Phosphoenolpyruvate is a metabolic checkpoint of anti-tumor T cell responses

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EXTENDED EXPERIMENTAL PROCEDURES

$CD4^+$ T cell reconstitution in Rag1-KO mice.

For measuring tumor growth rates of control Braf/Pten and HK2-OE Braf/Pten melanoma cells *in vivo*, Rag1-KO mouse was reconstituted with $4x10^6$ $CD4^+$ T cells. Two days later, $2x10^5$ control or HK2-OE Braf/Pten melanoma cells were suspended in 50μl of PBS and then injected subcutaneously into left and right flanks of reconstituted Rag1-KO mice, respectively. Tumors were isolated and analyzed two weeks post engraftment.

Tumor Digestion and Tumor Interstitial Fluid Isolation

Braf/Pten and B16 melanomas were minced in HBSS containing collagenase IV (Sigma-Aldrich, St. Louis, MO) and DNase (Sigma-Aldrich, St. Louis, MO) and digested at 37°C for 1h. The digested materials were then filtered and single cell suspensions were incubated with ACK lysis buffer (Invitrogen, Grand Island, NY) for 2 min to lyse red blood cells. The cells were then re-suspended in complete RPMI for subsequent analyses. Isolation of tumor interstitial fluid was performed as described (Haslene-Hox et al., 2011) and the glucose levels were determined using glucose meters (Accu-Chek).

Intracellular Cytokine Staining, Phosphostaining and Flow Cytometry

For surface staining, cells were stained with antibodies in FACS buffer (PBS with 1% FBS and sodium azide). For intracellular cytokine staining, T cells were stimulated with plate-bound anti-CD3 antibody and soluble anti-CD28 antibody in the presence of Brefeldin A (eBiosciences, San Diego, CA). After fixation and permeabilization, cells were stained for intracellular cytokines. For phosphostaining, cells were stimulated by plate-bound anti-CD3 antibody and soluble anti-CD28 antibody. Cells were fixed in 4% paraformaldehyde and then incubated in cold methanol for 30 mins. After washing, cells were stained with antibodies for indicated phosphoproteins (Cell Signaling) and antibodies for surface staining. Antibodies for surface staining and intracellular cytokine staining were purchased from BD Biosciences (San Jose, CA) and eBiosciences (San Diego, CA). All experiments were conducted on a BD LSRII flow cytometer and analyzed with FlowJo software.

Cell Culture, 2-NBDG Uptake and Co-culture of Cancer Cells with T_H1 Cells

293T cells and B16 melanoma cells were maintained in DMEM media with 10% FBS, 1% glutamine and 1% penicillin-streptomycin. Braf/Pten melanoma cell lines was established by Dr. Marcus Bosenberg and maintained in DMEM media with 10% FBS, 1% glutamine and 1% penicillin-streptomycin. The HK2-overexpressing Braf/Pten melanoma cell line (HK2-OE) was established by stably transfecting Braf/Pten cells with HK2 overexpressing plasmid and selecting with puromycin for two weeks. For the 2-NBDG uptake assay, a single cell suspension of spleens or tumors was incubated with 100nM 2-NBDG in glucose-free media for 2 min at 37°C. The uptake of 2-NBDG in activated $CD4^+$ T cells was analyzed by flow cytometry. For co-culture experiments, cancer cells were plated and cultured overnight. Equal

numbers of T_H1 cells isolated from splenocytes of LCMV-infected mice were used. 2-NBDG uptake in co-culture experiments was performed as described above. For cytokine production, T_H1 cells were stimulated and analyzed as mentioned above.

Retrovirus Production, Concentration, and Transduction

293T cells were transfected with Eco-helper plasmid and either control or PCK1-overexpression MSCV vectors. Cell culture supernatant containing retrovirus was collected at 48 and 72 hours post-transfection. Retrovirus-containing supernatant was concentrated with a Retro-X concentrator (Clontech, Mountain View, CA). Purified $CD4^+$ T cells were activated by anti-CD3/anti-CD28 in the presence of 10 ng/ml IL-2 for 24h. These activated $CD4^+$ T cells were then spin-transduced with concentrated retrovirus at 2,000xg for 60 minutes at 30 °C.

Enzyme-Linked Immunosorbent Assay and Phosphoenolpyruvate Fluorometric Assay

IFNγ was purchased from eBioscience (San Diego, CA) and used for ELISA according to the manufacturer's instructions. For TGFβ ELISA (eBioscience, San Diego, CA), T_H1 cells were culture in serum-free RPMI media with the indicated concentration of glucose for 5h or in X-vivo 15 media (Lonza, Basel, Switzerland) with the indicated glycolytic inhibitors for 18h. The supernatants were analyzed by ELISA according to manufacturer's instructions. The phosphoenolpyruvate fluorometric assay kit was purchased from Cayman Chemical (Ann Arbor, MI) and the assay was performed according to manufacturer's instructions.

RNA purification, quantitative real-time RT-PCR, RNA-sequencing and bioinformatic analysis

Total RNA was extracted with Trizol (Invitrogen) and RNeasy Mini Kit (Qiagen, Valencia, CA). Purified DNA-free RNA was subjected to cDNA synthesis and those cDNAs were analyzed by quantitative real-time RT-PCR as described before (Ho et al., 2014). Purified DNA-free RNA was also subjected to mRNA isolation and library construction. Libraries were sequenced on an Illumina HISEQ 2500 (Illumina, San Diego, CA). The total RNA count-based differential expression protocol was adapted for this analysis. Mappable data were counted using HTSeq, and imported into R for differential expression analysis using the DESeq2 package. A minimum expression filter (mean of counts per gene > 10) was applied prior to further analysis. Significantly differentially expressed genes for each pairwise comparison between conditions was defined as a 1.5 fold change with an FDR (Benjamini Hochberg) ≤ 0.05. The RNA-seq data can be download with the accession number SRA SRP058700.

Glycolytic Metabolites Analysis by Liquid Chromatography Q-exactive Mass Spectrometry (LC-QE-MS)

Splenocytes were pooled from twelve C57/B6 mice (Jackson Laboratory) and $CD4^+$ T cells were isolated by magnetic bead negative selection (Miltenyi Biotec, San Diego, CA). Isolated cells were pre-incubated with 50mM 2-deoxyglucose, 100mM iodoacetate, 1mM oxalate, or vehicle for 10min, and then

stimulated for 1h or 5h on plates coated with 1μg/ml anti-CD3 and 1μg/ml anti-CD28. For each condition and time-point triplicate pellets of $4x10^6$ cells were lysed in 80% methanol on dry ice. Lysates were cleared of cell debris and then dried to completion by SpeedVac (Thermo Scientific, Waltham, MA). The dried samples were processed and analyzed by LC-MS as described previously (Liu et al., 2014).

Adoptive T Cell Transfer

B16 melanoma cells ($2x10^5$) were engrafted subcutaneously into male C57BL/6 mice. Trp-1 $CD4^+$ or Pmel $CD8^+$ T cells transduced with control or PCK1-containing retrovirus. These transduced Trp-1 $CD4^+$ T cells ($2x10^6$) or Pmel $CD8^+$ T cells ($1x10^6$) were adoptively transferred into tumor-bearing mice through intravenous injection on day 7 and day 14 post-tumor engraftment. Tumor size was measured every two days after transfer and calculated as length x width of the tumor. Mice with tumors bigger than $400mm^2$ were euthanized. For functional analysis of transferred Trp-1 cells, B16 tumor-bearing mice were adoptively transferred with Trp-1 cells ($2x10^6$). 3 days later, single cell suspension of tumors, draining lymph nodes, and spleens were prepared and the function of transferred Trp-1 cells and the maturation markers of tumor-associated macrophages were determined by FACS analysis.

Calcium Flux Assay

$CD4^+$ T cells were loaded with Fluo-4AM (1μM) and Fura-Red (1μM) (Molecular Probe, Grand Island, NY) in the presence of antibodies that recognize CD4, CD44 or PSGL1 at 37^oC for 15 min. After washing with PBS, cells were re-suspended in culture media in the presence of the indicated glycolytic inhibitors. Calcium flux was determined as the ratio between Fura-4AM and Fura-Red fluorescence intensity. For measuring calcium efflux from the ER, cells were re-suspended in EGTA-containing (2mM) culture media to chelate extracellular calcium.

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