Targeting immunosuppressive classical monocytes prevents immunotherapy resistance

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Introduction

Immune checkpoint inhibitors have revolutionized the treatment of lung cancer. However, most patients fail to respond or acquire resistance over time. Understanding resistance mechanism to immune checkpoint blockade in NSCLC is critical to developing new therapeutic strategies and improving patient survival. In this study, we evaluated the immune cell infiltration in anti-PD-1+anti-CTLA-4 combination therapy resistant tumors. Previously derived murine lung cancer cells lines from primary lung lesions of the *Kras$^{LA1/+}$p53$^{R172HΔg/+}$* genetically engineered mouse model were used (3). The mesenchymal 344SQ tumors were found to have lower CD8 T-cell infiltration and an increase in exhaustion markers to compared to epithelial tumors (4). Here we found several immunosuppressive cell types including T-regs, MDSC, neutrophils, and monocytes in the IO resistant 344SQ tumors. We then assessed the role of the immune cells in contributing to resistance to combination anti-PD-1+anti-CTLA-4 therapy by *in vivo* depletion. We found that addition of anti-Ly6C to the combination of anti-PD-1+anti-CTLA-4 was capable of complete tumor eradication. We confirmed this triple combinatorial approach in multiple models and found similar results. Increased infiltration of CD115+CD14+Ly6C+ monocytes were found in the anti-PD-1+anti-CTLA-4 combination therapy resistant tumors. Two distinct monocytes populations were characterized in immunotherapy resistant tumors based upon Ly6C staining. The Ly6C- monocytes differentiate into dendritic cells whereas the Ly6C+ differentiate into M2 macrophages. Dendritic cells derived from tumor-infiltrating monocytes had high levels of antigen presentation and robust B7 signaling. Triple combination of anti-PD1+anti_CTLA+anti-LyC tumors show high levels of Ly6C- monocytes as well as monocyte-derived dendritic cells. Additional clinical data from several lung cancer patient datasets shows a strong correlation between monocytes and dendritic cells. This study suggests that immunotherapy resistance is associated with progenitor monocytes and if we can control the differentiation process of monocytes, we can have a lasting impact on the ability of the immune system to control tumor growth.

Background

Nivolumab plus ipilimumab show improvement in NSCLC patients

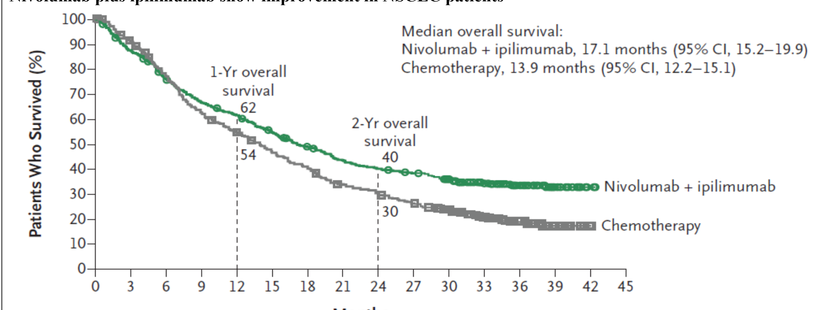


Fig 1. Overall survival in advanced non-small-cell lung cancer patients in open-label, phase 3 trial with patients treated with nivolumab plus ipilimumab or chemotherapy. First line treatment of nivolumab plus ipilimumab resulted in a longer duration of overall survival than chemotherapy independent of PD-L1 expression level. Hellmann et al. *The New England Journal of Medicine*. 2019.

Results

Dual PD-1/CTLA4 blockade leads to long term acquired resistance due to infiltrating immunosuppressive cells

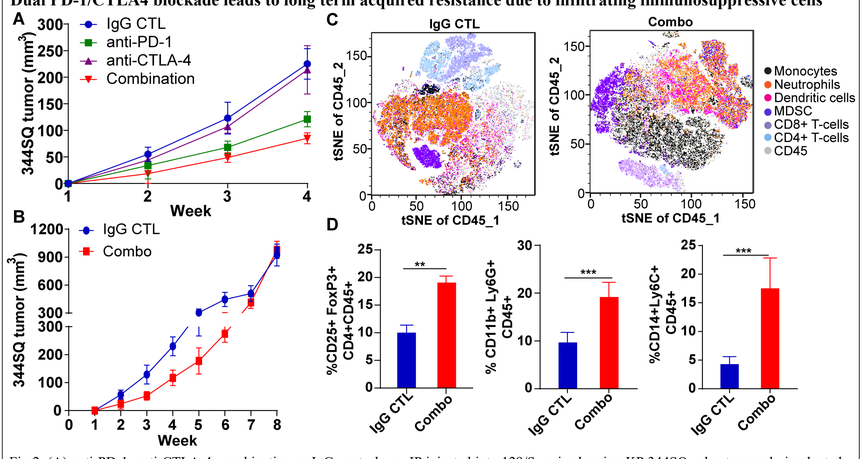


Fig 2. (A) anti-PD-1, anti-CTLA-4, combination or IgG control was IP injected into 129/Sv mice bearing KP 344SQ subcutaneously implanted tumors for 4 weeks. (B) Combination anti-PD-1_CTLA-4 or IgG control was IP injected in 129/Sv mice bearing KP 344SQ tumors for 8 weeks. (C) Flow cytometry tSNE CD45 plots from 344SQ tumors treated with combination anti-PD-1_CTLA-4 or IgG control for 8 weeks. (D) Enrichment of CD45+CD4+CD25+FoxP3+ T-regulatory cells, CD45+CD3-CD11b+Ly6G+ neutrophils, and CD45+CD3-CD14+Ly6C+ monocytes from combination therapy resistant tumors. *p* values were calculated with *t*-test, n.s., no significant difference; *p<0.05; **p< 0.01; ***p<0.001; ****p<0.0001.

Anti-PD-1_CTLA-4 treatment resistance is eradicated by addition of anti-Ly6C antibody treatment

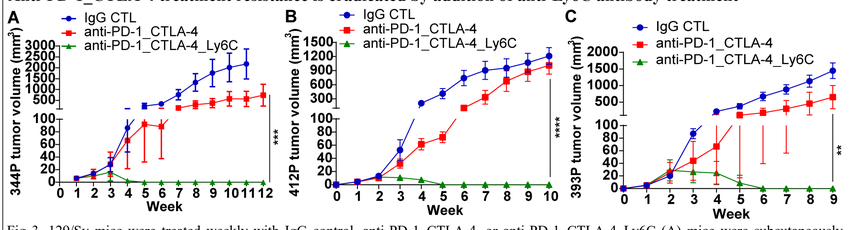


Fig 3. 129/Sv mice were treated weekly with IgG control, anti-PD-1_CTLA-4, or anti-PD-1_CTLA-4_Ly6C (A) mice were subcutaneously implanted with 344P cells, (B) 412P cell, and (C) 393P cells.

Monocyte infiltration in anti-PD-1/CTLA-4 resistant tumor are mediated by IFN-g and the CCL2/CCR2 axis

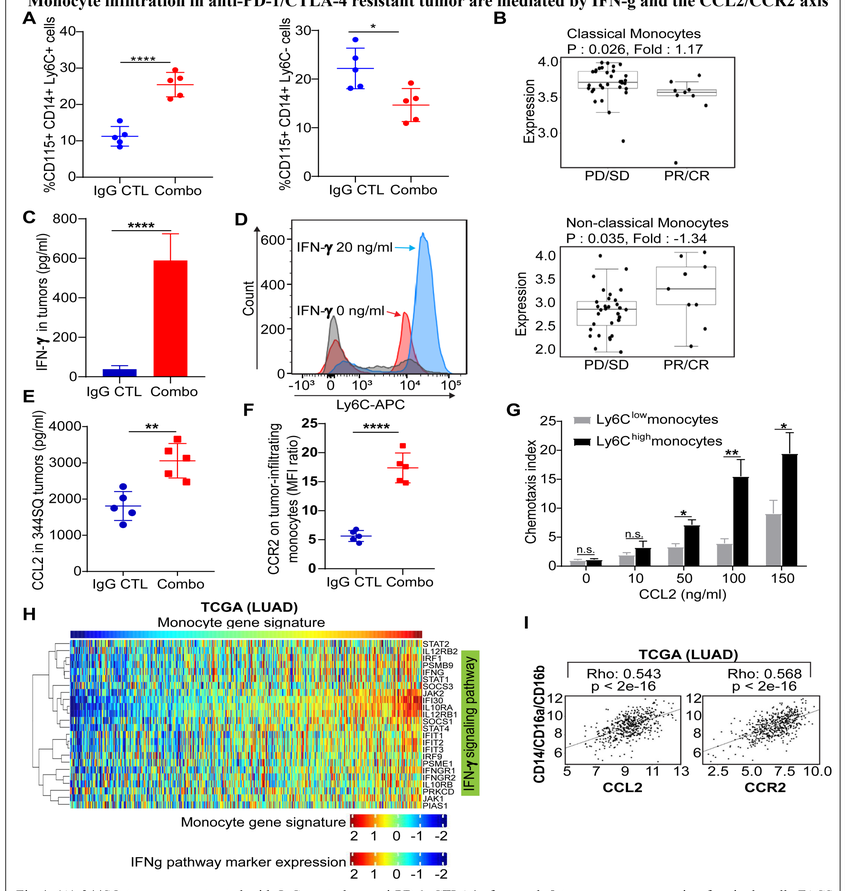


Fig 4. (A) 344SQ tumors were treated with IgG control or anti-PD-1_CTLA4 after week 8 tumors were processing for single cells FACS analysis. Percentage of CD115+CD14+Ly6C+ and CD115+CD14+Ly6C- monocytes was compared and found to be much higher in combination resistant tumors. (B) Classical monocyte and non-classical monocytes expression in melanoma patients stratified based on response of progressive disease/stable disease (PD/SD) or progressive response/complete response (PR/CR). (C) IFN-g concentration from tumors treated with IgG control or combination therapy resistant tumors. (D) Monocytes sorted from 344SQ tumors were treated with IFNg for 12 hours. Expression of Ly6C was evaluated over time.
(E) Tumors from 344SQ tumors were treated with IgG control or combination therapy over week 8. Total levels of CLL2 (F) and CCR2 expression on the monocytes were determined via FACS analysis. (G) Transwell migration assays were completed on sorted monocytes from 344SQ tumors. Sorted monocytes were added to top chamber and transmigration was determined. (H) Heat map of mRNA levels between monocyte gene signature and IFNg pathway in TCGA lung adenocarcinoma. (I) Correlation between CD14/CD16a/CD16b and CCL2 or CCR2 TCGA-lung adenocarcinoma.

Tumor-infiltrating monocytes trans-differentiation into dendritic cells via Ly6C blockade

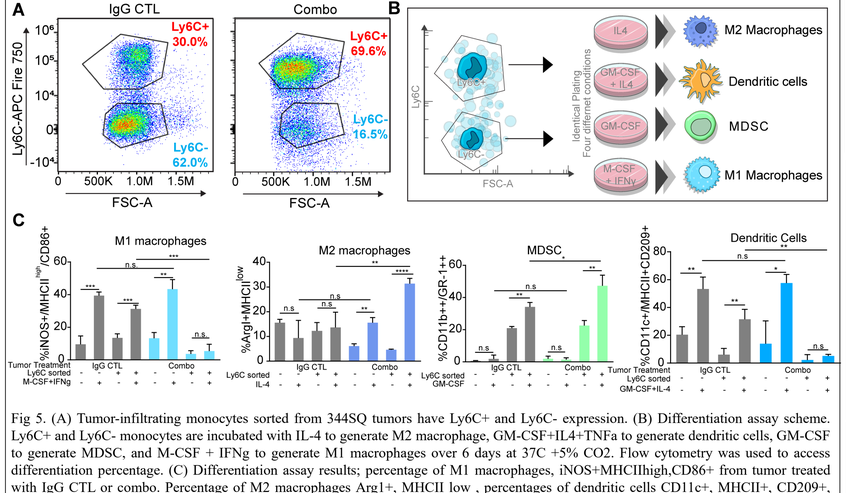


Fig 5. (A) Tumor-infiltrating monocytes sorted from 344SQ tumors have Ly6C+ and Ly6C- expression. (B) Differentiation assay scheme. Ly6C+ and Ly6C- monocytes are incubated with IL-4 to generate M2 macrophage, GM-CSF+IL4+TNFa to generate dendritic cells, GM-CSF to generate MDSC, and M-CSF + IFNg to generate M1 macrophages over 6 days at 37C +5% CO2. Flow cytometry was used to access differentiation percentage. (C) Differentiation assay results; percentage of M1 macrophages, iNOS+MHCIIhigh,CD86+ from tumor treated with IgG CTL or combo. Percentage of M2 macrophages Arg1+, MHCII low , percentages of dendritic cells CD11c+, MHCII+, CD209+, percentage of MDSC CD11b++GR1++. Monocytes were Ly6C sorted -/+.

Monocyte-derived dendritic cells enhance antitumor immunity of anti-PD-1_CTLA-4 treatment via antigen-presentation

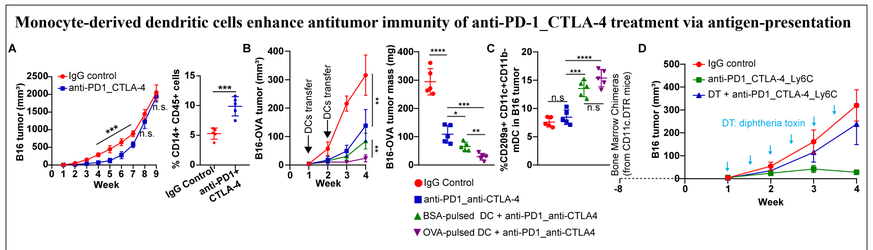


Fig 6. (A) Left:C57BL/6 mice were treated weekly with combined anti-PD-1_CTLA-4 or their IgG control beginning on day 7 after subcutaneous melanoma B16 cell injection for 8 weeks. B16 tumor growth plotted over time. (right) B16 tumors were processed for single cell FACS analysis. Percentage of CD45+CD14+ monocytes from tumors treated at week 8. (B) left; B16-OVA melanoma cells subcutaneously injected in C57BL/6 mice. Day 7 mice were treated with IgG control, anti-PD-1_CTLA-4, or BSA pulsed DC and anti-PD-1_CTLA-4 or OVA-pulsed DC and anti-PD-1_CTLA-4. Pulsed OVA or BSA dendritic cell were administered via tail via on week 1 and week2. (middle) at week 4 mice were euthanized and tumor volume weighed. (C) B16-OVA tumors were processe d for FACS analysis. Percentage of CD209a+CD11c+CD11b- mature dendritic cells shown. (D) Dendritic cells from CD11c-DTR mice were transferred to C57BL/6 to generate chimerical mice. Mice were treated with diphtheria toxin weekly starting on week1. Mice were also treated with anti-PD-1_CTLA-4_Ly6C.

Loss of Flt3/PU.1 in classical Ly6C+ monocytes in the anti-PD-1/CTLA4 resistant tumors prevents differentiation into antigen presenting cells

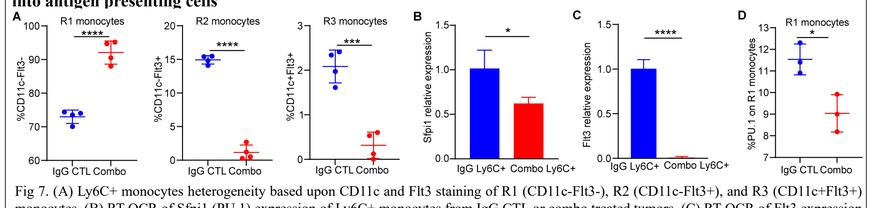


Fig 7. (A) Ly6C+ monocytes heterogeneity based upon CD11c and Flt3 staining of R1 (CD11c-Flt3-), R2 (CD11c-Flt3+), and R3 (CD11c+Flt3+) monocytes. (B) RT-QCR of Sfpi1 (PU.1) expression of Ly6C+ monocytes from IgG CTL or combo treated tumors. (C) RT-QCR of Flt3 expression of Ly6C+ monocytes from IgG CTL or combo treated tumors. (D) Percentage of PU.1 on R1 monocytes from (A) from IgG CTL or combo treated tumors.

Tumor-derived dendritic cells play a vital role in maximizing the antitumor immunity of anti-PD-1_CTLA-4 treatment

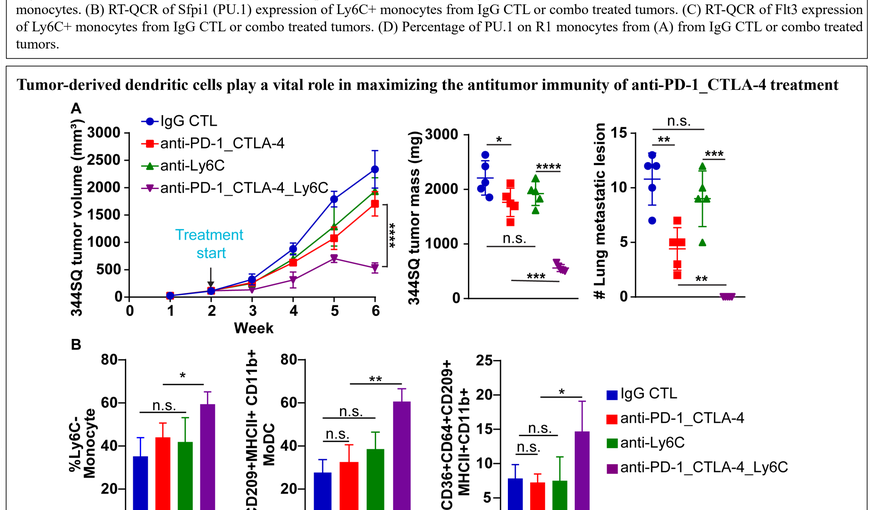
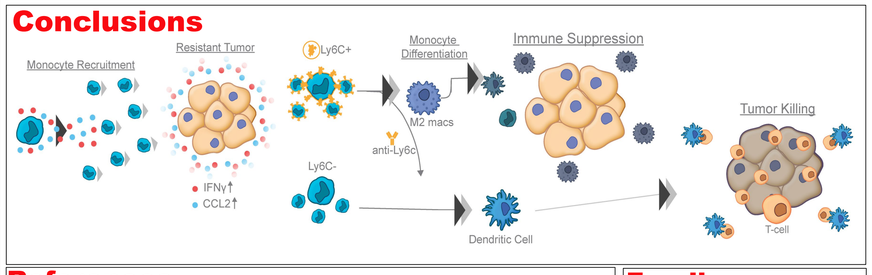


Fig 8. (A) 344SQ tumor bearing 129/Sv mice were treated weekly with IgG control, anti-PD-1_CTLA-4, anti-Ly6C, or anti-PD-1_CTLA-4_Ly6C beginning on week2 after subcutaneous cell injection for 4 weeks. At week 6 mice were euthanized and to measure tumor weight (middle) and # of lung metastatic lesion (right). Tumors from (A) were harvested to prepare single cell suspensions for FACS analysis. (B) FACS analysis reveal percentage of CD45+CD3-CD14+Ly6C- monocytes (left), percentage of CD45+CD3-CD209+MHCII+CD11b+ monocyte derived dendritic cells (middle), percentage of CD45+CD36+CD64+CD209+MHCII+CD11b+ cells (right). The results were analyzed using ANOVA test. n.s. no significant difference; *p<0.05; **p< 0.01; ***p<0.001; ****p<0.0001.

Conclusions



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