***OncoImmunology***

***Identification of TTLL8, POTEE, and PKMYT1 as immunogenic cancer-associated antigens and potential immunotherapy targets in ovarian cancer***

Supplementary Materials:

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**Supplementary Figure 1. Sequence motifs for HLA-A01:01 and HLA-A02:01.** Images representing the known peptide sequence motifs for HLA-A01:01 and HLA-A02:01 binders. Images are from the MHC Motif Atlas (http://mhcmotifatlas.org).

**Supplementary Figure 2. CT antigen expression in normal and cancer tissues.** (A) A heatmap representing the mRNA expression of 24 CT antigens across all normal tissues in the GTEx Portal (www.gtexportal.org). Note: Transcript levels of RNF103-CHMP3 are not shown as the transcript-level data was not found in the GTEx Analysis Release V8. (B) mRNA expression of 16 CT antigens in ovarian cancer tumors and corresponding normal tissue. (C) Protein expression of 16 CT antigens as determined from the Human Protein Atlas database. Data is shown as the percentage of positively stained ovarian tumors. ND: not determined. (D) qPCR for POTEE mRNA following treatment of OVCAR-5 cells with indicated doses of 5-aza-2′-deoxycytidine (DAC) (n=3 technical replicates, bars represent the mean).

**Supplementary Figure** **3. CT antigens activate peptide-specific T cell responses in patient TILs.** (A) Flow cytometry gating strategy. Sequential gating strategy showing: Initial cell population based on forward and side scatter, Single cell selection using FSC-H vs FSC-A, Doublet exclusion using SSC-H vs SSC-A, Live cell selection using Live/Dead stain, CD8+ T cell identification, and Analysis of TNFα and IFNγ expression. This gating strategy was consistently applied across all flow cytometry experiments. (B, C) ICS for IFNγ and (D, E) TNFα in CD8 T cells from A02:01+ patients 3 and 4 with HGSOC. T cells were stimulated with each indicated peptide prior to staining and assessed via flow cytometry.

**Supplementary Figure 4. Assessment of peptide-specific T cell cytotoxicity in OC patients**. (A) Analysis of custom tetramers generated via a peptide exchange system. Flow cytometry was used to assess the percentage of exiting peptide following the exchange reaction. (B, C) T cell cytotoxicity at 96-hour incubation with peptide-pulsed OVCAR-5 cells at 10:1 E:T ratio in patients 2 (B) and 3 (C). (D) T cell cytotoxicity at 48-hour incubation with IFNγ-pretreated OVCAR-5 cells at 10:1 E:T ratio in patients 1-3. OVCAR-5 cells were pretreated with 20ng/ml IFNγ for 24 hours prior to co-culture. Data were normalized to OVCAR-5 cells alone (0:1 E:T ratio) at 48 (D) and 96 hours (B, C). \*\*\*\*p<0.0001

**Supplementary Table 2. Clinical Summary of OC Patients Analyzed for TTLL8 and POTEE Expression by IHC.**

|  | Overall (N=107) |
| --- | --- |
| Age at Diagnosis |  |
| Median | 63.000 |
| Q1, Q3 | 56.500, 72.000 |
| Category |  |
| Malignant | 106 (99.1%) |
| Borderline Malignant Mixed | 1 (0.9%) |
| Malignancy Type |  |
| Epithelial | 50 (46.7%) |
| Primary Peritoneal | 45 (42.1%) |
| Fallopian Tube | 12 (11.2%) |
| Histology |  |
| Low Grade Serous | 1 (0.9%) |
| High Grade Serous | 91 (85.0%) |
| Mucinous | 4 (3.7%) |
| Endometrioid | 8 (7.5%) |
| Clear Cell | 3 (2.8%) |
| Stage |  |
| 1 | 12 (11.2%) |
| 2 | 5 (4.7%) |
| 3 | 68 (63.6%) |
| 4 | 22 (20.6%) |
| Grade |  |
| 1 | 4 (3.7%) |
| 2 | 11 (10.3%) |
| 3 | 92 (86.0%) |
| Platinum Chemo? |  |
| N-Miss | 17 |
| yes | 90 (100.0%) |
| Taxane Chemo? |  |
| N-Miss | 17 |
| no | 2 (2.2%) |
| yes | 88 (97.8%) |
| Surgery Occurence |  |
| Primary debulking | 102 (95.3%) |
| Interval debulking | 2 (1.9%) |
| Diagnostic procedure | 3 (2.8%) |
| Debulking Status |  |
| Optimal; no macroscopic disease | 34 (31.8%) |
| Optimal; macroscopic disease <1 cm | 27 (25.2%) |
| Optimal; macroscopic disease unknown | 20 (18.7%) |
| Sub-optimal | 23 (21.5%) |
| Missing | 3 (2.8%) |

**Supplementary Table 3. TTLL8 and POTEE antigens vs Histology of OC patients**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  |  | Negative (N=45) | Positive (N=59) | Total (N=104) |
| TTLL8 | Non-HGS | 10 (22.2%) | 6 (10.2%) | 16 (15.4%) |
| HGS | 35 (77.8%) | 53 (89.8%) | 88 (84.6%) |
|  |  | Little (N=51) | A Lot (N=55) | Total (N=106) |
| POTEE | Non-HGS | 7 (13.7%) | 9 (16.4%) | 16 (15.1%) |
| HGS | 44 (86.3%) | 46 (83.6%) | 90 (84.9%) |

P values for count data of TTLL8 and POTEE are 0.107 and 0.79 respectively according to Fisher's Exact Test.

**Supplementary Table 4. TTLL8 and POTEE antigens vs stage of OC patients**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  |  | Negative (N=45) | Positive (N=59) | Total (N=104) |
| TTLL8 | Early (1 & 2) | 12 (26.7%) | 5 (8.5%) | 17 (16.3%) |
| Advanced (3 & 4) | 33 (73.3%) | 54 (91.5%) | 87 (83.7%) |
|  |  | Little (N=51) | A Lot (N=55) | Total (N=106) |
| POTEE | Early (1 & 2) | 5 (9.8%) | 12 (21.8%) | 17 (16.0%) |
| Advanced (3 & 4) | 46 (90.2%) | 43 (78.2%) | 89 (84.0%) |

P values for count data of TTLL8 and POTEE are 0.017 and 0.115 respectively according to Fisher's Exact Test

# **Supplementary Methods**

## Cell ***Lines***

The OVCAR-5 cell line was obtained from the ​NCI-Frederick Cancer DCTD Tumor/Cell Line Repository. The cell line was tested for mycoplasma and authenticated using the commercial service (​CellCheck, ​IDEXX Bioresearch). OVCAR-5 cells were cultured in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS) (M7201-127, Cardinal Healthcare). The OVCAR-5 cell line is of female origin.

## Ovarian cancer tissue microarray – immunohistochemistry

Tissue microarrays (TMAs) were supplied from the Mayo Clinic Biospecimen Resource for Ovarian Cancer Research under IRB 20-001221 and stained with support from the Mayo Clinic Pathology Resource Core. TMAs were stained using the Roche Benchmark Ultra automated staining platform with TTLL8 antibody (1:250, Sigma, HPA016882) or POTEE (1:250, Sigma, HPA043260).

## Ovarian cancer tissue microarray – survival analysis

The stained TMAs were scored by a gynecologic pathologist on a scale from 0-3, reflecting staining intensity for 106 patients for POTEE and 104 patients for TTLL8 in triplicate. Intensity was reported as 0=Negative, 1=Weak, 2=Intermediate, or 3=Strong. Associations between staining and categorical variables were assessed via Fisher’s Exact test. The association with overall survival and time to progression was assessed via Kaplan Meier plots and log-rank tests. Cox regression models were used to adjust these time-to-event analyses for stage and debulking status. Overall survival was defined as the time from diagnosis to death. Progression-free survival was defined as the time from diagnosis to the first recurrence, persistent disease, or death. Data acquisition and analysis were blinded.

## Antibodies

The following antibodies were used in this study: CD3-violetFlour 500 (#85-0038, Tonbo Biosciences), CD4-PerCP-Cy5.5 (#65-0048, Tonbo Biosciences), CD8-Alexa Fluor 700 (#344724, BioLegend), PD-1-APC (#329908, BioLegend), TNF-α-BV421 (#502932, BioLegend), IFN-γ-PE (#502509, BioLegend), W6/32 (# 311428, Biolegend), and IgG (#400166, Biolegend).

## mRNA expression analysis

OVCAR-5 cells were treated with 5-aza-2′-deoxycytidine (DAC) (Sigma, MO) for 7 days with DAC. Total RNA was extracted using the phenol-chloroform method. Reverse transcription of 2 μg total RNA was carried out using the High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). qPCR was performed with human TaqMan probes and FastPROBE qPCR No-ROX Master Mix (Cytek Biosciences) on a Roche LightCycler 480 Real-Time PCR System.

## Statistics

All statistical analyses were performed using GraphPad Prism (GraphPad, La Jolla, CA). Sample sizes were determined based on previous experience with the individual experiment. The mean and the standard error of the mean (s.e.m) indicating variance are reported for all graphs. For experiments making one comparison, data was analyzed using a two-tailed Mann-Whitney U test to account for the non-normal distribution of the data. For experiments with more than one comparison, one-way ANOVA with Tukey’s multiple comparisons post-test was used. A log2 transformation was applied to the data before analysis, where the standard deviations were significantly different. Differences were considered significant if p< 0.05.