Higher Antioxidant Thiol Levels Contribute to Increased Persistence and Improved Anti-Tumor T Cell Function

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Supplementary Material and Methods

SUPPLEMENTRY METHODS

***Fluorescence microscopy*.** Human PBMCs were stained with MitoTracker Red FM (500 nM; Life Technologies, Grand Island, NY), following which cells were concentrated on the microscopic slides using a cytospin centrifuge. A coverslip was placed over the cells in mounting media containing DAPI. Five different fields were imaged in both red and DAPI channels for each sample using the Olympus BX61 (Olympus, Center Valley, PA) fluorescent microscope. ImageJ software (National Institutes of Health) was used to analyze the images.

GSH/GSSG determination. Total GSH and GSSG ratio were measured in triplicate in sorted populations using the fluorescence-based glutathione assay kit, as per the manufacturer's protocol (BioVision, Milpitas, CA).

Measurement of oxidative phosphorylation and glycolytic flux. Mitochondrial oxygen consumption or glycolytic flux was measured using the XF 24 analyzer (Seahorse Bioscience, MA). The human PBMCs or TIL1383i TCR^+ were used for assay. Cells were either kept 3 days in the presence of rapamycin or without any treatment. One group of cells was treated with L-NAC (5 mM) for 30 minutes. The cells were suspended in XF media (with 1 mM sodium pyruvate and 25 mM glucose, 2mM L-glutamine) and immobilized on a poly-D lysine-coated (Sigma-Aldrich Co., MO) XF24 cell culture plate before measurement of the OCR. A similar experiment was setup for analysis of ECAR using glucose-free XF media. Each experiment was repeated thrice and each treatment group was run in multiple wells.

Activation induced T cell death. PBMCs were stimulated using plate-bound anti-CD3 (10 μg/mL) antibody (clone: OKT3). Cells were later re-stimulated for 4 h with or without different apoptosis-inducing stimuli, including anti-CD3 (for TCR engagement; 10 μg/mL), lectin phytohemagglutinin (PHA; 1 μg/mL), H_2O_2 (50 μM), and staurosporine (1 μM). When using TCR transduced cells (TIL1383i TCR) cells were pulsed with human tyrosinase ($hTyr_{368-376}$) peptide (YMDGTMSQV) loaded on T2 cells. Peptide was purchased from Genzyme Corporation (Cambridge, MA). Where indicated, PBMCs/TCR transduced cells were cultured in the presence or absence of rapamycin (250 nM) for 3 days or treated with L-NAC (5mM) for 30 minutes. Apoptosis was measured by staining for Annexin-V and 7-AAD according to the manufacturer's protocol, followed by flow cytometry.

Measurement of mitochondrial membrane potential. Mitochondrial membrane potential was detected by staining with 20nM dihexyloxacarbocyanine iodide ($DiOC_6$; Molecular Probes, Eugene, OR) for 15 min at 37ºC, as described previously (1).

Assessment of intracellular reactive oxygen species (ROS) and reactive nitrogen species (RNS). Dihydroethydium (DHE; Sigma, St. Louis, MO) was used for the measurement of ROS production. Cells were incubated with 1 μM of DHE for 37ºC for 10 min (2). Intracellular nitric oxide was measured by staining with 1 μm 4-amino-5-methylamino-2',7'-difluoroflourescein diacetate (DAF) (Invitrogen) for 10 min at 37ºC (3).

***Glucose consumption assay*.** Cells were stained with fluorescently-labeled deoxyglucose analog, 2NBDG (Cayman Chemicals, Ann Arbor, MI). Staining was done according to manufacturer's protocol. Cells were washed and stained with other fluorochrome-labeled antibodies and acquired by flow cytometry. All analysis was done on viable cells.

T cell proliferation by CFSE dilution. CFSE, dissolved in DMSO, was added to T cells at a final concentration of 1 μM. Cells were incubated with CFSE at 37°C for 7 min. Incorporation of CFSE into cell membranes was stopped by adding 5 volumes of ice-cold medium with FBS (7).

***Real-time PCR*:** The relative expression of genes involved in oxidative stress was determined in each of the four RNA samples, from different donors, with or without treatment by rapamycin for 5 days. A RT^2 Profiler^{TM} Human Oxidative Stress and Anti-oxidant Defense PCR Array (Cat. No. PAHS-065, SA Biosciences Corp, Frederick, MD) that profiles the expression of 84 genes related to oxidative stress was used for the same. A relative change in the expression of each gene ≥ 2-fold in rapamycin-treated cultures, compared to the control cultures from 2 different donors, was considered to be significant. Real time for individual genes was done using Sso advance SYBR green (Bio-rad Hercules, CA). Primers sequences for individual genes have been given in Supplemental Table 1.

***Western blot*.** Cells were lysed using radioimmunoprecipitation assay (RIPA) buffer with freshly added protease inhibitors, following which samples were subjected to SDS-PAGE using 4-12% NuPAGE gels and Western blot (Millipore, Burlington, MA). Dilutions and sources of primary antibodies were as follows: anti-TRX-1 (1:1000; Cell Signaling Technologies, Danvers, MA), anti-TRX-2 (1:1000; Abcam, Cambridge, MA), and anti-GAPDH (1:6000; Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti- HIF1a (1:1000; Millipore, Burlington, MA) and anti-β-actin (1:5000; Millipore, Burlington, MA) HRP-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology, Inc.

Image stream analysis. Human $CD8^+$ TCR transduced cells (TIL1383i TCR) cells were stained with CD62L and Mito-Tracker Red FM (100 nM; Life Technologies, Grand Island, NY). Experiment was reproduced using human $CD8^+$ TCR transduced cells (TIL1383i TCR) cells were stained with CD62L and Mito-Tracker green (50 nM; Life Technologies, Grand Island, NY). In both the experiments were repeated 2-3 times. Cells were acquired on Image stream **(**Amnis Corporation, Seattle, WA). Data from image stream was analyzed on IDEAS v5.0 (Amnis Corporation, Seattle, WA). Data for was analyzed by gating on either $CD62L^{hi}$ ^{or} $CD62L^{lo}$ cells and then comparing Mito-Tracker intensity.

References

1. Banki K, Hutter E, Gonchoroff NJ, Perl A. Elevation of mitochondrial transmembrane potential and reactive oxygen intermediate levels are early events and occur independently from activation of caspases in Fas signaling. J Immunol. 1999;162:1466-79.
2. Gulow K, Kaminski M, Darvas K, Suss D, Li-Weber M, Krammer PH. HIV-1 trans-activator of transcription substitutes for oxidative signaling in activation-induced T cell death. J Immunol. 2005;174:5249-60.
3. Koncz A, Pasztoi M, Mazan M, Fazakas F, Buzas E, Falus A, et al. Nitric oxide mediates T cell cytokine production and signal transduction in histidine decarboxylase knockout mice. J Immunol. 2007;179:6613-9.
4. Franco R, Panayiotidis MI, Cidlowski JA. Glutathione depletion is necessary for apoptosis in lymphoid cells independent of reactive oxygen species formation. J Biol Chem. 2007;282:30452-65.
5. Sebastia J, Cristofol R, Martin M, Rodriguez-Farre E, Sanfeliu C. Evaluation of fluorescent dyes for measuring intracellular glutathione content in primary cultures of human neurons and neuroblastoma SH-SY5Y. Cytometry A. 2003;51:16-25.
6. Gelderman KA, Hultqvist M, Holmberg J, Olofsson P, Holmdahl R. T cell surface redox levels determine T cell reactivity and arthritis susceptibility. Proceedings of the National Academy of Sciences of the United States of America. 2006;103:12831-6.
7. Lyons AB. Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution. J Immunol Methods. 2000;243:147-54.

Supplemental Table 1: PCR primers sequences

Accession No.	Symbol	Forward	Reverse
Human Primers			
NM_001191	BCL-XL	5'-CTTGGATGGCCACTTACCTGA-3'	5'-TCTTCTGGTCATTTCCGACTGAAG-3'
NM_001752	Catalase	5'-CTATCCTGACACTCACCGCC-3'	5'-GGCTTCTCAGCATTGTACTTGTC-3'
NM_001256799	GAPDH	5'-GAAGATGGTGATGGGATTTC-3'	5'-GAAGGTGAAGGTCGGAGTC-3'
NM_006516	GLUT-1	5'-GGCATTGATGACTCCAGTGTT-3'	5'-ATGGAGCCCAGCAGCAA-3'
NM_001145412	NRF-2	5'-GCCGCTTGGAGGCTCATCTCA-3'	5'-GCAATTCTGAGCAGCCACTTTATTCT-3'
NM_000454	SOD-1	5'-AGGGCATCAATTTCGAGC-3'	5'-GCCCACCGTGTTTTCTGGA-3'
NM_000636	SOD-2	5'-CTGCTGGGGATTGATGTGTGG-3'	5'-TGCAAGCCATGTATCTTTCAGT-3'
NM_012473	TRX-2	5'-CTGGTGGCCTGACTGTAACAC-3'	5'-GTTGACCACTCGGTCTTGAAA-3'
NM_002610	PDK-I	5'-CGGATCAGAAACCGACACA-3'	5'-GGATCAGAAACCGACACA-3'
NM_001199898	PDK-II	5'-CATGATGTCCAGGAGGCTCT-3'	5'-AAAGAGATCAACCTGCTTCCC-3'
NM_000188	HK-II	5'-TGGAGGGACCAACTTCCGTGTGCT-3'	5'-TCAAACAGCTGCGTGCCACTGC-3'
NM_002654	PKM-I	5'-GTCTGAATGAAGGCAGTCCC-3'	5'-TCCGGATCTCTTCGTCTTTG-3'
NM_182470	PFK-II	5'-TGTCGCTTATGGCTGCCGTGT-3'	5'-AGCGGGGTGACACTATTGCGT-3'
NM_181054	HIF-1α	5'-CATAAAGCTTGCAACATGGAAGGT-3'	5'-.ATTTGATGGGTGAGGAATGGGTT-3'
NC_012920.1	12S rRNA	5'-CCTCCCCAATAAAGCTAAAA-3'	5'-GCTATTGTGTGTTCAGATAT-3'
NM_032609	COX II	5'-TTAATTCTAGGACGATTGGGC-3'	5'-CTGAACCTACGAGTACACCG-3'
NM_005015	CYT B	5'-GGGGGTTGTTTGATCCCGTTT-3'	5'-GGGGCCACAGTAATTACAAA-3'
NM_020142	ND4	5'-AAGTCATCAAAAGCTATTA-3'	5'-CTTACATCCTCATTACTATTC-3'
NM_013261	PGC1α	5'-AAAGGATGCGCTCTCGTTCA-3'	5'-GGAATATGGTGATCGGGAACA-3'
NM_003201	TFAM	5'-AATGGATAGGCACAGGAAACC-3'	5'-CAAGTATTATGCTGGCAGAAGTC-3'
NM_001101	β-Actin	5'-CTGGAACGGTGAAGGTGACA-3'	5'-AAGGAACTTCCTTGAACAATGCA-3'
Mouse primers			
NM_013820	HK-II	5'-GGAACCGCCTAGAAATCTCC-3'	5'-GGAGCTCAACCAAAACCAAG-3'
NM_010431	HIF-1α	5'-ACGTTCTGTTATGAGGCTCACC-3'	5'-TGACTTGATGTTCATCGTCCTC-3'
NM_021514	PFK-II	5'-AGCATTCATACCTTGGGCAT-3'	5'-CCATGAAGAGCATCATGCAG-3'
NM_011660	TRX-1	5'-CGTGGTGGACTTCTCTGCTACGTGGT-3'	5'-GGTCGGCATGCATTTGACTTCACAGT-3'
NM_011434	SOD-1	5'-TGGGGACAATACACAAGGCTGT-3'	5'-TTTCCACCTTTGCCCAAGTCA-3'
NM_144548	IL-23R	5'-TTCAGATGGGCATGAATGTTTCT-3'	5'-CCAAATCCGACGTGTTGTTCTAT-3'
NM_013542	GzmB	5'-GCCCACAACATCAAAGAACAG-3'	5'-AACCAGCCACATAGCACACAT-3'
NM_010556	IL-3	5'-GGGATACCCACCGTTTAACCA-3'	5'-AGGTTTACTCTCCGAAAGCTCTT-3'
NM_001159562	IL-1rn	5'-GCTTGCTGGGTACTTACAA-3'	5'-CCAGACTTGGCACAAGACAGG-3'
NM_007393	β-Actin	5'-ACGTAGCCATCCAGGCTGGTG-3'	5'-TGGCGTGAGGGAGAGCAT-3'