Biomarkers and Immune Monitoring

O1
Combinatorial CD8+ and PD-L1+ cell densities correlate with response and improved survival in non-small cell lung cancer (NSCLC) patients treated with durvalumab
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Background
Immunotherapies have improved patient responses and survival, though not all patients benefit. Effective biomarkers may help to improve outcomes. Durvalumab is a human IgG1 monoclonal antibody that inhibits PD-L1 binding to PD-1 and CD80, restoring antitumor immunity [1, 2]. PD-L1 expression on tumor or tumor-infiltrating immune cells measured manually with different immunohistochemistry (IHC) assays can enrich for patients responding to anti-PD-1/PD-L1 agents. Tumor-infiltrating cytotoxic CD8+ T cells may also have potential predictive utility for therapeutic response. We explored automated image analysis of CD8+ and PD-L1+ cell densities in baseline tumor biopsies to determine whether CD8+ and PD-L1+ cell densities could better identify patients most likely to respond to durvalumab than PD-L1 IHC alone.

Methods
CP1108/NCT01693562 was a nonrandomized phase I/II trial evaluating durvalumab in advanced NSCLC and other solid tumors [3]. By 29APR2016, 304 previously treated NSCLC patients, median 3 prior lines, received 10 mg/kg of durvalumab q2w ≤12 months. Baseline archived or fresh tumor biopsies were analyzed for PD-L1 (Ventana/SP263) and CD8 (Ventana/SP239) by IHC. For the marker combination, slides were scored using the product of PD-L1+ and CD8+ cell densities with Definiens’ Developer XD 2.1.4 software. For PD-L1 alone, ≥25% tumor cells stained for PD-L1 at any intensity were scored positive. Clinical outcomes (ORR, PFS and OS) were analysed based on CD8+ and PD-L1+ densities (n = 163 available) and PD-L1 alone in pre-treatment biopsies using a discovery (n = 84) and validation (n = 79) set. Datasets were matched on baseline PD-L1 status, histology, ECOG, lines of therapy, and response.

Results
Patients with high pretreatment CD8+ and PD-L1+ densities (prevalence = 36%) had better ORR, OS, and PFS compared to those with low CD8+ and PD-L1+ densities (Fig. 1), as well as high PD-L1 expression alone.

Conclusions
Automated image analysis of CD8+ and PD-L1+ cell densities in baseline tumor biopsies may identify patients with improved outcomes to durvalumab.

Trial Registration
ClinicalTrials.gov identifier NCT01693562.

References
1. MedImmune/AstraZeneca. Data on file.
2. Ibrahim R, Stewart R, Shalabi A: PD-L1 blockade for cancer treatment: MED14736. Semin Oncol 2015, 42:474–483.
3. Rzizi NA, Brahmer JR, Ou SH, Segal NH, Khleif S, Hwu WJ, et al: Safety and clinical activity of MED14736, an anti-programmed cell death-ligand 1 (PD-L1 antibody, in patients with non-small lung cancer (NSCLC). J Clin Oncol 2015, 33(Suppl.):Abstract 8032.
There is no standard second-line therapy for advanced urothelial cancer. Although paclitaxel, docetaxel, and vinflunine are commonly used, they provide limited clinical benefit. KEYNOTE-045 compared the efficacy and safety of the anti–PD-L1 antibody pembrolizumab versus investigator-choice chemotherapy as second-line therapy for advanced urothelial cancer that progressed or recurred following first-line platinum-based chemotherapy. Baseline characteristics were generally balanced between arms, with 87.3% with visceral disease, 34.3% with liver metastases, 1.1% with ECOG PS 2, and 32.8% with 75 mg/m² and 320 mg/m² Q3W. Randomization was stratified by ECOG PS (0/1 vs 2) and treatment-related AEs support pembrolizumab as a new standard of care for advanced urothelial cancer that progressed or recurred following first-line platinum-based chemotherapy. Pembrolizumab demonstrated a statistically significant OS benefit over chemotherapy in the second-line advanced urothelial cancer setting, making it the first therapy to demonstrate a survival benefit over an active comparator in this population. The superior OS combined with the lower rate of any-grade and high-grade treatment-related AEs support pembrolizumab as a new standard of care for advanced urothelial cancer that progressed on/after platinum-based chemotherapy.

### Methods
Eligible patients were enrolled regardless of PD-L1 expression and randomized 1:1 to pembrolizumab 200 mg Q3W for 24 months or investigator’s choice of paclitaxel 175 mg/m² Q3W, docetaxel 75 mg/m² Q3W, or vinflunine 320 mg/m² Q3W. Randomization was stratified by ECOG PS (0/1 vs 2), liver metastases (yes vs no), hemoglobin level (<10 vs ≥10 g/dL), and time from last chemotherapy dose (<3 vs ≥3 months). The study had a group sequential design to control for type I error. Primary endpoints were OS and PFS (RECIST v1.1 by blinded, independent central review). ORR was a key secondary endpoint. Differences in OS and PFS were assessed in the intention-to-treat population using the stratified log-rank test.

### Results
Between November 5, 2014 and November 13, 2015, 542 patients from 29 countries were enrolled: 270 in the pembrolizumab arm, 272 in the chemotherapy arm. As of September 7, 2016, median follow-up was 9.0 months; 49 (18.4%) patients remained on pembrolizumab and 3 (1.2%) patients remained on chemotherapy. Baseline characteristics were generally balanced between arms, with 87.3% with visceral disease, 34.3% with liver metastases, 1.1% with ECOG PS 2, and 32.8% with <3 months since most recent chemotherapy. Pembrolizumab significantly improved OS over chemotherapy (HR 0.98, P = 0.042) (Table 1). There was no difference in PFS (HR 0.98, P = 0.42) (Table 1). ORR was significantly improved with pembrolizumab (21.1% vs 11.4%) (Table 1). Pembrolizumab was associated with fewer any-grade (60.9% vs 90.2%) grade 3-5 treatment-related AEs (15.0% vs 49.4%). 4 patients in each arm died due to treatment-related AEs.

### Conclusions
Pembrolizumab demonstrated a statistically significant OS benefit over chemotherapy in the second-line advanced urothelial cancer setting, making it the first therapy to demonstrate a survival benefit over an active comparator in this population. The superior OS combined with the lower rate of any-grade and high-grade treatment-related AEs support pembrolizumab as a new standard of care for advanced urothelial cancer that progressed on/after platinum-based chemotherapy.

### Clinical Trials: Cutting-Edge (Completed Trials)

#### O2

**Keynote-045:** open-label, phase III study of pembrolizumab versus investigator’s choice of paclitaxel, docetaxel, or vinflunine for previously treated advanced urothelial cancer

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Journal for ImmunoTherapy of Cancer 2016, 4(Suppl 2):O2

### Background
There is no standard second-line therapy for advanced urothelial cancer. Although paclitaxel, docetaxel, and vinflunine are commonly used, they provide limited clinical benefit. KEYNOTE-045 compared the efficacy and safety of the anti–PD-L1 antibody pembrolizumab versus investigator-choice chemotherapy as second-line therapy for advanced urothelial cancer that progressed or recurred following first-line platinum-based chemotherapy.

### Table 1 (abstract O2) Efficacy in KEYNOTE-045

| End point | Pembrolizumab | Chemotherapy |
|-----------|---------------|--------------|
| OS, no. of events | 155 | 179 |
| Median (95 % CI), months | 10.3 (8.0-11.8) | 7.4 (6.1-8.3) |
| HR (95 % CI) | 0.73 (0.59-0.91) | P = 0.0022 |
| PFS, no. of events | 218 | 219 |
| Median (95 % CI), months | 2.1 (2.0-2.2) | 3.3 (2.3-3.5) |
| HR (95 % CI) | 0.98 (0.81-1.19) | P = 0.42 |
| ORR (95 % CI) | 21.1 % (16.4-26.5) | 11.4 % (7.9-15.8) |
| Treatment difference, % (95 % CI) | 9.6 (3.5-15.9); P = 0.0011 |
O3 Efficacy and safety of nivolumab plus ipilimumab in metastatic urothelial carcinoma: first results from the phase I/II CheckMate 032 study
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Journal for Immunotherapy of Cancer 2016, 4(Suppl 2):O3

Background
Nivolumab is a programmed death-1 (PD-1) immune checkpoint inhibitor associated with clinical benefit in previously treated patients with metastatic urothelial carcinoma [1]. Preclinical and clinical data indicate that the combination of nivolumab plus ipilimumab, an anti-cytotoxic T lymphocyte antigen-4 (CTLA-4) antibody, can improve antitumor activity in other tumor types. Here, we report the first efficacy and safety results of combined nivolumab plus ipilimumab given at two different dosing schedules in CheckMate 032, an open-label, multicenter, phase I/II study of patients with metastatic urothelial carcinoma who progressed after prior platinum-based therapy.

Methods
Patients with locally advanced or metastatic urothelial carcinoma previously treated with platinum-based therapy were included in the study. Patients were treated with either of two combination schedules, nivolumab 1 mg/kg + ipilimumab 3 mg/kg (N1I3) or nivolumab 3 mg/kg + ipilimumab 1 mg/kg (N3I1) every 3 weeks for four cycles, followed by nivolumab 3 mg/kg every 2 weeks; or they were treated with nivolumab monotherapy 3 mg/kg (N3) every 2 weeks. All patients were treated until disease progression or unacceptable toxicity. The primary endpoint was investigator-assessed objective response rate (ORR) by RECIST v1.1. Secondary endpoints included safety and duration of response (DoR).

Results
Minimum follow-up was 3.9 months in the N1I3 (n = 26) group, 14.5 months in the N3I1 group (n = 104), and 13.8 months in N3 group (n = 78). ORR was 38.5% (95% confidence interval [CI], 20.2-59.4), 26.0% (95% CI, 17.9-35.5), and 25.6% (95% CI, 16.4-36.8) in the N1I3, N3I1, and N3 groups, respectively. Median DoR has not been reached in any treatment group. The frequency of drug-related grade 3-4 adverse events was 30.8% (N1I3), 31.7% (N3I1), and 23.1% (N3). Treatment-related adverse events led to discontinuation in 7.7% (N1I3), 13.5% (N3I1), and 3.8% (N3) of patients. One death was reported in the N3I1 group (pneumonitis) and two deaths were reported in the N3 group (pneumonitis and thrombocytopenia).

Conclusions
Second-line treatment with N1I3 may provide the most favorable benefit-risk ratio among the regimens studied. If these interim results are confirmed with longer follow-up, further development of the N1I3 combination in metastatic urothelial carcinoma is warranted.

Trial Registration
ClinicalTrials.gov identifier NCT01928394.

References
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O4 Coinhibition & Costimulation

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Journal for Immunotherapy of Cancer 2016, 4(Suppl 2):O4

Background
While antibody blockade of the CTLA-4 and PD-1 pathways has emerged as an effective treatment modality for cancer, the majority of patients do not derive long-term benefit, suggesting a need for targeting of additional immune checkpoints. Employing our unique computational algorithms to define new members of the B7/CD28 family, we identified PVRIG, which is expressed by multiple subsets of T and NK cells. We report here its expression pattern, functional characterization, and anti-tumor activity of blocking antibodies targeting this molecule.

Methods
Utilizing Compugen’s Predictive Discovery platform we identified PVRIG as a potential novel immune checkpoint, after which a retroviral cell screening library was used to identify its cognate binding counterpart. Target effects on T cell modulation were assessed with primary and tumor-derived T cell assays, taking advantage of target overexpression, knockdown, and antagonist antibody approaches. Antibodies against the human protein were screened for their ability to enhance T cell activation in vitro, while antibodies targeting the mouse orthologue were assessed in vivo for effects on tumor growth inhibition in syngeneic models.

Results
A PVRIG-Fc-fusion protein was found to bind PVRLG, with binding specificity confirmed both by ELISA and flow cytometry analysis. PVRIG demonstrated unique expression kinetics upon T cell activation, with detection of the target on memory T cells, as well as on NK cells and γδ T cells. A panel of high affinity human antibodies with the ability to block interaction of PVRIG with PVRLG were generated, which when tested in vitro were shown to enhance activation of both primary CD4+ and tumor-derived CD8+ T cells through a PVRLG-dependent mechanism. The lead antibody, COM-701, is
currently in preclinical development. Since COM-701 is not mouse cross-reactive, in vivo studies were conducted with a surrogate blocking anti-mouse PVRIG antibody. When combined with anti-PD-L1 blockade, anti-mouse PVRIG inhibits growth of established tumors in both the CT26 and MC38 colorectal cancer models. Combination testing with additional immune checkpoint inhibitors, as well as in PVRIG knockout mice, is ongoing.

Conclusions

We describe the identification of PVRIG as a novel immune checkpoint on T cells, as well the development of a high affinity antagonistic antibody, COM-701, that is currently in preclinical development. COM-701 is able to enhance human T cell activation, and a surrogate antibody with similar characteristics shows synergy with PD-L1 in vivo in multiple syngeneic models. Overall, our data demonstrate the utility of targeting PVRIG in addition to other B7 family checkpoints for the treatment of cancer.

**Combinations: Immunotherapy/Immunotherapy**

**O5**

**Preliminary efficacy from a phase I/II study of the natural killer cell–targeted antibody lirilumab in combination with nivolumab in squamous cell carcinoma of the head and neck**

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**Background**

Natural killer (NK) cells and the innate immune system play a critical role in immunosurveillance, control of tumor growth, and metastasis. NK-cell activation is negatively regulated by inhibitory killer-cell immunoglobulin-like receptors (KIRs); therefore, blocking KIR function may potentiate an anti-tumor immune response and complement other immuno-oncology therapies that enhance T cell activity. We present preliminary efficacy results in patients with squamous cell carcinoma of the head and neck (SCCHN) from a phase I/II study of lirilumab, a fully human monoclonal antibody that blocks inhibitory KIRs on NK cells, in combination with nivolumab, a fully human IgG4 monoclonal antibody that targets the PD-1 receptor, in patients with solid tumors (NCT01714739).

**Methods**

During dose escalation, patients with advanced solid tumors who progressed after ≥1 prior therapy received lirilumab 0.1–3.0 mg/kg once every 4 weeks (Q4W) plus nivolumab 3.0 mg/kg Q2W. Cohort expansion was initiated at the maximum dose of lirilumab 3.0 mg/kg Q4W plus nivolumab 3.0 mg/kg Q2W in patients with advanced solid tumors. Key study endpoints include safety (primary), objective response rate (ORR), disease control rate (DCR), duration of response (DOR), and biomarker assessments.

**Results**

As of the August 30, 2016 data cutoff, 159 patients were treated with the lirilumab plus nivolumab combination. Treatment-related adverse events (TRAEs) and grade 3–4 TRAEs were reported in 72% (114/159) and 15% (24/159) of patients, respectively. Discontinuations due to TRAEs occurred in 8% (12/159). Of the 41 patients with SCCHN treated, 29 were evaluable for response. In this heavily pretreated, checkpoint inhibitor–naïve group, ORR was 24% (7/29; confirmed and unconfirmed) and DCR was 52% (15/29). Maximum reduction in target lesions is presented in Fig. 2 for 26 patients with available tumor assessments. Two patients classified as stable disease per RECIST v1.1 showed unconventional responses, with 100% and 37% reductions in target lesions. Among evaluable patients, five (17%) had reductions in tumor burden >80%. Responses appear durable, with the median DOR not reached (Fig. 3). Updated efficacy and preliminary biomarker analyses (including PD-L1 and HPV status) will be presented.

**Conclusions**

Preliminary efficacy of lirilumab plus nivolumab in patients with advanced platinum-refractory SCCHN demonstrates clinical benefit, with encouraging response rates that were deep and durable responses in some patients. This combination demonstrated a manageable safety profile similar to that observed with nivolumab monotherapy. Further evaluation of this novel combination of an NK-cell inhibitor and an immune checkpoint inhibitor is ongoing.

**Trial Registration**

ClinicalTrials.gov identifier: NCT01714739

**Fig. 2 (abstract O5).** Maximum percent reduction in target lesions from baseline

**Fig. 3 (abstract O5).** Percent change from baseline in target lesions over time
Phase II study of intratumoral plasmid interleukin 12 (pIL12) with electroporation in combination with pembrolizumab in stage III/IV melanoma patients with low tumor infiltrating lymphocytes: Alain Algazi1, Katy Tsai1, Michael Rosenblum1, Prachi Nandoskar1, Robert HI Andtbacka2, Amy Li1, John Nonomura1, Kathryn Takamura1, Mary Dwyer3, Erica Browning4, Reneta Taita5, Chris Twitty6, Sharron Gargosky7, Jean Campbell7, Carmen Ballesteros-Merino8, Carlo B. Bifulco9, Bernard Fox10, Mai Le10, Robert H Pierce11, Adil Daud1
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Journal for ImmunoTherapy of Cancer 2016, 4(Suppl 2):006

Background
Low tumor infiltrating lymphocytes (TILs) are predictive for poor response to immunotherapy with anti-PD-1/PD-L1 agents. We have shown that melanoma patients with a low frequency of PD-1hiCTLA-4+ TIL are unlikely to respond to pembrolizumab (Daud 2016). Intratumoral electroporation of pIL-12 (IT-pIL12-EP) leads to an IFN-g signature suggestive of increased TIL as well as regression in both treated and untreated lesions. We hypothesize that combination IT-pIL12-EP and pembrolizumab will improve clinical outcomes in this low-response population. Preliminary results from a multi-center, phase II, open-label trial testing this hypothesis are presented.

Methods
Melanoma stage III/IV patients with accessible lesions were consented and enrolled if they had a TIL status of hiCTLA-4+ in the CD45 + CD8 + CD3+ gate by flow cytometry (FC). Patients were treated with pembrolizumab (200 mg every 3 weeks) concurrently with IT-pIL12-EP on days 1, 5 and 8 every 6 weeks. Patients were evaluated for overall response rate (ORR) every 12 weeks by RECISTv1.1. Pre and post-treatment blood and tumor specimens were collected, and analyzed for immune phenotyping, gene expression, TCR diversity, and changes in the tumor microenvironment with multispectral immunohistochimistry.

Results
Interim ORR data is available on 15 patients. 13/15 patients had a frequency of PD-1hiCTLA-4+ TIL of <22% (low TIL status), phenotypes associated with a low probability of response to anti-PD-1 (Daud 2016). These 15 patients age 39-89 years, were 53% male, 66% stage III and 34% stage IV. Treatment was well tolerated; 38% of adverse events (AE) were classified as treatment site reactions (grade 1-2) that resolved. One SAE of cellulitis resolved with 5d antibiotics. One grade 3 AE of diarrhea resulted. The ORR was 40% (4CR, 2PR) by RECISTv1.1. Analysis of tumor biopsies and blood demonstrated meaningful immunological changes including an increased number and ratio of CD8+PD-L1+ and CD8+FoxP3+ TIL, tumoral RNA signatures indicating an increase in CD8 and IFN-g-related gene expression and concordant immune phenotypes in the periphery.

Conclusions
The combination IT-pIL12-EP with pembrolizumab in patients with an anti-PD-1 non-responsive phenotype engendered a 40% clinical response with associated positive immune-based biomarker data and an excellent safety profile. These data suggests that IT-pIL12-EP modulates the tumor microenvironment to enable an effective anti-PD-1 mAb response in patients otherwise unlikely to respond.

Acknowledgements
We thank Merck and Oncosec for supporting this trial with pembrolizumab and IT-pIL-12, respectfully.

Trial Registration
ClinicalTrials.gov identifier: NCT02493361

References
1. Daud AI, Loo K, Pauli ML, Sanchez-Rodriguez R, Sandoval PM, Taravati K, et al: Tumor immune profiling predicts response to anti-PD-1 therapy in human melanoma. J Clin Invest 2016, 126(9):3447–3452.

Adoptive Cellular Therapy

P1
Chemo-immunotherapy with cyclophosphamide and tumor reactive CD4+ T cells lead to destruction of tumor vasculature and eventual tumor eradication
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Journal for ImmunoTherapy of Cancer 2016, 4(Suppl 2):P1

Background
CD4+ T cells are critical components of anti-tumor immunity and play a pivotal role in orchestrating anti-tumor immune responses. Mounting evidence from preclinical and clinical studies indicates that CD4+ T cells in combination with chemotherapy can control tumor growth and recurrence. CD4+ T cells are suggested to mediate tumor rejection mechanisms that include cytotoxic effects on tumor cells, inhibition of angiogenesis, and reprogramming of the tumor microenvironment.

Methods
In this project, we set out to study the cellular and molecular mechanisms underlying the therapeutic effect of chemo-immunotherapy in the form of cyclophosphamide (CTX) and tumor specific CD4+ T cells in a murine model of colorectal cancer. Mice were injected subcutaneously with colorectal cancer cells. When the tumor reached 140-160 mm2 in area, mice were injected with a low dose of cyclophosphamide followed by adoptive transfer of tumor reactive CD4+ T cells.

Results
In a murine model of colorectal cancer, we show that the combination therapy of CTX and tumor reactive CD4+ T cells resulted in enhanced necrosis of tumor cells in vivo, leading to eventual eradication of advanced tumors. By using immunofluorescence staining and blood perfusion imaging, we demonstrated that the combination therapy leads to destruction of the established tumor vasculature and reduced blood supply to tumor tissue. Furthermore, we assessed blood vessel permeability in the tumor tissue and found that the combination therapy increased extravasation of Evans blue dye, suggesting an increase in vascular permeability.

Conclusions
In summary, our findings suggest that the combination therapy of CTX + CD4+ T cells leads to destruction of the tumor vasculature, resulting in extensive necrosis of tumor tissue and eventual tumor regression. These findings may provide new insights into mechanisms of tumor rejection by CD4+ T cells.

P2
Preclinical development of tumor-infiltrating lymphocyte therapy for pancreatic cancer
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Journal for ImmunoTherapy of Cancer 2016, 4(Suppl 2):P2

Background
Immunotherapy has become an effective cancer therapy, particularly in the case of checkpoint blockade and adoptive T cell therapy (ACT). ACT exploits the presence of tumor-infiltrating lymphocytes (TIL) by exponentially expanding their numbers ex vivo and re-infusing them into the patient in an autologous setting. With the effectiveness of
TIL therapy already well established in multiple phase II studies in melanoma, there is a push to translate it to other cancers in dire need of improved therapies. Pancreatic ductal adenocarcinoma (PDAC) is one such cancer for which the current therapy, surgery and chemotherapy, provides an overall 5-year survival rate of only 5%. The presence of TIL is correlated with increased survival in PDAC, which suggests that TIL could effectively control the disease and provides a rationale to test TIL therapy in this setting.

Methods
To assess the feasibility, we characterized the immune component of PDAC, explored the ability to grow and expand TIL from tumor fragments, and analyzed the clonality of these expanded TIL.

Results
Flow cytometry analysis detected low, CD4-rich T cell infiltration. These TIL were able to be expanded ex vivo and the addition of an agonistic anti-41BB antibody to the cultures preferentially increased total TIL outgrowth, particularly that of CD8+ TIL. The success rate of TIL growth was increased from 23% to 50% for cultures grown without and with anti-41BB respectively. Sequencing of the T cell receptor CDR3-beta chain found specific T cell clones enriched at the tumor site in comparison to the blood. IHC staining for MHC class I (MHCI) on PDAC tumor samples showed that it is widely expressed but at low levels generally.

Conclusions
In conclusion, it is possible to expand CD8+ T cells from PDAC bearing TCR sequences highly enriched in the tumor. Additionally, expanded TIL would be able to target tumor cells as they are shown to express MHCI. Although there are barriers yet to overcome, the initial data suggest the feasibility of TIL therapy for PDAC.

References
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Biomarkers and Immune Monitoring

P4
Evaluation of anticancer immunity in patients with thyroid cancer with a focus towards developing effective combination immunotherapy
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Journal for Immunotherapy of Cancer 2016, 4(Suppl 2):P4

Background
Thyroid cancer is the most common endocrine-related cancer with 64,330 diagnoses expected this year. While the majority of these cancers are curable, almost 2% of these cancers are anaplastic thyroid cancers, which are highly aggressive and almost uniformly lethal. At the same time, the thyroid is known for being inherently immunogenic. For these reasons and due to an active surgical practice providing regular resections of thyroid cancers, we undertook a study of thyroid cancer with the idea of developing an immunotherapy for this disease.

Methods
We have developed a thyroid cancer tumor bank to complement our Oral, Head and Neck Cancer Program. This tumor bank cryopreserves enzymatically isolated viable cells from resected tumors (n = 16). We are also attempting to develop primary cell lines and are isolating and assessing autologous tumor-specific functions of tumor-infiltrating lymphocytes (TIL) (n = 7).

Results
To date we have established 3 tumor cell lines from thyroid cancer specimens and identified PD-L1 expression on 2 of 2 tested. While
numbers are small, preliminary analyses suggest that TIL cultures can be generated from 85% of thyroid cancer specimens and that autologous tumor-reactive TIL can be detected in 43% (n = 7) of thyroid cancers. Since not every tumor appears to contain TIL capable of recognizing autologous tumor, strategies to prime tumor-specific T cells represents an area of interest. DPV-003 is a microvesicle vaccine, DrIbble, that contains more than 80 proteins that are overexpressed by thyroid cancer (TCGA provisional RNASeq n = 509 pts). The vaccine also contains a number of DAMPs and agonist activity for multiple TLRs packed into stable double membrane microvesicles that are targeted to CLEC9A+ antigen presenting cells. We are also developing a second thyroid-specific DrIbble vaccine from a cell line derived from an anaplastic thyroid cancer.

Conclusions
Almost half of thyroid cancers evaluated, including one anaplastic thyroid cancer, contain T cells capable of recognizing autologous cancer cells and secreting IFN-g. However, the other 50% of thyroid cancers appear to lack tumor-reactive T cells and may benefit from combination immunotherapy strategies that include a vaccine.

Acknowledgements
Support: Steve and Cindy Harder, Robert W. and Elsie Franz, Wes and Nancy Lematta, Lynn and Jack Loacker, and The Chiles foundation (BAF).

PS
Development and clinical translation of 89Zr-Df-IAB22M2C for detecting CD8+ T cells for immunotherapy applications
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Journal for Immunotherapy of Cancer 2016, 4(Suppl 2):PS

Background
Immunotherapies are changing the landscape for cancer treatment; however, the field is hampered by the lack of biomarkers that can be used for patient selection and for monitoring treatment responses rapidly and non-invasively. To address this need, ImaginAb is developing 89Zr-Df-IAB22M2C, an ~80 kDa minibody (Mb) with high affinity to the CD8 glycoprotein (binding EC50 = 0.4 nM) conjugated with desferrioxamine (DF) and radiolabeled with the positron emitting radionuclide Zirconium-89 (89Zr; T1/2 = 78.4 hours) for imaging CD8+ T cells in humans.

Methods
A comprehensive preclinical program that included evaluation of the in vitro and in vivo pharmacodynamics of IAB22M2C (unconjugated Mb), Df-IAB22M2C (conjugated Mb intermediate), Zr-Df-IAB22M2C (Zr chelated, conjugated non-radiolabeled form of final drug) and 89Zr-Df-IAB22M2C (radioactive final drug product) was conducted to demonstrate the safety and potential efficacy of the probe.

Results
In vitro studies using human PBMCs from 10 individual human donors showed no measurable or reproducible impact on proliferation, activation or depletion of CD8+ T cells and no consistent release of cytokines when donor CD8+ T cells were exposed to soluble or immobilized Mb protein. Studies that evaluated the effect of saturating concentrations of 89Zr-Df-IAB22M2C on proliferation and viability of CD8+ T cells in vitro, also showed no impact on these parameters. Preclinical imaging and biodistribution studies demonstrated favorable pharmacokinetics and the ability of 89Zr-Df-IAB22M2C to detect infiltrating CD8+ T cells in a mouse hu-PBMC NSG™ GvHD model and in Matrigel® plugs implanted with different numbers of human CD8+ T cells. Radiation dosimetry studies conducted in hu-CD34 NSG™ mice and the results GLP dosimetry analysis showed that on average, the organs receiving the largest dose equivalent were the kidneys at 8.0 rem/mCi (2.2 mSv/Mbq) followed by the liver at 7.9 rem/mCi (2.1 mSv/Mbq) and LLI wall at 6.5 rem/mCi (1.8 mSv/Mbq). A GLP toxicology study was conducted in cynomolgus monkeys that included multiple dose cohorts of Zr-Df-IAB22M2C and a vehicle control. The results showed that doses up to 25 mg/kg of Zr-Df-IAB22M2C administered weekly to cynomolgus monkeys did not result in any treatment-related findings in survival, clinical signs, body weights, food consumption, ophthalmic examinations, electrocardiography, blood pressure, heart rate, clinical and anatomic pathology, peripheral blood lymphocyte population, and cytokine levels.

Conclusions
89Zr-Df-IAB22M2C has the desired sensitivity and safety profile for imaging CD8+ T cells and the first-in-human studies will commence in the Q4 2016.

P6
High dose interleukin-2 (HD IL-2) select trial in melanoma: a tissue and blood collection protocol to identify predictive biomarkers of benefit to HD IL-2 in patients with advanced melanoma
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Journal for Immunotherapy of Cancer 2016, 4(Suppl 2):P6

Background
HD IL-2 provides objective responses in 15-20% and durable complete remission in 5-8% of patients with metastatic melanoma (MM). We previously identified a gene expression-based tumor subclass characterized by immune related genes (Class 2; C2) associated with durable response to HD IL-2 compared to the remaining tumors that overexpressed lineage-associated genes (Class 1; C1). The primary objective of the HD IL-2 select trial in melanoma was to prospectively validate this favorable gene expression signature (C2). Secondary objectives were to seek serum and tissue biomarkers of durable response.

Methods
170 patients with MM were enrolled at 15 Cytokine Working Group sites from 2010 to 2014. All patients had formalin-fixed paraffin-embedded (FFPE) tumor tissues identified and blood drawn prior to HD IL-2. Tumor assessments used WHO criteria and investigator-assessed outcomes. RNA extracted from FFPE tumor tissues was used for whole transcriptome profiling by RNA sequencing (114 samples yielded sufficient RNA, 101 passed default Quality Control (QC)). Pre-treatment serum from 114 patients served as the test set and was analyzed using matrix-assisted laser desorption/ionization (MALDI
and machine-based learning algorithms to identify a predictive protein expression signature.

Results
Thirty-one of 170 pts (18.2%) responded, and median overall survival was 21.3 months, with a 40 month median follow-up. Analysis of RNAseq from 101 patients whose specimens passed QC showed that a C2 signature was associated with response to HD IL-2 (normalized enrichment score 1.70, false discovery rate 0.004). Using MALDI, a protein expression signature enriched for acute phase proteins (including CRP, IL-6, and SAA) was defined in the pre-treatment serum and used to classify 39 patients into group A (non-acute phase protein expression) and 75 patients in group B (acute phase protein expression). Complete response rate in group A was 21% and zero in group B (p = 0.0001). Two-year PFS rate was 29% in group A compared to 4% in group B (p = 0.0005).

Conclusions
In this prospective biomarker validation study, HD IL-2 produced durable remissions and prolonged survival in patients with MM. A tumor-associated gene expression signature enriched for immune-related genes was associated with response. Additionally, preliminary data with a serum protein signature appears to identify patients most likely to have a complete response.

Trial Registration
ClinicalTrial.gov identifier NCT01288963.

P7 Pharmacodynamic gene expression changes from talimogene laherparepvec (T-VEC) plus ipilimumab in a phase Ib study for metastatic melanoma
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Journal for ImmunoTherapy of Cancer 2016, 4(Suppl 2):P7

Background
T-VEC is a herpes simplex virus type 1-based oncolytic immunotherapy designed to selectively replicate in tumors, produce GM-CSF, and stimulate antitumor immune responses. Ipilimumab is a checkpoint inhibitor that promotes T cell activation by blocking negative signal transmission in pre-clinical models. Both agents have demonstrated activity in advanced melanoma. Based on the potential complementary MOA of the agents, tumor cell lysis and antigen presentation (T-VEC) in combination with T cell checkpoint inhibition, we hypothesized that improved efficacy was possible when the agents are used in combination. Because the safety profiles are non-overlapping, the combination was not anticipated to have significant increased toxicity. To address these hypotheses, a phase Ib/II study evaluating the safety and efficacy of T-VEC plus ipilimumab for Stage III-IV metastatic melanoma was initiated. The phase Ib study was completed (N = 19) with no DLTs (primary endpoint) or new safety signals with combination treatment, and an ORR of 50% [1]. Phase IIb also included biomarker analyses investigating potential pharmacodynamic markers for T-VEC monotherapy and in combination with ipilimumab.

Methods
Nineteen patients received T-VEC at $10^8$ PFU/mL at week 1, then $10^8$ PFU/mL Q2W from week 4. Ipilimumab was given at 3 mg/kg Q3W starting at week 6 for 4 infusions. Peripheral blood was obtained (Paxgene RNA) at baseline and at weeks 4, 6, 9, and 15. Gene expression (Agilent Microarray) was analyzed for changes in expression level with treatment. Pharmacodynamic markers were identified with a linear mixed effects model. False discovery was controlled with permutation testing.

Results
Gene expression was measured in 16 patients in phase Ib. Most treatment effects on expression were seen after ipilimumab treatment, but there were a few effects in the initial T-VEC phase that passed false discovery controls. These T-VEC effects included SELV, SYNPO, ZBTB32, IQCF2, CDC27, KLK1, PRR20B, CHST6, and IGH. ZBTB32 has been reported to control the proliferative burst of virus-specific natural killer cells responding to infection. The combination effects were enriched for genes involved in lymphoid tissue structure and development and immune cell trafficking. 185 of these genes had signs of a T-VEC effect in the monotherapy phase. These included increases in G2HMM, PDCD1, CDBB, CDBA, and CTLA4 and decreases in IL18, IRAK3, and TXNRD1.

Conclusions
This hypothesis-generating microarray analysis identified genes up-regulated in circulating peripheral blood cells after T-VEC monotherapy and combination treatment. We plan to further evaluate these genes and other potential pharmacodynamic markers in phase II.

Trial Registration
ClinicalTrials.gov identifier NCT01740297.

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Clinical Trials in Progress

P8 Phase I study of alpha-tocopherlyoxyacetic acid in patients with advanced cancer: immune response and pharmacokinetics
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Journal for ImmunoTherapy of Cancer 2016, 4(Suppl 2):P8

Background
Alpha-tocopherlyoxyacetic acid (α-TEA) targets tumor cell mitochondria to release reactive oxygen species (ROS) that induce immunogenic cell death (ICD), antigen release, and enhanced antigen cross-presentation in pre-clinical models. α-TEA is being evaluated for safety and tolerability in a first-in-human phase I trial in patients with advanced cancers (NCT02192346). Tumor types in the ongoing trial include renal cancer, esophageal adenocarcinoma, thyroid cancer, duodenal cancer, and squamous cell carcinoma of the head and neck.

Methods
α-TEA lysine salt is administered orally to patients and given daily in escalating doses for 28 days. Immune monitoring of peripheral whole blood was conducted for all 12 patients at baseline, and at 1 week and 4 weeks post-treatment. Plasma levels of α-TEA have been determined so far in patients receiving 2.4 mg/kg and 4.8 mg/kg α-TEA at 1, 4, 8, and 24 hours after the first dose. Additional samples were
evaluated on days 8, 15, 22, and 29 before the planned α-TEA dose on those days.

Results
Twelve patients have been treated so far at 2.4 mg/kg and 4.8 mg/kg dose levels. Eight patients have stable disease, lasting from 1 to 22+ months. One patient showed more than a 2-fold increase in the number of activated (CD38+ HLA-DR+) effector CD8+ T cells 1 week post-treatment. A second patient showed more than a 2-fold increase in the number of activated effector memory CD8+ T cells 4 weeks post-treatment. Both patients experienced stable disease over 5 and 22 months, respectively. Evaluation of α-TEA levels at the 2.4 mg/kg and 4.8 mg/kg doses revealed a proportional increase in α-TEA plasma levels over a 28-day interval without any indication that steady state plasma levels were reached. Of the 12 patients, 6 developed atrial fibrillation (AF) after starting α-TEA. The earliest event occurred 7 days post-treatment, but AF was more common 29-56 days post-treatment. Four of the 6 patients had a medical history of AF. These were grade 2 events by CTCAE 4.0 criteria and managed with appropriate medication without further sequelae.

Conclusions
α-TEA treatment resulted in stable disease in 80% of patients lasting between 1 and 22+ months. AF was observed commonly in patients with a medical history of AF, and was managed with appropriate medication. No clinically meaningful grade 3 or 4 toxicities were observed. Plasma α-TEA levels increased proportionally without any indication that steady state levels were achieved. α-TEA may function through enhancing pre-existing CD8+ T cell-mediated anti-tumor activity.
relapsed/refractory iNHL (n = 15 per group). Intratumoral CDX-301 25ug/kg is injected into a palpable lymph node for 9 days, followed by 2Gy local radiotherapy on day 9 and 10 to the target lymph node. To activate local DCs, poly-ICLC 2 mg is injected on day 10, 14, 17, and weekly thereafter for a total of 8 treatments.

**Results**

Exploratory endpoints include measuring induction of systemic tumor-specific immune response in pre- and post-vaccine blood and tissue samples. Using flow cytometry and CyTOF, we have confirmed that CD1c+ (BDCA1) and CD141+ (BDCA3) DCs home to treated tumors following treatment with Flt3L and T cells attain a mature effector phenotype. Tissue from initial bx is being sequenced, and candidate neoantigens being determined in silico; these neoantigens are then being synthesized and tested for potential to activate patient pre- and post-vaccination T cells.

**Conclusions**

This trial is in process.

**Trial Registration**

ClinicalTrials.gov identifier NCT01976585.

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**P11**

Preliminary safety and efficacy data for radiotherapy and PD-L1 checkpoint blockade in metastatic non-small cell lung cancer: is timing everything?

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*Journal for ImmunoTherapy of Cancer* 2016, 4(Suppl 2):P11

**Background**

Inhibition of the PD-1/PD-L1 checkpoint pathway can induce rapid and durable responses in patients with non-small cell lung cancer (NSCLC). Unfortunately the majority of patients fail to respond and there is interest in exploring combinatorial strategies to improve response rates. One such strategy is combining checkpoint inhibition with radiotherapy (RT). We report here our pre-clinical data for combining radiotherapy with PD-L1 checkpoint blockade. These data demonstrate a clear influence of the sequencing of combinatorial therapy on its efficacy. Based on these data, we have initiated a clinical trial testing sequencing strategies of radiotherapy with PD-L1 inhibition in patients with metastatic NSCLC.

**Methods**

Using syngeneic mouse tumor models, we tested the synergy of combining RT with PD-L1 inhibition and the influence of the sequencing of these therapies on efficacy. Based on this preclinical work, we have initiated a phase II clinical trial testing this combinatorial strategy with three cohorts. The three cohorts are concurrent therapy, radiotherapy followed by PD-L1 checkpoint blockade, and PD-L1 blockade followed by radiotherapy. We report here the preliminary safety, efficacy, and correlative science data from interim analysis of the safety run-in for this trial.

**Results**

In studies using syngeneic mouse tumor models, we find that PD-L1 inhibition provides no added benefit to radiotherapy alone when administered after radiotherapy. Conversely, priming of the immune system with anti-PD-L1 prior to RT provides significant synergy of the combinatorial therapy. Our clinical trial has completed enrollment to the safety-run in of 6 patients. In total, 2 patients experienced grade 3 dose limiting toxicities meeting the criteria for completion of the safety-run in without the need for dose de-escalation. One patient experienced asymptomatic grade 3 lymphopenia and one patient experienced both grade 3 lymphopenia and grade 3 failure to thrive. At twelve weeks post-treatment initiation, 83% of patients experienced response or disease stability. Three patients (50%) had abscopal responses by RECIST criteria, two patients (33%) had stable disease, and one patient (17%) had progressive disease.

**Conclusions**

Pre-clinical data suggests that sequencing may be key to the success of combinatorial strategies of PD-L1 blockade and RT. The safety run-in for combining PD-L1 checkpoint inhibition with RT suggests that the combination is safe and tolerable in metastatic NSCLC. The trial will continue to accrue to evaluate different sequencing strategies for combining RT and PD-L1 checkpoint blockade. Further study is needed to evaluate the efficacy and optimal sequencing of RT + PD-L1 checkpoint blockade.

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**Combinations: Immunotherapy/Immunotherapy**

**P12**

Tissue factor is a novel oncotarget in triple negative breast cancer and BRAF-mutated melanoma for immunotherapy using a second generation ICON (L-ICON) in monotherapy and combination therapy

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*Journal for ImmunoTherapy of Cancer* 2016, 4(Suppl 2):P12

**Background**

The objective of this study is to identify tissue factor (TF) as a novel oncotarget for triple negative breast cancer (TNBC) and BRAF-mutated melanoma, both of which are very difficult to treat in clinic, and to develop a novel TF-targeting agent for immunotherapy. To achieve this goal, Hu developed a second generation TF-targeting ICON, named L-ICON, which consists of only the light chain (1-152 aa) of FVII fused to an IgG1Fc. The effects of L-ICON were evaluated as monotherapy or combination therapy with interleukin 15 (IL-15) for the malignancies.

**Methods**

TF expression was determined by immunohistochemistry or by flow cytometry. L-ICON protein (GenBank accession no. KY760097) and replication-deficient adenoviral vectors have been developed. Binding activity of L-ICON was determined. Its ADCC effect was evaluated by an ADCC effector assay and coagulation activity by FVII chromogenic activity assay. L-ICON efficacy in monotherapy and combination therapy with IL-15 was tested in mouse models of murine and human breast cancer (4 T1 and TNBC MDA-MB-231) and melanoma (B16F10 and BRAF mutated SK-Mel-28).

**Results**

TF is over-expressed on TNBC cells and the tumor neovascularature in over 85% of TNBC patients (n = 14) when using standard paraffin-embedded tumor tissues or in nearly 60% of TNBC patients (n = 157) when employing tissue microarray slides. Importantly, TF expression is not detected in normal breast tissues. L-ICON has several important improvements over its first generation ICON, including (i) more than 50% reduction in molecular mass, (ii) complete elimination of coagulation activity, (iii) stronger binding activity to TNBC and (iv) more effective as monotherapy in vivo in orthotopic and subcutaneous mouse models of
human TNBC (MDA-MB-231) and murine cancer 4 T1 (an animal stage IV human breast cancer) and B16F10. L-ICON monotherapy and combination with IL-15 were effective for the treatment of SK-Mel-28 in SCID mouse models.

Conclusions
TF is a novel biomarker and oncogarget in TNBC and BRAF- mutated melanoma. L-ICON, a novel TF-targeting ICON, was effective in monotherapy and combination therapy with IL-15 for the treatment of murine and human TNBC and melanoma in vitro and in vivo in preclinical mouse models.

Acknowledgements
This work was partly supported by a startup fund from OSUMC, a Seed Award from the OSU CCS TT Program, a Phase I L-Pilot Award from OSU CCTS via NCATS Award Number UL1TR001070 and the Dr. Ralph and Marian Falk Medical Research Trust. IL-15 was obtained from the NC1 Preclinical Repository. Z.H. is the inventor of L-ICON and its uses (US Patent Application # 62/082,891).

P13
Beta-adrenergic blockade improves the immunotherapeutic response to melanoma
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Journal for Immunotherapy of Cancer 2016, 4(Suppl 2):P13

Background
Recent developments in immunotherapy have made enormous strides towards expanding the scope of cancer treatment by targeting a patient's own immune cells. Despite these advances, malignant melanoma remains a significant clinical issue with a high proportion of patients remaining unresponsive to therapy and improved, but still low, complete response rates. The body's response to stress is closely integrated with the immune response, yet few cancer treatment strategies account for the relationship between these biological systems. When the stress response is activated, neurotransmitters, including norepinephrine, which bind β-adrenergic receptors (BARs) located on the surface of immune cells, are released, leading to regulation of various immune cell functions. BAR signaling can be prevented pharmacologically with BAR antagonists (β-blockers) and considerable literature suggests that these drugs, which are commonly prescribed for other indications including hypertension, are associated with positive outcomes in cancer patients. We examined the effects of BAR blockade on the efficacy of two immunotherapies approved to treat metastatic melanoma: IL-2, which promotes T cell proliferation and anti-PD-1, which impacts T cell activation.

Methods
C57BL/6 J mice with established B16.F10 melanomas were treated with β-blockers and immunotherapy (anti-PD-1, IL-2 or anti-PD-1/IL-2) and tumor growth was monitored throughout each treatment regimen. The accumulation of immune cells within the tumors and lymphoid tissues were evaluated by flow cytometry at multiple time points following treatment.

Results
Blockade of BAR signaling beginning after tumors were established had no significant impact on tumor growth. In contrast, attenuation of tumor growth by each immune-based therapy was improved in the presence of β-blockers. We observed significantly extended survival in mice treated with anti-PD-1 or anti-PD-1/IL-2 combined with β-blockers compared to immunotherapy only mice. Most importantly, the combination of β-blockers, anti-PD-1 and IL-2 produced a highly significant delay in tumor growth and prolonged survival compared to anti-PD-1/IL-2 without β-blockers.

Conclusions
Blocking BAR signaling improved the efficacy of at least two types of immunotherapy, but was most effective when administered with dual-immunotherapy. We suggest that each therapeutic component may improve a unique aspect of the immune response to maximally delay melanoma progression. Due to the availability of all three components for use in humans, this therapeutic regimen can potentially be clinically translated to expand the population of metastatic melanoma patients who experience long term benefits from immune-based therapies.

Acknowledgements
This work was supported by CURE Grant SAP #4100072562 (Pennsylvania Department of Health) and NIH/NCI S T22 CA60395 (KMK). IL-2 was generously provided by Prometheus Laboratories Inc.
Novel IL-2/mAb complexes mediate potent anti-tumor immunity which is augmented with anti-PD-1 mAb therapy

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Journal for ImmunoTherapy of Cancer 2016, 4(Suppl 2):P15

Background
Recent success and FDA approval of immune checkpoint inhibitors (CI) in a growing number of cancers are transforming cancer treatment and revitalizing interest in immunotherapies. However, while efficacy is observed in patients with advanced metastatic diseases treated with CI, not all patients respond and most responses are incomplete. Preclinical studies suggest that combinations of additional modalities will provide opportunities to improve patient responses. As both IL-2 and CI therapy can independently augment anti-tumor immunity in patients, likely in mechanistically distinct ways, we hypothesized we could improve anti-tumor immunity by combining IL-2 and anti-PD-1 mAb therapy.

Methods
To improve IL-2 efficacy and therapeutic index, we generated novel anti-IL-2 mAbs which, when complexed with IL-2 (IL-2/mAb) offer advantages over standard IL-2 therapy [1-3]. First, binding to an anti-IL-2 mAb increases IL-2 half-life and biological activity. Second, depending on the epitope at which the mAb binds to IL-2, antibody binding can modulate which IL-2 receptor subunits (alpha, beta, or gamma) are engaged. Antibodies that interfere with binding of IL-2Ra can reduce activation of high IL-2Ra-expressing cell types, such as suppressive Tregs, and steer activity toward cell types expressing only IL-2Rβ and γ. In this way, these complexes may have more effective anti-tumor activity [1-3]. We screened antibody phage libraries to identify antibodies that shift IL-2 receptor binding and activity differentially on different cell types in vitro and in vivo. Complexes of these antibodies were tested in vivo for their effects on T cell frequency and activation, and in a subcutaneous Lewis lung carcinoma model for their ability to mediate anti-tumor immunity, both alone and in combination with anti-PD-1 mAb.

Results
In normal mice, IL-2/mAb complexes potently expanded CD8+ T cells and NK cells with minimal expansion of Tregs. As single agent therapy, IL-2/mAb complexes or anti-PD-1 mAb reduced tumor growth, although most mice succumb to tumor growth eventually. Combination of IL-2/mAb complexes with anti-PD-1 mAb therapy resulted in durable, complete responses in nearly half of the mice.

Conclusions
While immune based therapies such as anti-PD-1 mAb can be highly effective in select patients, even in those patients that obtain clinical benefit, disease may recur. Our results suggest that the addition of IL-2/mAb complexes to therapy with anti-PD-1 mAb could broadly increase the percentage of patients deriving benefit from immune-based therapy.

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C15 The combination of an IL-15/IL-15Ralpha complex (ALT-803) and anti-PD-1 mAb leads to superior anti-tumor immunity in a murine lung tumor model

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Journal for ImmunoTherapy of Cancer 2016, 4(Suppl 2):P16

Background
Administration of antibodies that block the PD-1/PD-L1 pathway has demonstrated unprecedented success in mediating clinical responses in patients with advanced cancer. These antibodies are thought to act by blocking the ability of PD-L1 to mediate an inhibitory signal to PD-1 expressing T cells during antigen-recognition. These antibodies are now FDA-approved for multiple cancers including non-small cell lung cancer (NSCLC) in patients with disease that has progressed during or after platinum-based chemotherapy. In these patients, one in five patients can attain a clinical response while on checkpoint inhibitor therapy. While promising, this therapy fails to induce a durable clinical response in most patients. To overcome this limitation, we hypothesized that combinatorial therapy with anti-PD-1 mAb and a lymphocyte growth factor would more effectively augment the expansion of tumor-reactive lymphocytes. This would also provide a means to not only remove inhibitory pathways but directly augment the function of tumor-reactive lymphocytes. We chose to use an IL-15/IL-15Rα complex (ALT-803) composed of an IL-15 mutant (N72D) that was pre-associated with the soluble IL-15Rα/Fc fusion protein. This superagonist complex has been shown to potently expand and activate CD8+ T cells and NK cells in various animal models.

Methods
To assess the efficacy of combination therapy, we injected C57BL/6 mice subcutaneously with Lewis lung carcinoma. Mice with established tumors were treated with anti-PD-1 mAb and/or IL-15/IL-15Rα complex, and we monitored tumor progression and changes in immune cell populations in the periphery and tumor.
Results
The combination of anti-PD-1 mAb and the IL-15/IL-15Ra complex was substantially more effective at inducing complete responses compared with administration of either agent alone. Effective therapy was associated with the expansion of CD8+ T cells and NK cells, and the acquisition of the ability of CD8+ T cells to produce IFNγ after activation. Interestingly, in vitro, IFNγ led to upregulation of both MHC and PD-L1 on tumor cells, suggesting a mechanistic basis for the improved efficacy of the combination therapy.

Conclusions
Our results suggest that the efficacy of anti-PD-1 mAb therapy may be improved by co-administration of the IL-15/IL-15Ra complex. Our results also suggest a mechanistic basis for why the combination may be superior to single agent therapy. To determine if this combination would be of value in human patients, we have initiated a phase Ib/II clinical trial (NCT02523469) to assess the combination of anti-PD-1 mAb (nivolumab) in combination with ALT-803 in patients with refractory advanced NSCLC.

P17
Functional dichotomy of PI3K isoforms in CD4 T cells provides a strategy for selectively targeting regulatory T cells to enhance anti-tumor immunotherapy
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Journal for ImmunoTherapy of Cancer 2016, 4(Suppl 2):P17

Background
The PI3K-Akt signaling pathway modulates diverse biological responses including signaling, proliferation and survival of T cells. The identification of a signaling pathway, which differentially regulates regulatory T cells (Tregs) and conventional T cells (Tconvs), is crucial for selectively modulating these two subsets. The differential role of class IA PI3K isoform in regulating the survival and apoptosis of Tregs and Tconvs has not been elucidated yet.

Methods
For in vitro experiments sorted Tregs and Tconvs were labeled with CellTrace™ Violet Cell Proliferation stain (VCT) according to the manufacturer’s protocol (Life Technologies, NY). Cells were stimulated with and without inhibitors. For in vivo experiments C57BL/6 Mice were injected subcutaneously (s.c.) with TC-1 tumor cells and monitored for development of tumors. Vaccine was given weekly s.c. For therapeutic experiments vaccine was given weekly throughout the experiment. CAL-101 treatment was provided on the day when tumor size reached 3-4 mm 5-6 day before vaccination.

Results
Here, we report that PI3Kd is sufficient for TCR downstream signaling, proliferation, and survival for either Tconvs or Tregs. In Tregs, however, PI3Kδ is a dominant isoform, where Tregs are fully dependent on PI3Kδ to regulate these properties as PI3Kα and PI3Kβ do not play any role in these biologic processes. On the other hand, in Tconvs, the two other isoforms, PI3Kα and PI3Kβ combined, provide redundant pathway to PI3Kδ in the regulation of TCR signaling, proliferation and survival. This redundant role provided by PI3Kα and PI3Kβ isoforms to PI3Kδ in Tconvs offers a selective therapeutic approach to inhibit Tregs, where by inhibiting PI3Kδ, signaling, proliferation, and survival are inhibited in Tregs, while PI3Kα and PI3Kβ, will provide a path for Tconvs to proliferate and function.

Importantly, we demonstrate that our findings translate to therapeutic efficacy in vivo, where the inhibition of PI3Kδ, enhanced anti-tumor efficacy of antigen-specific vaccine by decreasing the suppressive Tregs and increasing the number of vaccine-induced CD8+ T cells, thus showing synergetic therapeutic effect against tumors. Our findings provide a strategy for the selective targeting of Tregs in the frame of cancer combination immunotherapy.

Conclusions
These findings provide a new insight into CD4 T cell biology and offer a new strategy for selective targeting of Tregs in the frame of development of anti-cancer immunotherapies.
Acknowledgements
Funding supported by the Merck Investigator Studies Program.

Trial Registration
ClinicalTrials.gov identifier: NCT02586207

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Diet, Exercise and/or Stress and Impact on the Immune System

P19
Exercise training reduces splenic accumulation of MDSCs and delays tumor progression in a therapeutic breast cancer model

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Journal for ImmunoTherapy of Cancer 2016, 4(Suppl 2):P19

Background
Epidemiological studies show a correlation between physical activity and cancer-related mortality [1]. However, the contribution of immune mediated anti-tumor immunity to the beneficial effects of exercise has yet to be defined [2]. We sought to investigate if forced running would have a therapeutic benefit in mice bearing a poorly immunogenic breast cancer and investigate the immunological changes occurring in response to exercise.

Methods
On day 0 Balb/c mice were inoculated with 4 T1 breast cancer cells subcutaneously in the right flank (n = 6/group). Starting on day 7, once tumors were palpable, mice were subjected to 30 minutes of forced treadmill running (18 cm/sec) five days per week. Control mice remained sedentary throughout the study. Analysis of immune cells in spleen and tumor was performed at day 17 and 32 and spontaneous lung metastases were evaluated at day 32.

Results
We observed a significantly delayed primary tumor growth (tumor volume on day 31: 1167 ± 174 mm3 in sedentary versus 847 ± 124 mm3 in exercised mice, p < 0.01) and a tendency for reduced metastatic burden in the lungs of exercised compared to sedentary mice. The progressive marked increase in myeloid-derived suppressor cells (MDSCs) and splenomegaly seen in sedentary 4 T1 tumor-bearing mice was less pronounced in exercised mice. This difference was significant on day 17; with spleen weight (520 ± 110 mg in sedentary versus 330 ± 30 mg in exercised mice, p < 0.01) and MDSC frequency in spleen leukocytes (22.7 ± 2.6% in sedentary versus 14.3 ± 2.7% in exercised mice, p < 0.001) were significantly lower in exercised mice compared to sedentary mice. Furthermore, on day 32, the CD8+ T cell/Treg and CD8+ T cell/MDSC ratio showed a tendency to increase in tumors from exercised mice.

Conclusions
Our data demonstrate that exercise can slow tumor progression in a therapeutic setting. While the mechanisms of this effect require further investigation, the observed decrease in the...
The proportion of immunosuppressive immune cells in spleen and tumor of exercised mice is likely to play a role. Importantly, the ability of exercise to reduce immunosuppression locally and systematically supports testing exercise in combination with immunotherapy as a therapeutic modality that can increase responses without increasing toxicity.

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Not Listed – Other

P20 Systemic immunotherapeutic efficacy of an immunocytokine, NHS-muIL12, in a superficial murine orthotopic bladder cancer model
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Journal for ImmunoTherapy of Cancer 2016, 4(Suppl 2):P20

Background
Interleukin-12 is one of the most powerful proinflammatory cytokines capable of supporting T and NK cell function, inducing IFNγ while driving a Th1 adaptive immune response. Its success as an antitumor agent in preclinical models has yet to be realized in a clinical setting due to systemic toxicity. An IL-12 delivery system has been developed to maximize deposition of the cytokine directly in the tumor microenvironment (TME), while mitigating the dose-limiting systemic effects.

Methods
NHS-IL12 is a novel immunocytokine, consisting of two molecules of human or murine IL-12 fused to a tumor necrosis-targeting human IgG1 (NHS76). NHS76 recognizes exposed chromatin-DNA found in necrotic human/murine tumors. Previous studies have shown selective tumor uptake of NHS-IL12 in necrotic subcutaneous murine tumors. Urothelial bladder cancer is known to respond favorably to immunotherapeutic agents due to many somatic mutations and TILs, and response to Bacillus Calmette-Guerin (BCG).

Results
We evaluated the use of NHS-muIL12 in a murine orthotopic bladder cancer model. MB49Luc cells, instilled into the bladder form superficial, multifocal tumors which can be monitored with a luciferase-based intravitral imaging system. NHS-muIL12 is a very potent anti-tumor agent in both MB49 tumor models, reducing tumor volume in a dose-dependent manner. In the intravesical bladder model, antitumor effects were seen at 2.5 mg/kg administered as three separate systemic injections. Mice were cured of tumor when treated at 20 mg/kgx3 NHS-muIL12 with durable tumor-free long-term survival. Immune analyses revealed TAA-specific CTLs and IFN-γ responses, indicating the development of a specific anti-tumor immune response. An immune memory response protected mice following re-challenge with MB49 tumor cells. Anti-tumor efficacy required CD4+ or CD8+ T cells as depletion of either abrogated the anti-tumor effects. Evaluation of TILs by FACS, revealed that NHS-muIL12 significantly reduced the number of immune suppressive cells such as MDSCs, 24 hours post-treatment, which continued to the end of the study. Immunofluorescence showed correlative treatment-related modulation of CD4+ and CD8+ T cells as well as MDSCs and Tregs within the TME. Gene expression of RNA from bladder tumors, identified various immune components with immunosuppressive or immune potentiating roles, modulated by NHS-muIL12 treatment.

Conclusions
These data support the possibility that NHS-muIL12 abrogates an immune-suppressive response within the TME, which might permit T cells to execute their antitumor effects. NHS-huIL12 (MSB0010360N; M9241), is currently being evaluated against solid tumors in a phase I clinical trial (NCT01417546).

Acknowledgements
We acknowledge the kind contribution of NHS-muIL12 from EMD Serono, Billerica, MA.

Therapeutic Cancer Vaccines

P21 Intracellular trafficking of self-assembled immune signals
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Journal for ImmunoTherapy of Cancer 2016, 4(Suppl 2):P21

Background
We recently exploited electrostatic interaction to design self-assembling nanostructures comprised entirely from peptide antigens and toll-like receptor (TLR) agonists as adjuvants. These materials simplify vaccine composition and exhibit unique properties such as direct control over the absolute and relative concentrations of each component and co-delivery of the signals to antigen presenting cells. In pre-clinical models of melanoma, this approach leads to significantly enhanced anti-tumor immunity. Here, we study how the physicochemical features (e.g., peptide charge) and relative concentration of each component impact the internalization, trafficking, and processing of the immune signals in antigen presenting cells.

Methods
FITC-labeled SIINFEKL peptide was modified with three or nine arginines, for use as a cationic anchor to support self-assembly with a polyanionic nucleic acid-based TLR3 agonist, polyIC. Hollow capsules built from these signals were synthesized by coating a sacrificial CaCO3 core with alternating layers of modified SIINFEKL and PolyIC. After deposition, the core was removed using EDTA and capsules were washed with buffer, resulting in stable capsules formed from immune signals. Capsule size was determined by image analysis and component loading levels were determined by fluorimetry using FITC-labeled peptide and Cy5-labeled TLRa. Stability studies were carried out by incubating capsules in media as a function of different pH and ionic strengths. For uptake and trafficking studies, murine splenocytes were isolated and treated with different concentrations of capsules. Cells were analyzed by flow cytometry and imaging in the presence or absence of inhibitors of endocytic processes and during staining with markers for surface proteins and intracellular organelles.

Results
Capsules loaded with FITC-SIINFEKL-R9 and PolyIC were 1-2 μm in diameter and exhibited similar size and shape for 2 weeks when in buffer. These materials exhibited tunable loading with a composition of 15.5% peptide and 84.5% TLRa used for trafficking studies. FITC-SIINFEKL-R9 and PolyIC capsules were efficiently internalized through energy dependent processes (i.e., endocytosis) when incubated with primary dendritic cells within 1 hour of treatment. These effects were also found to be dose-dependent and did not impact viability of treated cells.

Conclusions
Initial studies reveal capsules comprised of FITC-SIIN-R9 and PolyIC are uptaken by primary immune cells quickly and effectively. Ongoing studies will assess the uptake of capsules by endocytosis in the presence of inhibitors to decipher the endocytic pathway and trafficking of capsules through lysosomes and endosomes.
Acknowledgements
This work was supported in part by NSF CAREER # 1351688 and Alliance for Cancer Gene Therapy # 15051543.

P22
Analysis of B and T cell responses in non-small cell lung cancer (NSCLC) patients enrolled in a phase II trial of cyclophosphamide with allogenic DRibble vaccine (DPV-001)

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Journal for ImmunoTherapy of Cancer 2016, 4(Suppl 2):P22

Background
DRibble vaccines are microvesicles derived from proteasome-blocked autophagosomes. The DPV-001 DRibble vaccine is derived from an adenocarcinoma and a mixed histology cancer cell line. By mass spectrometry they contain more than 130 potential NSCLC antigens, many as prospective altered-epitope ligands, which could intensify their immunogenicity. In preclinical models, DRibble immunotherapy provided significant cross-protection against 8 of 9 tumors tested. Additionally, Dribble vaccines are effective in treating established tumors in preclinical combination immunotherapy models. We hypothesize that the efficacy of DRibbles’ vaccination can be attributed to their capacity to present tumor-derived short-lived proteins (SLiPs) and defective ribosomal products (DRiPs) that are typically not processed and presented by professional antigen presenting cells. These SLiPs and DRiPs embody a prospective pool of tumor antigens against which the host may be less tolerant.

Methods
Thirteen definitively-treated stage III NSCLC patients were vaccinated at 3-week intervals. Patients were randomized such that some patients’ intradermal vaccines were combined with administration of imiquimod or GM-CSF as an adjuvant. PBMCs and serum were collected at baseline and at each vaccination. For one patient, PBMCs from the baseline visit and week 12 were tested against that patient’s autologous tumor cell line to measure increased tumor specific T cell activation. Studies are currently underway to evaluate changes in TCR repertoires. CD4+ and CD8+ T cells from multiple time points were sorted and TCR sequencing is being performed to look at alterations in the T cell repertoire. The primary outcome measure of this clinical trial was to discover if vaccine alone, vaccine plus imiquimod, or vaccine plus GM-CSF generated the greatest number of strong antibody response.

Serum from the baseline visit and week 12 was analyzed for increased antibody response to >9000 human proteins using ProtoArrays and Microsphere Affinity Proteomics. Where sufficient tumor was available, whole exome sequencing was done to evaluate whether antibody and T cell responses were directed to mutations, altered peptide ligands or overexpressed normal proteins.

Results
Compared to vaccination alone or vaccination with GM-CSF, vaccination with DPV-001 plus imiquimod significantly (p < 0.05) increased the number of antibody responses that were four-fold higher at week twelve. In the one patient where autologous tumor was available, vaccination increased the tumor-specific release of TNF-alpha by peripheral blood CD4 T cells.

Conclusions
Based on these studies, future trials will combine the adjuvant imiquimod with DRibble vaccine. Trial Registration ClinicalTrials.gov identifier: NCT01909752

P32
An open-label phase I/IIa escalating dose study to evaluate safety and T cell immunogenicity of PDS0101 in subjects with cervical intraepithelial neoplasia (CIN) and high-risk HPV infection

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Journal for ImmunoTherapy of Cancer 2016, 4(Suppl 2):P32

Background
Current HPV vaccines are effective at preventing infection. However, there are no therapeutic vaccines to treat the infection or commonly associated diseases e.g. CIN, cervical, anal and oral cancers. A therapy that is simple, effective and safe enough to be administered to CIN and early-stage cancer patients could be important in achieving the goal of effective cancer prevention and treatment of pre-metastatic cancer. We assessed whether PDS0101, a combination of modified multi-epitope HPV16 peptides (HPVmix) and escalating doses of the synthetic Versamune® T cell activating platform could facilitate antigen cross-presentation and safe immune activation leading to strong HPV-specific CD8+ T cell induction in CIN.

Methods
Safety and immunogenicity were assessed in an open label dose escalation study. Groups of 3-6 subjects received either low dose (1 mg), medium dose (3 mg) or high dose (10 mg) of Versamune® cationic lipid with 2.4 mg of HPVmix. Each subject received one SC dose every 3 weeks for a total of 3 doses. T cell response was evaluated by IFN-γ and granzyme-b ELISpot using blood drawn from the subjects pre-vaccination, 2 weeks after each vaccination and 90 days after vaccination 3. The trial is registered at ClinicalTrials.gov (number NCT02065973).

Results
No serious adverse events were reported. No IND safety reports were submitted. No subjects withdrew. Strong HPV-specific T cell responses occurred at all 3 doses, even in those subjects with low pre-vaccination T cell responses. PDS0101 vaccination led to strong T cell responses evaluated by both IFN-γ and granzyme-b ELISpot. Conclusions: PDS0101 is safe and effectively performs antigen cross-presentation as demonstrated by HPV-specific T cell responses, including inducing active cytolytic T cells. Clinical benefit in CIN2/3 and cancer will be evaluated in larger phase II trials.

Trial Registration ClinicalTrials.gov identifier: NCT02065973
Tumor Microenvironment

**P24**

Effects of TLR7 agonist imiquimod (IMQ) onto BCC of the skin application dynamic changes associated with ICR in breast cancer and to clinical trial of the combination of IMQ and radiotherapy (RT), to de- serial FNA tumor biopsies from breast cancer patients treated on a injection (ICR) preceding complete remission as shown in a random- 

**Background**

Application of TLR7 activator imiquimod (IMQ) onto BCC of the skin

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**Journal for ImmunoTherapy of Cancer 2016, 4(Suppl 2):P24**

**Conclusions**

ICR signature in tumors before IMQ-RT treatment is positively correlated with complete local response, which validates the ICR hypothesis in metastatic breast cancer. Systemic response consistent with induction and/or boosting of adaptive immunity is predicted by significant enrichment of immune signature.

**Acknowledgements**

1IROICA161891

**Trial Registration**

ClinicalTrials.gov identifier: NCT01421017

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3. P25

Immunoscore as a prognostic marker in stage I-III colon cancer: results of a SITC-led global validation study

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**Journal for ImmunoTherapy of Cancer 2016, 4(Suppl 2):P25**

**Background**

Increasing evidence has illustrated that enhanced lymphocytic infiltration is a powerful prognostic marker in colon cancer (CC). The Immunoscore (IM) methodology was developed as a standardized assay to quantify the in situ immune cell infiltrate.

**Methods**

The Society for Immunotherapy of Cancer (SITC) led an international consortium, initiated with 23 expert centers from 17 countries, to evaluate the Immunoscore in routine clinical settings. CC patients (pts) stages II/III with no prior neo-adjuvant treatment were included in this study. Overall, 3855 pts split into a training set (TS), internal validation set (IVS), and external validation set (EVS) were quantified for IM using...
immunohistochemistry with CD3/CD8 antibodies and digital pathology quantification of whole slide sections. All statistical analyses were pre-defined and performed by external statisticians. The primary endpoint was time-to-recurrence (TTR); multivariate analyses were performed using Cox models adjusted for IM, age, gender, T-stage, N-stage, and stratified by participating center.

Results
Across centers, the median recurrent follow-up was 126.6 months. Pt characteristics: 51.5% male, median age 69 years, and 17%/54%/29% stage I/II/III, respectively. Among pts with stages I-III CC in the TS, TTR was shorter among 152 pts (22%) with Low-IM CC vs. 548 pts with High-IM CC (HR [95% CI], 0.41 [0.28-0.61]; P < 0.0001). In the WS, TTR was also shorter among 155 pts with Low-IM CC vs. 481 pts with High-IM CC (0.41 [0.27-0.65]; P < 0.0001). In the EVS, TTR was also shorter among 225 pts with Low-IM CC vs. 744 pts with High-IM CC (0.51 [0.38-0.68]; P < 0.0001). These results were independent of age, sex, tumor stage, and sidedness. Among secondary objectives, Immunoscore groups (High, Int, Low) predicted time to recurrence in the TS (HR[1.0-1.37]; P < 0.0001), IVS (0.27 [0.14-0.53]; P < 0.0001), and EVS (0.33 [0.22-0.49]; P < 0.0001). In stage II CC pts (1433), the difference in TTR was significant between the Low and High-Immunoscore groups (0.36 [0.23-0.56]; P < 0.0001). In multivariate models, Immunoscore grouping (2, 3, or 5) was significant (C-index : 0.73 [0.66-0.80], all P < 0.0001). Multivariate models including MSI and sidedness were performed and will also be presented. Reproducibility of the IM assay was validated across centers.

Conclusions
The primary and secondary endpoints of the global Immunoscore study were reached. Overall, TTR was significantly longer in pts with stages I-III CC defined as High-IM. Moreover, a subgroup of patients with high-risk stage II CC was also identified by Low-IM.

Acknowledgements
This initiative was supported by a variety of sources, including funding from Definiens, Prometheus, and a grant from the Czech Ministry of Health, 15-28188A and League against cancer.

P26
Defining critical features of the immune microenvironment in melanoma
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Journal for ImmunoTherapy of Cancer 2016, 4(Suppl 2):P26

Background
Precise biomarkers are urgently needed to characterize the tumor immune microenvironment, both for prognostication and to predict the benefit of immuno-therapeutic intervention. HLA-DR on tumor cells and Ki67 on cytotoxic (CD8+) T cells have been proposed as biomarkers of anti-PD1 activity. Multiplex immunohistochemistry (mIHC) allows for automated quantitation of phenotypes and spatial distributions of immune cell populations within formalin fixed paraffin embedded (FFPE) tissues.

Methods
In order to test whether mIHC can better characterize the tumor immune microenvironment, we screened databases at the Herbert Irving Cancer Center (HICC) at Columbia University for early stage melanoma patients with available FFPE primary melanoma tissue and documented clinical follow up. We identified a preliminary population of 31 stage II-III melanoma patients diagnosed between 2000 and 2012, with characteristics shown in Fig. 9 for whom pathology from the primary biopsy was shown. Clinical follow up was available on 18 patients of whom 9 patients were alive with no evidence of recurrence, 1 had died of another malignancy, and 7 had died of melanoma. 15 patients had more than 24 months of survival information available but no detailed clinical information. 5 μm slides from either the primary biopsy or subsequent wide local excision procedure were stained using Opal multiplex IHC for DAPI, CD3 (LN10, Leica), CD8 (4B11, Leica), CD68 (KP1, Biogenex), SOX10 (BC34, Biocare), HLA-DR (LN-3, Abcam) and Ki67 (MB1, Abcam). Cell phenotypes within representative fields pre-selected by a trained dermato-pathologist and were visualized using the Mantra quantitative pathology workstation (Perkin Elmer), and analysis of spatial distribution of CD3 + CD8+ cells analyzed as shown in Figs. 10 and 11 using inForm® image analysis software (Perkin Elmer), and Spotfire software (TIBCO).

Results
CD3 + CD8+ cells are closer to both tumor (SOX10+) and CD68+ cells when they express HLA-DR (p < 0.001). Conversely, CD3 + CD8+ cells are significantly farther from Sox10+ cells when they express Ki-67. Among patients with clinical follow up, CD3 + CD8+ cells in non-recurrent patients were closer to SOX10+HLA-DR+ cells than they were in recurrent patients (p < 0.001).

Conclusions
If proximity is a surrogate for interaction, these data may indicate that HLA-DR expression enhances interaction with T cells for both CD68+ infiltrating cells and Sox10+ tumor cells. In addition, CD3+CD8+ cells were closer to SOX10+HLA-DR+ cells in patients who did not recur, which is interesting in light of recent data showing that expression of HLA-DR by tumor cells increases likelihood of response to anti-PD1. Further staining and analysis of annotated tumor samples from the complete HICCC cohort 2000-2012 is ongoing and results will be updated at time of presentation.

Fig. 9 (abstract P26). Demographic Characteristics of Melanoma
Imprime is a soluble, intravenously (iv) administered β-glucan PAMP (pathogen-associated molecular pattern). As a PAMP, Imprime triggers innate immune function, including direct tumor killing, repolarization of the immunosuppressive tumor microenvironment (flipping immunosuppressive M2 macrophages to an anti-tumor M1 state), and T cell expansion and activation via dendritic cell maturation and antigen presentation. Clinically, Imprime has demonstrated promising efficacy in clinical trials when combined with tumor-targeting or anti-angiogenic antibodies. Phase II studies with pembrolizumab are starting in both metastatic triple negative breast cancer and metastatic melanoma. Herein, we have employed multiplexed immunofluorescence to profile the immune microenvironment in preclinical tumor tissues.

**Methods**

The B16F10 experimental metastasis model was used to interrogate Imprime’s anti-tumor activity in vivo. B16F10 melanoma cells were injected into the tail vein of syngeneic C57BL/6 mice, seeding the lungs with B16 foci. Outgrowth of these metastatic foci was assessed after treatment with the tumor-targeting anti-tryp1 antibody TA99, Imprime, or the combination. At various times post tumor injection, lungs were examined via multiplexed immunofluorescence (IFC) for markers of immune infiltration and activation. IFC was performed using 7-color staining (Opal technology, PerkinElmer) combined with in situ hybridization (RNA-Scope, AC). Images were acquired with the Vectra3 multispectral imaging system and cells segmented using Inform (PerkinElmer). Imaging data were transformed into “fcs” files and analyzed using Flowjo flow cytometry software (Treestar). Relational parameters such as immune cell clustering and tumor infiltration were performed via custom algorithms in R.

**Results**

TA99 alone suppressed the outgrowth of B16 lung metastases by 54% when compared to vehicle treatment. The combination of Imprime with TA99 reduced the number of metastases even more profoundly (96% vs vehicle). IFC analyses showed that Imprime specifically accumulates in the tumor stroma, binds to macrophages and elicits increased iNOS production, indicating the re-polarization of these macrophages to a more M1-like, inflammatory state. Imprime-treatment also triggered the formation of large immune cell clusters, possibly representing resolved tumor nests or the establishment of tertiary lymphoid tissues, both of which have been identified as predictors of successful anti-tumor immune responses. Finally, Imprime treatment and localization at the tumor site corresponds with substantial upregulation of the gene M1x-1 a type 1 interferon-responsive gene.

**Conclusions**

Imprime is a potent immunomodulator that induces a coordinated immune attack in vivo demonstrated by immune cell binding, M1 re-polarization and a type-1 interferon signature that coincides with reduced outgrowth of established lung metastases.
Methods
Transgenic C57BL/6-PTEN(fl/fl) mice were injected with a retrovirus expressing PDGFb and cre recombinase, inducing tumorigenesis as previously described. In this model, with a median survival of 80 days post-tumor induction (D80), convergence to a stereotyped subset of genomic rearrangements occurs by approximately D35. Intracranial osmotic pumps filled with mDX400 or isotype control antibody solution were implanted at the tumor site for 14-day windows spanning (D28-D42) or following (D42-D56) this developmental transition, then removed. Tumor burden was monitored by bioluminescence (luciferase reporter), and mice were sacrificed upon presentation of tumor-related morbidity. Tissue was formalin-fixed for histopathology and cryopreserved for gene expression analysis.

Results
During both treatment windows, tumor burden decreased differentially in mDX400-treated mice. While survival time between mDX400- and isotype-treated mice was nearly identical for D28-D42 (both median D70), for mice treated between D42-D56, median survival differed (D88 vs. D68), but without statistical significance between the groups (p = 0.25). Interestingly, the D42-D56 mDX400 group produced several "long-term survivors", who lived up to 158 days with stable tumor burden. While substantial T cell infiltration was detected in the end-stage tumors of both mDX400- and isotype-treated mice by immunohistochemistry (CD3e), expression of immune signaling pathways (e.g., Fc receptor and Toll-like receptor families, phagosome/lysosome components), was significantly higher among three long-surviving mDX400-treated mice than in three isotype-treated mice.

Conclusions
Our pilot study of mDX400 administration by CED identified an impact on tumor burden during and following therapy, but a lack of survival benefit for D28-D42 treatment. While additional experiments are needed to statistically evaluate survival benefit for the later treatment window, differentially high intratumoral expression of genes reflecting immune activation among mDX400-treated, long-surviving mice demonstrates that molecular study in this model may elucidate intratumoral conditions associated with response to anti-PD1 blockade in glioma.

Acknowledgements
This pre-clinical study is supported by Merck & Co. Investigator-Initiated Sponsored Projects grant LKR146174.