Hk2	TGATCGCCTGCTTATTCACGG	AACCGCCTAGAAATCTCCAGA
<i>Pfkfb1</i>	18639	Pfkfb1	ATGAGCTGCCCTATCTCAAGT	GTCCCGGTGTGTGTTTCACAG
<i>G6pd2</i>	14380	G6pd2	CACAGTGGACGACATCCGAAA	AGCTACATAGGAATTACGGGCAA
<i>Slc2a1</i>	20525	Glut1	CAGTTCGGCTATAACACTGGTG	GCCCCGACAGAGAAGATG
<i>Ldhd</i>	52815	Ldhd	CTGAAGGCAGTTGTAGGGAGC	GGAACACCTTGATTGTAGCACAG
<i>Cs</i>	12974	Cs	GGACAATTTTCCAACCAATCTGC	TCGGTTCATTCCCTCTGCATA
<i>Sod1</i>	20655	Sod1	AACCAGTTGTGTTGTCAGGAC	CCACCATGTTTCTTAGAGTGAGG
<i>Sod2</i>	20656	Sod2	CAGACCTGCCTTACGACTATGG	CTCGGTGGCGTTGAGATTGTT
<i>Ndufa6</i>	67130	Ndufa6	TCGGTGAAGCCCATTTTCAGT	CTCGGACTTTATCCCGTCCCTT
<i>Sdhc</i>	66052	Sdhc	GCTGCGTTCTTGCTGAGACA	ATCTCCTCCTTAGCTGTGGTT
<i>Uqcrb</i>	67530	Uqcrb	GGCCGATCTGCTGTTTCAG	CATCTCGCATTAACCCAGTT
<i>Cox4i1</i>	12857	Cox4i1	ATTGGCAAGAGAGCCATTCTAC	CACGCCGATCAGCGTAAGT
<i>Atp5fb1</i>	11947	Atp5b	ACGTCCAGTTCGATGAGGGAT	TTTCTGGCCTCTAACCAAGCC
<i>Ucp2</i>	22228	Ucp2	GGCATTACGGGCAACATT	CTGGTGGTGGTCGGAGATA
<i>Slc25a27</i>	74011	Ucp4	TACTAAAACACGGCTGCAAGTC	GCATGAAACATCCCTCTATTTTTAT
<i>Slc25a14</i>	20523	Ucp5	CATCTACAGAGCTGCCTACTTC	TCACACTCTGGGCAATCATC
<i>Slc25a4</i>	11739	Ant1	GTGATGCTCAGGTATCCATCCA	CACAGTTCTCAAAGCACAGCG
<i>Nos2</i>	18126	iNOS	TCATTGTACTCTGAGGGCTGAC	CTTTGCCACGGACGAGAC
<i>Ptgs2</i>	29225	Cox2	AGCCCACCCCAACACAGT	AAATATGATCTGGATGTCAGCACATATT
<i>Ido1</i>	15930	Ido1	GGGCTTTGCTCTACCACATC	AAGGACCCAGGGGCTGTAT
<i>Icam1</i>	15894	Icam1	GTGATGCTCAGGTATCCATCCA	CACAGTTCTCAAAGCACAGCG
<i>Vegfa</i>	22339	Vegfa	AAAAACGAAAGCGCAAGAAA	TTTCTCCGCTCTGAACAAGG
<i>Acod1</i>	66141	Acod1	TTTGGGGTCGACCAGACTTC	CCATGGAGTGAACAGCAACAC

4.15. Agilent Seahorse XF Analyzer

We employed Agilent Seahorse XF Analyzer to evaluate the metabolic features of MSCs. MSCs were washed with PBS, trypsinized, scraped, centrifuged (8°C, 8 min, 170 g), and counted using the Bürker chamber. Cells were seeded into a 96-well plate (1.5×10^4 cells). The measurement was prepared according to the following protocol:

- Hydrate the cartridge one day prior to the measurement by adding 200 µl distilled water in each well, covering with parafilm, and putting into an incubator without CO₂ at 37°C
- Discard water from the cartridge and add 200 µl of warmed calibrant and incubate 1 h in an incubator without CO₂ at 37°C
- Wash the cells with Seahorse XF DMEM medium, and 180 µl of Seahorse XF DMEM was added to each well and incubated for 1 h
- Resuspend the metabolic inhibitors from Seahorse XF Real-Time ATP Rate Assay Kit (Agilent Technologies, catalog no: 103592-100) in Seahorse XF DMEM: 15 µM oligomycin, 5 µM rotenone and antimycin A
- Add 20 µl of oligomycin into Port A and 22 µl of rotenone plus antimycin A into Port B of the cartridge
- Transfer the cartridge into the instrument to start the calibration process (15 min)
- Wash the cells again with Seahorse XF DMEM
- Put the cartridge on the cell plate instead of the calibrant
- Start the measurement

Data was normalized to protein content per cell using BCA method. BCA method (Bicinchoninic acid assay) was performed as follows: the cells were washed with PBS, 100 µl of solution (Reagent A, Reagent B, Reagent C; 24:1:25) was added, and incubated for 30 min at 60°C. The absorbance was then measured using spectrophotometer ELx800 at 562 nm.

4.16. Statistical analysis

Statistical analysis was performed using GraphPad Prism 8. The statistical significance was estimated with one-way ANOVA with Holm-Sidak's multiple comparisons test and two-way ANOVA with Tukey's or Sidak's comparisons test.

5. Results

5.1. Part 1 – The effect of cold exposure on MSCs

5.1.1. Establishment of cold acclimation model

One of the adaptations to a cold environment in rodents is an increased activity of thermogenic adipocytes. This can be achieved by the expansion of fully thermogenic adipocytes within the BAT or transdifferentiation of white adipocytes within WAT into thermogenic beige adipocytes. The expansion of BAT can be considered a hallmark of acclimation to cold temperatures; therefore, we first measured the weight of BAT of mice exposed to 8°C for one day (referred to as 1D-cold) or 14 days (referred to as 14D-cold) to assess the dynamics of response to cold in mice. As expected, 14D-cold mice had significantly increased BAT weight (Fig.8), suggesting that these animals acclimated to the cold environment. Short-term cold exposure did not result in increased BAT weight.

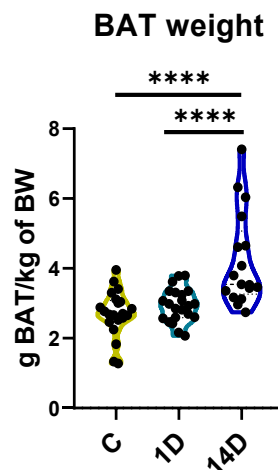


Figure 8. The weight of BAT isolated from mice exposed to cold temperatures. The weight of BAT is shown as g of BAT per g of mouse body weight (each dot represents one mouse, mean $\pm$ standard deviation (SD), data from 5 independent experiments, $n=17-22$, one-way ANOVA with Holm-Sidak's multiple comparisons test, **** = $p < 0.0001$). BAT...brown adipose tissue, BW...body weight, C...control MSCs, 1D...1D-cold MSCs, 14D...14D-cold MSCs.

As was described in the Literature overview, the adaptations of adipose tissue in response to cold environment are accompanied by concurrent changes in the composition of immune cells within the adipose tissue, which differentially contribute to the beiging process. Previous research in our laboratory also showed that cold exposure leads to alterations in cytokine levels within BAT and WAT (Vasek et al., 2024). We wanted to see whether the same alterations in cytokine levels can be observed in our mouse model of cold acclimation. We, therefore, measured levels of several cytokines using ELISA in pWAT and BAT (Fig.9a,b). Surprisingly, pWAT exhibited significantly increased levels of all measured cytokines during cold exposure, while BAT exhibited significantly decreased levels. Next, we wondered whether cytokine levels were affected in other tissues. ELISA was performed in homogenates from iWAT, asWAT,

thymus, and spleen (Fig.9c,d + Fig.10). Cytokine levels in iWAT and asWAT were less affected by cold exposure than in pWAT and BAT. Similarly, cytokine levels in the spleen were almost unaffected. In contrast, cytokine production within the thymic tissue was significantly increased in the case of IL-1 β , TNF α , and IL-7. IL-6 and IL-2 production also had an increasing trend in 14D-cold mice. We created heat maps of cytokine levels in different tissues to summarize our findings and demonstrate the tissue-specific response to cold exposure (Fig.11).

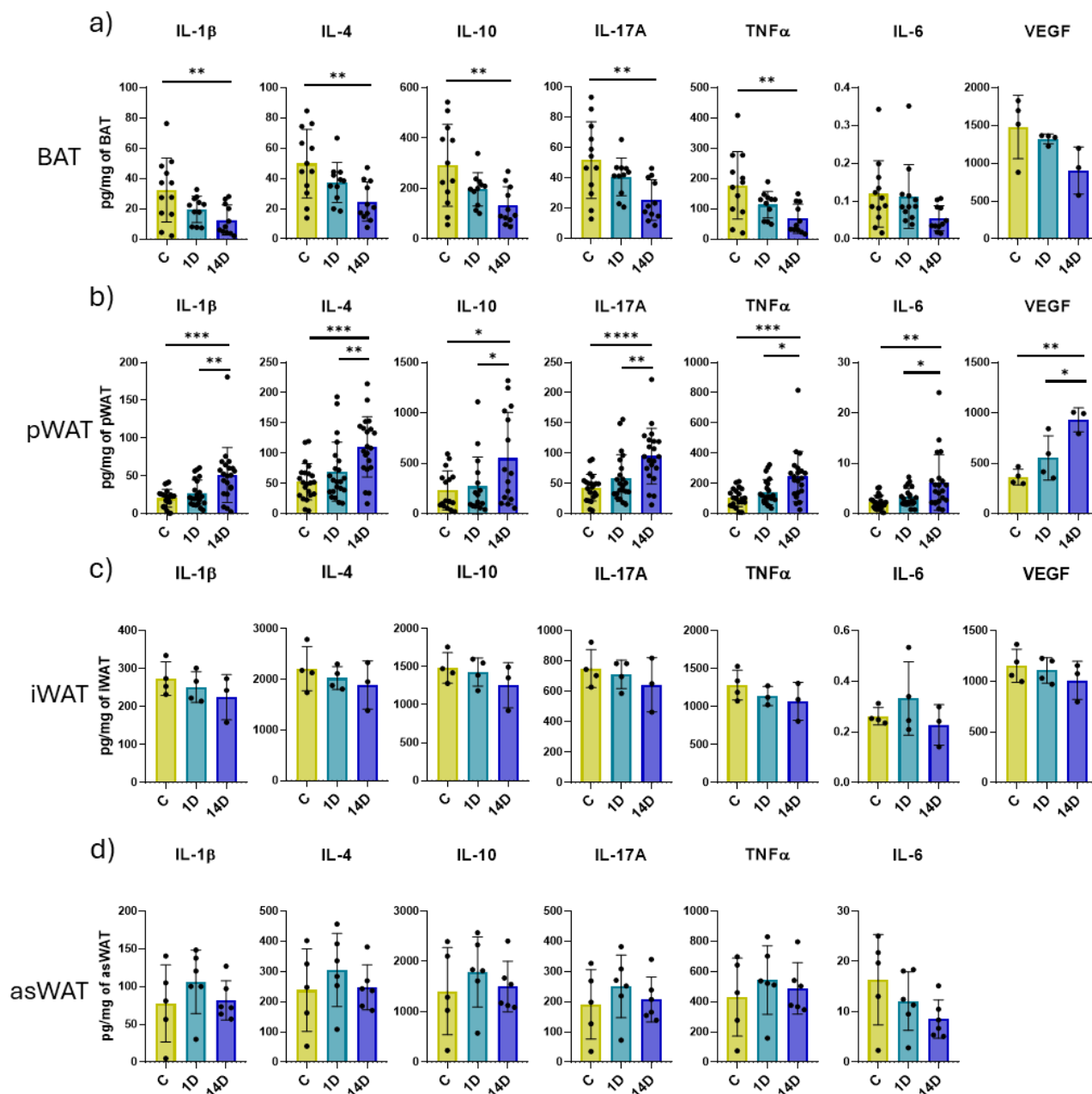


Figure 9. Cytokine and VEGF levels within BAT, pWAT, iWAT, and asWAT measured with ELISA. Cytokine levels in BAT (a), pWAT (b), iWAT (c), and asWAT (d). The concentration of cytokines in the tissue is shown as pg of a cytokine per mg of tissue weight. Each dot represents one mouse (BAT – data from 3 independent experiments, pWAT – data from 4-5 independent experiments, asWAT, iWAT – data from 1 experiment, VEGF levels – data from 1 experiment, n=3-22; one-way ANOVA with Holm-Sidak's comparisons test, * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$). BAT...brown adipose tissue, pWAT...perigonadal white adipose tissue, iWAT...inguinal white adipose tissue, asWAT...anterior subcutaneous white adipose tissue, IL...interleukin, TNF α ...tumor necrosis factor- α , VEGF...vascular endothelial growth factor; C...control MSCs, 1D...1D-cold MSCs, 14D...14D-cold MSCs.

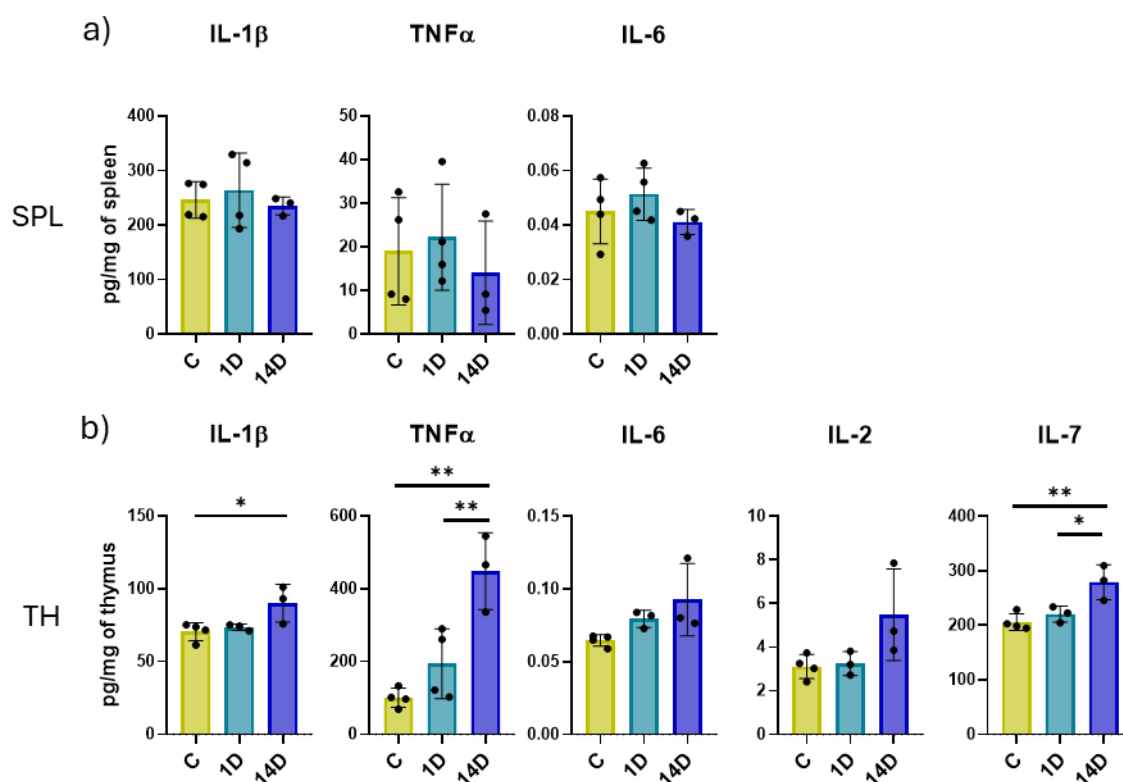


Figure 10. Cytokine levels in the spleen and thymus. Cytokine levels were measured in the spleen (a) and thymus (b). The concentration of cytokines in the tissue is shown as pg of a cytokine per mg of tissue weight. Each dot represents one mouse (data from 1 experiment, n=3-4; one-way ANOVA with Holm-Sidak's comparisons test, * = $p < 0.05$; ** = $p < 0.01$). SPL...spleen, TH...thymus, IL...interleukin, TNF α ...tumor necrosis factor- α , C...control MSCs, 1D...1D-cold MSCs, 14D...14D-cold MSCs.

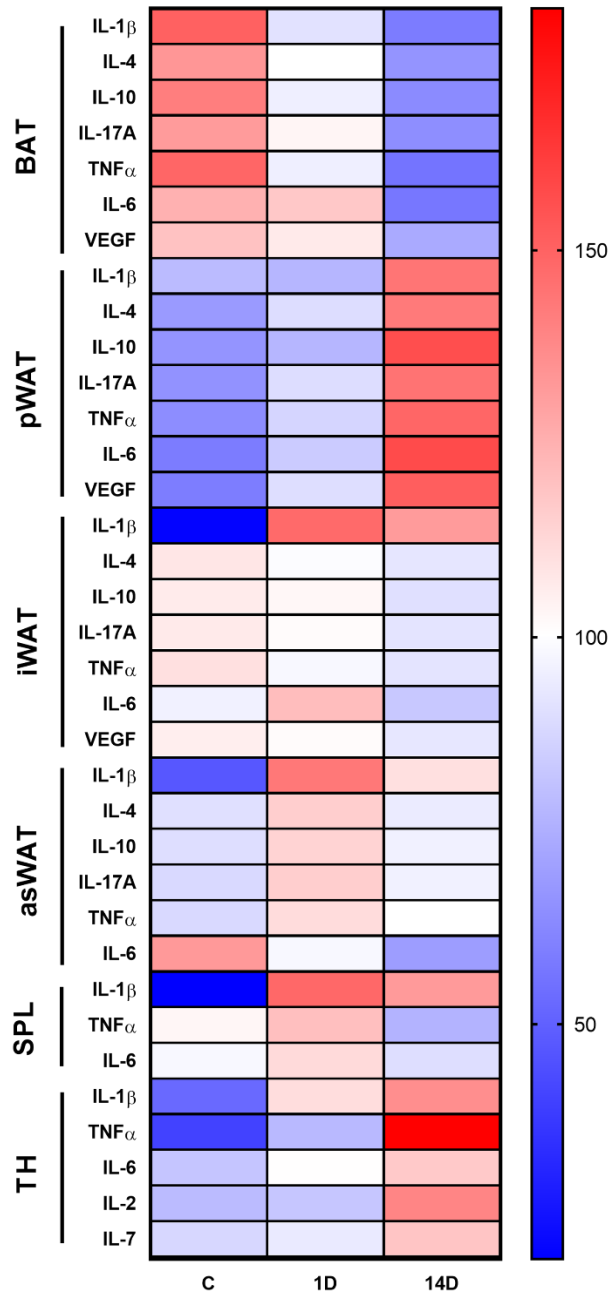


Figure 11. Heat maps showing differential cytokine levels in different tissues. The data from all performed ELISAs were averaged and normalized to the mean. 100% was defined as the average of all means in the dataset, and the color codes represent percentages. The heat map was created in GraphPad Prism 8. BAT...brown adipose tissue, pWAT...perigonadal white adipose tissue, iWAT...inguinal white adipose tissue, asWAT...anterior subcutaneous white adipose tissue, SPL...spleen, TH...thymus, IL...interleukin, TNF α ...tumor necrosis factor- α , VEGF...vascular endothelial growth factor, C...control MSCs, 1D...1D-cold MSCs, 14D...14D-cold MSCs.

5.1.2. Characterization of MSCs derived from cold-exposed mice

Having described cold-induced changes in cytokine levels in adipose tissue depots, we wondered if and how these changes impact MSCs isolated from the adipose tissue.

MSCs are characterized by three minimal criteria proposed by Dominici et al. (Dominici et al., 2006). We inspected all criteria to ensure that MSCs isolated from cold-exposed mice still share the same characteristics as MSCs isolated from mice housed at RT. The surface marker expression was evaluated by FACS staining. Fig.12 shows a representative gating strategy for MSC discrimination in experiments describing MSC phenotype and metabolic properties. The minimal human MSC markers are CD73, CD90, and CD105 (Dominici et al., 2006). In our phenotypic analysis, we additionally employed CD29 and CD44. We stained MSCs cultures for CD31, CD34, CD11b, and CD45 to exclude contaminating cells of hematopoietic origin or endothelial cells. This combination of markers has proven effective in defining murine MSCs previously in our laboratory (Hajkova et al., 2017; Porubska et al., 2021). MSCs isolated from mice exposed 1 day to a cold environment and mice exposed 14 days to a cold environment expressed all MSC markers, and, at the same time, they lacked the expression of non-MSC markers (Fig.13). MSCs isolated from 1D-cold mice had slightly enhanced expression of CD44 and CD90.

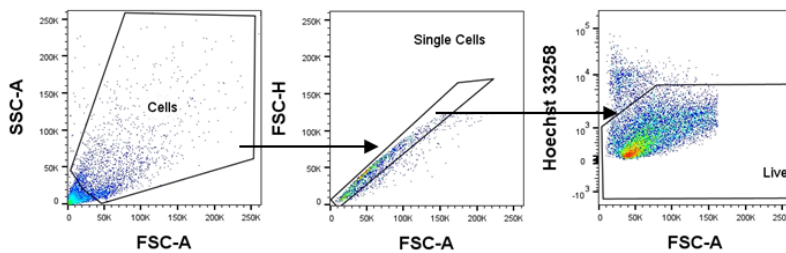


Figure 12. Representative gating strategy to determine MSCs. Cells were determined based on forward-scatter (FSC-A) and side-scatter (SSC-A). Singlets were eliminated based on forward-scatter (FSC-A, FSC-H). Dead cells were eliminated based on Hoechst 33258. As we have characterized MSCs in our cultures, no additional markers were necessary.

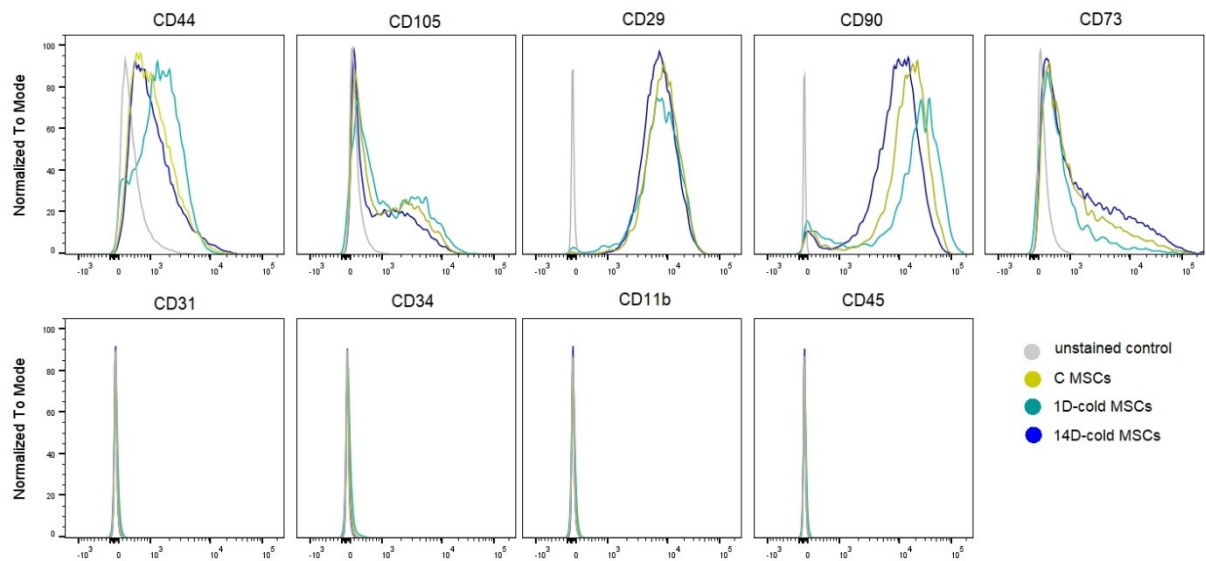


Figure 13. Characterization of MSCs isolated from cold-exposed mice. Gray lines in histograms represent a negative control for all depicted markers, and colored lines represent samples: C MSCs (yellow), 1D-cold MSCs (teal), and 14D-cold MSCs (blue). Overlaid histograms are normalized to mode.

The second criterion for MSC characterization is plastic adherence in culture. Fig.14a shows a representative picture of C MSC adherence; cold MSCs adhered similarly (not shown). Lastly, MSCs must be capable of multilineage differentiation. We differentiated MSCs by culturing them under adipogenic, chondrogenic, and osteogenic conditions. Both control MSCs and cold-derived MSCs were able to differentiate into all tested cell types with no recognizable differences (Fig.14b)

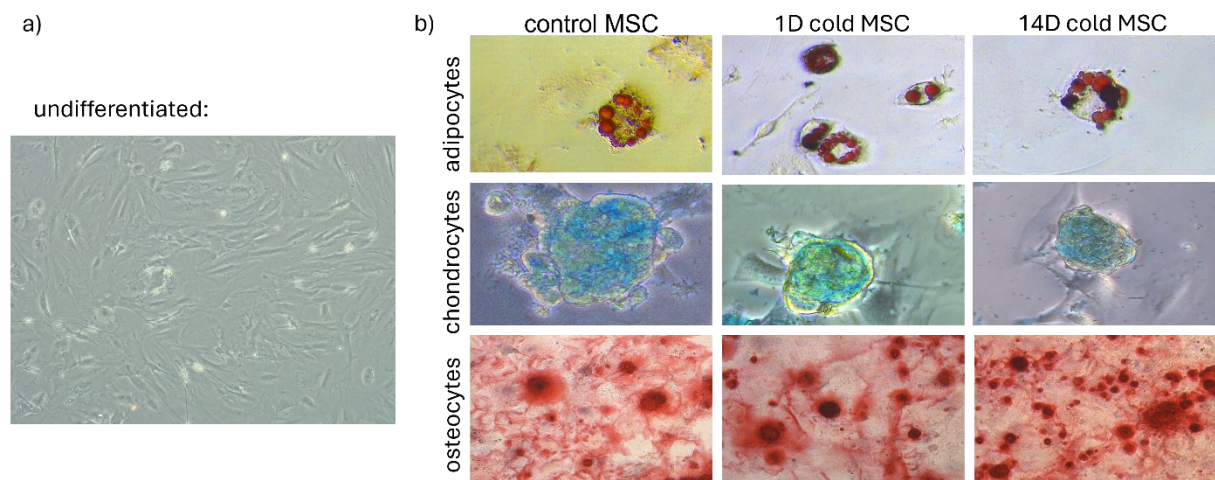


Figure 14. Differentiation of MSCs. (a) shows undifferentiated C MSCs adhering to the bottom of a tissue culture flask, (b) shows representative pictures of differentiated MSCs into adipocytes stained with Oil Red O (detects neutral lipids in lipid droplets), chondrocytes stained with Alcian Blue (detects glycosaminoglycans in cartilages), and osteocytes stained with Alizarin Red S (detects calcium depots within bone tissue). All images were taken with 100x magnification.

The results obtained from this section allowed us to conclude that MSCs isolated from mice exposed to cold temperatures meet the criteria for being characterized as MSCs.

5.1.3. Metabolic properties of MSCs derived from cold-exposed mice

MSC metabolic state is closely related to their immunomodulatory activity. Therefore, we decided to employ diverse methods to describe the metabolic properties of MSCs derived from cold-exposed mice. First, we measured glucose uptake by MSCs after 24h incubation in a glucose-free medium (Fig.15a). To evaluate mitochondrial functionality, we stained MSCs with MitoTracker Red, which detects active mitochondria based on the mitochondrial membrane potential (Fig.15b). 1D-cold MSCs exhibited significantly increased glucose uptake compared to control and 14D-cold MSCs. In contrast, the mitochondrial membrane potential was decreased, indicating a partial metabolic switch toward glycolysis in those cells. Next, we assessed the production of ROS by MSCs. There was a tendency for increased ROS production in MSCs derived from cold-exposed mice; however, it was insignificant (Fig.15c).

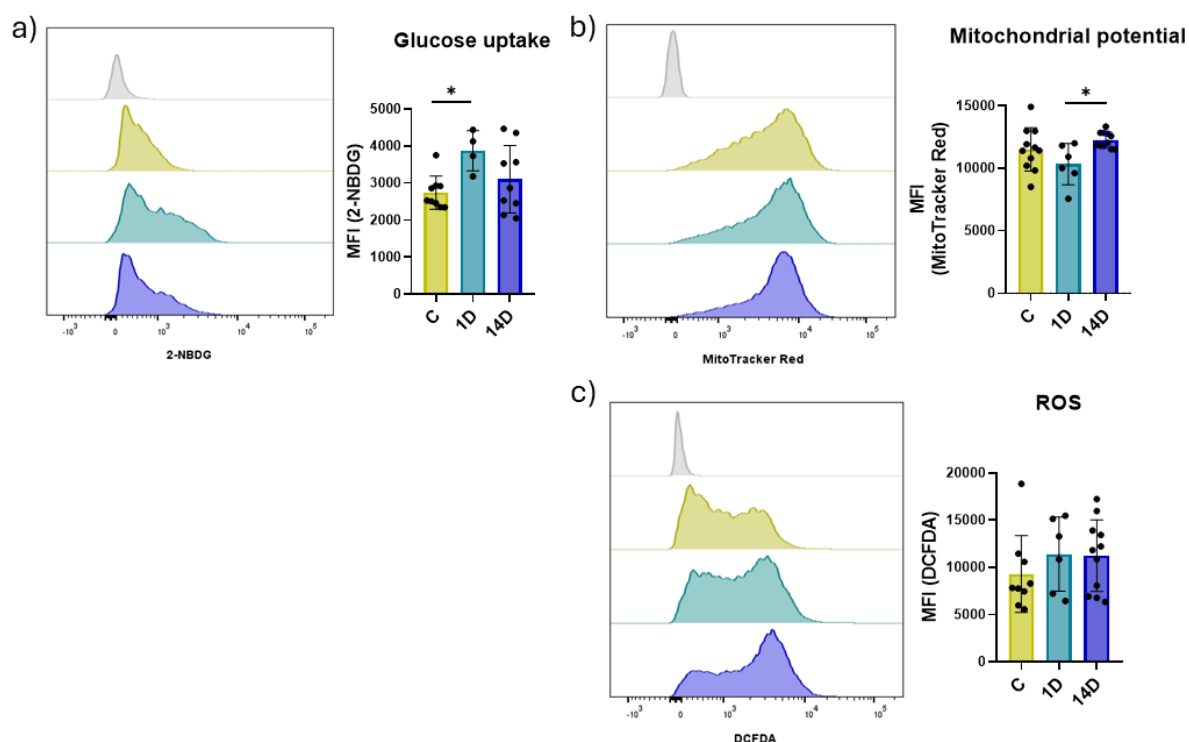


Figure 15. Flow cytometric analysis of glucose uptake, mitochondrial membrane potential, and ROS production. Mean fluorescence intensity of 2-NBDG (a), MitoTracker Red (b), and DCFDA (c) in MSCs. Histograms on the left show a negative control and fluorescence intensity of experimental groups. Each dot represents one MSC sample pooled from 2 mice, mean $\pm$ SD, data from 4-5 independent experiments, $n=4-11$, one-way ANOVA with Holm-Sidak's multiple comparisons test, * = $p < 0.05$. MFI...mean fluorescence intensity, 2-NBDG...2-(N(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose, DCFDA...2',7'-dichlorofluorescein diacetate, ROS...reactive oxygen species, C...control MSCs, 1D...1D-cold MSCs, 14D...14D-cold MSCs.

We wanted to corroborate the data obtained from flow cytometry with a different approach, so we employed the Seahorse Analyzer. Specifically, we employed a kit detecting real-time ATP production rate from glycolytic and mitochondrial metabolism to examine the metabolic state of MSCs. Seahorse assay was performed in collaboration with Mgr. Miriama Sikorová from the laboratory of doc. RNDr. Marie Hubálek Kalbáčová, Ph.D. As shown in Fig.16, we observed a trend of enhanced glycolytic rate in 1D-cold MSCs demonstrated by increased ECAR, with a lower rate of mitochondrial metabolism demonstrated by reduced OCR. However, this experiment was conducted only once, and the data were not consistent; therefore, additional repetitions are necessary. Nevertheless, the results obtained from the Seahorse analysis repeated our flow cytometry data, illustrating the changes in MSC metabolism following short-term cold exposure.

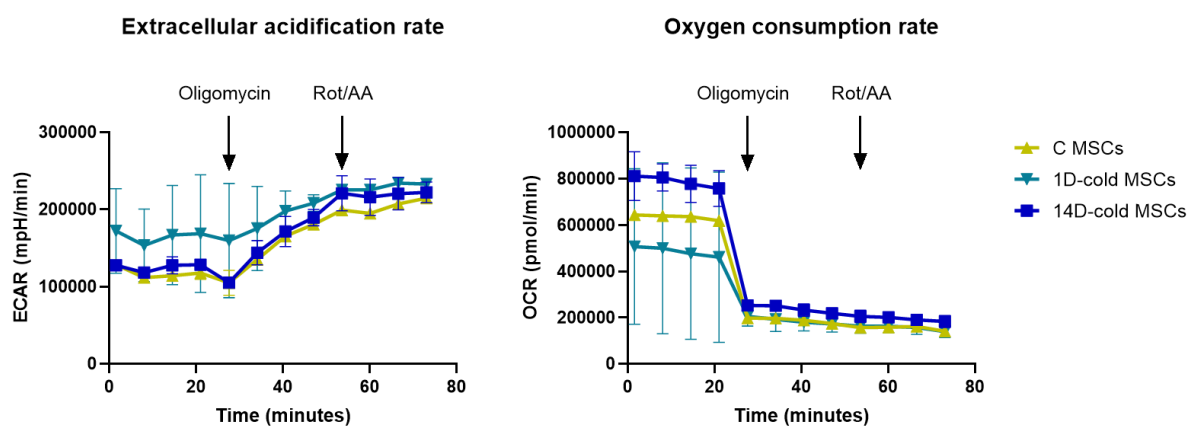


Figure 16. Analysis of MSC metabolism using Seahorse assay. The graphs show the real-time extracellular acidification rate and oxygen consumption rate in basal conditions and following treatment with metabolic inhibitors (oligomycin – inhibitor of ATP synthase, rotenone plus antimycin A – inhibitors of Complexes I and III). Arrows indicate the timing of treatment with inhibitors. Data is normalized per cell count. ECAR...extracellular acidification rate, OCR...oxygen consumption rate, Rot...rotenone, AA...antimycin A.

The WST assay can be employed to describe the metabolic activity of cells. We observed no significant differences in the metabolic activity of cold MSCs compared to controls, only a slight increase in 1D and 14D-cold MSCs (Fig.17). 24-hour stimulation with LPS and IFN γ did not result in elevated cellular metabolism.

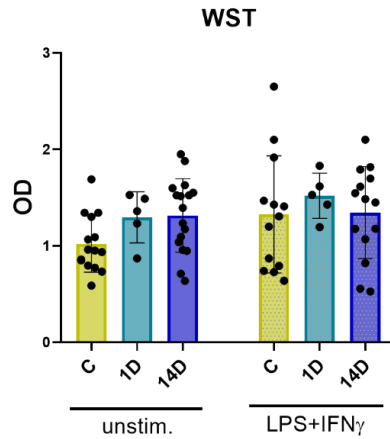


Figure 17. The metabolic activity of MSCs measured with the WST assay. The graph shows the optic density of the colored product generated during the WST reaction (450 nm) in unstimulated MSCs and MSCs stimulated for 24 hours with LPS and IFN γ . The optic density is proportional to the activity of succinate dehydrogenases. Each dot represents one MSC sample pooled from 2 mice, mean $\pm$ SD, data are from 4-6 independent experiments, $n=5-17$, two-way ANOVA with Tukey's multiple comparisons test (between groups) or Sidak's multiple comparisons test (unstim. versus stimulated groups), results are not significant. WST...water-soluble tetrazolium, OD...optic density, LPS...lipopolysaccharide, IFN γ ...interferon γ , unstim...unstimulated, C...control MSCs, 1D...1D-cold MSCs, 14D...14D-cold MSCs.

We observed a clear inconsistency in our data, especially in the WST data. During the culture of MSCs, we noticed an apparent difference in the proliferation rate of individual MSC groups. We hypothesized that this could be the underlying cause of the vast variability in our results, as highly proliferating cells will differ in their cellular properties from cells with lower growth and proliferation. Thus, we tested the effect of cell density in the culture on the metabolic properties of control MSCs. Fig.18 clearly shows that cells cultured at low densities compared to cells cultured at high densities have different metabolic qualities. The production of ROS decreased with fewer MSCs in the culture.

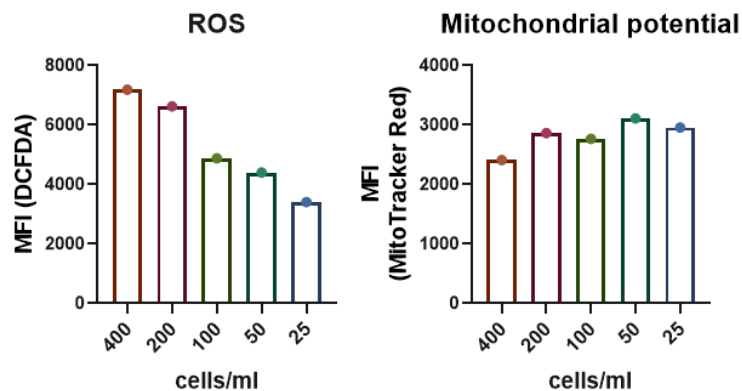


Figure 18. The effect of cell density on the metabolic properties of MSCs. The graphs show mean fluorescence intensity of DCFDA on the left and MitoTracker Red on the right in MSCs cultured at different concentrations. The X-axis indicates the cell density in cells per ml. Data are from 1 experiment, $n=1$. MFI...mean fluorescence intensity, ROS...reactive oxygen species, DCFDA...2',7'-dichlorofluorescein diacetate.

Lastly, the expression of genes involved in various metabolic pathways was examined using qPCR. Fig.19 shows the relative expression of genes involved in glycolysis and oxidative phosphorylation, genes encoding antioxidative proteins, and uncoupling proteins. 1D-cold MSCs showed upregulated expression of NADH:ubiquinone oxidoreductase subunit a6 (*Ndufa6*) and a trend toward increased expression of 6-phosphofructo-2-kinase/fructo-2,6-bisphosphatase 1 (*Pfkfb1*), glucose-6-phosphate dehydrogenase (*Gapd*), or succinate dehydrogenase complex subunit c (*Sdhc*) compared to control MSCs. 14D-cold MSCs exhibited increased expression of UCP4 and 5 (encoded by *Slc25a27* and *Slc25a14*, respectively) compared to control MSCs. UCP5 was significantly upregulated also in 1D-cold MSCs.

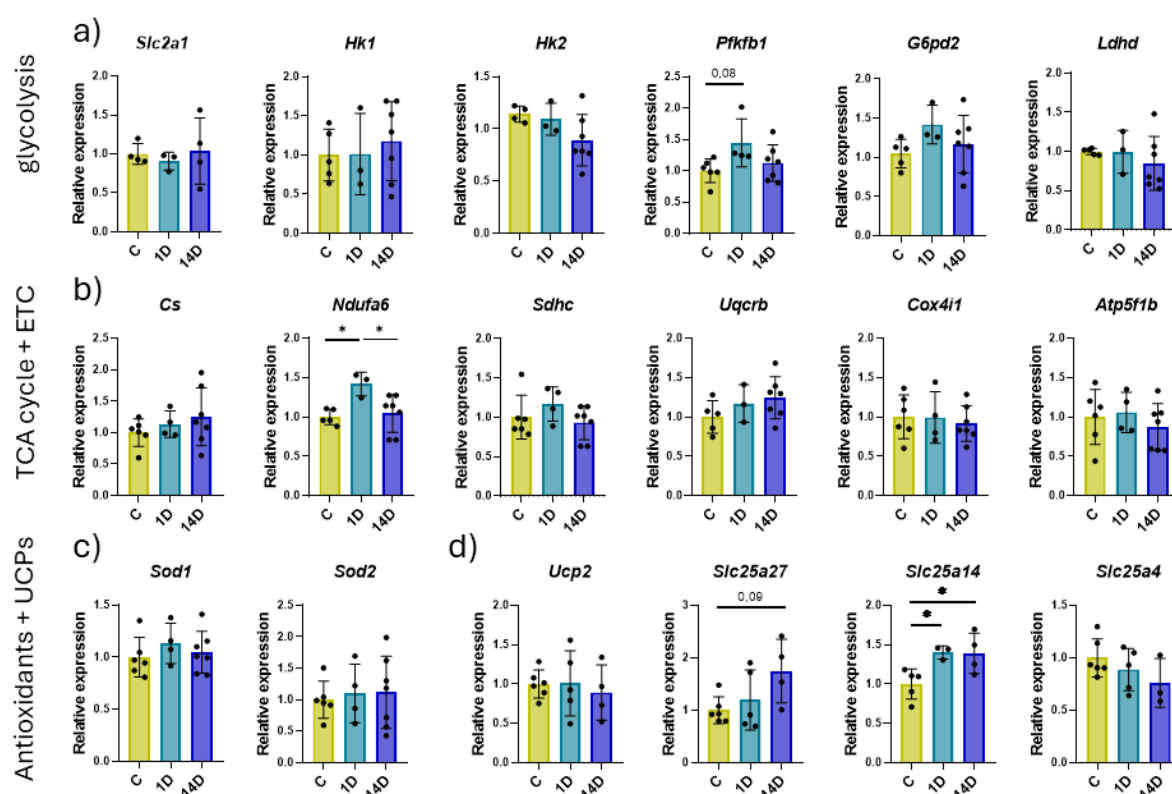


Figure 19. Expression of metabolism-related genes. The graphs show the relative expression of genes involved in glycolysis (a), oxidative phosphorylation (b), antioxidative activity (c), and uncoupling activity (d) measured by qPCR. *Actb*, *Rpl13a*, and *B2m* were used as housekeeping genes. Each dot represents cDNA isolated and transcribed from one MSC sample (data from 2-4 independent experiments, $n=3-7$, mean $\pm$ SD, one-way ANOVA with Holm-Sidak's multiple comparisons test, * = $p > 0.05$). *Slc*...solute carrier family X member X, *Hk*...hexokinase, *Pfkfb1*...6-phosphofructo-2-kinase/fructo-2,6-bisphosphatase 1, *G6pd*...glucose-6-phosphate dehydrogenase, *Ldhd*...lactate dehydrogenase d, *Cs*...citrate synthase, *Ndufa6*...NADH:ubiquinone oxidoreductase subunit a6, *Sdhc*...succinate dehydrogenase complex subunit c, *Uqcrb*...ubiquinol-cytochrome c reductase binding protein, *Cox4i1*...cytochrome c oxidase subunit 4i1, *Atp5f1b*...ATP synthase F1 subunit beta, *Sod*...superoxide dismutase, *Ucp*...uncoupling protein, C...control MSCs, 1D...1D-cold MSCs, 14D...14D-cold MSCs, TCA...tricarboxylic acid cycle, ETC...electron transport chain.

5.1.4. Immunomodulatory properties of MSCs derived from cold-exposed mice

NO is a key molecule in murine MSC immunomodulation (G. Ren et al., 2008). Therefore, we measured NO production by cold MSCs after 48-hour stimulation with LPS and IFN γ . Intriguingly, 14D-cold MSCs produce significantly more NO than C and 1D-cold MSCs (Fig.20a). We also evaluated the expression of iNOS, the enzyme responsible for NO production, on a transcriptomic level. *Nos2* (encoding iNOS) expression is upregulated in MSCs after 48-hour stimulation, as shown in Fig.20b. We noticed a decrease in *Nos2* expression in 14D-cold MSCs after 48-hour stimulation compared to stimulated C and 1D-cold MSCs.

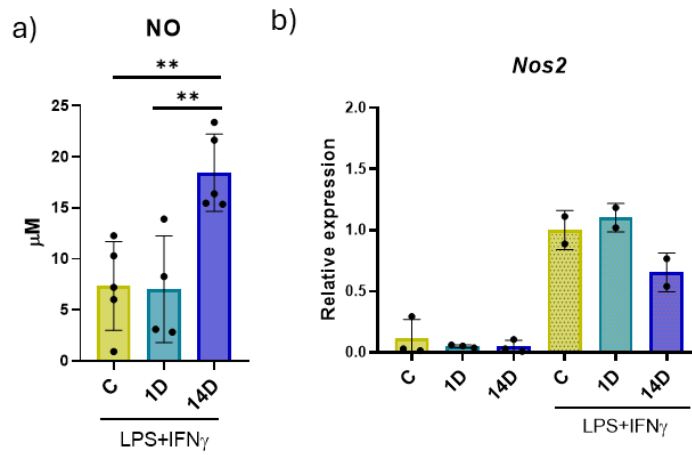


Figure 20. NO production by MSCs after inflammatory stimulation. (a) the extracellular production of NO was established using the Griess reaction; the graph shows the concentration of NO produced in μ M after 48-hour stimulation with LPS and IFN γ (each dot represents one MSC sample pooled from 2 mice, data from 3 independent experiments, $n=4-5$, mean $\pm$ SD, one-way ANOVA with Holm-Sidak's multiple comparisons test, ** = $p < 0.01$). (b) shows the relative expression of *Nos2* (gene encoding iNOS) after 48-hour stimulation with LPS and IFN γ , *Actb* and *Rpl13a* were used as a housekeeping control (data from 1-2 independent experiments, $n=2-3$, mean $\pm$ SD; expression relative to stimulated C). NO...nitric oxide, iNOS...inducible nitric oxide synthase, *Nos2*...nitric oxide synthase 2, LPS...lipopolysaccharide, IFN γ ...interferon- γ , unstim...unstimulated, C...control MSCs, 1D...1D-cold MSCs, 14D...14D-cold MSCs.

Finally, qPCR was used to evaluate the expression of other genes involved in MSC immunomodulatory activity following 48-hour or 24-hour inflammatory stimulation (Fig.21). The expression of all measured immunomodulatory factors was increased after stimulation. *Icam1* and *Ptgs2* (encoding cyclooxygenase 2) expression significantly increased in all MSC groups, *Ido1* in C and 1D-cold MSCs after 48-hour stimulation. Interestingly, *Ido1* expression in stimulated MSCs was significantly downregulated in 14D-cold MSCs compared to C and 1D-cold MSCs. Also, *Vegf* expression is dramatically raised in stimulated 1D and 14D-cold MSCs compared to stimulated C MSCs. Although 48-hour stimulation increased the expression of *Ptgs2* and *Icam1*, no differences were observed among MSC groups. Similarly, the expression of aconitate decarboxylase 1 (*Acod1*) was elevated after 24-hour stimulation; however, no significant differences were observed among MSC groups.

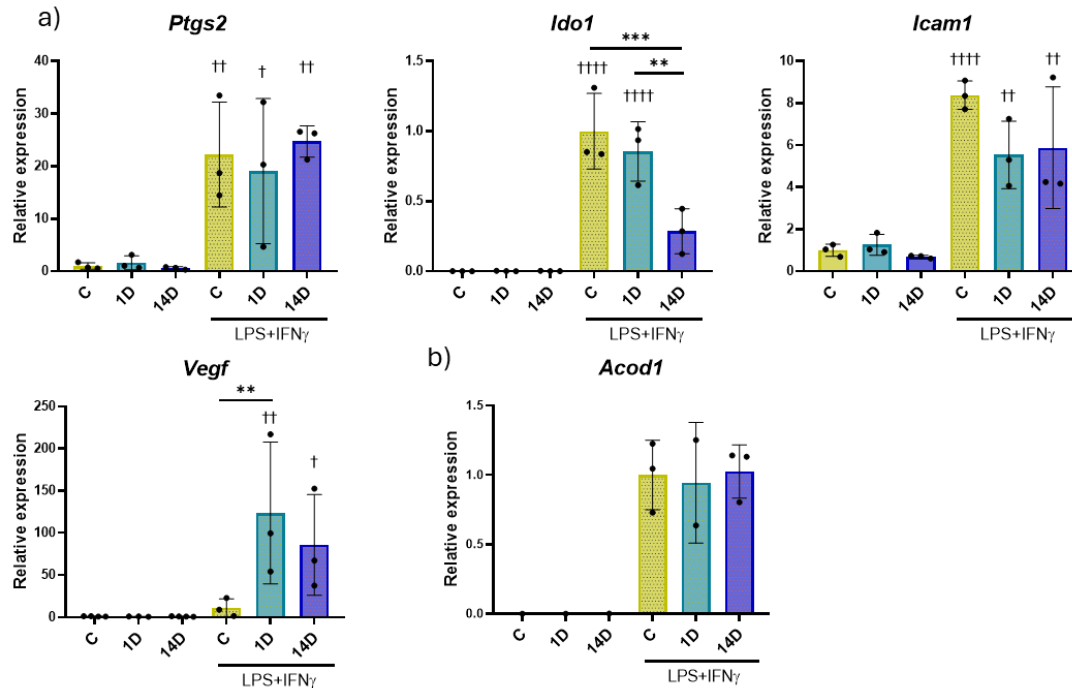


Figure 21. Expression of immunomodulatory genes. The relative expression of genes involved in MSC immunomodulation was assessed by qPCR. MSCs were stimulated with LPS and IFN γ for 48 (a) or 24 (b) hours. *Actb* and *Rpl13a* were used as housekeeping genes. The gene expression is relative to unstimulated control MSCs (except *Acod1* and *Ido1*, where it is relative to stimulated control). Each dot represents cDNA isolated and transcribed from one MSC sample (data from 1-2 independent experiments, $n=2-3$, mean $\pm$ SD, two-way ANOVA with Tukey's multiple comparisons test (between groups) or Sidak's multiple comparisons test (unstimulated versus stimulated groups), significance between unstimulated and stimulated samples is demonstrated by daggers, $\dagger = p < 0.05$; $\dagger\dagger = p < 0.01$; $\dagger\dagger\dagger = p < 0.0001$; asterisks indicate significance between MSC groups, $** = p < 0.01$; $*** = p < 0.001$). *Ptgs2*...prostaglandin-endoperoxide synthase 2, *Ido1*...indoleamine-2,3-dioxygenase 1, *Icam1*...intercellular adhesion molecule 1, *Vegf*...vascular endothelial growth factor, *Acod1*...aconitate decarboxylase 1, LPS...lipopolysaccharide, IFN γ ... interferon- γ , C...control MSCs, 1D...1D-cold MSCs, 14D...14D-cold MSCs.

5.1.4.1. Analysis of splenocytes in co-culture with MSCs derived from cold-exposed mice

Due to an increased NO production by 14D-cold MSCs, we hypothesized that these cells could possess enhanced immunosuppressive activity toward immune cells, specifically T cells. So, we performed co-culture experiments of MSCs with splenocytes, as explained in Chapter 4.11.4, and analyzed the functional properties of T cells. The representative gating strategy used to determine CD4⁺ and CD8⁺ T cells is shown in Fig. 22. First, we evaluated the effect of control and 14D-cold MSCs on the activation state of T cells based on the expression of CD25 and CD69. No differences were observed in the expression of CD25 or CD69 in CD4⁺ and CD8⁺ T cells after co-culture with control and 14D-cold MSCs (Fig.23).

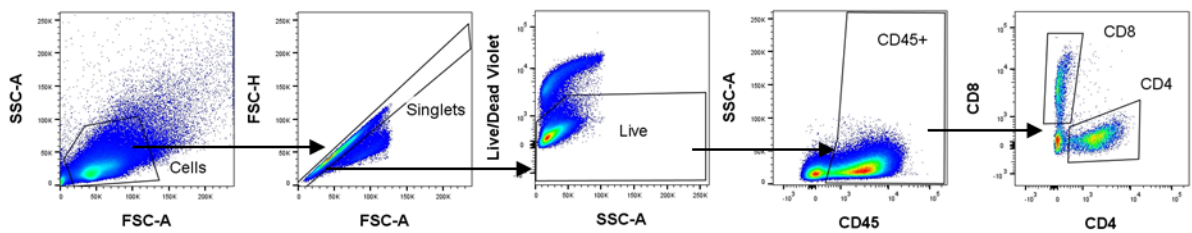


Figure 22. Representative gating strategy to determine CD4⁺ T cells and CD8⁺ T cells. Cells were determined based on FSC-A and SSC-A. Singlets were eliminated based on forward-scatter (FSC-A, FSC-H). Dead cells were eliminated based on Hoechst 33258 (extracellular staining) or Live/Dead Violet (intracellular staining). Live cells were gated on the expression of CD45, and then CD4 expression determined CD4⁺ T cells, and CD8 expression determined CD8⁺ T cells.

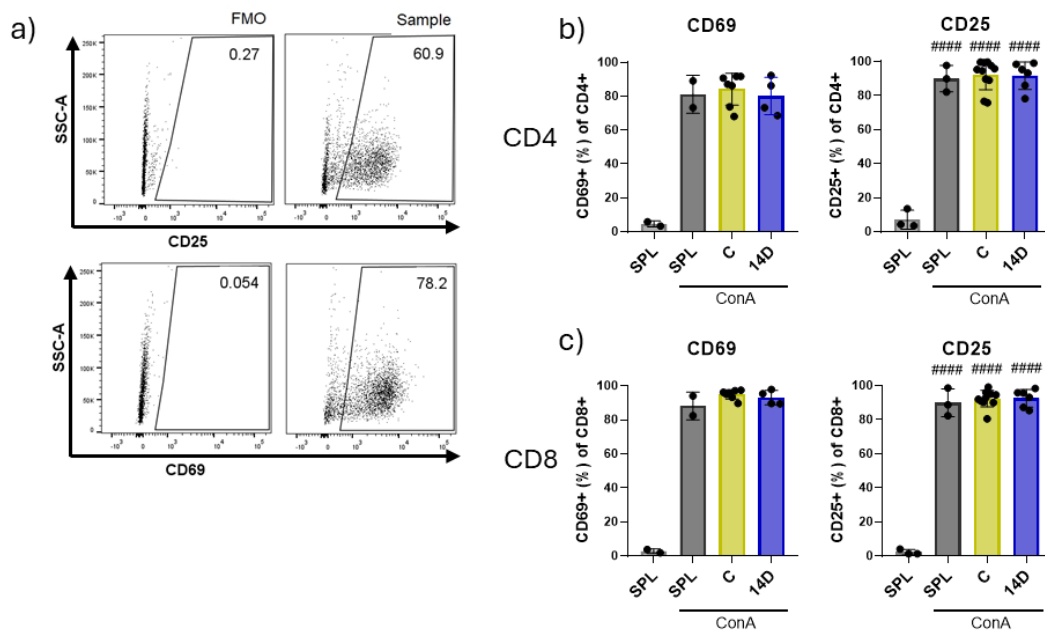


Figure 23. The effect of MSCs on the expression of activation markers CD25 and CD69 by T cells. (a) shows representative gating of the positive population based on FMO control. The expression of CD25 and CD69 was measured in CD4⁺ (b) and CD8⁺ (c) T cells after 48-hour co-culture with MSCs and activation with concanavalin A. Each dot represents one MSC sample pooled from 2 mice, data from 2 (CD69) and 3 (CD25) independent experiments, $n=2-11$, mean $\pm$ SD, one-way ANOVA with Holm-Sidak's multiple comparisons test, number signs indicate significance to unstimulated SPL, ##### = $p < 0.0001$). SPL...splenocytes, ConA...concanavalin A, C...splenocytes co-cultured with C MSCs, 14D...splenocytes co-cultured with 14D-cold MSCs.

MSCs have a great ability to suppress T-cell proliferation. We assessed the effect of cold MSCs on T-cell proliferation by Ki67 staining. Our results show that all MSCs had a minor impact on the proliferation of activated T cells (Fig.24). 1D-cold MSCs exhibited a slightly impaired ability to suppress CD4⁺ T-cell proliferation. However, there is a considerable inconsistency between individual experiments.

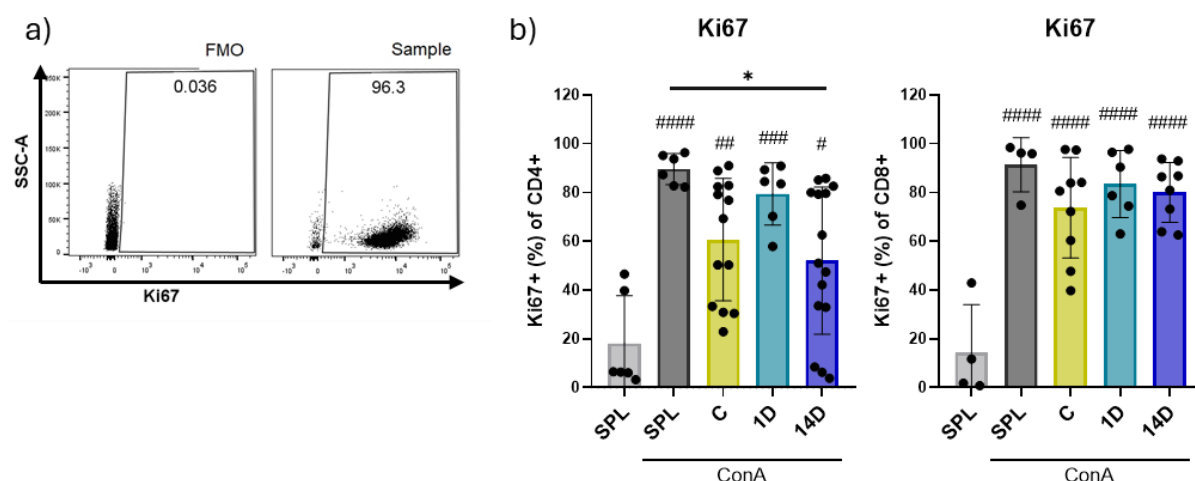


Figure 24. The effect of MSCs on T-cell proliferation. The proliferation rate of concanavalin A-activated CD4⁺ and CD8⁺ T cells in a co-culture with MSCs for 72h was evaluated by Ki67 staining. (a) shows representative gating of the positive population based on FMO control, (b) shows the percentage of T cells positive for Ki67. Each dot represents one sample (data from 6 independent experiments, $n=4-15$, mean $\pm$ SD, one-way ANOVA with Holm-Sidak's multiple comparisons test, number signs indicate significance to unstimulated SPL, # = $p < 0.05$; ## = $p < 0.01$; ### = $p < 0.001$; ##### = $p < 0.0001$; other significances are demonstrated by asterisks, * = $p < 0.05$). SPL...splenocytes, ConA...concanavalin A, C...splenocytes co-cultured with C MSCs, 1D...splenocytes co-cultured with 1D-cold MSCs, 14D...splenocytes co-cultured with 14D-cold MSCs.

To observe whether MSCs limit or induce specific subsets of CD4⁺ T cells, we stained splenocytes for the expression of FOXP3 and ROR γ t. Fig.25 shows that MSCs had no impact on the expression of FOXP3 by Th cells. On the other hand, C and 1D-cold MSCs increased the expression of ROR γ t in T cells, but the increase is significant only in 1D-cold MSC co-culture.

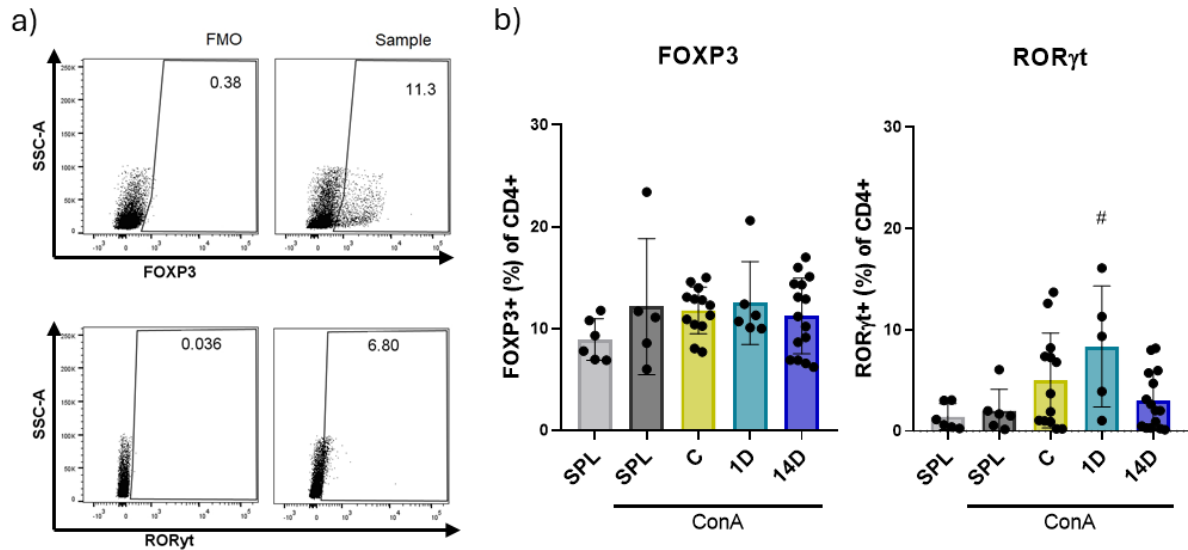


Figure 25. Expression of master transcription factors of Treg and Th17 cells. FOXP3 and RORγt expression by CD4⁺ T cells was assessed by flow cytometry. (a) shows representative gating of the positive population based on FMO control. (b) shows the percentage of CD4⁺ T cells positive for given markers. Each dot represents one sample (data from 6 independent experiments, n=5-15, mean +/- SD, one-way ANOVA with Holm-Sidak's multiple comparisons test, number signs indicate significance to unstimulated control, # = p < 0.05). FOXP3...Forkhead box P3, RORγt...retinoic acid receptor-related orphan receptor C, SPL...splenocytes, ConA...concanavalin A, C...splenocytes co-cultured with C MSCs, 1D...splenocytes co-cultured with 1D-cold MSCs, 14D...splenocytes co-cultured with 14D-cold MSCs.

Intracellular expression of cytokines by CD4⁺ and CD8⁺ T cells was assessed by flow cytometry. Fig.26 shows no differences between C, 1D-cold, and 14D-cold MSCs in their ability to suppress the expression of IL-2 and IL-17A by either T cell subset. 1D-cold MSCs exhibited an enhanced ability to promote IL-10 production by CD8⁺ T cells compared to 14D-cold MSCs.

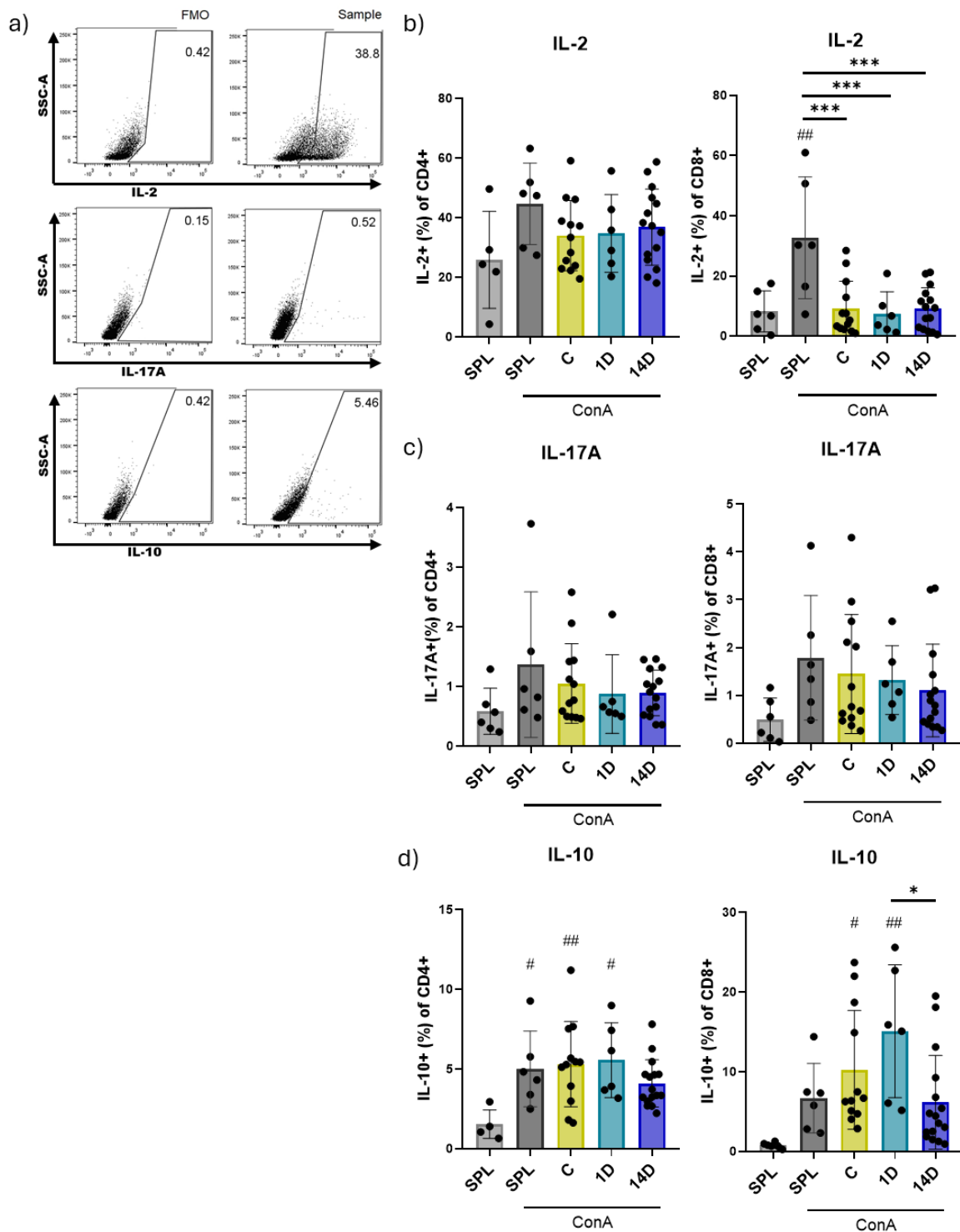


Figure 26. Intracellular expression of IL-2, IL-17A, and IL-10 by T cells in co-culture with MSCs. (a) shows representative gating of the positive population based on FMO control. The graphs show the percentage of concanavalin A-activated CD4⁺ and CD8⁺ T cells expressing IL-2 (b), IL-10 (c), and IL-17A (d). Each dot represents one sample (data from 4-6 independent experiments, $n=4-16$, mean $\pm$ SD, one-way ANOVA with Holm-Sidak's multiple comparisons test, number signs indicate significance to unstimulated SPL, # = $p < 0.05$; ## = $p < 0.01$; asterisks demonstrate other significances, * = $p < 0.05$; *** = $p < 0.001$). SPL...splenocytes, ConA...concanavalin A, IL...interleukin, C...splenocytes co-cultured with C MSCs, 1D...splenocytes co-cultured with 1D-cold MSCs, 14D...splenocytes co-cultured with 14D-cold MSCs.

5.2. Part 2 – The effect of reduced culture temperature on MSCs

In the previous section, we observed the impact of cold-induced changes in the adipose tissue on the qualities of isolated MSCs. We believe that the single factor of reduced temperature also affects MSC properties. Therefore, we compared the metabolic and immunomodulatory properties of MSCs cultured at standard temperature (37°C) with MSCs cultured at reduced temperature (34°C) for short-term (1 day) or prolonged (3 days) periods.

5.2.1. Metabolic properties of MSCs cultured at reduced temperature

First, we performed flow cytometry to analyze mitochondrial membrane potential and ROS production by MSCs in basal conditions and after priming with inflammatory stimuli (Fig.27). In basal conditions, mitochondrial potential was rather unchanged; however, in prolonged culture (24h), the mitochondrial potential significantly increased with the duration of exposure to reduced temperature. ROS production was significantly increased in 1D and 3D MSCs compared to controls. 24h inflammatory stimulation was accompanied by a significant decrease in mitochondrial potential in all MSC groups. Moreover, stimulated C MSCs had significantly reduced mitochondrial potential compared to stimulated 1D and 3D MSCs. Stimulation did not substantially alter the ROS production in any MSC group.

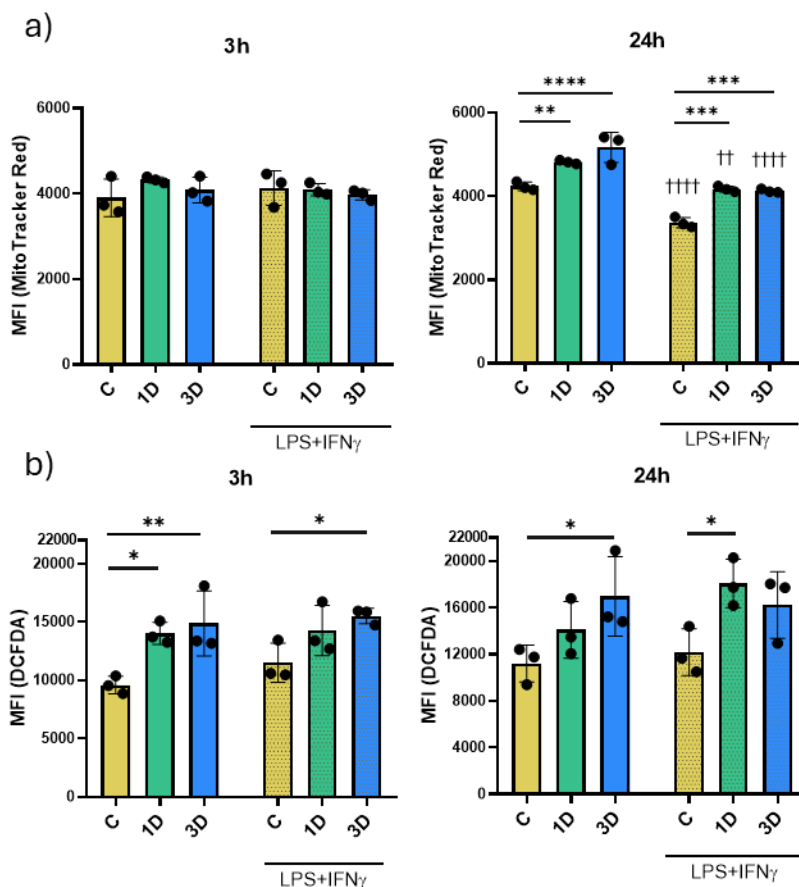


Figure 27. Metabolic properties of MSCs cultured at 34°C. The graphs show mean fluorescence intensity of MitoTracker Red to evaluate the mitochondrial membrane potential (a), and DCFDA to evaluate ROS production (b) by MSCs in basal conditions or following 3-hour and 24-hour stimulation with LPS and IFN γ . Each point represents one MSC sample (data from 3 independent experiments, $n=3$, mean $\pm$ SD, two-way ANOVA with Tukey's multiple comparisons test (between groups) or Sidak's multiple comparisons test (unstimulated versus stimulated groups), significance between unstimulated and stimulated samples is demonstrated by daggers, $\dagger\dagger = p < 0.01$; $\dagger\dagger\dagger = p < 0.0001$; asterisks indicate significance between MSC groups, $* = p < 0.05$; $** = p < 0.01$; $*** = p < 0.001$; $**** = p < 0.0001$). MFI...mean fluorescence intensity, 2-NBDG...(2-(N(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-deoxyglucose, DCFDA...2,7-dichlorodihydrofluorescein diacetate, LPS...lipopolysaccharide, IFN γ ...interferon- γ , ROS...reactive oxygen species, C...control MSCs, 1D...MSCs cultivated 1 day at 34°C, 3D...MSCs cultivated 3 days at 34°C.

Seahorse analysis uncovered additional aspects of MSC metabolism in response to reduced culture temperature. Fig.28a shows ECAR and OCR of MSCs unstimulated and stimulated with LPS and IFN γ for 24 hours. 3D MSCs exhibited increased ECAR and OCR in a steady state and after stimulation, suggesting that these cells are overall more metabolically active than C and 1D MSCs. This can also be demonstrated by increased ATP production by 3D MSCs, as shown in Fig.28b. Thanks to the Seahorse assay, we could discriminate ATP produced by glycolysis and oxidative phosphorylation. In 3D MSCs, both glycolysis and oxidative phosphorylation contributed to enhanced ATP production. The opposite effect was observed for 1D MSCs, which had decreased ECAR, OCR, and ATP production compared to C MSCs.

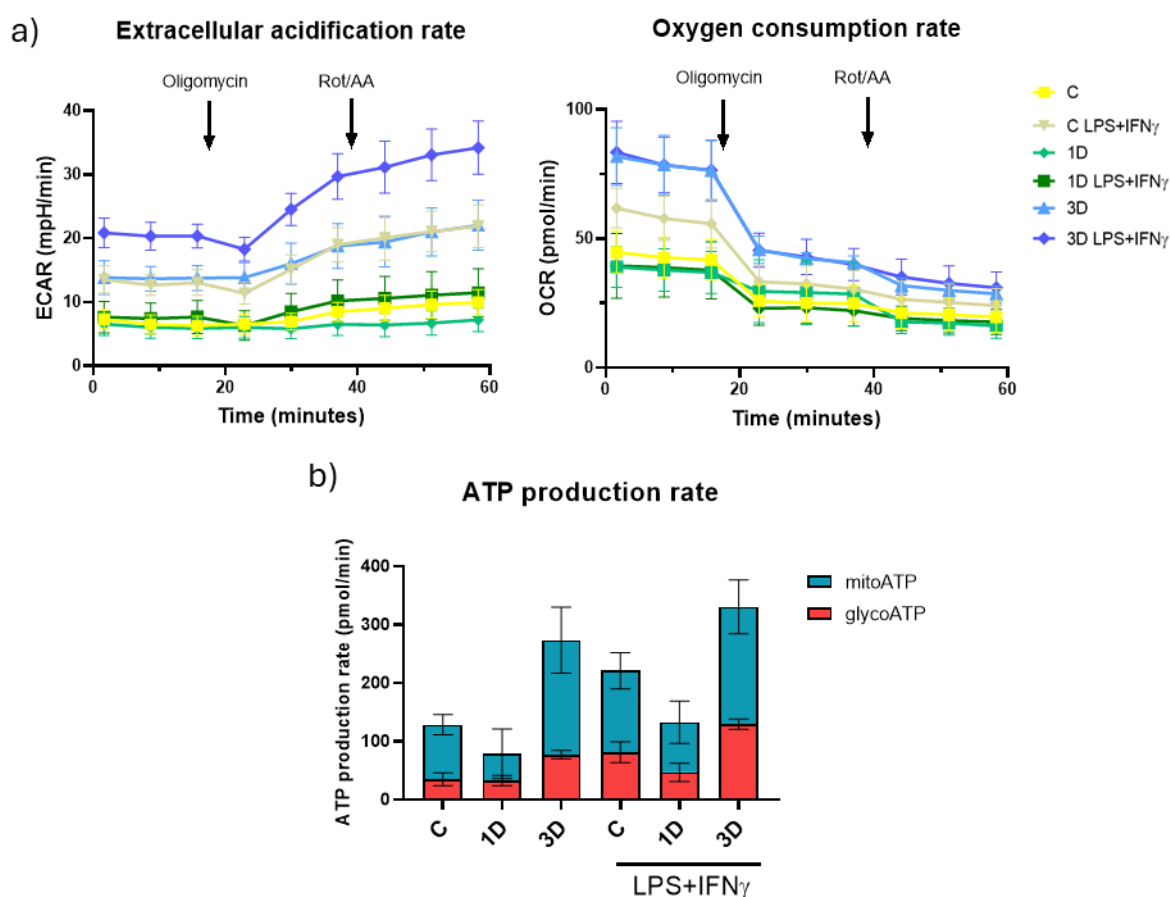


Figure 28. Evaluation of metabolic properties of MSCs cultured at 34°C by Seahorse assay. The graphs in (a) show real-time extracellular acidification rate (change of pH per min) and oxygen consumption rate (pmol produced per min) in basal conditions and following treatment with metabolic inhibitors (oligomycin – inhibitor of ATP synthase, rotenone plus antimycin A – inhibitors of Complexes I and III). (b) shows the quantification of real-time ATP production in pmol/min discriminated between the ATP produced via glycolytic metabolism and ATP produced via oxidative metabolism. Arrows indicate the timing of treatment with inhibitors. ECAR...extracellular acidification rate, OCR...oxygen consumption rate, Rot...rotenone, AA...antimycin A, mitoATP...ATP produced in mitochondrial metabolism, glycoATP...ATP produced in glycolysis, C...control MSCs, 1D...MSCs cultivated 1 day at 34°C, 3D...MSCs cultivated 3 days at 34°C.

Metabolic activity of MSCs was also evaluated using WST assay, again under basal conditions and after inflammatory stimulation. Fig.29 shows that in both steady state and inflammatory stimulation, 3D MSCs exhibited slightly decreased metabolic activity. Inflammatory priming for 3 hours or 24 hours did not alter the metabolic state of MSCs in any experimental group.

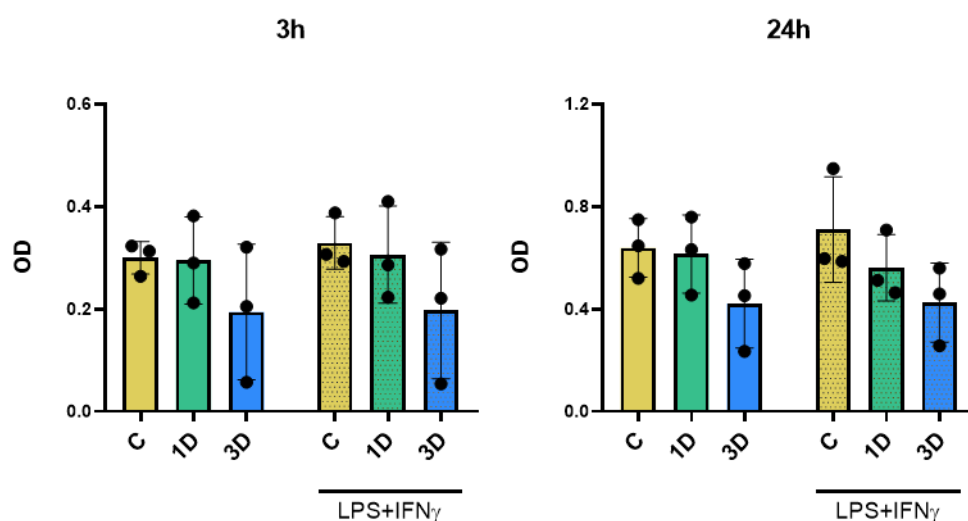


Figure 29. Evaluation of MSC metabolic activity using WST assay. The optic density of a formazan product generated in the WST reaction represents the activity of succinate dehydrogenase in MSCs. The graph shows optic density in unstimulated MSCs and after 3-hour or 24-hour stimulation of MSCs with LPS and IFN γ . Each point represents one MSC sample (data from 3 independent experiments, $n=3$, mean $\pm$ SD, two-way ANOVA with Tukey's multiple comparisons test (between groups) or Sidak's multiple comparisons test (unstimulated versus stimulated groups), results not significant). OD...optic density, LPS...lipopolysaccharide, IFN γ ...interferon- γ , C...control MSCs, 1D...MSCs cultivated 1 day at 34°C, 3D...MSCs cultivated 3 days at 34°C.

5.2.2. Immunomodulatory properties of MSCs cultured at reduced temperature

Similarly to the first part of this project, the immunomodulatory potential of MSCs cultured at 34°C was determined by evaluating NO production after stimulation, measured via intracellular FACS staining. Short-term stimulation with inflammatory factors did not lead to differential NO production among MSC groups. On the other hand, after prolonged inflammatory stimulation, NO production by 3D MSCs significantly increased compared to unstimulated 3D MSCs. 3D MSCs also expressed significantly more NO than 1D MSCs (Fig.30).

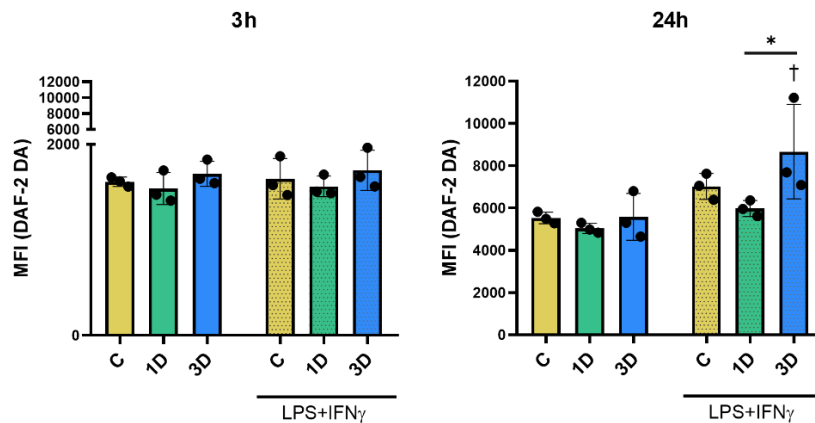


Figure 30. NO production by MSCs cultured at reduced temperature. NO production by MSCs was evaluated by intracellular staining with DAF-2 DA. The graphs show mean fluorescence intensity of DAF-2 DA in a steady state and after 3-hour and 24-hour stimulation with LPS and IFN γ . Each point represents one MSC sample (data from 3 independent experiments, mean $\pm$ SD, two-way ANOVA with Tukey's multiple comparisons test (between groups) or Sidak's multiple comparisons test (unstimulated versus stimulated groups), significance between unstimulated and stimulated samples is demonstrated by daggers, $\dagger = p < 0.05$; asterisks indicate significance between MSC groups, $* = p < 0.05$). MFI...mean fluorescence intensity, DAF-2 DA...diaminofluorescein-2 diacetate, NO...nitric oxide, LPS...lipopolysaccharide, IFN γ ...interferon- γ , C...control MSCs, 1D...MSCs cultivated 1 day at 34°C, 3D...MSCs cultivated 3 days at 34°C.

5.2.2.1. Analysis of splenocytes in co-culture with MSCs cultured at reduced temperature

As we observed some differences in the metabolic properties and NO production in MSCs cultured at reduced temperature, we decided to test whether they also possess improved immunomodulatory activity. We co-cultured MSCs with splenocytes and analyzed T-cell proliferation, cytokine production, and expression of transcription factors. The representative gating strategy for determining CD4 $^{+}$ and CD8 $^{+}$ T cells is shown in Fig.22. Positive populations were gated based on corresponding FMO controls (for the representative dot plots, see Fig.24-26).

While all MSC experimental groups mitigated CD4⁺ T-cell proliferation, no differences could be observed among individual MSC groups (Fig.31b). A similar trend was also observed for the suppression of CD8⁺ T-cell proliferation. To further examine the ability of MSCs to skew CD4⁺ T-cell polarization, we stained them for the expression of FOXP3 and ROR γ t. Compared to stimulated T cells, the presence of all MSC groups in a co-culture led to a slight increase in FOXP3 and ROR γ t expression. However, no difference was observed among MSC groups (Fig.31a).

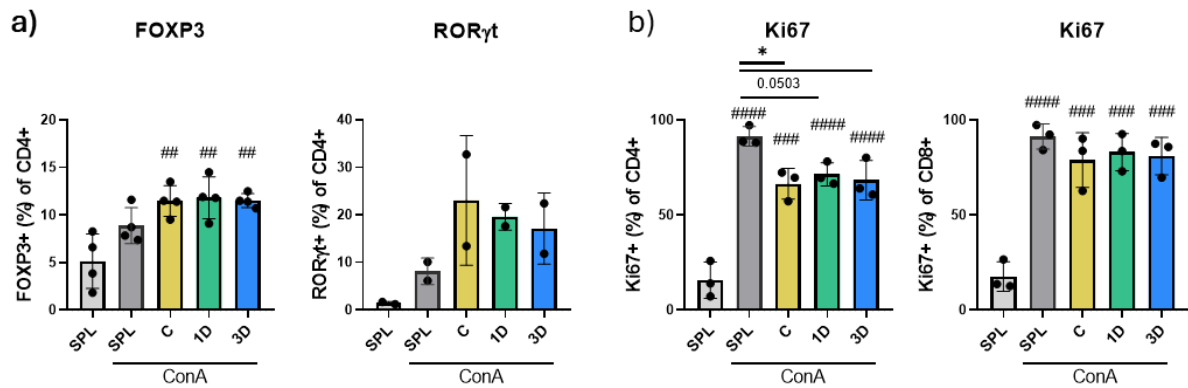


Figure 31. Expression of transcription factors and proliferation rate of T cells co-cultured with MSCs cultured at reduced temperature. The expression of FOXP3 and ROR γ t in CD4⁺ T cells is demonstrated as a percentage of positive cells assessed by flow cytometry (a). CD4⁺ and CD8⁺ T cell proliferation was assessed based on Ki67 staining and is depicted as a percentage of cells positive for Ki67 (b). Each point represents one sample (data from 2-3 independent experiments, $n=2-4$, one-way ANOVA with Holm-Sidak's multiple comparisons test; significance to unstimulated SPL is depicted by number signs, ## = $p < 0.01$; ### = $p < 0.001$; ##### = $p < 0.0001$; other significances are depicted by asterisks, * = $p < 0.05$). SPL...splenocytes, ConA...concanavalin A, FOXP3...forkhead box protein 3, ROR γ t...retinoic acid receptor related orphan receptor C, C...splenocytes co-cultured with C MSCs, 1D...splenocytes co-cultured with MSCs cultivated 1 day at 34°C, 3D...splenocytes co-cultured with MSCs cultivated 3 days at 34°C.

Lastly, we evaluated the cytokine production by CD4⁺ and CD8⁺ T cells (Fig.32). Co-culture of MSCs with activated T cells resulted in a slight decrease in the expression of IL-2 by CD4⁺ and CD8⁺ T cells, as well as IL-17A and IL-10 production by CD4⁺ T cells. The production of IL-10 by CD8⁺ T cells increased in co-culture with 3D MSCs, while IL-17A production decreased in co-culture with 1D MSCs, although not significantly.

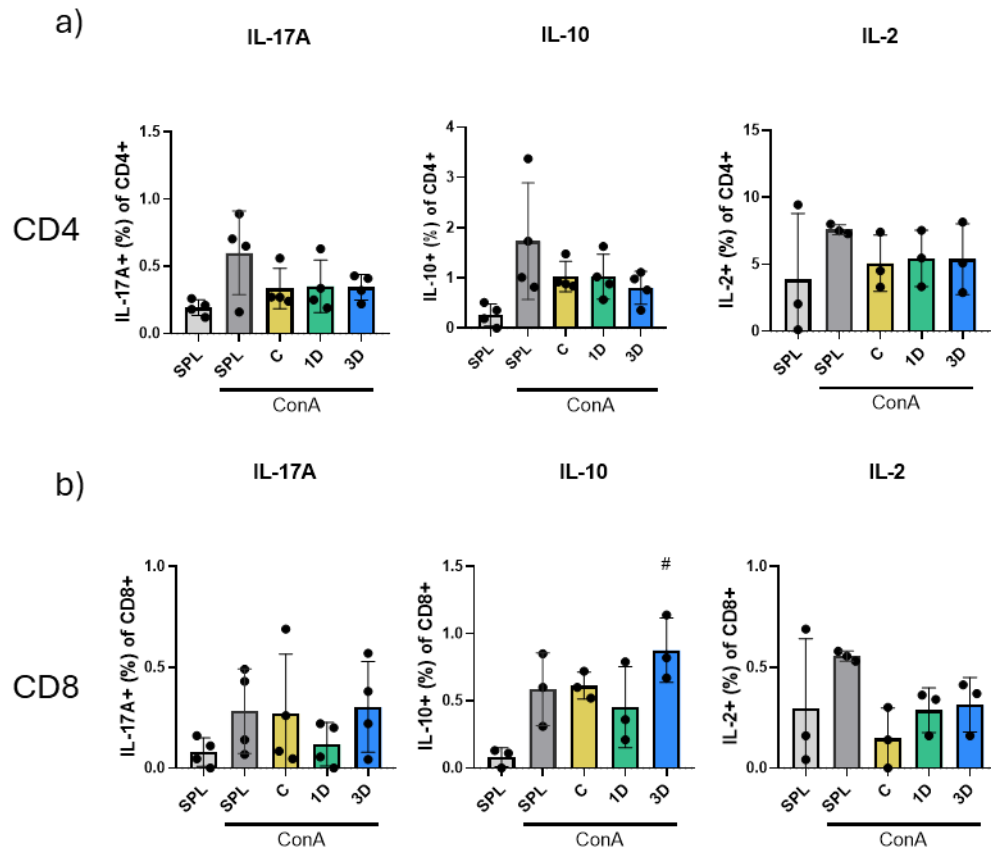


Figure 32. Cytokine production by MSCs cultured at reduced temperature evaluated by FACS staining. The graphs show the percentage of CD4⁺ (a) and CD8⁺ (b) T cells positive for IL-10, IL-17A, and IL-2 expression. Each point represents one sample (data from 3 independent experiments, $n=3-4$, mean $\pm$ SD, one-way ANOVA with Holm-Sidak's multiple comparisons test, significance to unstimulated SPL is depicted with number signs, # = $p < 0.05$). IL...interleukin, SPL...splenocytes, ConA...concanavalin A, C...splenocytes co-cultured with C MSCs, 1D...splenocytes co-cultured with MSCs cultivated 1 day at 34°C, 3D...splenocytes co-cultured with MSCs cultivated 3 days at 34°C.

6. Discussion

The first part of the diploma project focused on cold exposure and mechanisms of acclimation to cold. First, we observed the dynamics of cold adaptation in mice. Previous research in our laboratory showed that 14 days of cold exposure significantly increased the weight of BAT in rats (Vasek et al., 2024). The same observation was made in mice exposed 14 days to 4°C, suggesting that 14-day-long cold exposure leads to BAT expansion and acclimation, which corresponds to previous publications focusing on cold acclimation reporting that long-term (from 10 days to 2 weeks) exposure to cold temperatures leads to BAT expansion and activation (Labbé et al., 2016; Marvanova et al., 2023). So, we can consider 14D-cold mice acclimated.

As shown in the study by Vasek et al., cold exposure in rats is associated with local alterations in cytokine levels within adipose tissue depots (Vasek et al., 2024). Therefore, ELISA was used to determine cytokine levels in adipose tissue in our mouse cold adaptation model. In the beginning, we evaluated cytokine levels in BAT. To our surprise, the cytokine production pattern observed in mice differed from that observed in rats. These inter-species differences could be explained by differential thermoregulation in mice and rats. Rats have a lower surface-to-volume ratio, lower basal metabolic rate, and better insulation, leading to an enhanced capacity to retain heat compared to mice. Following exposure to temperature below the thermoneutral zone, rats exhibit a lower increase in metabolic rate than mice (reviewed in Hankenson et al., 2018). Even though no differences were observed between mice and rats in the expansion of BAT after long-term cold exposure, the metabolic activity of BAT could differ.

Further, we observed different patterns of cytokine production among the mouse adipose tissue depots in response to cold. sWAT, vWAT, and BAT diverge in many aspects, including the content of thermogenic versus energy-storing adipocytes (Wu et al., 2012), metabolic capacity (Rosell et al., 2014), innervation (Jiang et al., 2017; Nguyen et al., 2014), vascularization (Xue et al., 2009), production of soluble factors and hormones (Deshmukh et al., 2019), and response dynamics to cold stimulation (Rosell et al., 2014). Moreover, concurrently with ELISA performed on adipose tissue homogenates, flow cytometry was employed to analyze immune cell composition of those tissues. It revealed substantial differences among adipose tissue depots in the abundance of immune cell subsets. While pWAT is populated mainly by macrophages, iWAT and BAT contain more diverse immune compartments with a higher abundance of CD4⁺ T cells (unpublished data from our laboratory). The differential composition of immune cells among adipose tissue depots could explain the differences observed in the levels of soluble factors within those tissues. Future research should focus on cells responsible for cytokine production and their involvement in cold acclimation.

However, based on previous observations of our laboratory and others, we would expect different results. For instance, IL-17-producing $\gamma\delta$ T cells were implicated in the regulation of NST (Hu et al., 2020; Kohlgruber et al., 2018), plus IL-17A and IL-17F levels were increased in BAT of rats after 14 days of cold exposure (Vasek et al., 2024). Similarly, type 2 immune response cytokines were associated with thermogenesis in sWAT (Qiu et al., 2014).

IL-6 secreted by adipocytes is also important for NST and beiging (Kristóf et al., 2019). Besides cytokines, VEGF participates in the regulation of BAT function (Sun et al., 2014). Since the cytokines and other soluble factors are involved in BAT activation and NST, we would expect their increased levels in mice exposed to long-term cold. Selecting other time points could provide additional information regarding the kinetics of cytokine production after cold exposure.

We wanted to see whether cold affects cytokine production in other immune relevant tissues, so we performed ELISA on thymus and spleen homogenates. The trend of increased cytokine production in the spleen after 1 day of cold exposure could be potentially explained by increased cytokine production by immune cells. 1-day-long cold exposure increased the production of IL-17A and TNF α by splenic $\gamma\delta$ T cells in rats (Vasek et al., 2024). We expect a similar situation in our mouse model. The increased cytokine production in the thymus of cold-acclimated mice remains to be explained.

To summarize, after 14 days of exposure to cold temperatures, mice expand BAT and acclimate to the changed environment. This is accompanied by changes in the cytokine levels in several tissues, which are tissue and species-specific.

Next, we wanted to examine whether the described changes in cytokine levels in adipose tissue during cold exposure affect resident MSCs. To ensure that MSCs isolated from cold-exposed mice exhibit the characteristics of MSCs isolated from control mice, we performed phenotypic and functional characterization of MSCs. As Dominici et al. proposed, three minimal criteria must be fulfilled to define MSCs (Dominici et al., 2006). First, using flow cytometry, we confirmed that MSCs isolated from cold-exposed mice (1D-cold and 14D-cold MSCs) share the surface marker expression with MSCs isolated from control mice (C MSCs). We noticed a higher expression of CD90 and CD44 in 1D-cold MSCs. CD44 is involved in MSC migration through its interaction with hyaluronic acid (Zhu et al., 2006). Decreased CD90 expression was correlated with impaired suppressive capacity of human MSCs (Campioni et al., 2009). Therefore, upregulation of those markers might boost the migratory and immunosuppressive potential of 1D-cold MSCs.

Second, MSCs derived from cold-exposed mice adhered to a plastic surface during cultivation in the same manner as control MSCs. Third, MSCs derived from cold-exposed mice could differentiate into adipocytes, chondrocytes, and osteocytes. Taken together, MSCs isolated from cold-exposed mice can be characterized as MSCs.

MSCs possess great immunomodulatory capacity (reviewed in G. Yang et al., 2023). Their immunomodulatory and regenerative capacity is triggered by inflammatory stimulation, culture under hypoxic conditions, or in 3D spheroids (Gangadaran et al., 2023; Kadle et al., 2018). These conditions influence MSC metabolism and consequently increase their immunomodulatory capacity (reviewed in H. Li et al., 2022). To see whether cold-induced changes in the adipose tissue microenvironment stimulated MSC metabolism and immunomodulatory potential, we analyzed the metabolism of MSCs from cold-exposed mice.

Flow cytometric analysis suggested that 1D-cold MSCs exhibit an increased glucose uptake with decreased mitochondrial potential. This was accompanied by elevated ECAR and reduced OCR as measured by Seahorse assay. Increased glucose uptake demonstrated by increased protein expression of GLUT1 and GLUT2 transporters, together with increased ECAR and expression of several genes involved in glycolysis, was previously observed in murine MSCs following stimulation with TNF α and IFN γ . Further, the upregulation of glycolysis by preconditioning MSCs with oligomycin enhanced their immunosuppressive activity (Contreras-Lopez et al., 2021). On the other hand, IFN γ pretreatment decreased the activity of complex I of ETC and mitochondrial membrane potential in human MSCs (Y. Liu et al., 2018). Corresponding to the literature, our flow cytometry and Seahorse results suggest that 1D-cold MSCs undergo a partial switch from mitochondrial metabolism to glycolysis, potentially leading to enhanced immunosuppressive capacity.

However, glycolysis upregulation in 1D-cold MSCs was not corroborated by measuring the expression of genes involved in glycolysis. We evaluated the expression of *Slc2a1*, encoding GLUT1, using qPCR and observed no upregulation of *Slc2a1* expression in 1D-cold MSCs. Whether other GLUTs are involved in the increased glucose uptake in 1D-cold MSCs should be tested. A slight increase in the expression of 6-phosphofructo-2-kinase/fructo-2,6-bisphosphatase 1 (*Pfkfb1*) and glucose-6-phosphate dehydrogenase (*G6pd*) genes might indicate an augmented glycolysis rate. Glycolysis upregulation is accompanied by enhanced lactate efflux (Contreras-Lopez et al., 2021). However, the expression of lactate dehydrogenase D (*Ldhd*) was indifferent among MSC groups. On the other hand, ECAR, a measure of extracellular acidification, was elevated in 1D-cold MSCs. It is important to note that gene expression does not provide information about the activity of transcribed proteins; therefore, further validations are necessary to determine lactate dehydrogenase expression and activity in 1D-cold MSCs, for example, by measuring the concentration of lactate produced by MSCs or evaluating the expression of lactate transporters. Other genes encoding lactate dehydrogenase (*Ldha*, *Ldhb*) could also be tested.

We observed a decrease in mitochondrial membrane potential and OCR in 1D-cold MSCs. The reduction in mitochondrial metabolism suggested by these experiments was not confirmed on a transcriptomic level. The expression of genes encoding citrate synthase (*Cs*) and complexes of ETC (*Ndufa6*, *Sdhc*, *Uqcrb*, *Cox4i1*, *Atp5f1b*) was unchanged or even slightly increased in 1D-cold MSCs compared to C and 14D-cold MSCs. Further, we observed enhanced ROS production by 1D-cold and 14D-cold MSCs compared to controls. Liu et al. observed increased mitochondrial and total ROS production by MSCs after IFN γ stimulation. Treatment of stimulated MSCs with mitoquinone, which neutralizes ROS, resulted in impaired expression and activity of IDO, suggesting impaired immunomodulatory activity due to ROS inhibition (Y. Liu et al., 2018). Increased ROS production in 1D and 14D-cold MSCs was accompanied by elevated expression of UCP proteins, namely UCP5, encoded by *Slc25a14*, and, in 14D-cold MSCs, also UCP4, encoded by *Slc25a27*. UCP proteins play a role in regulating oxidative stress (Hoang et al., 2012); however, the specific role of UCP4 and UCP5 in adipose tissue and MSCs remains unexplained.

The activity of succinate dehydrogenase, measured by the WST assay, exhibited an increasing trend in 1D and 14D-cold MSCs, pointing to enhanced metabolic activity of those MSCs. Unfortunately, there is a clear inconsistency in the data. This inconsistency could have arisen from the differential growth of individual MSC groups observed during MSC expansion. Cell density has been shown to impact the differentiation potential, proliferative capacity, and metabolic state of human MSCs, with MSCs cultured at low densities exhibiting higher use of glycolysis than MSCs cultured at high densities (Y. Liu et al., 2015). We also observed a difference between low and high cell density of MSC culture, specifically in the amount of ROS produced. Lower ROS production when culturing MSCs at low densities correlates with higher use of glycolysis, as described by Liu et al (Y. Liu et al., 2015). We believe that different cell densities among MSC groups at the time of analysis resulted in different cellular and metabolic states of MSCs, which could have impacted our results and caused high variability in the data.

Finally, the immunomodulatory potential of MSCs derived from cold-exposed mice was evaluated. iNOS and NO production represent an essential mechanism of MSC-mediated immunosuppression (Sato et al., 2007). Therefore, the observation of significantly increased NO production by stimulated 14D-cold MSCs prompted the hypothesis that those MSCs might possess enhanced immunosuppressive capacity compared to C and 1D-cold MSCs. We tried to corroborate this finding by measuring the expression of the iNOS enzyme on the transcriptomic level using qPCR. Surprisingly, after 48 h long stimulation, the expression of *Nos2* was lower in 14D-cold MSCs than in C and 1D-cold MSCs. The existence of a negative feedback loop preventing excessive NO production could potentially explain the decrease in *Nos2* expression in 14D-cold MSCs at this time point (Smith et al., 2012). *Nos2* expression in murine MSCs stimulated with IFN γ and TNF α increased during the first 36 hours of stimulation (Yu et al., 2024), so an earlier time point would be better to prove the enhanced expression of *Nos2* in 14D-cold MSCs. Moreover, Western blot analysis showed the induction of iNOS expression already 12 hours after MSC stimulation with IFN γ and TNF α (Yu et al., 2024). The differences in iNOS expression in MSCs might be detected at earlier time points.

MSCs employ several additional molecules to suppress the immune response (H. J. Lee et al., 2021; G. Ren et al., 2010; Vasandan et al., 2016). Inflammatory stimulation of all MSC groups also resulted in the upregulation of genes involved in immunomodulation (*Ptgs2*, *Icam1*, *Ido1*, *Acod1*). *Ido1* expression was significantly lower in stimulated 14D-cold MSCs, but, similarly to *Nos2* expression, prolonged stimulation could have triggered a negative feedback loop, resulting in the downregulation of *Ido1* expression. Interestingly, MSC groups differ in their expression of *Vegf*, a factor implicated in MSC-mediated angiogenesis (Beegle et al., 2016). 1D-cold and 14D-cold MSCs might, therefore, possess enhanced pro-angiogenic activity, which could play a role in the thermogenic activation of adipose tissue.

The immunomodulatory activity of MSCs was tested in co-cultures with splenocytes. Even though we did not observe any difference in the activation of CD4⁺ and CD8⁺ T cells based on the expression of activation markers CD25 and CD69, we noticed a difference in the proliferation rate of T cells based on Ki67 staining. Ki67 is routinely used in our laboratory as a cell proliferation marker (Porubska et al., 2021). 14D-cold MSCs significantly decreased

CD4⁺ T-cell proliferation, while 1D-cold MSCs exhibited poor suppressive ability. But again, huge variability in the data should be noted.

C and 1D-cold MSCs tended to enhance ROR γ t expression by CD4⁺ T cells while having no effect on the expression of FOXP3. Even though most of the literature reported MSC-mediated suppression of Th17-cell differentiation with decreased IL-17 production and ROR γ t expression (Ghannam et al., 2010; Luz-Crawford et al., 2013), some reports demonstrated an increase in ROR γ t expression in T cells in a co-culture with MSCs (Najar et al., 2019). These discrepancies could have arisen from different methodical approaches, which impact the conditions of co-cultures, such as the type of stimulation (W. Li et al., 2012; G. Ren et al., 2008), ratio between MSCs and T cells (Najar et al., 2019; Porubska et al., 2021), source of MSCs (Y. Liu et al., 2024), period of co-culture, or differentiation cytokines used. However, the increased ROR γ t expression in a co-culture with C and 1D-cold MSCs could not be confirmed by increased production of IL-17 by CD4⁺ T cells. IL-10 production by CD4⁺ T cells was unchanged, corresponding to the unchanged expression of FOXP3.

Although significant upregulation of NO production was observed in 14D-cold MSCs, this did not translate into enhanced ability to suppress T-cell responses compared to C and 1D-cold MSCs. We now wonder whether the increased NO production serves a different role besides modulating the T-cell response. NO could modulate another immune cell population, which was not tested in our project. It is also possible that NO regulates MSCs in an autocrine manner. NO was implicated in the regulation of stem cell metabolism. It shifts the metabolism of human induced pluripotent stem cells toward glycolysis, at least in part by inducing HIF-1 α expression (Caballano-Infantes et al., 2021). Further examination of the effect of NO on the metabolism of MSCs, their proliferation, and other stem cell properties is necessary to explain this observation. Of note, it was suggested that NO plays a role in the vascularization of BAT in rats after long-term exposure to cold temperatures (Korac et al., 2008). NO produced by MSCs derived from cold-acclimated mice could, therefore, function to regulate the homeostasis of WAT after cold exposure.

To conclude, we showed that mice exposed to long-term mild cold temperatures are acclimated and that cold exposure causes substantial changes in the cytokine profile of tissues. MSCs derived from room temperature-exposed and cold-exposed mice exhibit differences in their metabolic state, some immunomodulatory properties, and, most importantly, NO production. However, our hypothesis that MSCs derived from cold-exposed mice have improved immunomodulatory potential was not confirmed. The significance of enhanced NO production by MSCs derived from cold-acclimated mice is yet to be explained. The underlying cause of the observed alterations in metabolism and immunomodulatory properties between control and cold-derived MSCs remains unknown.

In the second part of this project, we focused on the effect of culturing MSCs at reduced temperature on their metabolism and immunomodulation. MSCs cultured at reduced temperature differ in their metabolic properties. Significantly increased mitochondrial membrane potential and ROS production were observed following exposure to reduced

temperature. The increase in ROS production in 1D and 3D MSCs contrasts with previous studies describing a reduction in ROS production in MSCs cultured at reduced temperatures (Stolzing et al., 2006; Tirza et al., 2020). However, we analyzed the metabolic parameters after relatively short exposure to reduced temperature compared with the studies mentioned, which could explain this discrepancy. After 3 days, MSCs could still be experiencing cellular stress from the changed environment, and prolonged exposure could result in cellular adaptation and stabilization of metabolic state. Moreover, mitochondrial membrane potential significantly decreased following 24-hour stimulation in all MSC groups, as described in the literature (Y. Liu et al., 2018). In agreement with the flow cytometry data, Seahorse analysis revealed elevated ECAR and OCR in 3D MSCs. This was accompanied by an overall increase in ATP production, suggesting increased metabolic activity of those cells. On the other hand, Seahorse analysis showed low ECAR and OCR in 1D MSCs compared to C MSCs, contrary to the data obtained from flow cytometry. This discrepancy could have arisen from the differential settings of the flow cytometry and Seahorse experiments. When preparing cells for flow cytometry, MSCs were kept at 34°C or 37°C until the measurement. Conversely, the Seahorse measurement was running at 37°C. The fact that 1D MSCs were transferred from 34°C to 37°C might have caused additional cellular stress, which could explain the low metabolic activity of those cells. Moreover, in contrast to the flow cytometry data, Seahorse analysis showed that stimulation of MSCs led to enhanced ECAR and upregulation of ATP production. OCR was either unchanged or decreased after stimulation, corresponding to the decrease in mitochondrial potential. Differential settings of those experiments could have caused this disagreement in the results. In contrast to the above-described observations, the WST assay showed decreased succinate dehydrogenase activity in 3D MSCs. WST assay is also used for the discrimination of cellular proliferation, so we could argue that a lower number of 3D MSCs caused the decrease in WST in 3D MSCs compared to other groups. However, this is unlikely, as already 3 hours after stimulation, a similar effect was observed. A possible explanation could be the existence of a “break” within the TCA cycle, leading to the accumulation of intermediates with various biological activities, including the inhibition of TCA cycle enzymes, as was described for itaconate in macrophages (Cordes et al., 2016).

The effect of reduced culture temperature on the immunomodulatory capacity of MSCs was also evaluated. Interestingly, prolonged stimulation with LPS and IFN γ substantially upregulated NO production in 3D MSCs, as evaluated by flow cytometry. Stimulated 3D MSCs produced more NO than stimulated C and 1D MSCs. This could lead to an assumption that reduced temperature augments MSC immunomodulatory potential. Unfortunately, co-culture experiments with splenocytes did not reveal significant differences among MSC groups in their ability to suppress T-cell proliferation, cytokine production, or expression of transcription factors. It remains to be determined whether a long-term culture of MSCs at reduced temperature, longer than examined in this project, impacts their immunomodulatory activity. Interestingly, even though we did not observe any difference among MSC groups in their ability to promote the expression of FOXP3 in CD4⁺ T cells, we noticed that all MSC groups increased the expression of FOXP3 in those cells. This contrasts with our observations from the first part of this project, where we did not see any increase in FOXP3 expression, even in the co-culture

with control MSCs. The reason might be the different settings of co-culture experiments; as for the experiments with MSCs isolated from cold-exposed mice, we employed a 1:10 ratio of MSCs to splenocytes, whereas for the experiments with MSCs cultured at reduced temperatures, we employed a 1:20 ratio. The impact of the ratio between co-cultured splenocytes and MSCs was reported. The increasing number of splenocytes in the co-culture enhanced the suppressive function of MSCs, probably due to the increased number of inflammatory mediators produced by splenocytes to activate MSCs (W. Li et al., 2012).

Overall, the second part of this project proved that MSCs can react to deviations in ambient temperatures, although the receptors and molecular mechanisms involved are unknown. Acute exposure to reduced temperatures altered the metabolic profile of MSCs but did not promote their immunosuppressive function.

We showed that MSCs respond to cold-induced changes in the microenvironment of adipose tissue and to cold itself. Further research should address the potential of those conditions for improving the immunomodulatory and regenerative potential of MSCs in transplantation medicine.

7. Summary

Part 1 – The effect of cold exposure on MSCs

- We confirmed that cold exposure (4°C) for 14 days leads to BAT expansion and adaptation to cold in mice.
- We showed that the response to cold exposure includes alterations in the cytokine profile of various tissues. Those alterations are tissue-specific, and further research should address the underlying cause for the patterns of cytokine production observed following cold exposure.
- MSCs isolated from the adipose tissue of mice exposed to acute (1D) and long-term (14D) cold share the characteristics of MSCs derived from mice kept at room temperature, and, therefore, can be defined as MSCs.
- 1D-cold MSCs differ in their metabolic profile from C and 14D-cold MSCs, with increased glycolytic metabolism.
- There was a huge variability in the obtained data, which we proposed to stem from differential growth among MSC groups, affecting their cellular properties.
- 14D-cold MSCs produced significantly more NO, a key immunomodulatory molecule, suggesting enhanced immunosuppressive potential.
- However, MSCs isolated from cold-exposed mice have similar capabilities to control T-cell activation, proliferation, survival, and polarization as C MSCs.
- It remains to be determined what the role of increased NO production in MSCs from cold-adapted mice could be.

Part 2 – The effect of reduced culture temperature on MSCs

- We showed that MSCs cultured at reduced temperature (34°C) possess different metabolic properties than MSCs cultured at standard conditions (37°C).
- We noticed an increased intracellular production of NO in 3D MSCs after stimulation, suggesting their enhanced suppressive potential.
- However, the immunomodulatory function of MSCs cultured at reduced temperature was similar to MSCs cultured at standard conditions.

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