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Research paper

CD4$^+$ T cell activation, function, and metabolism are inhibited by low concentrations of DMSO

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ABSTRACT

Dimethyl sulfoxide (DMSO) is a polar organic solvent used in a wide range of biological applications. DMSO is routinely used as a cryoprotectant for long-term cell freezing as well as to dissolve peptides and drugs for immune cell functional assays. Here, human CD4$^+$ T cell activation, cytokine production, proliferation, and metabolism were investigated after stimulation in the presence of 0.01% to 1%, DMSO, representing concentrations commonly used *in vitro*. Surface expression of the activation markers CD69, CD25 and CD154 after polyclonal activation of CD4$^+$ T cells was inhibited by 0.25% or higher concentrations of DMSO. The frequencies of IL-21$^+$, IL-4$^+$, and IL-22$^+$ CD4$^+$ T cells, following polyclonal activation were variably inhibited by DMSO at concentrations ranging from 0.25% to 1%, whereas IFNγ$^+$ cells were unaffected. CD4$^+$ T cell proliferation after anti-CD3 or antigen stimulation was inhibited by 0.5% DMSO and abolished by 1% DMSO. After polyclonal stimulation, glucose uptake was inhibited in the presence of 1% DMSO, but only minor effects on CD4$^+$ T cell respiration were observed. Consistent with the immune effects, the gene expression of early signaling and activation pathways were inhibited in CD4$^+$ T cells in the presence of 1% DMSO. Our study revealed that DMSO at concentrations generally used for *in vitro* studies of T cells impacts multiple features of T cell function. Therefore, we urge care when adding DMSO-containing preparations to T cell cultures.

1. Introduction

Dimethyl sulfoxide (DMSO) is a small amphiphilic molecule with a highly polar domain and two apolar methyl groups, and is extensively used in biological and medical research (Capriotti and Capriotti, 2012; Santos et al., 2003; Timm et al., 2013). DMSO accumulates in lipid bilayers of plasma membranes and forms water-permeable pores in cell membranes (de Ménorval et al., 2012). DMSO is used as a cryoprotectant for long-term freezing of various cell types (Egorin et al., 1998; Rall and Fahy, 1985). It affects the hydrophilic region of membrane lipids, decreasing membrane fluidity, and preventing ice crystal formation. Importantly, properly cryopreserved peripheral blood mononuclear cells (PBMCs) can be recovered and used in functional assays (Galeano Niño et al., 2016; Weinberg et al., 2009). DMSO has radical-scavenging and anti-inflammatory activity but is also considered as an inducer of oxidative stress (Duarte et al., 2004; Yuan et al., 2014). In immunological studies, peptides and proteins are often dissolved in DMSO, and various blood and immune cells are frozen in relatively high concentrations of DMSO (Galvao et al., 2014). *In vitro* functional cellular assays are frequently used to determine immunological effects in response to peptides or drugs. While DMSO effects in *Saccharomyces cerevisiae* and astrocytes are reported (Kakolyri et al., 2016; Sadowska-Bartosz et al., 2013; Yuan et al., 2014), the current literature only discusses toxic effects of DMSO on immune cells at concentrations above 1%. Notably, minor evidence for metabolic effects of DMSO exists (Galvao et al., 2014; Kahler, 2000).

In the scope of this study, we aimed to determine the effect of low DMSO concentration on functional assays that are regularly performed to evaluate T lymphocytes, and to couple this to T cell metabolism. We show that DMSO decreases activation and cell growth as well as cytokine secretion at very low concentration. Long-term incubation profoundly affects T cell biology at much lower concentration than reported previously.

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2. Materials and methods

2.1. CD4$^+$ T cell isolation and culture under DMSO exposure

Healthy donors were recruited from the Munich Diabetes Bioresource with informed consent and ethical committee approval (Ethical approval number 5049/11). Fresh peripheral blood mononuclear cells were isolated from heparinized blood using Ficoll-Hypaque density centrifugation and CD4$^+$ T cells isolated by MACS separation (Miltenyi Biotech) according to the manufacture's protocol. Cells were cultured in RPMI 1640 supplemented with 5% heat-inactivated human male AB serum, 2 mM L-Glutamine and 5 mM Glucose with increasing concentration of DMSO (Sigma-Aldrich) given as % *v*/v. CD4$^+$ T cells (10^6 per ml) were cultured in 96-well round-bottom plates (Costar) and stimulated by plate-bound anti-CD3 antibody (Biolegend; clone: UCHT1) or anti-CD3/CD28 Dynabeads [1:4 ratio] (Thermofisher). Plate bound anti-CD3 antibody was dissolved at a concentration of 5 µg/ml in PBS and 50 µL per well were added for 2–3 h at 37 °C. The supernatant was aspirated and wells washed twice with PBS prior to cell seeding.

2.2. Evaluation of live/dead cells

T cells were stained with an amine reactive fluorescent dye (Biolegend) performed according to manufacturer's protocol.

2.3. Early activation

CD4$^+$ T cells were cultured and stimulated with 5 µg/ml of a plate-bound anti CD3 antibody for 16 h (LEAF purified anti-human CD3; Biolegend), stained with anti-CD4 (anti-CD4-PerCP; BD Bioscience), anti-CD69 (anti-CD69-FITC; BD Bioscience), anti-CD25 (anti-CD25-PE; BD Bioscience), and anti-CD154 (anti-CD154-Brilliant Violet 605; Biolegend). Samples were analyzed by flow cytometry.

2.4. Proliferation assay

CD4$^+$ T cells were labelled with the cell proliferation dye eFluor450 (eBioscience). Briefly, 10 µM eFluor450 in PBS was added for 30 min at room temperature. The labelling reaction was terminated by addition of culture medium (RPMI 1640; Gibco) containing 15% heat-inactivated human male AB serum. Cells were incubated on ice for 5 min followed by three washing steps in culture medium. CD4$^+$ T cells were stimulated with plate-bound anti-CD3 antibody for 72 h in the presence and absence of DMSO. Alternatively, PBMC'S were stimulated with tetanus toxoid (Andreae-Noris Zahn AG, Frankfurt, Germany; diluted 1/500) for 5 days.

2.5. Short-term intracellular cytokine assay

CD4$^+$ T cells were stimulated with anti-CD3/CD28 Beads (Thermofisher) for 6 h. Brefeldin A was added for the last 4 h (10 µg/ml, Sigma-Aldrich). Cells were fixed and permeabilized using Cytofix/Cytoperm solution (BD Bioscience) according to the manufacturer's protocol. Subsequently, cells were stained: surface staining was performed with anti-CD4 (anti-human CD4-FITC; Biolegend). Intracellular staining was performed with antibodies anti-IL-4 (anti-human IL-4-Brilliant Violet 605; Biolegend), anti-IL-17A (anti-human IL-17A-Brilliant Violet 510; Biolegend), anti-IL-22-eFluor450 (eBioscience), anti-IFNγ (anti-human IFNγ-PE-Cy7; BD Bioscience) and anti-IL-21 (anti-human IL-21-PE; eBioscience).

2.6. Flow cytometric analysis

All samples were acquired within 24 h on a Becton Dickinson LSR Fortessa flow cytometer and analyzed with FlowJo software (Version 10; TreeStar Inc.). All conditions were analyzed in technical duplicates.

2.7. Bioenergetic measurements

Human CD4$^+$ T cell bioenergetics in the presence of increasing DMSO concentration was measured using the XF96 extracellular flux analyzer (Agilent Technologies). CD4$^+$ T cells (10^6 per ml) were cultured in RPMI containing 5 mM glucose, 2 mM L-glutamine and 5% heat-inacitvated human male AB serum and DMSO. Cells were polyclonally stimulated with anti-CD3/CD28 Dynabeads (Thermofisher) in 96-well culture plates (Costar) for 48 h. Prior to the measurement, T cells were transferred to the Seahorse 96-well culture microplate in XF media in the presence of the polyclonal stimulus, 5 mM glucose and different concentrations of DMSO. Subsequently, T cells were incubated in these conditions for additional 45 min at 37 °C in a non-CO_2 incubator. In order to ameliorate cell adhesion and distribution within a well, culture plates were centrifuged at 180 *g*. The seahorse XFe96 sensor cartridge was hydrated in a non-CO_2 37 °C incubator (Agilent XF Seahorse Calibrant) for 24 h prior to the measurement. Four baseline measurement cycles were recorded. To analyze oxygen consumption rate, 2 µM oligomycin, 1.5 µM carbonyl cyanide 4(trifluoromethoxy) phenylhydrazone (FCCP), 2.5 µM rotenone and 2.5 µM antimycin A were injected sequentially. Glycolysis was monitored in the presence of the glycolytic inhibitor 2-desoxyglucose (2-DG) [1 mM]. The experiments were performed with at least four technical replicates.

2.8. Statistical analysis

Statistical analysis was performed using Graph Pad Prism 7 software. Data are presented as mean. Multiple Comparisons were conducted using one-way analysis of variance (ANOVA) with the Greenhouse-Geisser correction and Dunnett's multiple comparison test, with individual variances computed for each comparison. Alternatively, a paired *t*-test was performed. A two-tailed p value < 0.05 was considered significant.

2.9. Differential gene expression analysis

Fastq files corresponding to each sample was aligned to the human transcriptome (Ensembl ver 91) using STAR (version 2.5.4a). Reads that mapped to more than one location in the transcriptome were discarded. The resulting bam files were used to generate a count matrix using featureCounts. Differentially expressed genes were identified using DESEQ2 by first merging technical replicates from the same samples and then filtering genes where the corrected *p*-value was < 0.05.

3. Results

3.1. The effect of DMSO on early CD4$^+$ T cell activation and cytokine production

Short-term (16 h) and long-term (72 h) exposure to DMSO concentrations up to 1% did not affect T cell viability (Supplementary Fig. 1). CD4$^+$ T cell function is often measured by response to non-specific and specific stimuli. The early response includes the upregulation of activation markers and, for memory T cells, the production of cytokines. The activation markers CD69, CD25 and CD154 were upregulated within 16 h post-stimulation with plate-bound anti-CD3 (Supplementary Fig. 2). The frequency of cells that expressed CD69 was decreased in the presence of 1% of DMSO (86.1 ± 7.7% of value without DMSO; $p = 0.0013$). CD25$^+$ T cells were attenuated in the presence of 0.5% DMSO (81.7 ± 6.1%; $p = 0.0002$) and CD154$^+$ T cells were already reduced in the presence of 0.1% DMSO (83.4 ± 9.7%; $p = 0.0125$) and almost completely abolished in the presence of 1% DMSO (9.7 ± 4.7%; $p = 0.0001$); (Fig. 1).

The effect of DMSO on human CD4$^+$ T cell cytokine production was

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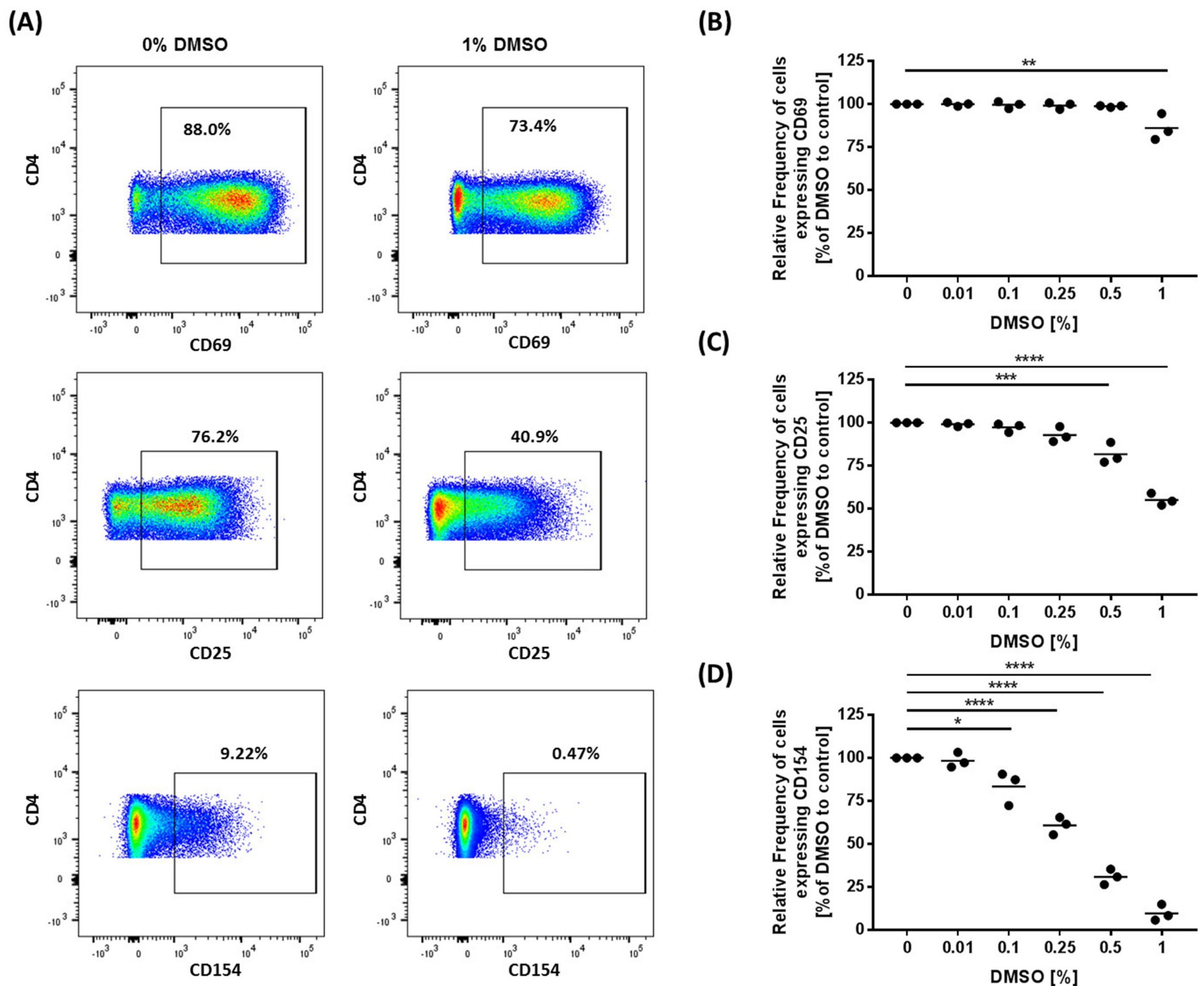

Fig. 1. The Effect of DMSO on CD4$^+$ T cell activation. CD4$^+$ T cells were stimulated by plate-bound anti-CD3 antibody in the presence of increasing DMSO concentrations and surface expression of the activation markers CD69 (B), CD25 (C), and CD154 (D) measured by flow cytometry after 16 h. Representative FACS plots at 0% and 1% DMSO are shown on the left and the quantitative data from three independent experiments using different donors expressed as the frequency of activation marker positive cells relative to the value at 0% DMSO is shown on the right. ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$; two-tailed one-way ANOVA.

determined after stimulation of T cells with anti-CD3/CD28 Dynabeads for 6 h with the addition of a transport inhibitor for the last 4 h before intracellular cytokine staining. The presence of DMSO resulted in a decrease in CD4$^+$ T cells positive for IL-4, IL-22, IL-21 and IL-17A, but no effect was observed on IFNγ positive CD4$^+$ T cells (Fig. 2). A DMSO concentration of 0.25% was sufficient to reduce the frequency of IL-4$^+$ cells (84.2 ± 4.2% of 0% DMSO value; $p = 0.0031$) and IL-21$^+$ cells (76.2 ± 8.8% of 0% DMSO value; $p = 0.0089$), and at 1% DMSO, the frequency of cytokine positive cells was reduced to 70.5 ± 3.5% of that observed without DMSO for IL-4 ($p = 0.0001$), 47.9 ± 14.0% for IL-21 (p = 0.0001) and 44.0 ± 17.0% for IL-22 ($p = 0.0004$).

3.2. DMSO inhibits CD4$^+$ T cell proliferation

CD4$^+$ T cell proliferation was investigated upon plate bound anti-CD3 stimulation for 72 h (Fig. 3A, Supplementary Fig. 3). Proliferation was affected already at a DMSO concentration of 0.25% (69.7% ± 10.0% of value without DMSO; $p = 0.021$), and was almost completely abolished at 1% DMSO (2.7% ± 0.3%; $p = 0.0001$). Antigen-specific T cell responses were also decreased in the presence of DMSO (Fig. 3B), with 0.5% DMSO reducing responses to 49% ± 22.7% of responses in the absence of DMSO ($p = 0.0016$). T cell proliferation at 72 h was almost completely recovered when DMSO was removed after 16 h (Supplementary Fig. 4).

3.3. DMSO affects cellular glucose uptake and increases glycolytic rates

The metabolism of a T cell is closely related to its function. In line with the inhibition of activation, the presence of 1% DMSO during plate-bound anti-CD3 stimulation reduced glucose uptake by CD4$^+$ T cells during 16 h of incubation (Fig. 4A). Mitochondrial respiration and glycolysis were investigated by extracellular flux analysis 48 h after polyclonal stimulation of CD4$^+$ T cells with anti-CD3/CD28 Dynabeads. Mitochondrial oxidative phosphorylation (OXPHOS), represented by ATP-linked respiration and maximal substrate oxidation, represented by maximal respiration, were unaffected by DMSO concentrations up to 1% (Fig. 4B). Basal and oligomycin-induced glycolytic rates, represented by extracellular acidification rates, were also not

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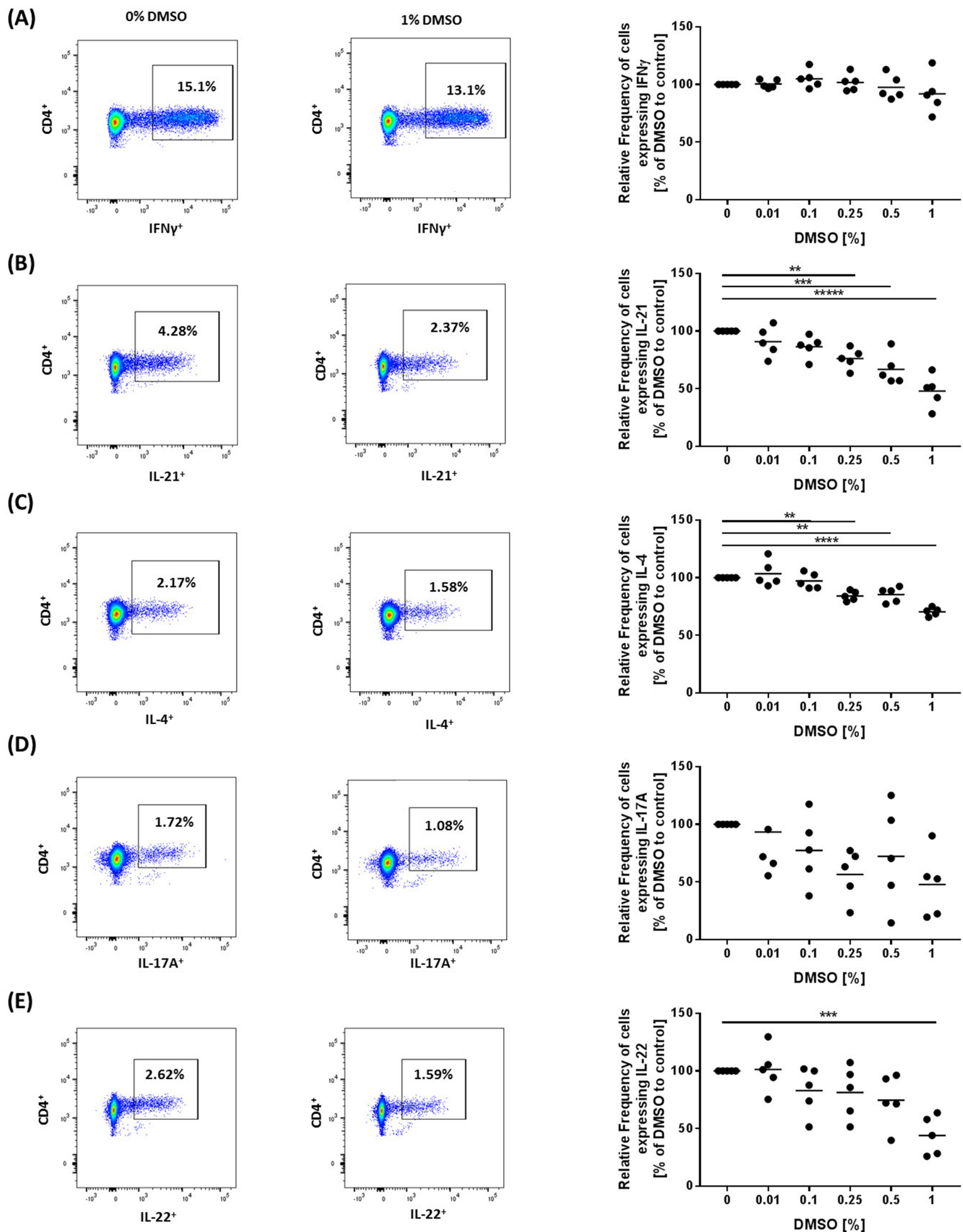

Fig. 2. Effect of DMSO on cytokine production by $CD4^+$ T cells. $CD4^+$ T cells were stimulated with anti-CD3/CD28 Dynabeads for 6 h in the presence of increasing DMSO concentrations. Brefeldin A was added for the last 4 h of culture before intracellular cytokine staining of cells and analysis by flow cytometry. Representative FACS plots are shown for IFNγ (A), IL-21 (B), IL-4 (C), IL-17A (D), and IL-22 (E) at 0% and 1% DMSO on the left and quantitative data from five independent experiments using different donors each performed with technical duplicates are shown on the right. The data on the right is expressed as the relative frequency of cytokine positive cells as compared to the value at 0% DMSO. ** $P \leq 0.01$; *** P ≤ 0.001; **** $P \leq 0.0001$; two-tailed one-way ANOVA.

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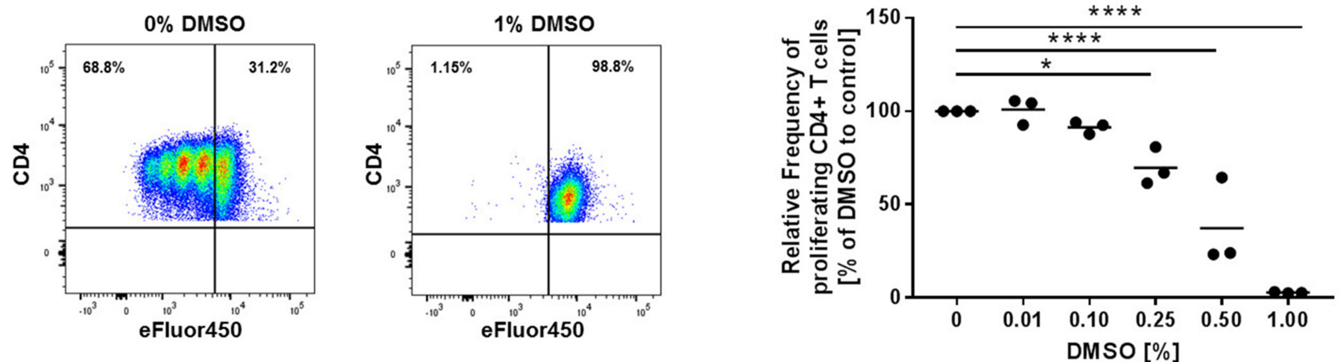

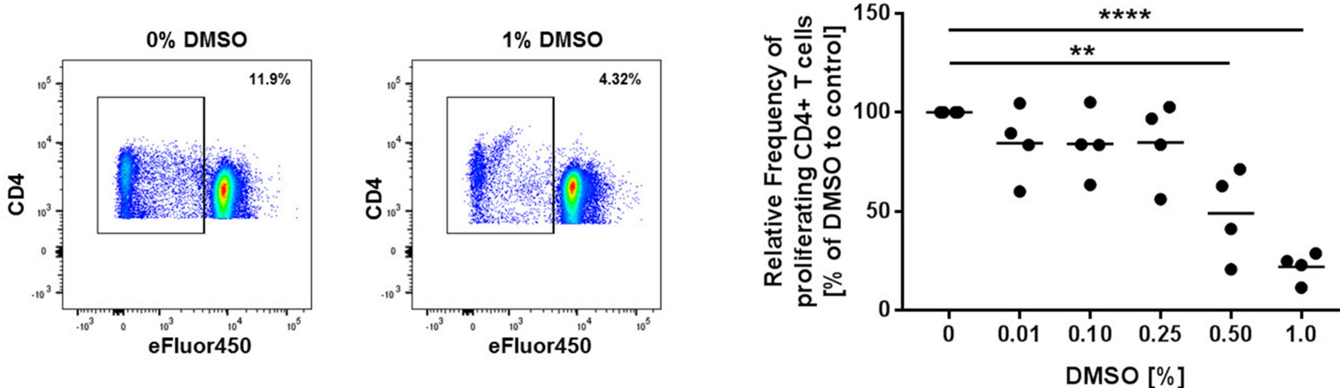

Fig. 3. DMSO inhibits $CD4^+$ T cell proliferation. $CD4^+$ T cells labelled with eFluor450 were stimulated with plate-bound anti-CD3 antibody (A) for 72 h and eFluor450-labelled peripheral blood mononuclear cells were stimulated with tetanus toxoid for 5 days (B); both experiments were performed in the presence of increasing DMSO concentrations. Representative FACS plots of the proliferation by $CD4^+$ T cells (eFluor450 dilution) in the presence of 0% and 1% of DMSO are shown on the left and the quantitative data from 3 (anti-CD3 stimulation) and 4 (tetanus toxoid stimulation) independent experiments using different donors, each with technical duplicates are shown on the right. The data on the right is expressed as the relative frequency of dye-diluted cells as compared to the value at 0% DMSO. * $P \le 0.05$; ** $P \le 0.01$; **** $P \le 0.0001$; two-tailed one-way ANOVA.

significantly affected by DMSO (Fig. 4C).

3.4. DMSO affects $CD4^+$ T cell activation and function pathways

RNA sequencing was performed on $CD4^+$ T cells that had been activated with anti CD3/CD28 Dynabeads in the presence or absence of 1% DMSO. Differential gene expression analysis using DESEQ2 identified 309 genes that are significantly up-regulated and 468 genes that are down-regulated in cells in the presence of 1% DMSO (*p*-value adjusted for multiple testing < 0.05; Supplementary table 1; Fig. 5A). Consistent with the data of protein and functional assays, KEGG pathway analysis of the down-regulated genes identified enrichment of cytokine-cytokine receptor interaction (hsa04060), T-cell receptor signaling (hsa04660), and NF-kappa B signaling (hsa04064) pathways. No pathway enrichment was observed for the up-regulated genes (Fig. 5B).

3.5. The solvent Acetonitrile minimally affects T cell proliferation

Acetonitrile may also be used to dissolve proteins and peptides. We, therefore, tested the effect of Acetonitrile on $CD4^+$ T cell proliferation (Fig. 6). No reduction in proliferation was observed at Acetonitrile concentrations up to 2% and in some but not all samples tested at a concentration of 4%.

4. Discussion and conclusion

The beneficial effects of DMSO as therapeutics for several human diseases and its utility in medical and biological research have been known since the 1960s (Capriotti and Capriotti, 2012). After its approval for clinical trials, the use of DMSO was restricted by the United States Food and Drug Administration (FDA) 'DMSO Ban: Was it Handled Properly?', 1966. Its use was again approved in 1978 for the treatment of interstitial cystitis ('FDA Set to Approve DMSO for Human Use', 1978). Despite this clinical use, there is a relative paucity of information regarding its biological effects on cells.

In this study, we show that DMSO affected $CD4^+$ T cell responses at a concentration of 0.25% and markedly impact function at 1%. These DMSO concentrations are generally used in experiments that involve the addition of antigen and other proteins to assays that measure T cell responses and function. Peptides, for example, are often dissolved in DMSO and added at 1 μl volumes to wells with 200 μl of cell medium volume, resulting in final concentrations of 0.5%. We demonstrate that activation, proliferation and cytokine secretion were affected by different thresholds of DMSO concentration. Hence, we urge that not only DMSO vehicle controls should be used but that DMSO concentrations should be kept below 0.25%, considering its immunological activity. Alternatively, inhibitory effects may be reduced by washing out DMSO after an activation phase, or the use of solvents such as Acetontrile,

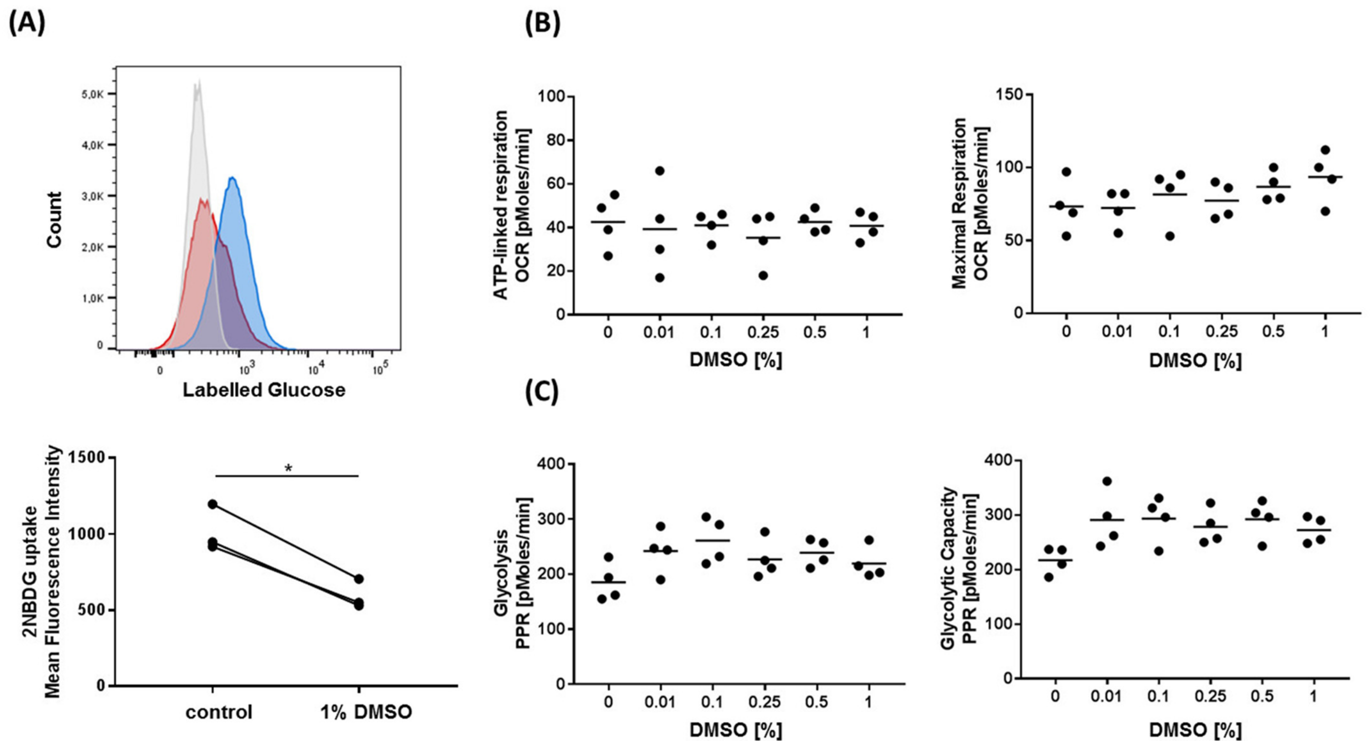

Fig. 4. Effect of DMSO on CD4$^+$ T cell metabolism: CD4$^+$ T cells were stimulated with anti CD3/CD28 beads in the presence of increasing DMSO concentration up to 1%. (A) Glucose uptake was measured using labelled glucose (2NBDG) after stimulation of CD4$^+$ T cells or 16 h without and in the presence of 1% of DMSO ($N = 3$). Representative FACS data is shown (gray curve represents the unstimulated control, blue curve cells without DMSO and the red curve the cell subsets measured in the presence of 1% of DMSO). The Mean Fluorescence Intensity of 2NBDG with and without DMSO is shown in the lower panel. (B) T cell OXPHOS rates (OCR) of ATP-linked respiration and maximal respiration, and (C) Glycolysis and glycolytic capacity of the CD4$^+$ T cells after 48 h stimulation with anti CD3/CD28 beads in the presence of increasing DMSO concentration. Each point represents one independent experiment from different donors ($N = 4$). * $P \leq 0.05$; paired *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

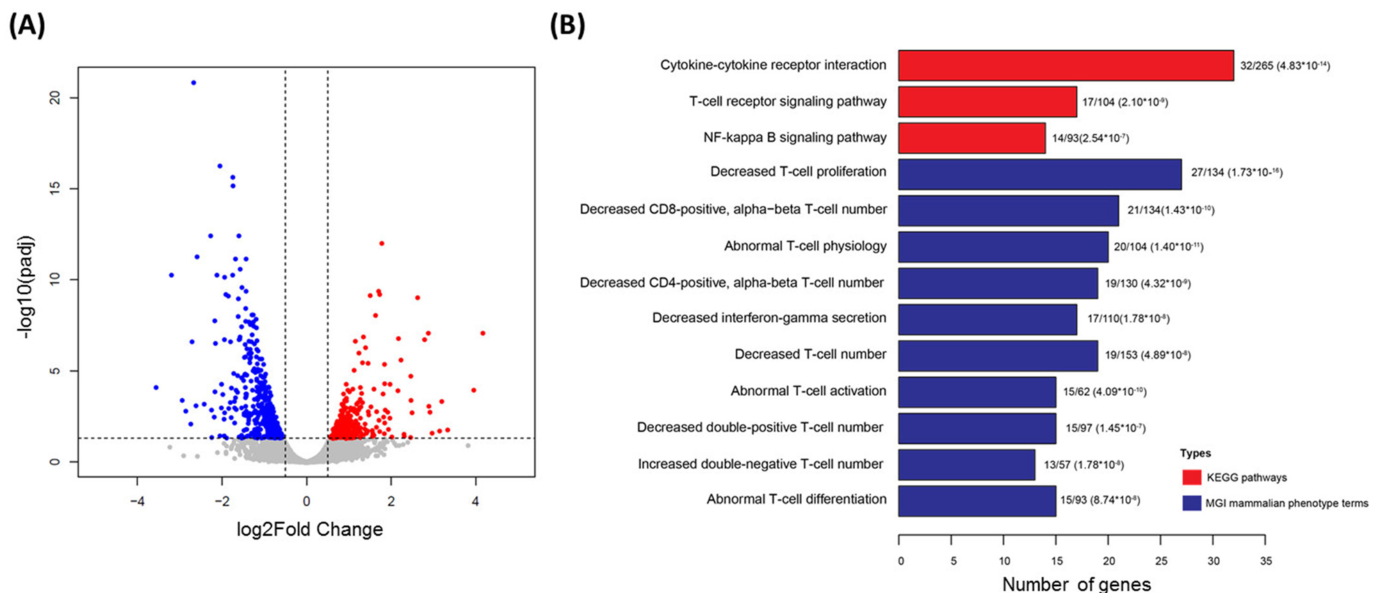

Fig. 5. Differentially expressed genes of CD4$^+$ T cells stimulated in the presence of DMSO. (A) Volcano plot of RNA-seq data comparing CD4$^+$ T cell stimulated with plate bound anti-CD3 in the presence and absence of 1% DMSO. Blue dots represents downregulated genes, red dots are upregulated genes ($P < 0.05$). Genes that are either not significant or show < 1–5 fold changes are indicated in gray. The horizontal broken line represents a *p* value of 0.05 and the vertical broken lines represent a 1.5-fold change. (B) Results of enrichment pathway analysis; The KEGG pathway enrichment analysis identified three pathways which are downregulated by DMSO. The Mammalian phenotype terms are indicated as blue bars. The p value and the number of differently expressed genes are listed for each pathway. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

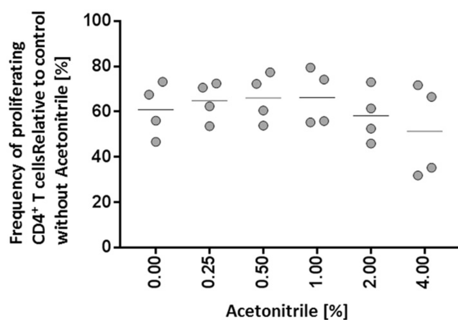

Fig. 6. Acetonitrile does not affect T cell proliferation at low concentrations. $CD4^+$ T cells labelled with eFluor450 were stimulated with plate-bound anti-CD3 antibody for 72 h. T cells were cultured in the presence of increasing Acetonitrile concentrations. The quantitative data from 4 independent experiments using different donors, each with technical duplicates are shown and expressed as the relative frequency of dye-diluted cells as compared to the value at 0% Acetontrile. * $P \leq 0.05$; ** $P \leq 0.01$; **** $P \leq 0.0001$; two-tailed one-way ANOVA.

which showed no effect on $CD4^+$ T cell activation at concentrations up to 2%. We did not test the effects of DMSO in T cell response assays in which antigen presenting cells are loaded with peptide and washed prior to presentation to T cells. However, we expect that $CD4^+$ T cell responses such assays are less likely to be affected by DMSO than in methods where T cells are directly exposed to DMSO.

Authors' contribution

LH, DL and AG acquired the data. LH and VS analyzed the data. LH and EB wrote the manuscript and LH, EB, MJ and AGZ were responsible for the experimental design. EB and AGZ designed the study. All authors received the final version of the manuscript. EB is responsible for the integrity of the work as whole.

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Conflict of interest

The authors declare that they have no competing interest.

Ethical standard

The Bioressource study was approved by the local Institutional Review Board (No. 5049/11) and performed in accordance with ethical standards.

Informed consent

Written informed consent was obtained prior to study inclusion.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2018.09.004.

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