Mesenchymal Stromal Cells Disrupt mTOR- Signaling and Aerobic Glycolysis During T-Cell Activation

MARTIN BÖTTCHER,a ANDREAS D. HOFMANN,a HEIKO BRUNS,a MARTINA HAIBACH,a ROMY LOSCHINSKI,a DOMENICA SAUL,a ANDREAS MACKENSEN,a KATARINA LE BLANC,b REGINA JITSCHIN,a DIMITRIOS MOUGIAKAKOSa

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ABSTRACT

Mesenchymal stromal cells (MSCs) possess numerous regenerative and immune modulating functions. Transplantation across histocompatibility barriers is feasible due to their hypo-immunogenicity. MSCs have emerged as promising tools for treating graft-versus-host disease following allogeneic stem cell transplantation. It is well established that their clinical efficacy is substantially attributed to fine-tuning of T-cell responses. At the same time, increasing evidence suggests that metabolic processes control T-cell function and fate. Here, we investigated the MSCs’ impact on the metabolic framework of activated T-cells. In fact, MSCs led to mitigated mTOR signaling. This phenomenon was accompanied by a weaker glycolytic response (including glucose uptake, glycolytic rate, and upregulation of glycolytic machinery) toward T-cell activating stimuli. Notably, MSCs express indoleamine-2,3-dioxygenase (IDO), which mediates T-cell suppressive tryptophan catabolism. Our observations suggest that IDO-induced tryptophan depletion interferes with a tryptophan-sufficiency signal that promotes cellular mTOR activation. Despite an immediate suppression of T-cell responses, MSCs foster a metabolically quiescent T-cell phenotype characterized by reduced mTOR signaling and glycolysis, increased autophagy, and lower oxidative stress levels. In fact, those features have previously been shown to promote generation of long-lived memory cells and it remains to be elucidated how MSC-induced metabolic effects shape in vivo T-cell immunity.

INTRODUCTION

Adoptive transfer of mesenchymal stromal cells (MSCs) has emerged as a promising option for treating particularly acute forms of graft-versus-host disease (GVHD) following stem cell transplantation. In GVHD the MSCs’ beneficial effects can be attributed to their fine-tuning of T-cell responses. At the same time increasing evidence highlights that metabolic signaling governs T-cell function and fate. Here, we show for the first time how MSCs suppress T-cell activation by modulating their metabolism. However, several of the observed MSC-mediated metabolic effects are linked to memory formation and longevity in T-cells. Our findings could therefore represent an explanation why MSC-transfer in GVHD patients does not lead to an increased risk for disease relapse or infectious complications.
formation [9]. Following antigen encounter, T-cells shift towards aerobic glycolysis for acquiring effector functions while memory and regulatory T-cells (Treg) utilize mainly oxidative phosphorylation (OXPHOS) for meeting energetic demands. Remarkably, interference within the metabolic framework allows controlling T-cell fate as exemplified by studies showing that blocking glycolysis hampers effector functions and promotes development of long-lived memory T-cells [10–13].

As yet, it remains unknown whether MSCs interfere with T-cell metabolism. Therefore, we sought out to investigate the MSCs’ impact on metabolic signaling in activated T-cells.

**METHODS**

**Cells**

T-cells were purified by magnetic bead-based selection (T-cell Isolation Kit II and Effector Memory T-cell Isolation Kit, Miltenyi Biotec, Bergisch Gladbach, Germany). MSCs were isolated from iliac crest bone marrow aspirates from healthy donors (approval number: 200_12), expanded as previously detailed while fulfilling uniformly minimal MSC criteria [3].

**Coculture Experiments**

MSC and T-cell cocultures were performed for 72 hours as previously detailed [6]. In selected experiments rapamycin (Sigma Aldrich, St. Louis, MO, USA), 1-methyl-DL-tryptophan (1-MT, Sigma-Aldrich), αi-tryptophan (Sigma-Aldrich) was applied.

**Adenosine 5′-triphosphate Levels**

Adenosine 5′-triphosphate (ATP) was assessed using a coloriometric ATP Assay Kit (Abcam, Cambridge, UK).

**Flow Cytometry**

Cells were stained following the manufacturers’ recommendations using fluorochrome-coupled antibodies (Supporting Information Table 1) and/or chemical dyes. Cells were analyzed using a FACS Canto II cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and the FlowJo Version 9.5 software (TreeStar, Ashland, OR, USA).

**DNA, RNA preparation, and Quantitative Polymerase Chain Reaction**

RNA/DNA were extracted from cell lysates (RNeasy Mini Kit, Qiagen, Hilden, Germany) and cDNA prepared (Superscript First Strand Synthesis System, Life Technologies, Carlsbad, CA, USA) using a Mastercycler Nexus (Eppendorf, Hamburg, Germany). The messenger RNA (mRNA) levels were quantified by quantitative polymerase chain reaction (qPCR; Quantitect First Strand Syntesis System, Qiagen, Hilden, Germany). Relative gene expression was determined by normalizing expression of target genes (Supporting Information Table 2) to 18S.

**Extracellular Flux Analysis**

Bioenergetics were determined using an XFe96 Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA, USA) as previously described [14].

**Figure 1.** Increased mTOR signaling and aerobic glycolysis during T-cell activation. (A) T-cells were cultured in presence or absence of TCR/CD28 triggering for 72 hours (n = 7). The ECAR indicating aerobic glycolysis was measured using an XFe96 flux analyzer. ECAR was measured upon injection of glucose (gluc) and 1 μM of the mitochondrial inhibitor oligomycin (oligo) yielding baseline glycolysis and glycolytic reserve capacity respectively. The reaction was terminated using 100 mM 2-Deoxy-d-Glucose. (B) Changes in the relative gene expression (mRNA) of glycolytic enzymes including hexokinase-1/2 (hk1/2), lactate dehydrogenase A (ldha), and glyceraldehyde-3-phosphate dehydrogenase (gapdh) were determined by quantitative PCR in T-cells (n = 5) stimulated and unstimulated cells (set as 1). (C) Density of the glucose transporters GLUT1 was assessed on T-cells (n = 6) by FACS based on the MFI. (D) Activation of the mTOR pathway was assessed based on the MFI of phosphorylated (active) mTOR (pmTOR) (n = 6). (E) Changes of the carnitine-palmitoyl-transferase-1 (cpt1a) relative gene expression (mRNA), which represents the rate-limiting enzyme for fatty acid oxidation (FAO), are shown for stimulated and unstimulated (set as one) T-cells (n = 5). (F) OCR as a surrogate for oxidative phosphorylation (OXPHOS) is measured in (stimulated and unstimulated) T-cells under basal conditions and in response to 40 μM of the FAO/cpt1a inhibitor etomoxir. Extent of OCR reduction represents an indicator for the FAO’s contribution to OXPHOS and finally to energy production. On that account decrease of OCR correlates with the cells’ energetic dependency on FAO. A paired t-test was used for data analysis. Bars indicate the standard error of the mean. * p < 0.05; ** p < 0.005; *** p < 0.001. Abbreviations: ECAR, extracellular acidification rate; MFI, median fluorescence index; mRNA, messenger RNA; MSC, mesenchymal stromal cells; mTOR, mechanistic target of rapamycin; pmTOR, phosphorylated mTOR; OCR, oxygen consumption rate.

**Fluorescence Microscopy**

Autophagy was visualized using an LC3 antibody (Merck Millipore, Billerica, MA, USA) and DAPI (Sigma Aldrich) for nuclear counter staining. Analysis was performed on a fluorescence microscope (Axio-Imager M2, Zeiss, Jena, Germany) at 630 × magnification using the AxioVision Software (Zeiss).

**Statistical Analyses**

Differences in means of continuous variables were evaluated using a paired t-test if not stated otherwise. All statistical analyses were performed using GraphPad Prism five

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RESULTS AND DISCUSSION

Metabolism critically controls T-cell function and fate [9]. Trig-
ering T-cell and CD28 co-stimulatory receptors elicits a marked glycolytic shift (Fig. 1A, Supporting Information Fig. 1). This metabolic switch is facilitated by upregulation of glycolytic molecules including hexokinase-1/2, lactate dehydro-
genase, and glyceraldehyde-3-phosphate dehydrogenase, increased expression of glucose transporters promoting glu-
cose uptake (Fig. 1B and C, Supporting Information Fig. 2), and as previously shown [15] mechanistic target of rapamycin (mTOR) signaling (Fig. 1D). Blocking aerobic glycolysis abro-
gates T-cell activation (Supporting Information Fig. 3). At the same time expression of carnitine-palmitoyl-transferase-1 a rate-limit for fatty acid oxidation (FAO) declines [11] and proportion of FAO-dependent OXPHOS (and ATP-production) accordingly decreases (Fig. 1E and F).

Since MSCs display a high density of glucose transporters, a substantial glucose influx, and perform aerobic glycolysis (Supporting Information Fig. 4), we were interested whether MSCs interfere with T-cell activation through substrate competition in analogy to the concept of TRegs controlling T-cell responses via IL-2 restriction [16]. In fact, glucose availability had no impact on MSCs’ suppressive activity (Supporting Information Fig. 5).

MSCs significantly interfered with mTOR signaling, paral-
leled by reduced upregulation of glycolytic enzymes and glu-
cose uptake mimicking effects of mTOR inhibitors (Fig. 2A-C, Supporting Information Figs. 6–10). This is in line with ex vivo and in vitro observations regarding MSC-related Treg induction [5, 6], which can also be achieved by pharmacological mTOR inhibition [17]. The T-cells’ overall metabolic turnover was reduced as implicated by lower ATP and OXPHOS levels (Fig. 2D, Supporting Information Fig. 11). Mitochondrial fitness as

**Figure 2.** MSCs interfere with mTOR signaling and glycolytic switch during T-cell activation. (A) T-cells were cultured in presence/absence of TCR/CD28 stimulation and allogeneic MSCs for 72 hours (n = 7). Activation of the mTOR signaling in T-cells was assessed based on the phosphorylation levels of mTOR (pmTOR MFI) (n = 6) (left panel) and of its downstream molecule ribosomal protein S6 kinase β-1 (pS6K1) (n = 4) (right panel). (B) Changes of the relative gene expression (mRNA) of glycolytic enzymes were determined by quantitative PCR in T-cells (n = 7-9) stimulated in presence of MSCs as compared to stimulated cells alone (set as 1). (C) ECAR was measured in activated T-cells following culture in presence or absence of MSC contact (n = 7), in response to glu-
cose (baseline glycolysis) and upon blocking the mitochondrial ATP generation by oligomycin. The resulting (compensatory) effect on ECAR following interference with mitochondrial energy metabolism represents the cells’ maximal glycolytic capacity. (D) ATP lev-
els were determined in lysates from T-cells cultured for three days under the indicated conditions (n = 5). (E) The OCR/ECAR ratio indicative for the energetic balance between OXPHOS and aerobic glycolysis was calculated for T-cells activated for 3 days in pres-
ence or absence of MSCs (n = 4). (F) Changes in the relative gene expression (mRNA) of carnitine-palmitoyl-transferase-1 (cpt1α) were determined by quantitative PCR in T-cells (n = 6) stimulated in presence of MSCs for three days as compared to stimulated T-cells alone (set as 1). A paired t-test was used for data analysis. Bars indicate the standard error of the mean. *, p < 0.05; **, p < 0.005; ***, p < 0.001. Abbreviations: ATP, adenosine 5’-triphosphate; ECAR, extracellular acidification rate; MFI, median fluo-
rescence index; mRNA, messenger RNA; MSC, mesenchymal stromal cells; mTOR, mechanistic target of rapamycin; pmTOR, phosphorylated mTOR; OCR, oxygen consumption rate.
indicated by the ability to generate and sustain an electrochemical gradient (\(=\Delta \Psi M\)) was not affected (Supporting Information Fig. 12). However, when analyzing the ratio between oxygen consumption rate (OCR, indicative for OXPHOS) and extracellular acidification rate (ECAR, indicative for aerobic glycolysis) we noticed an MSC-related skewing toward OCR suggesting a shift of energetic balance (Fig. 2E). This effect is additionally resembled by pharmacological mTOR inhibition (Supporting Information Fig. 13) \[10\] and further corroborated by findings that MSCs preserve carnitine-palmitoyl-transferase-1 (cpt1a) expression (Fig. 2F) \[18\].

Next, we tested whether MSC-mediated metabolic effects last upon MSC removal. T-cells previously activated in presence of MSCs retain reduced proliferative capacity (Supporting Information Fig. 14), mTOR responsiveness, glucose influx, and aerobic glycolysis (Fig. 3A and B). As anticipated, glycolytic molecules were not adequately upregulated (Supplemental Fig. 15). In view of a so-called epigenetic memory that is formed in activated T-cells, \[19\] the MSCs' impact on it remains to be elucidated.

Within its role as a metabolic master-regulator mTOR integrates nutrient sensing pathways \[20\]. Deprivation of amino acids causes mTOR inactivation (Fig. 2A) leading to autophagy via LC3 de-repression (Fig. 3C, Supporting Information Fig. 16), translational blockade via S6K inactivation (Fig. 2A), and inhibition of aerobic glycolysis (Fig. 2C) \[21\]. Recently, it was shown that tryptophan-depleting IDO disrupts a tryptophan sufficiency signal that stimulates mTOR \[22\]. MSCs employ among other mechanisms IDO for regulating T-cell responses \[2, 4, 6\] (Supporting Information Fig. 17). Tryptophan supplementation and IDO inhibition by 1-MT acting as a tryptophan mimetic \[22\] sustained activated T-cells' mTOR signaling together with increased glucose uptake and glycolytic capacities (Fig. 3D and E, Supporting Information Fig. 18).

Recent evidence indicates that mTOR inhibition \[12, 13\], disrupting glycolysis \[11\], and inducing autophagy (Fig. 3C)
positively impact T-cells’ durability and memory cell formation. Accordingly, T-cells activated in presence of MSCs expressed more CD62L and genes linked to memory response (Fig. 4A and B) [11, 12]. Furthermore, cellular oxidative stress levels are reduced together with increased thiol content, upregulation of antioxidants (Fig. 4C-E), and reduced mitochondrial oxidative stress (Supporting Information Fig. 19). These phenomena have been previously reported in context of mTOR inhibition and metabolic quiescence [11, 12, 24].

**CONCLUSIONS**

Taken together, MSCs impact the metabolic phenotype of activated T-cells [9]. The rapid increase of glycolysis is a prerequisite for proper T-cell activation. Therefore, MSC-mediated perturbation of mTOR signaling—a key regulator of glycolytic metabolism [20]—is in keeping with the T-cell suppressive activity of MSCs [4]. However, several of the MSC-mediated metabolic effects including mTOR inhibition [10, 12, 13], reduced glycolysis [11], and promotion of autophagy [23] are linked to memory formation and longevity in T-cells. These findings certainly require further exploitation especially in view of ongoing discussions regarding the risk for disease relapse and/or infectious complications upon adoptive MSC transfer in GvHD patients [7, 8]. Furthermore, combining pharmacological mTOR inhibition [25] and MSC-transfer might represent a promising strategy for merging their beneficial (metabolic) effects on T-cells in an additive fashion.

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**AUTHOR CONTRIBUTIONS**

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

REFERENCES


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