A population of ectoenzyme expressing T-cells is associated with immunotherapy resistance in metastatic melanoma patients.

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Abstract

Therapies targeting T-cell checkpoints have resulted in durable anti-tumor responses leading to FDA approval of immunotherapies for metastatic melanoma and an expanding list of other malignancies. Despite having unprecedented efficacy, PD1 and CTLA4 antagonist antibodies still fail to benefit many patients. Critically, reliable biomarkers differentiating patient response to immunotherapies are lacking. Using a unique computational approach to biomarker discovery, we recently identified and validated a novel population of peripheral blood T-cells predictive of resistance in nivolumab (αPD1) treated metastatic melanoma patients. This population is defined by the marker set CD3+CD4+CD127-GARP-CD38+CD39+. Based on the co-expression of CD38 and CD39, we have termed the population ectoenzyme expressing T-cells (T_{eee}). We have since found that increases in circulating T_{eee} were also associated with relapse in stage III/IV, resected melanoma patients treated with adjuvant combination ipilimumab (αCTLA4) and nivolumab. In patients, circulating T_{eee} frequencies positively correlated with the frequencies of immune suppressive populations (e.g. Tregs, MDSCs). Our ongoing characterization of this population showed an enhanced adenosine generating phenotype (i.e. $CD73^{high}$, $CD26^{low}$), a terminal exhaustion phenotype (i.e. TOX^{high}, $TCF1^{low}$), expression of inhibitory receptors (e.g. CTLA4, TIM3) and ligands (e.g. PDL1, B7-H4), and expression of immunosuppressive cytokines (e.g. IL-8, TGFβ). Supporting a mechanistic relationship to resistance, patient T_{eee} suppressed autologous T-cell proliferation and inflammatory function *in vitro*. Preliminary data using human tumors showed high frequencies of T_{eee} in tumor relative to the peripheral blood, with a similar phenotype. We have also demonstrated the existence of this population in several mouse tumor models. In mice, T_{eee} frequencies increased with tumor progression and negatively correlated with the overall tumor immune infiltrate. We continue to investigate the roles of this novel population of ectoenzymes expressing T-cells in ongoing experiments.

Figure 1

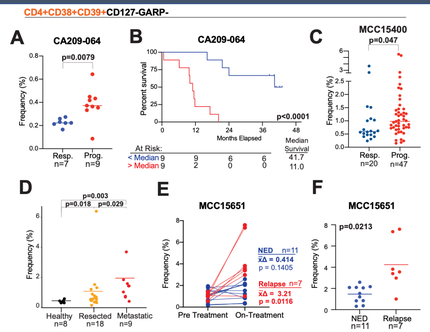


Figure 1. Increased frequencies of T_{eee} are associated with immunotherapy resistance in metastatic melanoma patients. Peripheral blood samples from metastatic melanoma patients were evaluated by flow cytometry. **(A,B)** Patients with stage III/IV active disease enrolled in clinical trial CA209-064 were treated with sequential nivolumab-ipilimumab. **(A)** The baseline frequency of T_{eee} as a percentage of the total CD3+ T-cell population were evaluated, comparing responding patients (partial or complete response) against progressing. Significance was determined by an unpaired t-test. **(B)** Patients were stratified by above or below median T_{eee} frequencies and survival curves plotted. Significance was determined by log-rank test. **(C)** Patients with stage III/IV active disease enrolled in clinical trial MCC15400 were treated with nivolumab monotherapy. The baseline frequencies of T_{eee} were evaluated, comparing responding patients against progressing. Significance was determined by an unpaired t-test. **(D)** Peripheral blood samples from demographic matched healthy donors, untreated stage III/IV resected melanoma patients and untreated stage III/IV active disease patients were evaluated by flow cytometry for the frequencies of $_{Teee}$. Significance was determined by one-way ANOVA with Tukey's post-hoc tests. **(E)** Stage III/IV, resected disease patients enrolled in clinical trial MCC15651 were treated with adjuvant combination nivolumab and ipilimumab. Paired baseline and on-treatment (week 12) frequencies of T_{eee} were evaluated. Paired t-tests were used to determine significance in patients with no evidence of disease (NED) (i.e. those that did not relapse) and relapsing patients, independently. **(F)** On-treatment frequencies of T_{eee} were compared in NED and relapsing patients. Significance was determined by an unpaired t-test.

Figure 2

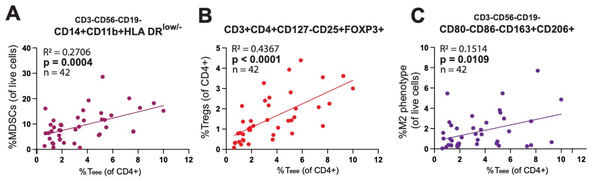


Figure 2. T_{eee} frequencies correlate with other peripheral blood immunosuppressive populations. Peripheral blood samples from metastatic melanoma patients treated with sequential nivolumab-ipilimumab or ipilimumab-nivolumab were evaluated by flow cytometry. The frequency of the T_{eee} population as percentage of the CD4+ population (x-axis) were evaluated in relation to: **(A)** myeloid derived suppressor cells (MDSCs), **(B)** Tregs, and **(C)** innate cells with an M2 phenotype (y-axis). Significance and coefficient of determination were determined by univariable linear regression.

Figure 3

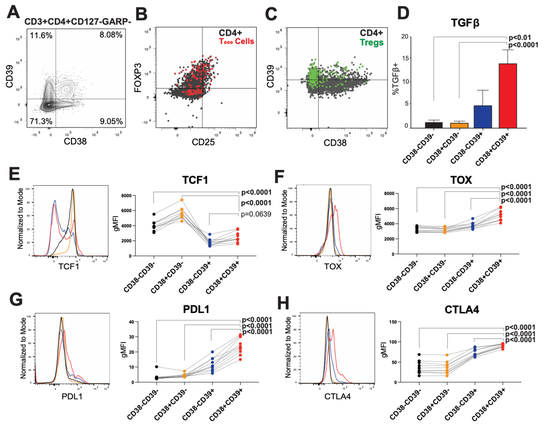


Figure 3. Phenotyping of T_{eee} in metastatic melanoma patient PBMC. Metastatic melanoma patient PBMC were evaluated by flow cytometry. **(A)** CD3+CD4+CD127-GARP- T-cells were gated and the representative graph of CD38 vs. CD39 generated. **(B)** Patient T-cells were activated via CD3/CD28 DynaBeads and the four CD38/CD39 quadrants of the CD3+CD4+CD127-GARP- population evaluated by intracellular flow cytometry for TGFβ expression. **(C)** A plot of CD25 expression (x-axis) and FOXP3 (y-axis) is shown with the total CD3+CD4+ cells shown in dark grey and the Teee cell population shown in red. **(D)** The expression of CD38 (x-axis) and CD39 (y-axis) is shown with the total CD3+CD4+ cells shown in dark grey and Tregs(CD127-CD25+FOXP3+) cells shown in red. **(E)** TCF1, **(F)** TOX, **(G)** PDL1, and **(H)** CTLA4 expression were evaluated in the four CD38/CD39 quadrants of the parent CD3+CD4+CD127-GARP- population. CD38-CD39- cells are shown in black, CD38-CD39+ in blue, CD38+CD39- in orange, and CD38+CD39+ (i.e. T_{eee}) in red. A representative histogram of expression is shown in each left panel, and analysis in 10 patient samples shown in the right panels. Significance was determined by repeated measures ANOVA with Tukey's post-hoc tests comparing all groups.

Figure 4

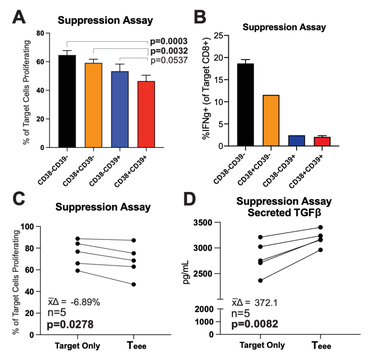


Figure 4. T_{eee} suppress autologous T-cells. (A,B) CD3+CD4+CD127-GAPR- T-cells were flow sorted into the CD38/CD39 quadrants and co-cultured with autologous, CellTrace Violet labeled CD3+ T-cells in the presence of CD3/CD28 DynaBeads for five days. The percent of **(A)** proliferating and **(B)** IFNγ expressing target T-cells were assessed by flow cytometry. **(C, D)** CellTrace Violet labeled T-cells from five patient PBMC samples were co-cultured with autologous, flow sorted T_{eee} or without ("Target Only"). Cultures were activated with CD3/CD28 Dynabeads for five days. **(C)** The percent of proliferating cells were assessed by flow cytometry. **(D)** The culture supernatants were assessed by a Luminex assay for secreted TGFβ concentrations. Significance was determined by paired t-tests.

Figure 5

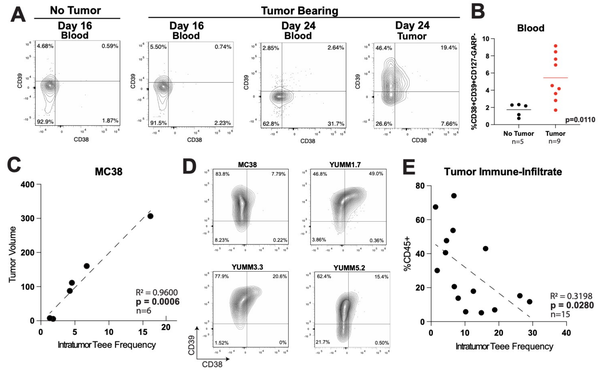
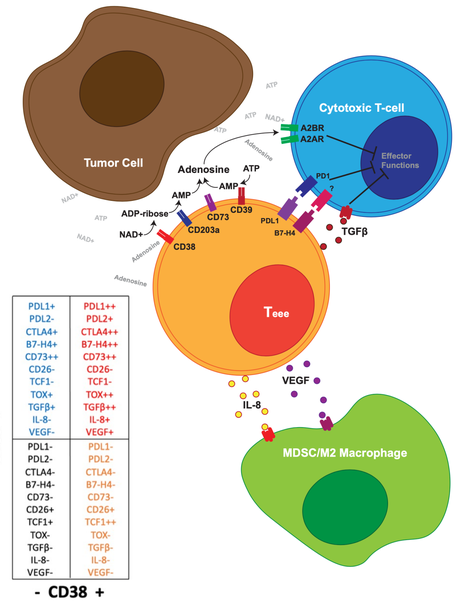


Figure 5. Frequencies of T_{eee} in murine tumors associate with increase tumor size and decreased immune infiltrate. (A,B,C) C57BL/6 mice were inoculated with the syngeneic MC38 cell line or a sham injection. Blood samples collected on days 16 and 24 and tumor samples from day 24 were evaluated by flow cytometry for the T_{eee} population. **(A)** Representative flow plots showing CD38 vs. CD39 expression in the CD45+CD3+CD4+CD127-GARP- population are shown. The plots from the tumor bearing mouse are all from the same mouse. **(B)** The frequency of peripheral blood T_{eee} in mice without tumor vs. those with were compared. Significance was determined by an unpaired t-test. **(C)** The tumor volume vs. T_{eee} as a percent of the tumor infiltrating CD45+ population is shown. Significance and coefficient of determination were determined by univariable linear regression. **(D)** Mice with different syngeneic tumors were evaluated for T_{eee} infiltrate in tumors. Representative plots of CD38 vs CD39 expression in the CD45+CD3+CD4+CD127-GARP- population are shown. **(E)** Tumor bearing mice were evaluated by flow cytometry for T_{eee} frequency vs. the percent of CD45+ (i.e. immune infiltrate) in the tumor specimen.

Hypothesized Model



Take-Aways

- Relatively high peripheral blood frequencies of CD3+CD4+CD38+CD39+CD127-GARP- cells are associated with immunotherapy resistance in melanoma patients.
- T_{eee} cells correlate with increased frequencies of immunosuppressive cell populations in patients.
- T_{eee} cells have a phenotype associated with immunosuppression including high expression of adenosine generating ectoenzymes, PDL1, TGFβ, and IL-8.
- T_{eee} cells suppress the function of autologous T-cells
- T_{eee} cells are present in tumor-bearing mice and negatively associated with overall immune-infiltrate into the tumor.

Acknowledgements

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