cancers

Acute conditioning of antigen-expanded CD8+ T cells via the GSK3β-mTORC axis differentially dictates their immediate and distal responses after antigen rechallenge

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Supplementary Materials

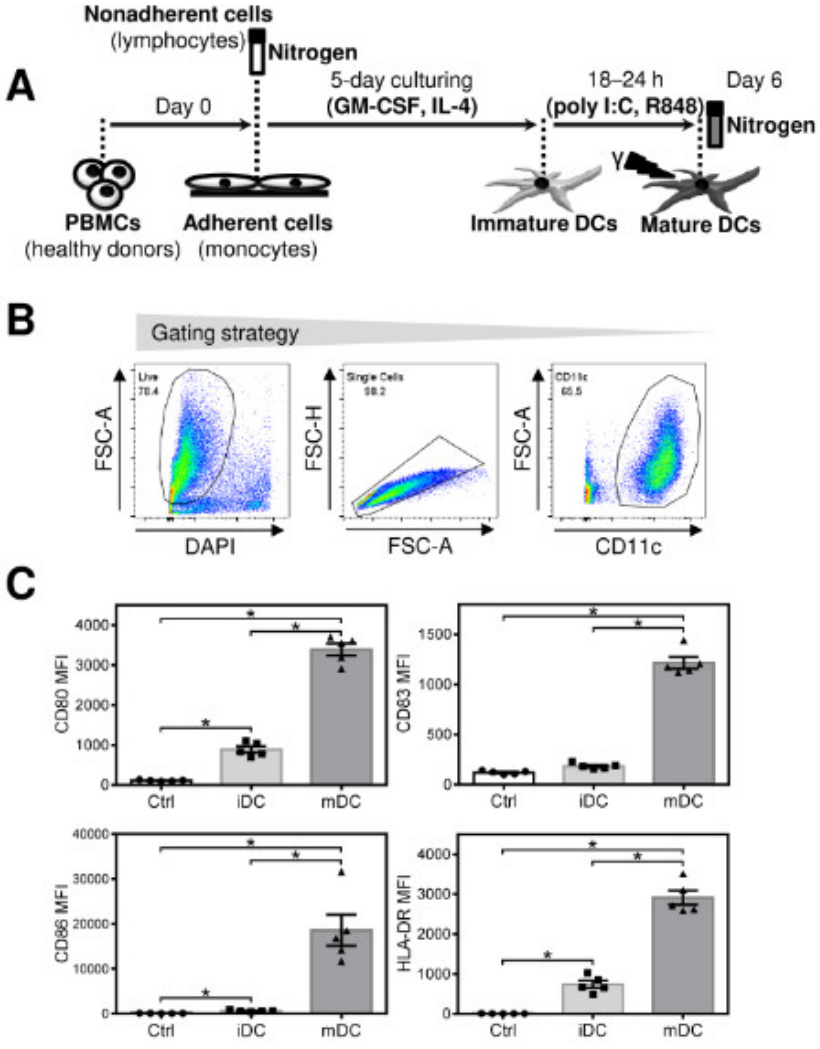

Figure 1. Preparation of mature monocyte-derived DCs. PBMCs from buffy coats were isolated and fractioned into adherent and nonadherent cells. The nonadherent fraction (lymphocytes) was cryopreserved and the adherent fraction (monocytes) was cultured for 4 days in the presence of GM-CSF and IL-4 (1,000 IU/ml). The cells were supplemented with fresh GM-CSF and IL-4 and cultured for 1 day. The cells were transferred to fresh medium with cytokines, and the concentration was

adjusted to 1 × 10^6 cells/ml. The culture was supplemented with poly I:C (25 μg/ml) and R848 (10 μg/ml), and the cells were allowed to mature for 18–24 h. The cells were harvested and γ-irradiated [32 Gy], and their maturation was determined by flow cytometry through the surface expression of DC maturation markers. (**A**) Schematic of DC preparation. (**B**) The gating strategy used to analyze flow cytometric data. (**C**) Intensities of the maturation marker staining (MFIs) of DCs ($CD11c^+$ cells) with no marker antibody used (Ctrl), cells not matured (iDC) or matured (mDC) with poly I:C/R848. In **C**, bars represent the mean of values determined in each group, and the significance of differences among the tested groups is indicated (*$P < 0.05$, $n = 5$ donors, 1-way ANOVA with the Tukey post-test).

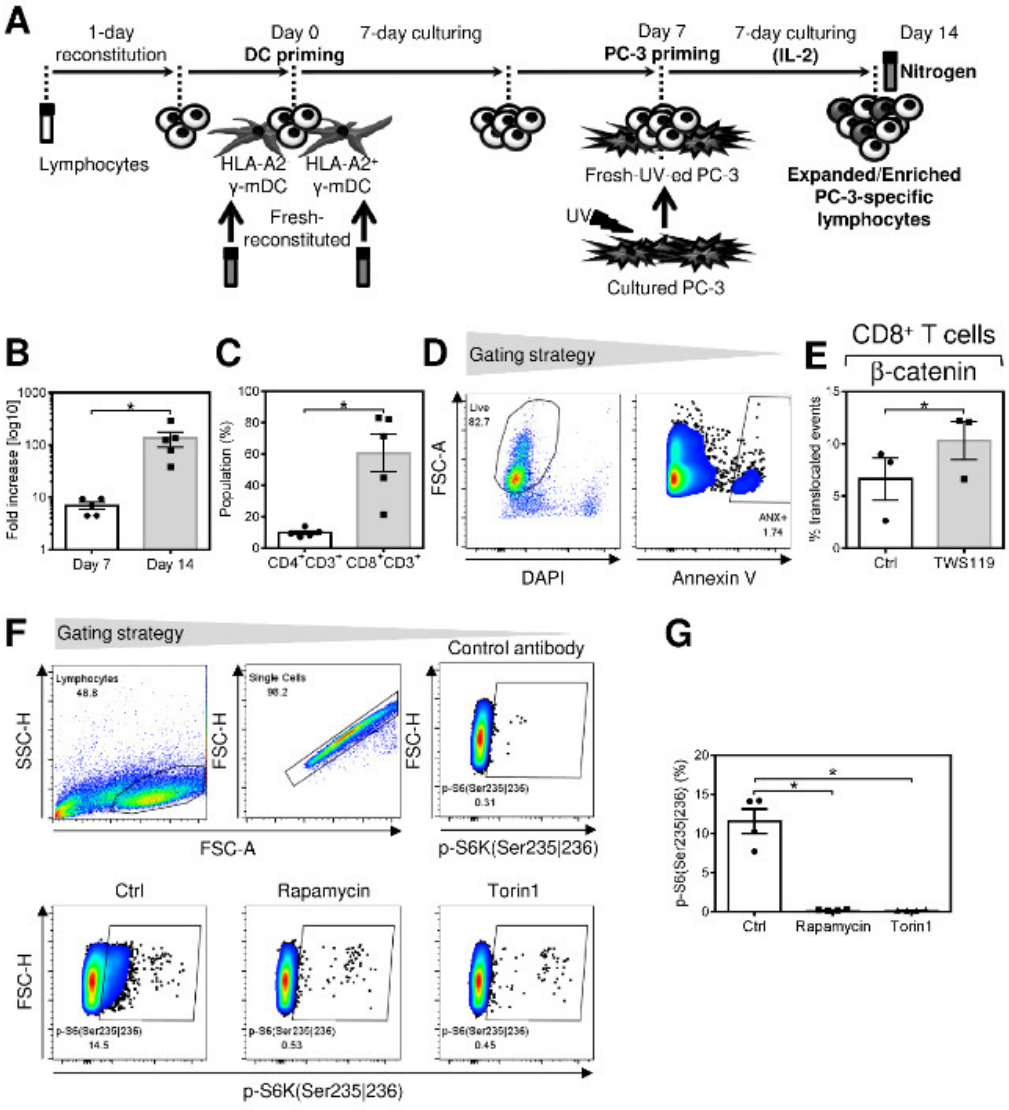

Figure 2. Enrichment and expansion of antigen-specific $CD8^+$ T cells, annexin V staining, β-catenin translocation, and phospho flow. (**A**) Schematic of enrichment and expansion of antigen-specific $CD8^+$ T cells. The cryopreserved buffy coat-isolated lymphocytes were reconstituted and cultured overnight. The cells were pelleted and resuspended in medium and cocultured in flat-bottom 48-well plates with a 1:1 ratio mix of γ-irradiated DCs from two HLA-A2-incompatible donors (5:1 ratio of lymphocytes and DCs) for 7 days. The DC-primed lymphocytes were then added to adherent UV-irradiated PC-3 cells in flat-bottom 48-well plates and cultured in the media with IL-2 for 7 days. On day 14 of culture, the DC/PC-3/IL2-expanded lymphocytes were cryopreserved. (**A**) Schematic of the enrichment and expansion of $CD8^+$ T cells. (**B**) Fold increase of the 14-day re-expanded lymphocytes on day 7 and day 14 of culture. (**C**) The proportions of $CD4^+$ and $CD8^+$ populations of T cells in the

14-day re-expanded culture. (**D**) The gating strategy used to analyze flow cytometry data of DAPI and annexin V stained cells. (**E**) Impact of TWS119 on β-catenin nuclear localization in the 14-day-expanded lymphocytes. The 14-day-expanded lymphocytes were reconstituted and cultured overnight (18–24 h) with IL-2 (80 IU/ml). The cells were pelleted, resuspended in IL-2-containing medium (80 IU/ml) supplemented with TWS119 (7 μM), and cultured for 18–24 h. The β-catenin nuclear localization in $CD8^+$ T cells ($CD8^+CD3^+$ cells) was determined by an imaging flow cytometer (ImageStreamX MKII) as the proportion of cells with β-catenin translocated to the nucleus. (**F**–**G**) Impact of rapamycin and Torin 1 on Ser235|236 phosphorylation of ribosomal protein S6 in the 14-day-expanded lymphocytes. The 14-day-expanded lymphocytes were reconstituted and cultured overnight (18–24 h) with IL-2 (80 IU/ml). The cells were pelleted, resuspended in IL-2-containing medium (80 IU/ml) supplemented with 100 nM rapamycin or 100 nM Torin1, and cultured for 3 h. The proportion of the phospho-Ser235|236 ribosomal protein $S6^+$ lymphocytes (p-S6K(Ser235|236) was determined by flow cytometry. (**F**) The gating strategy used to analyze flow cytometry data of phospho-Ser235|236 ribosomal protein S6-stained lymphocytes. A fluorescent-labeled IgG antibody was used as a negative control. (**G**) The evaluated proportions of the phospho-Ser235|236 ribosomal protein $S6^+$ lymphocytes (p-S6(Ser235|236)). In **B**–**C**, **E,** and **G**, bars represent the mean of values determined in each group. In **B**–**C**, and **E**, the significance of differences between the groups is indicated (*$P < 0.05$, **B**–**C**: $n = 5$ donors, **E**: $n = 3$ donors, paired 2-tailed Student's t test). In **G**, the significance of differences among the groups is indicated (*$P < 0.05$, $n = 4$ donors, 1-way ANOVA with the Tukey post-test).