# HHS Public Access

Author manuscript

*Cancer Immunol Res*. Author manuscript; available in PMC 2019 November 15.

Published in final edited form as:

*Cancer Immunol Res*. 2018 February ; 6(2): 189–200. doi:10.1158/2326-6066.CIR-17-0356.

# Robust antitumor responses result from local chemotherapy and CTLA-4 blockade

**Charlotte E. Ariyan**[1], **Mary Sue. Brady**[1], **Robert H. Siegelbaum**[2], **Jian Hu**[1], **Danielle M. Bello**[1], **Jamie Green**[1], **Charles Fisher**[3], **Robert A. Lefkowitz**[4], **Kathleen S. Panageas**[5], **Melissa Pulitzer**[7], **Marissa Vignali**[10], **Ryan Emerson**[10], **Christopher Tipton**[10], **Harlan Robins**[10], **Taha Merghoub**[6], **Jianda Yuan**[6], **Achim Jungbluth**[7], **Jorge Blando**[9], **Padmanee Sharma**[9], **Alexander Y. Rudensky**[8], **Jedd D. Wolchok**[#6,8], **James P. Allison**[#9]

[1]Department of Surgery, Memorial Sloan Kettering Cancer Center

[2]Department of Radiology, Memorial Sloan Kettering Cancer Center

[3]Department of Anesthesia, Memorial Sloan Kettering Cancer Center

[4]Department of Radiology, Memorial Sloan Kettering Cancer Center

[5]Department of Statistics, Memorial Sloan Kettering Cancer Center

[6]Department of Medicine, Memorial Sloan Kettering Cancer Center

[7]Department of Pathology, Memorial Sloan Kettering Cancer Center

[8]Department of Immunology, Memorial Sloan Kettering Cancer Center

[9]Department of Immunology, MD Anderson Cancer Center

[10]Adaptive Biotechnology

[#] These authors contributed equally to this work.

## Abstract

Clinical responses to immunotherapy have been associated with augmentation of preexisting immune responses, manifested by heightened inflammation in the tumor microenvironment. However, many tumors have a non-inflamed microenvironment, and response rates to immunotherapy in melanoma have been <50%. We approached this problem by utilizing immunotherapy (CTLA-4 blockade) combined with chemotherapy to induce local inflammation. In murine models of melanoma and prostate cancer, the combination of chemotherapy and CTLA-4 blockade induced a shift in the cellular composition of the tumor microenvironment, with infiltrating $CD8^+$ and $CD4^+$ T cells increasing the CD8/Foxp3 T-cell ratio. These changes were associated with improved survival of the mice. To translate these findings to a clinical setting, 26 patients with advanced melanoma were treated locally by isolated limb infusion with the nitrogen mustard alkylating agent melphalan followed by systemic administration of CTLA-4 blocking antibody (ipilimumab) in a phase II trial. This combination of local chemotherapy with systemic

**Corresponding Author** Charlotte Ariyan, MD, PhD, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, NY 10065, Phone: (212)-639-6280, ariyanc@mskcc.org.
JDW and JPA contributed equally to this work

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

checkpoint blockade inhibitor resulted in a response rate of 85% at 3 months (62% complete and 23% partial response rate), and a 58% progression-free survival at one year. The clinical response was associated with increased T-cell infiltration, similar to that seen in the murine models. Together, our findings suggest that local chemotherapy combined with checkpoint blockade–based immunotherapy results in a durable response to cancer therapy.

### Keywords

melanoma; immunotherapy; ipilimumab; isolated limb infusion; phase II clinical trial

## Introduction

The treatment of cancer has been transformed by the therapeutic success of "checkpoint blockade," first demonstrated using a neutralizing antibody against CTLA-4, a prototypic negative regulatory molecule that limits T-cell activation. This approach has produced durable antitumor responses in patients with melanoma, lung cancer, kidney cancer, and ovarian cancer (1, 2). Although some patients with melanoma appear to have been cured by checkpoint blockade with responses over ten years in duration, the rate of complete responses to CTLA-4 blockade remains low and the progression-free survival (PFS) is 2.8 months (1). Similarly, PD-1 blockade has a response rate of about 30%, and PFS is 6.9 months, with only 35% of patients progression-free at one year (3, 4). Combinations of CTLA-4 with PD-1 blockade have demonstrated improved response rates, yet the progression-free survival (PFS) is 11.5 months (5). Therefore, improved therapeutic strategies are still needed.

Previous work has demonstrated that certain chemotherapies require interaction with the innate and adaptive immune mechanisms for optimal response and may directly affect immune parameters. For example, the efficacy of anthracyclines and oxaliplatins partially relies on intact TLR (Toll-like receptor) signaling and signaling through SIR1α via calreticulin (6, 7). Furthermore, cyclophosphamide can partially deplete regulatory T (Treg) cells (8) whereas gemcitabine inhibits myeloid suppressor cells and enhances cross presentation (9, 10). Melphalan, widely used for cancer chemotherapy, causes reactive oxygen species (ROS)–dependent apoptosis and enhances antigen presentation and release of the IL8 and CCL2 (11, 12). Thus, we sought to augment the therapeutic response to checkpoint blockade in melanoma by creating an *in vivo* "vaccine" through combination with a chemotherapy that would induce rapid cell death and local inflammatory responses at the site of the tumor.

Supporting this idea, preclinical studies have demonstrated that the effectiveness of CTLA-4 blockade is enhanced by low-dose gemcitabine in a mesothelioma model (13) and by low-dose melphalan in a myeloma model in mice (12). However, a clinical trial of a combination of chemotherapy (dacarbazine) with CTLA-4 blockade (ipilimumab) demonstrated response rates similar to those seen with CTLA-4 blockade alone (14). On the other hand, the combination of ipilimumab phased with two different schedules of chemotherapy for non-

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

small cell lung cancer showed a PFS advantage over chemotherapy alone (15). Thus, the therapeutic utility of chemotherapy and immunotherapy combinations remains unclear.

Local delivery of chemotherapeutic agents into the tumor enables a high dose of chemotherapy without systemic side effects. In recurrent melanoma, local chemotherapy with melphalan has been used for over half a century for patients with in-transit disease, defined as metastatic disease presenting between the primary tumor and the nodal basin. This regional chemotherapy, melphalan, is delivered by isolated limb infusion or isolated limb perfusion. Although this treatment has a response rate of 50% in prospective trials, isolated limb infusion(ILI) remains a palliative treatment, as disease recurs in most patients within a year of treatment. In multiple trials, the progression-free survival after ILI is only 8 months (16, 17), and the 5-year survival is only 20% (18), as most patients die of systemic disease. It is interesting to note that the addition of tumor necrosis factor-α (TNFα) to melphalan in Europe has resulted in increased number of patients that respond, but their time-to-progression has increased only modestly, but has increased time to progression only modestly, to a median of 13 months.(19)

This study analyzed the response to local chemotherapy with melphalan in a poorly immunogenic B16 murine model of melanoma. We found that CTLA-4 blockade synergized with melphalan in this model as well as with gemcitabine in a the TRAMPC2 murine model of prostate cancer, TRAMPC2. This combination treatment resulted in a tumor environment enriched for effector $CD4^+$ and $CD8^+$ T cells. We translated this approach to the clinic in a phase II clinical trial for patients with recurrent melanoma, treating these patients with local melphalan chemotherapy via isolated limb infusion (ILI) and systemic CTLA-4 blockade. This therapeutic approach resulted in a dramatic clinical improvement, with; the progression-free survival and response rates superseding those seen with limb infusion or CTLA-4 blockade alone.

## Materials and Methods

### Cell lines and mice

The B16 cell line expressing ovalbumin was a gift from Michael Curran (MD Anderson Cancer Center., Houston TX), TRAMP C2 cells were obtained from N.M. Greenburg (Baylor College of Medicine, Houston, TX) and utilized as previously described (20). Each cell line was expanded once and a bank of the cell line was kept in liquid nitrogen until needed for an experiment. Each experiment ultilized a single aliquot from the bank. These cells were mycoplasma free but were not authenticated in the past year. C57/Bl6 mice, age 6–8 weeks, were purchased from Jackson Laboratory. Mice were bred and housed under protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Sloan Kettering Cancer Center. All animal procedures were approved and performed in accordance with the guidelines of the IACUC.

For melanoma allograft experiments, tumor cells (10,000 B16OVA cells) were injected subcutaneously in the flank of the male C57BL/6 mice 8–10 weeks of age. Tumors were measured every 3–4 days using Vernier calipers (Fisher Scientific). When tumors became palpable, melphalan (Sigma) at low dosage (1.5 mg/kg) was injected into the tumor as a

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

single dose (21). On the same day and 3, 6, and 9 days later, the mice received an intraperitoneal injection of monoclonal antibody (mAb) to mouse CTLA-4 (100 μg; clone 9H10, BioXCell) or hamster IgG isotype (100 μg, BioXCell).

For experiments with TRAMP mice, gemcitabine (Eli Lilly) at 120 mg/kg was injected intraperitoneally into mice every third day for five doses as previously described (22). Anti–mouse CTLA-4 was administered as described above. For CD8 depletion, monoclonal 2.43 (0.5 mg; BioXcell) was injected intraperitoneally 4 days prior to tumor injection and 4 and 14 days after tumor injection. 24.G2, SPAS-1 H-2D$^b$ tetramer PE (used at 1.1 μg/ml) was obtained from the MSKCC core facility.

### Phase II clinical trial

The phase II trial of systemic ipilimumab in combination with local melphalan for patients with in transit melanoma was approved by the institutional review board (IRB) at MSKCC (clinicaltrials.gov ()). This trial included patients with unresectable stage IIIB-IV melanoma with recurrent melanoma consisting of in-transit disease, defined as recurrence between the primary site and draining nodal basin, alone or in combination with distant disease. No patients had a prior limb infusion or perfusion, and the trial accrued patients from 2011–2015. Patients without history of previous immunotherapy treatment underwent an isolated limb infusion as previously described (melphalan 7.5–10 mg per liter limb volume and dactinomycin 500 μg) (23). Toxicity from limb infusion was measured by the Wieberdink toxicity scale (24). If the toxicity from limb infusion was  3, the patient was given systemic treatment with ipilimumab at 10 mg/kg x 4 doses, starting from day 7–21 after isolated limb infusion. Patients without systemic toxicity and with evidence of clinical benefit were eligible for maintenance ipilimumab (10 mg/kg every 3 months for the remaining time period of 2 years). The primary endpoint of the trial was PFS at one year. Secondary endpoints were response rates at 3 months, safety, and correlative immune studies. Response and progression were defined by the immune-related response criteria. Research biopsies and blood were taken just prior to limb infusion, after limb infusion (7–15 days) and 3 weeks after the last dose of ipilimumab. The trial was later amended to allow research blood draws with each dose of ipilimumab. Data were locked in May of 2016 to allow analysis as accrual slowed secondary to approval of systemic immunotherapies. Adverse events were graded by the Common Terminology Criteria for Adverse Events (CTCAE), version 4.

### Analysis of tumor and blood samples

Human blood and tumor were processed in the Immune Monitoring Core at MSKCC. Whole blood from patients was drawn in Vacutainer or Cell Preparation Tubes (CPT) containing sodium heparin (BD Vacutainer). Peripheral blood mononuclear cells (PBMCs) were collected after Ficoll gradient separation. Fresh tumor samples were mechanically dissociated and frozen for the analyses. Two patients had the biopsies performed while on steroids for autoimmune complications. The samples were analyzed in two batches.

Immune cell phenotypes were analyzed by staining with CD3 Pacific Blue, ICOS PE-Cy7 (eBioscience), CD4 ECD (Beckman Coulter Inc.), CD25 APC-Cy7, CD8 PerCP (BD Bioscience), and Foxp3 APC antibodies (eBioscience). Cells were analyzed on an LSR II

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Fortessa flow cytometer (BD Biosciences). Analysis was accomplished with FlowJo (version 9.1, Treestar, Inc). Isotype controls included the appropriate fluorochrome-conjugated mouse IgG1a or IgG2a (Dako). Cytokine in patient sera were analyzed by Meso Scale Discovery Th1/Th2 human 10-plex kit. The plates were measured on the MSD Sector Imager 2400 plate reader.

RNA extraction from tumors were performed using the RNeasy Tissue Kit (Qiagen) followed by quantification and QC with the Aglient 2100 Bioanalyzer. DNA extraction from PBMCs and tumors was performed using the DNeasy blood and Tissue Kit (Qiagen). region using the ImmunoSEQ™ Assay (Adaptive Biotechnologies, Seattle, WA) as previously described(25–27).

Multiplex IHC was performed on paraffin-embedded tissue, which was stained for CD4, CD8, PD-L1, and CD68. For multiplexed IF staining, the Opal protocol staining method (28) was used as reference with the following markers: CD4 (1:25, CM153BK, Biocare) with visualization using fluorescein AF-488 (1:50); CD8 (1:200, M7103, Dako) with visualization using AF-594 (1:50); CD68 (1:100, M0876, Dako) with visualization using AF-647; and PD-L1 (1:100, E1L3N, Cell Signaling Technology) with visualization using AF-555 (1:50). Nuclei were subsequently visualized with DAPI (1:2000). All of the sections were mounted for histological analysis using Vectashield Hardset 895 mounting media. A detailed methodology for multispectral analysis has been described (28). Each of the individually stained sections (CD4/AF-488, CD8/AF-594, CD68/AF-647, PD-L1/AF-555, and DAPI) were utilized to establish the spectral library of fluorophores required for multispectral analysis. The slides were scanned using the Vectra slide scanner (PerkinElmer) under fluorescent conditions. For each marker, the mean fluorescent intensity per case was then determined as a base point from which the positivity threshold and positivity calls could be established. Finally, a co-localization algorithm (Inform / PerkinElmer) was used to determine the percent of PD-L1$^+$ cells within each cellular subset (CD4$^+$, CD8$^+$, CD68$^+$, and tumor cells). Five random areas on each sample were analyzed at 20x magnification by a pathologist blinded to sample identity. MHC staining was performed using the monoclonal antibody LGII-612.14, generated by Dr. Soldano Ferrone and as previously described.(29)

## Statistical Analyses of TCR-β sequencing results

Clonality was defined as 1- Peilou’s evenness[i] and was calculated on productive rearrangements by: $1 + \frac{\sum_i^N p_i \log_2(p_i)}{\log_2(N)}$ where $p_i$ is the proportional abundance of rearrangement $i$ and $N$ is the total number of rearrangements. Clonality values range from 0 to 1 and describe the shape of the frequency distribution: clonality values approaching 0 indicate a very even distribution of frequencies, whereas values approaching 1 indicate an increasingly asymmetric distribution in which a few clones are present at high frequencies. Statistical analysis was performed in R version 3.2.

The fraction of T cells in formalin-fixed, paraffin-embedded tissue samples was calculated by normalizing TCR-β template counts to the total amount of DNA usable for TCR sequencing, where the amount of usable DNA was determined by PCR-amplification and

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

sequencing of several housekeeping genes that are expected to be present in all nucleated cells (25–27, 30).

RNA was isolated from paraffin-embedded tissue from 38 available samples of pretreatment, post limb infusion and post ipilimumab samples. Nine samples did not have enough RNA and were discarded, the remaining 29 samples were run on the nanostring system (NanoString Technologies, Seattle, WA, USA). Calculation of gene expression was performed with the nCounter technology, which allows for normalization of data in log-transformed data. Differences in expression were compared from 3 planned time points.

### Statistical Methods

In murine studies, differences between groups were examined by unpaired *t*-test (Prism) and survival by Kaplan- Meier analysis. ImmuneSEQ software (Adaptive Biotechnologies) was used to analyze TCR repertoire data. The nanostring data were analyzed with the nSOLVER program, which calculates differences in gene expression on a log2 scale, with t-statistic calculated in means of log-transformed data. The planned enrollment of the phase II clinical trial was 39 patients in a single-stage design. This cohort size gives 90% power to detect an improvement in one-year progression-free survival in patients with advanced stage melanoma from 30% to 50% at a significance level of 10%. Sixteen patients were needed to be progression free at one year to demonstrate acceptable effectiveness. By May of 2016, accrual had slowed and because we had at least 2 years follow-up on 96% of patients, we locked the data, and the trial has been closed to accrual. Survival was estimated by Kaplan-Meier analysis.

## Results

### Chemotherapy and CTLA-4 blockade improve antitumor immunity in preclinical models

Melphalan treatment can induce the release of inflammatory cytokines and apoptosis (11). We therefore explored the effect of melphalan on a broader set of immunological parameters. Melphalan treatment of B16 melanoma cells i*n vitro* increased expression of MHC class I by 2.5 fold and PD-L1 (CD274) by 7 fold, but did not affect MHC class II expression (Fig. 1A). This provided additional evidence that melphalan may lead to changes of immunogenicity within the tumor that would allow responses to immunotherapy.

We next assessed the efficacy of local chemotherapy in combination with CTLA-4 blockade induced by a mAb. Mice were injected with B16 melanoma cells expressing OVA (B16OVA). Once tumors were established, the mice were treated with a single dose of intratumoral melphalan chemotherapy, alone or combined with CTLA-4 blockade. The combination of CTLA-4 blockade and melphalan improved survival (Fig. 1B). The median survival time for CTLA-4 blockade alone was 25 days and was not reached for the combination ($P < 0.05$). Survivors in the combination therapy group of one experiment ($n =$ 4) were rechallenged with B16 tumor cells and given no further treatment; the B16 cells failed to grow tumors in 6 out of 7 rechallenged mice, whereas all 5 of control mice grew tumors, suggesting that the single combination treatment generated long-term antitumor immunity.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Immune cell phenotypes in the spleen, lymph node, and tumors were analyzed on day 12 after initiation of CTLA-4 blockade, and the tumor samples were significantly different (Fig. 1C). Tumors in the combination treatment group had fewer Foxp3$^{+}$ Treg cells (Fig. 1D) and an increase in the CD8/Treg ratio. These findings were supported by results from systemic treatment with gemcitabine and CTLA-4 blockade in TRAMP2 mice, a model of prostate tumors (Supplementary Fig. S1), where the combination of chemotherapy and CTLA-4 blockade again improved survival and resulted in improved tumor-specific immunity. To investigate the role of IFN in the generation of antitumor immunity, we utilized IFN$\gamma^{-/-}$ mice. Although the combination of melphalan and CTLA-4 blockade improved the median survival over melphan alone, no mice were cured. This suggests the importance of host IFN$\gamma$ in obtaining long-term antitumor immunity.

## Clinical benefit of local chemotherapy combined with CTLA-4 blockade

Given the improved outcomes between local melphalan and CTLA-4 blockade observed in a mouse model, we conducted a phase II trial of isolated limb infusion of melphalan and dactinomycin followed by CTLA-4 blockade in the form of systemic ipilimumab administration (Fig. 2A). The study cohort consisted of 26 patients with in-transit melanoma with or without systemic disease (Stage IIIB-IV melanoma) (Table 1; Fig. 2A). Tumors of the majority patients in this cohort were either “wild type”, defined as negative for *NRAS*, *BRAF*, and *KIT* mutations (35%), or carried an *NRAS* mutation (35%). Tumors in 15% of patients carried a *BRAF* V600E mutation. Previous treatment included surgery alone or in combination with adjuvant interferon. None of the patients had prior anti-CTLA-4 or anti-PD-1 therapy, and no patient underwent a lymph node dissection at time of infusion.

Ipilimumab (10mg/kg) was administered at an average of 11 days after limb infusion. Fourteen patients (54%) received all 4 of the planned induction doses of ipilimumab; the average number of doses was 3.

Of the 26 patients, 22 (85%) had a response in the limb, with 16 complete responses (62%) and 6 partial responses (23%) (Fig. 2B). At one year, 58% of patients were progression free. With a median follow-up of survivors of 36 months, the median PFS and median survival was not reached (Fig. 2C). Of the patients who progressed in the first year, 55% relapsed in the extremity, and 44% recurred distally. Responses occurred outside of the treated limb, and both of the evaluable patients with Stage IV disease had a complete response of at least two years’ duration; one patient had distant subcutaneous disease, and a post-treatment biopsy did not reveal any viable tumor. The response of the second patient with Stage IV disease is shown in Fig. 2D. This patient had seven metastatic lesions in the leg, nodal metastasis, and lung metastasis. After combination therapy, all disease has regressed in this patient, with a complete response of over 24 months’ duration.

Although numbers are small and therefore statistical significance cannot yet be gauged, when patients are categorized into “low” and “high” burden of disease based upon number and size of lesions, the low burden of disease had a higher response rate (16). Among the “high” burden group ($n = 7$), 57% had a complete response and 29% had partial response, whereas 14% had disease that progressed. In “low” tumor burden group ($n = 17$), 71% had a complete, and 29% had a partial response.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

We observed no increase in limb toxicity, classified by the Wieberdink scale of limb toxicity, with the combination treatment, beyond that seen with limb infusion alone (Supplementary Table S1). Treatment-related adverse events are listed in Table 2. Non-immune related events included the two deaths noted above with acute coronary syndrome and a pulmonary embolus; although these were not thought to be related to treatment, both were included in analysis of PFS. One patient (4%) had *E. coli* sepsis from a urinary tract infection (grade 4), another patient had grade 4 pneumonia, atrial fibrillation, and a retroperitoneal bleed. Immune-related adverse events included diarrhea in 74% of patients (grade 3 diarrhea in 12% of patients), colitis in 12%, and an additional pneumonia (grade 3) in one patient (4%). Four patients (15%) had long-term adrenal insufficiency.

## Combined isolated limb infusion and ipilimumab increases inflammation

Gene expression of tumors after isolated limb infusion was analyzed using NanoString and nCounter technology. This analysis revealed the upregulation of many genes related to innate and adaptive immunity and chemotaxis. For example, we observed a 12-fold increase in TREM1, an approximately 10-fold increase in CCL3L1 and CCL3, and an approximately 9-fold increase in TNFRSF10C mRNAs (Fig. 3; Table 3; Supplementary Data Table S2). In addition, mRNAs encoding several costimulatory ligands (CD80, CD86, and ICOS ligand) and costimulatory receptor ICOS increased significantly, as did the expression of a number of MHC class I and II genes (HLA-A, -B, -DPA1, -DPB1, -DRB3, -DMA, -DMB, and -DRA). After combination treatment with limb infusion and ipilimumab, gene expression analysis revealed increased expression of genes encoding cytotoxic T cell–effector molecules including granzymes (GZMA, GZMH, GZMK), interferon-γ (IFNγ), and perforin (PRF1), as well as ICOS (Table 3). Circulating cytokines were detected in the serum from patients and demonstrated a significant increase in IL8 and IL6 after isolated limb infusion, and a significant increase in IFNγ, IL17, and TNF after the ipilimumab treatment (Fig. 4A), in agreement with the gene expression analysis.

## Treatment is associated with activation of T cells and immune tumor infiltration

The observed immune activation and clinical response support the idea that the isolated limb infusion induces an inflammatory tumor microenvironment, and that subsequent CTLA-4 blockade can mobilize cytotoxic effectors. To further investigate this idea, we examined immune cell phenotypes in patients' blood and tumor samples. Flow cytometric analysis of peripheral blood leukocytes demonstrated an increase in proliferating $CD4^+$ and $CD8^+$ T cells after the first ipilimumab treatment (Fig. 4B). $CD4^+$ $ICOS^+$ cells increased by 3.9 fold compared to pretreatment after the first dose (Fig. 4C). We observed no significant change in amount of negative regulators of immune cells activation (TIM3, LAG3) on the peripheral blood mononuclear cells (Supplemental Fig. S3). We also analyzed the tumors, although the high response rate meant that many biopsies were too small to allow for flow cytometric analyses, and limited our statistical power. The $CD8^+$/Treg ratio and the percentage of ICOS $^+CD4^+$ T cells was not significantly increased after combination treatment, (Fig. 4D). Although the change in the immune phenotype was clear in the preclinical models, the small numbers of biopsies may have precluded a meaningful result. The amount of TIM3 or LAG3 on tumor cells did not change significantly.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

The tumor microenvironment in the pretreatment and post-treatment biopsies was analyzed by standard H&E staining and multiplex immunohistochemistry. As shown in Fig. 4E, F, and G, the untreated tumors had few T cells ($CD4^+$ and $CD8^+$) and little expression of inhibitory ligands such as PD-L1. However, after treatment with limb infusion and ipilimumab, tumors showed increased infiltration with both $CD4^+$ and $CD8^+$ T cells and an increase in PD-L1 expression, consistent with an increase in inflammation in the tumor as seen in preclinical models.

### T-cell receptor fraction is increased in tumors with combination treatment

To further quantify T cells in the tumor and peripheral blood, we performed high-throughput sequencing of the CDR3 variable region of TCRβ chains. In the tumors, the T-cell fraction, defined as the fraction of nucleated cells that corresponded to T cells, increased from pretreatment to post limb infusion and CTLA-4 blockade (Fig. 5A), confirming the IHC results above. Frequencies of T cells before treatment were low in all patients, and did not differ significantly between patients. Although the peripheral blood did not demonstrate much change after treatment, the frequency of T cells in the tumors increased among patients who achieved 1-year PFS, the primary endpoint of the trial (Fig. 5B), supporting the requirement for T cells in the durable response.

## Discussion

Although immunotherapy is a treatment option for melanoma, many patients still do not respond to the treatment. In this study, we demonstrated that, in a mouse model of melanoma, combining local chemotherapy with CTLA-4 blockade enhanced the inflammatory environment within the tumor mass and improved survival. In a phase II trial for patients with advanced melanoma, we showed that isolated limb infusion of melphalan and dactinomycin combined with systemic CTLA-4 blockade (ipilimumab) had high response rate, and responses were durable. The combination treatment induced a shift to an inflammatory microenvironment within the tumor and resulted in an influx of T cells.

In preclinical models, CTLA-4 blockade cures mice of immunogenic tumors, but curing less immunogenic tumors requires that CTLA-4 blockade be combined with other therapeutic modalities such as GM-CSF or Flt-3 ligand vaccine (31, 32). Consistent with these observations, we observed that CTLA-4 blockade alone had only a modest effect on the growth of the poorly immunogenic B16 melanoma and on survival of the host mice, but the combination of CTLA-4 blockade with local melphalan administration resulted in improved survival.

Our phase II clinical trial demonstrated an increased rate and durability of response with the combination of local chemotherapy and CTLA-4 blockade. The patients, who had stage IIB-IV disease, had a response rate of 85%. The one-year PFS was 58%, and median survival was not reached at 36 months of median follow-up, which compares favorably to published series demonstrating a PFS of 8 months(16, 33), or 13 months with melphalan and TNFα (19). The PFS is also better than that seen with ipilimumab alone. The PFS for ipilimumab 3 mg/kg alone is 2.9 months, and although survival does appear to be better with 10 mg/kg of ipilimumab (34), PFS remains below what we saw with the combination therapy. The

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

response rate to ipilimumab alone (10 mg/kg) in patients with unresectable Stage III disease was <10% in all prospective randomized trials (35). Therefore, our trial, although small, showed durable increase in efficacy in response to the combination of treatments, and supports further investigation of local therapies to enhance antitumor responses.

The adverse events were primarily immune-related. The two on-treatment deaths (acute coronary syndrome and pulmonary embolus) occurred in patients with prior history of cardiac disease and obesity. These comorbidities are something to consider when screening patients for this aggressive treatment. Grade 3 or 4 immune-related adverse events, were similar to those published for adjuvant ipilimumab at 10 mg/kg (36), except for an increase in endocrine dysfunction, the majority of which were grade 1–2.

As knowledge about the tumor microenvironment has expanded, so have the approaches to combinations of treatments with immunotherapy. For example, a trial of ipilimumab with GM-CSF demonstrated improved overall survival over ipilimumab alone, albeit without a difference in PFS, which was 3.1 months for both groups (37, 38). Preclinical work has demonstrated that local therapies such as radiation or cryotherapy can enhance the effects of CTLA-4 blockade (20, 39). However, despite encouraging initial reports (40), a clinical trial of 22 patients failed to demonstrate synergy of radiation with CTLA-4 blockade; only 18% of patients had a partial response (41). While we await the results of larger trials of radiation therapy, other combinations are under investigation. T-VEC is a modified herpes virus that expresses GM-CSF, and early reports of a trial of 19 patients receiving intratumoral TVEC in combination with CTLA-4 blockade demonstrated a 50% response rate and a 22% complete response rate (42). An additional combination under investigation is an IDO inhibitor with CTLA-4 blockade. This combination has been effective in preclinical models, and early results of a clinical trial are promising (43, 44). The efficacy of limb infusion and ipilimumab, with a complete response rate of 62% and a one-year PFS of 58%, validates the continued efforts to study this combination as another means to improve immunotherapy responses.

In our study, the tumors after treatment have $CD8^+$ T cells and an increased number of conventional $CD4^+$ T cells, which suggests that these cells have participate in an important role in tumor eradication. The accumulation of $CD4^+$ T cells is accompanied by an increase in IFN$\gamma$ in the patients' serum, suggesting a correlation between $CD4^+$ T cells and IFN$\gamma$. The gene expression data also suggest that the tumors have an increase in MHC II at this time, which could be a target for cytolytic $CD4^+$ T cells. Previous work has demonstrated the importance of $CD4^+$ effector T cells in responses to CTLA-4 blockade (45). In addition, patients had an increase in ICOS and ICOSL gene expression and in $CD4^+ICOS^+$ T cells. This is consistent with previous work demonstrating the power of ICOS engagement in treatment with CTLA-4 blockade and in tumor eradication (45–48). Together these results support the importance of $CD4^+$ T cells in addition to the $CD8^+$ T-cell response in the antitumor response generated by the combination of isolated limb infusion and ipilimumab.

The tumor biopsies, although limited secondary to rapid responses which precluded a tumor biopsy, demonstrated tumor inflammation by flow cytometry, multiplex IHC, and TCR sequencing. Together, the study provides evidence that an antitumor response to

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

immunotherapy combined with chemotherapy does not necessarily depend on the pretreatment tumor expressing inhibitory markers or being overtly inflamed. The starting inflammatory environments in patients in the study were similar. This contrasts with responses to immunotherapy alone, where responses have been associated with the presence of increased immune cells prior to treatment (49, 50). For example, response to PD1 blockade was associated with pre-existing PD-L1 expression and $CD8^+$ T cells in the tumors (51). In our study, the pretreatment tumors had few T cells and little PD-L1 expression, with no difference in pretreatment T cells between responding and non-responding lesions. After treatment, however, the tumors had an influx of $CD4^+$ cells and $CD8^+$ cells, as well increased PD-L1 expression. This also contrasts with previous results with CTLA-4 blockade combined with radiation, where increased PD-L1 expression was a marker of resistance (41). In our trial with local chemotherapy, PD-L1 expression increased during the antitumor response. We hypothesize the difference is the context of PD-L1 expression, which in this study is occurring in the setting of inflammation of a tumor.

The future of immunotherapy lies in combinations that would allow responses in additional tumor subtypes. As discussed above, multiple strategies, including radiation and local viral injections, are under investigation. This study provides evidence that local chemotherapy combined with CTLA-4 blockade provides a durable and rapid response in tumors that were not overtly inflamed. This suggests local chemotherapy is an additional tool to augment the immune system against cancer. Given the success of systemic combination therapy for melanoma, particularly with combination of CTLA-4 and PD-1 blockade, there will be little role for limb infusion and CTLA-4 blockade as a first line treatment. The limb infusion is more invasive, requires a hospitalization, and brings associated risks. The power of this treatment will be for patients in whom systemic immunotherapy alone has failed. Further studies will classify the results in this high-risk population, clarify the mechanism of this response, and clarify the capacity to expand this strategy to other tumor types.

In conclusion, this study provides evidence that local chemotherapy combined with CTLA-4 blockade provides a durable and rapid response. This suggests local chemotherapy is an additional tool to augment the immune system against cancer. We believe that combinations represent the future of immunotherapy, as they are a means to obtain responses in additional tumor subtypes. As discussed above, multiple strategies, including radiation and local viral injections, are under investigation. The challenges ahead include clarifying the mechanism of this response and expanding this strategy to other tumor types.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

The authors would like to honor Dr. Lloyd Old who was an early and enthusiastic supporter of this project and clinical trial, and all the patients that participated in the clinical trial. We also thank Janet Novak of Memorial Sloan Kettering Cancer Center for her editorial assistance.

Supported by the Ellen and Gary Davis Foundation, Society of Surgical Oncology Clinical Investigator Award, Cycle for Survival, Cancer Center Support Grant P30 CA008748 from the National Institutes of Health (CA, AR).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Ludwig Center at MSKCC (AR), Hilton-Ludwig Cancer Prevention Initiative Funding by the Conrad N. Hilton Foundation and Ludwig Cancer Research (AR). This study was also funded by Bristol Myers Squibb by a research grant.

## References

1. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med. 2010;363:711–23. [PubMed: 20525992]
2. Prieto PA, Yang JC, Sherry RM, Hughes MS, Kammula US, White DE, et al. CTLA-4 blockade with ipilimumab: long-term follow-up of 177 patients with metastatic melanoma. Clin Cancer Res. 2012;18:2039–47. [PubMed: 22271879]
3. Hodi F, Kluger H, Sznol M, Carvajal, Lawrence D, Atkins M, et al. Durable, long-term survival in previously treated patients with advanced melanoma (MEL) who received nivolumab (NIVO) monotherapy in a phase I trial. Proceedings of the 107th Annual Meeting of the American Association for Cancer Research; 2016 Apr 16–20; New Orleans, LA Philadelphia (PA): AACR; 2016 Abstract nr CT001. 2016.
4. Ribas A, Hamid O, Daud A, Hodi FS, Wolchok JD, Kefford R, et al. Association of Pembrolizumab With Tumor Response and Survival Among Patients With Advanced Melanoma. JAMA. 2016;315:1600–9. [PubMed: 27092830]
5. Larkin J, Hodi FS, Wolchok JD. Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. N Engl J Med. 2015;373:1270–1.
6. Ma Y, Kepp O, Ghiringhelli F, Apetoh L, Aymeric L, Locher C, et al. Chemotherapy and radiotherapy: cryptic anticancer vaccines. Seminars in immunology. 2010;22:113–24. [PubMed: 20403709]
7. Obeid M, Panaretakis T, Tesniere A, Joza N, Tufi R, Apetoh L, et al. Leveraging the immune system during chemotherapy: moving calreticulin to the cell surface converts apoptotic death from "silent" to immunogenic. Cancer research. 2007;67:7941–4. [PubMed: 17804698]
8. North RJ. Cyclophosphamide-facilitated adoptive immunotherapy of an established tumor depends on elimination of tumor-induced suppressor T cells. J Exp Med. 1982;155:1063–74. [PubMed: 6460831]
9. Suzuki E, Kapoor V, Jassar AS, Kaiser LR, Albelda SM. Gemcitabine selectively eliminates splenic Gr-1+/CD11b+ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity. Clin Cancer Res. 2005;11:6713–21. [PubMed: 16166452]
10. Nowak AK, Lake RA, Marzo AL, Scott B, Heath WR, Collins EJ, et al. Induction of tumor cell apoptosis in vivo increases tumor antigen cross-presentation, cross-priming rather than cross-tolerizing host tumor-specific CD8 T cells. J Immunol. 2003;170:4905–13. [PubMed: 12734333]
11. Dudek-Peric AM, Ferreira GB, Muchowicz A, Wouters J, Prada N, Martin S, et al. Antitumor immunity triggered by melphalan is potentiated by melanoma cell surface-associated calreticulin. Cancer Res. 2015;75:1603–14. [PubMed: 25762540]
12. Mokyr MB, Kalinichenko TV, Gorelik L, Bluestone JA. Importance of the B7–2 molecule for low dose melphalan-induced acquisition of tumor-eradicating immunity by mice bearing a large MOPC-315 tumor. J Immunol. 1998;160:1866–74. [PubMed: 9469448]
13. Lesterhuis WJ, Salmons J, Nowak AK, Rozali EN, Khong A, Dick IM, et al. Synergistic effect of CTLA-4 blockade and cancer chemotherapy in the induction of anti-tumor immunity. PLoS One. 2013;8:e61895. [PubMed: 23626745]
14. Robert C, Thomas L, Bondarenko I, O'Day S, Weber J, Garbe C, et al. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. N Engl J Med. 2011;364:2517–26. [PubMed: 21639810]
15. Lynch TJ, Bondarenko I, Luft A, Serwatowski P, Barlesi F, Chacko R, et al. Ipilimumab in combination with paclitaxel and carboplatin as first-line treatment in stage IIIB/IV non-small-cell lung cancer: results from a randomized, double-blind, multicenter phase II study. J Clin Oncol. 2012;30:2046–54. [PubMed: 22547592]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

16. Muilenburg DJ, Beasley GM, Thompson ZJ, Lee JH, Tyler DS, Zager JS. Burden of disease predicts response to isolated limb infusion with melphalan and actinomycin D in melanoma. Ann Surg Oncol. 2015;22:482–8. [PubMed: 25192683]
17. Brady MS, Brown K, Patel A, Fisher C, Marx W. A phase II trial of isolated limb infusion with melphalan and dactinomycin for regional melanoma and soft tissue sarcoma of the extremity. Ann Surg Oncol. 2006;13:1123–9. [PubMed: 16791448]
18. Raymond AK, Beasley GM, Broadwater G, Augustine CK, Padussis JC, Turley R, et al. Current trends in regional therapy for melanoma: lessons learned from 225 regional chemotherapy treatments between 1995 and 2010 at a single institution. J Am Coll Surg. 2011;213:306–16. [PubMed: 21493111]
19. Deroose JP, Eggermont AM, van Geel AN, de Wilt JH, Burger JW, Verhoef C. 20 years experience of TNF-based isolated limb perfusion for in-transit melanoma metastases: TNF dose matters. Ann Surg Oncol. 2012;19:627–35. [PubMed: 21879272]
20. Waitz R, Fasso M, Allison JP. CTLA-4 blockade synergizes with cryoablation to mediate tumor rejection. Oncoimmunology. 2012;1:544–6. [PubMed: 22754781]
21. Mokyr M, Kalinichenko T, Gorelik L, Bluestone J. Realization of the therapeutic potential of CTLA-4 blockade in low-dose chemotherapy-treated tumor bearing mice. Cancer research. 1998;58:5301–4. [PubMed: 9850053]
22. Nowak AK, Robinson BW, Lake RA. Gemcitabine exerts a selective effect on the humoral immune response: implications for combination chemo-immunotherapy. Cancer research. 2002;62:2353–8. [PubMed: 11956096]
23. Brady MS, Brown K, Patel A, Fisher C, Marx W. A phase II trial of isolated limb infusion with melphalan and dactinomycin for regional melanoma and soft tissue sarcoma of the extremity. Annals of surgical oncology. 2006;13:1123–9. [PubMed: 16791448]
24. Wieberdink J, Benckhuysen C, Braat RP, van Slooten EA, Olthuis GA. Dosimetry in isolation perfusion of the limbs by assessment of perfused tissue volume and grading of toxic tissue reactions. European journal of cancer & clinical oncology. 1982;18:905–10. [PubMed: 6891640]
25. Robins HS, Campregher PV, Srivastava SK, Wacher A, Turtle CJ, Kahsai O, et al. Comprehensive assessment of T-cell receptor beta-chain diversity in alphabeta T cells. Blood. 2009;114:4099–107. [PubMed: 19706884]
26. Carlson CS, Emerson RO, Sherwood AM, Desmarais C, Chung MW, Parsons JM, et al. Using synthetic templates to design an unbiased multiplex PCR assay. Nat Commun. 2013;4:2680. [PubMed: 24157944]
27. Robins H, Desmarais C, Matthis J, Livingston R, Andriesen J, Reijonen H, et al. Ultra-sensitive detection of rare T cell clones. J Immunol Methods. 2012;375:14–9. [PubMed: 21945395]
28. Stack EC, Wang C, Roman KA, Hoyt CC. Multiplexed immunohistochemistry, imaging, and quantitation: a review, with an assessment of Tyramide signal amplification, multispectral imaging and multiplex analysis. Methods (San Diego, Calif). 2014;70:46–58.
29. Temponi M, Kekish U, Hamby CV, Nielsen H, Marboe CC, Ferrone S. Characterization of anti-HLA class II monoclonal antibody LGII-612.14 reacting with formalin fixed tissues. J Immunol Methods. 1993;161:239–56. [PubMed: 8505553]
30. Kirsch I, Vignali M, Robins H. T-cell receptor profiling in cancer. Mol Oncol. 2015;9:2063–70. [PubMed: 26404496]
31. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. Science. 1996;271:1734–6. [PubMed: 8596936]
32. Curran MA, Allison JP. Tumor vaccines expressing flt3 ligand synergize with ctla-4 blockade to reject preimplanted tumors. Cancer research. 2009;69:7747–55. [PubMed: 19738077]
33. Turley RS, Raymond AK, Tyler DS. Regional treatment strategies for in-transit melanoma metastasis. Surg Oncol Clin N Am. 2011;20:79–103. [PubMed: 21111960]
34. Wolchok JD, Weber JS, Maio M, Neyns B, Harmankaya K, Chin K, et al. Four-year survival rates for patients with metastatic melanoma who received ipilimumab in phase II clinical trials. Ann Oncol. 2013;24:2174–80. [PubMed: 23666915]
35. Ariyan C Safety and clinical activity of combining systemic ipilimumab with isolated limb infusion. J Clin Oncol. 2014;32:abstr 9078.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

36. Eggermont AM, Chiarion-Sileni V, Grob JJ, Dummer R, Wolchok JD, Schmidt H, et al. Adjuvant ipilimumab versus placebo after complete resection of high-risk stage III melanoma (EORTC 18071): a randomised, double-blind, phase 3 trial. Lancet Oncol. 2015;16:522–30. [PubMed: 25840693]
37. Hodi FS, Lee S, McDermott DF, Rao UN, Butterfield LH, Tarhini AA, et al. Ipilimumab plus sargramostim vs ipilimumab alone for treatment of metastatic melanoma: a randomized clinical trial. JAMA. 2014;312:1744–53. [PubMed: 25369488]
38. Fong L, Kwek SS, O'Brien S, Kavanagh B, McNeel DG, Weinberg V, et al. Potentiating endogenous antitumor immunity to prostate cancer through combination immunotherapy with CTLA4 blockade and GM-CSF. Cancer Res. 2009;69:609–15. [PubMed: 19147575]
39. Demaria S, Kawashima N, Yang AM, Devitt ML, Babb JS, Allison JP, et al. Immune-mediated inhibition of metastases after treatment with local radiation and CTLA-4 blockade in a mouse model of breast cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2005;11:728–34. [PubMed: 15701862]
40. Postow MA, Callahan MK, Barker CA, Yamada Y, Yuan J, Kitano S, et al. Immunologic correlates of the abscopal effect in a patient with melanoma. N Engl J Med. 2012;366:925–31. [PubMed: 22397654]
41. Twyman-Saint Victor C, Rech AJ, Maity A, Rengan R, Pauken KE, Stelekati E, et al. Radiation and dual checkpoint blockade activate non-redundant immune mechanisms in cancer. Nature. 2015;520:373–7. [PubMed: 25754329]
42. Puzanov I, Milhem MM, Minor D, Hamid O, Li A, Chen L, et al. Talimogene Laherparepvec in Combination With Ipilimumab in Previously Untreated, Unresectable Stage IIIB-IV Melanoma. J Clin Oncol. 2016;34:2619–26. [PubMed: 27298410]
43. Holmgaard RB, Zamarin D, Li Y, Gasmi B, Munn DH, Allison JP, et al. Tumor-Expressed IDO Recruits and Activates MDSCs in a Treg-Dependent Manner. Cell Rep. 2015;13:412–24. [PubMed: 26411680]
44. Gibney G, Hamid O, Gangadhar T, Lutzky J, Olszanski A, Gajewski T, et al. Preliminary results from a phase 1/2 study of INCB024360 combined with ipilimumab (ipi) in patients (pts) with melanoma. J Clin Oncol. 2014;32:5S:abstr 3010.
45. Quezada SA, Simpson TR, Peggs KS, Merghoub T, Vider J, Fan X, et al. Tumor-reactive CD4(+) T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. J Exp Med. 2010;207:637–50. [PubMed: 20156971]
46. Ng Tang D, Shen Y, Sun J, Wen S, Wolchok JD, Yuan J, et al. Increased frequency of ICOS+ CD4 T cells as a pharmacodynamic biomarker for anti-CTLA-4 therapy. Cancer Immunol Res. 2013;1:229–34. [PubMed: 24777852]
47. Chen H, Fu T, Suh WK, Tsavachidou D, Wen S, Gao J, et al. CD4 T cells require ICOS-mediated PI3K signaling to increase T-Bet expression in the setting of anti-CTLA-4 therapy. Cancer Immunol Res. 2014;2:167–76. [PubMed: 24778280]
48. Carthon BC, Wolchok JD, Yuan J, Kamat A, Ng Tang DS, Sun J, et al. Preoperative CTLA-4 blockade: tolerability and immune monitoring in the setting of a presurgical clinical trial. Clinical cancer research : an official journal of the American Association for Cancer Research. 2010;16:2861–71. [PubMed: 20460488]
49. Spranger S, Koblish HK, Horton B, Scherle PA, Newton R, Gajewski TF. Mechanism of tumor rejection with doublets of CTLA-4, PD-1/PD-L1, or IDO blockade involves restored IL-2 production and proliferation of CD8(+) T cells directly within the tumor microenvironment. J Immunother Cancer. 2014;2:3. [PubMed: 24829760]
50. Ji RR, Chasalow SD, Wang L, Hamid O, Schmidt H, Cogswell J, et al. An immune-active tumor microenvironment favors clinical response to ipilimumab. Cancer Immunol Immunother. 2012;61:1019–31. [PubMed: 22146893]
51. Tumeh PC, Harview CL, Yearley JH, Shintaku IP, Taylor EJ, Robert L, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. Nature. 2014;515:568–71. [PubMed: 25428505]

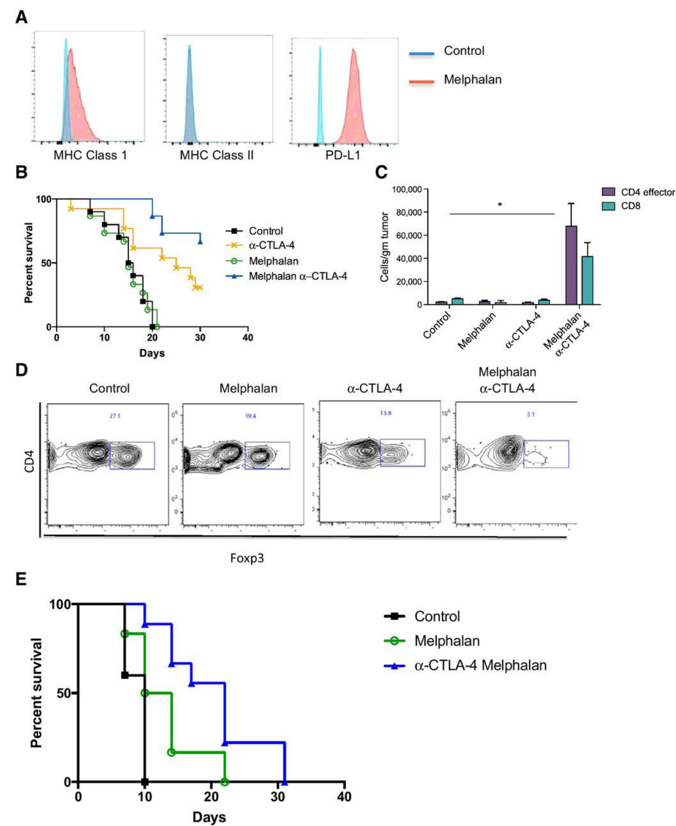


**Fig. 1.**

Melphalan and CTLA-4 blockade in a model of melanoma. (**A**) B16 cells were treated in vitro with melphalan or vehicle control at 50 μM. After 24 hours, the expression of MHC class I, MHC class II, and PD-L1 was assessed by flow cytometry. This is a representative figure from the experiment performed 3 times. Fold change was calculated based upon mean fluorescence intensity change from untreated to treated. (**B**) Melphalan synergizes with CTLA-4 blockade. Mice with palpable allografted B16 tumors were treated with single intratumoral dose of melphalan, alone or followed by CTLA-4 blockade, 100 μg IP, every 3 days for 4 doses. Control mice received intratumoral injection of vehicle control and IP injection of isotype antibody. Shown are pooled data of 3 separate experiments (3–5 mice per treatment group per experiment, four experiments). (**C,D**) Combination therapy enhances the inflammatory environment of the tumor. (**C**) CD4 effector and $CD8^+$ cells per gram of tumor in B16 tumors from the four treatment groups, $P < 0.05$ for combination versus control treatment. (**D**) CD4 and FoxP3 expression on cells from tumors from the four treatment groups. Experiments were performed 3 times; shown is representation of one experiment. (**E**) $IFN\gamma R^{-/-}$ mice had an improved median survival for melphalan plus CTLA-4 blockade over control or melphalan treated mice (combination ($n = 9$), median survival 22 days, control ($n = 5$), median survival 10 days, melphalan ($n = 6$), 12 days, $P < 0.05$). However, no combination mice were cured of tumors, suggesting the importance of IFNγ on the host.



Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

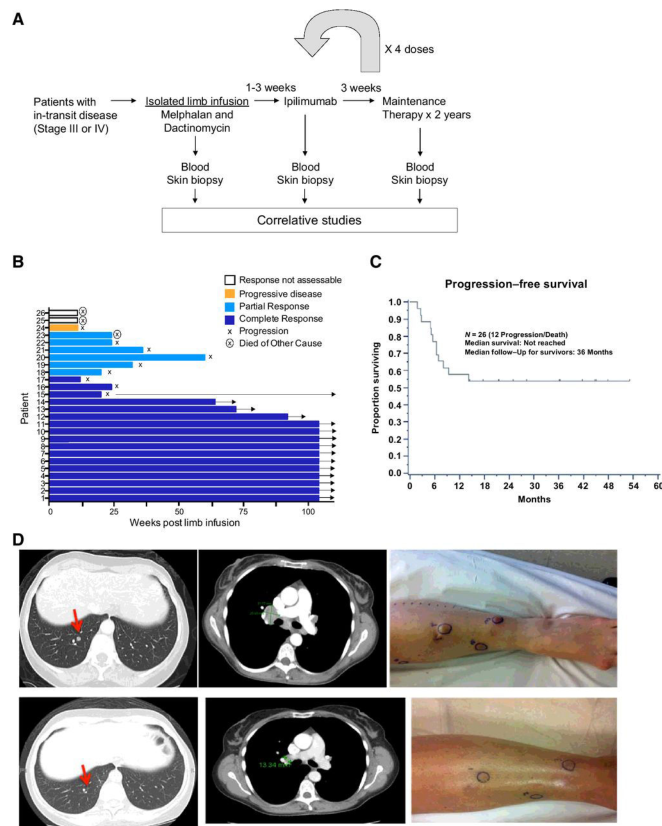


**Fig. 2.**

Clinical benefit from combination limb infusion and CTLA-4 blockade. (**A**) Schema of the phase II clinical trial. (**B**) Swimmer plot of all patients, showing responses in the melphalan-treated limb. (**C**) PFS for patients, including the two patients who died prior to the evaluation point. (**D**) Example of durable response both in and outside of the limb of the infusion. This patient had an unknown primary melanoma and presented with multiple subcutaneous nodules of the limb, with groin adenopathy, biopsy-proven lung metastasis, and thoracic adenopathy. Three years after limb infusion and systemic ipilimumab, the patient remains free of disease (lower panel).



Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

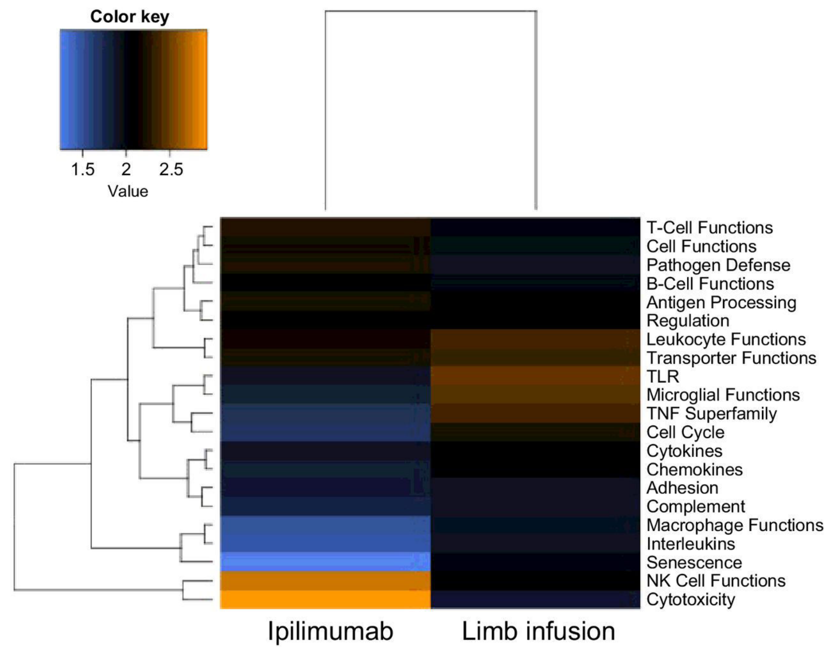


**Fig. 3.**

Nanostring analysis of tumors after isolated limb infusion (ILI) and after combination limb infusion and ipilimumab. Gene expression was compared from available pretreatment biopsies ($n = 11$) to post ILI ($n = 4$) and post-IPI ($n = 14$) biopsies. Tumor biopsies were taken just prior to ILI, after ILI (7–15 days) and 3 weeks after the last dose of Ipilimumab. The gene expression pattern after ILI favored up-regulation of costimulatory ligands, and innate immune function, while after combination treatment, there was increased expression of cytotoxic function, particularly granzymes, IFNγ, perforin and ICOS.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript



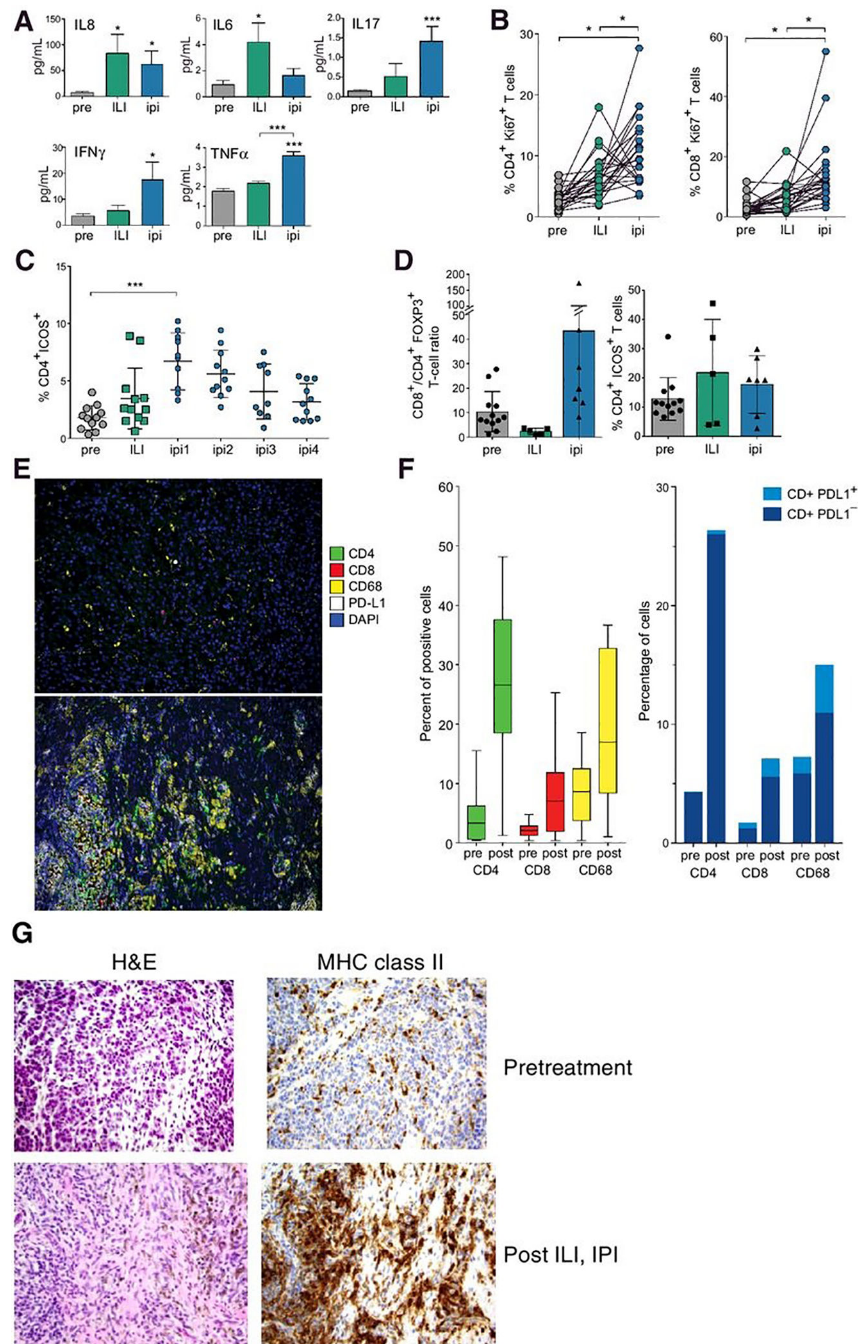


**Fig. 4.**

Analysis of cytokines and immune cell phenotypes in the phase II trial. (**A**) Increase in serum cytokines after combination therapy. The cytokines indicated were quantitated by Meso Scale Discovery assay in serum taken pretreatment (pre), after isolated limb infusion (ILI), and after the initial ipilimumab (ipi) treatment. *, $P < 0.05$; ***, $P < 0.001$ compared with pretreatment or as indicated by brackets. Note the increase in cytotoxic cytokines after combination treatment consistent with nanostring data (**B-D**) Changes in T cells in blood and tumor after ILI and ipilimumab treatments, assessed by flow cytometry. (**B**) Increase in

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

percentage of $CD4^+$ $Ki67^+$ and $CD8^+$ $Ki67^+$ T cells, of total $CD45^+$ cells, in peripheral blood mononuclear cells after ILI and after the first dose of ipilimumab. (**C**) Increase in percentage of $CD4^+$ $ICOS^+$ cells after ILI and after the each of the first (of 4) ipilimumab doses. (**D**) Analysis of tumor samples demonstrating an increase in the ratio of $CD8^+$ T cells to $CD4^+FOXP3^+$ T cells (left) and the percentage of $CD4^+ICOS^+$ T cells (right). The bars shown mean values and standard deviation. (**E,F**) Immune infiltration in tumors after isolated limb infusion and ipilimumab treatment. (**E**) Representative multiplex immunohistochemistry at 20x showing CD4 (green), CD8 (red), CD68 (yellow), and PD-L1 (white) on samples of a tumor before treatment (top) and after isolated limb infusion and ipilimumab (bottom). (**F**) Quantitation of the immunohistochemical results from all available tumors. Note the increase in $CD4^+$, $CD8^+$, and $CD68^+$ cells. Left, box-and-whisker plot showing the median, interquartile range, and range (excluding outliers) for cells positive for CD4, CD8, and CD68. Right, mean percentage of positive cells, with dark green indicating the PD-L1-positive subset. **G**) H&E and immunohistochemistry of a single tumor, demonstrating the pretreatment melanoma tumor cells with a paucity of immune cells but with MHC II expression (top), and an increase in both immune cells and MHC after ILI and IPI (bottom).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript



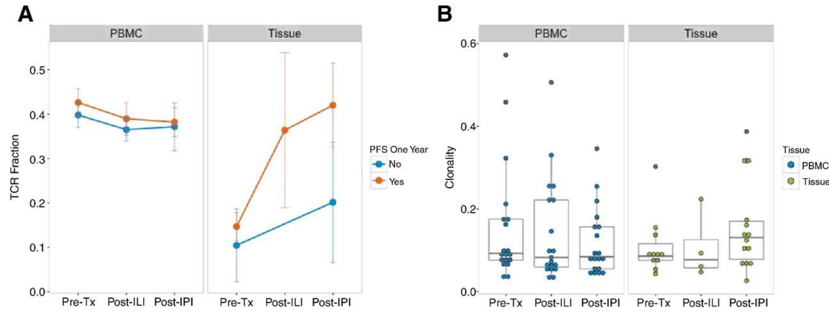


**Fig. 5.**

TCRβ seqencing. (**A**) TCR fraction in PBMCs and in tumor at baseline (Pre-TX), after limb infusion (post-ILI) and after the fourth dose of Ipilimumab (Post-IPI). Note that there is not a difference in the starting T cells between patients that derived benefit (PFS at one year versus not). Although there was not an overall change in T cells in the periphery, there T cells in the tumors of patients with PFS at one year were somewhat increased, although the change was not significant. (**B**) Box-and-whisker plots of the clonality of the TCR fraction. The clonality did not change in the PBMC; however, there was a significant increase in clonality, or a less even distribution of T cell clones, in the tumor versus the PBMC ($P =$ 0.015, by Wiloxon signed rank test), after combination treatment.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

**Table 1.**

Characteristics of the 26 patients in the Phase II trial.

| Characteristic | Number (%) |
|---|---|
| **Gender** | |
| Female | 10 (38) |
| Male | 16 (62) |
| **Stage** | |
| IIIB | 11 (42) |
| IIIC | 12 (46) |
| IV | 3 (12) |
| **Mutation Status** | |
| BRAF V600E | 4 (15) |
| NRAS | 9 (35) |
| WT | 9 (35) |
| Unknown | 4 (15) |
| **Melanoma Subtype** | |
| Acral | 3 (12) |
| Cutaneous | 19 (73) |
| Unknown Primary | 4 (15) |
| **High Tumor Burden (>50 lesions or one >3 cm)** | |
| Yes | 7 (27) |
| No | 19 (73) |

Author Manuscript
Author Manuscript
Author Manuscript
Author Manuscript

**Table 2.**

Number of patients affected by adverse events

| | Any | Grade 3 or 4 |
|---|---|---|
| Treatment-Related Adverse Event | | 10 (38%) |
| Any Adverse Event | | 13 (50%) |
| Diarrhea | 16 (62%) | 3 (12%) |
| Colitis | 3 (12%) | 3 (12%) |
| Fatigue | 18 (70%) | 1 (4%) |
| Pruritus | 17 (26%) | 2 (8%) |
| Rash | 18 (70%) | 1 (4%) |
| Nausea | 8 (31%) | 0 (0%) |
| Anorexia | 11 (42%) | 2 (8%) |
| Headache | 10 (38%) | 0 (0%) |
| Arthralgia | 4 (15%) | 1 (4%) |
| Myalgia | 9 (35%) | 1 (4%) |
| Pneumonitis | 1 (4%) | 0 (0%) |
| Elevated liver function test result * | 16 (62%) | 1 (4%) |
| Hypothyroidism | 5 (19%) | 0 (0%) |
| Hypoadrenal | 7 (27%) | 2 (8%) |
| Increased lipase | 3 (0%) | 0 (0%) |

* includes any increase in aspartate aminotransferase (AST) or alanine aminotransferase (ALT)

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

**Table 3.**

Genes upregulated in tumors after treatment relative to pretreatment. Shown are the 25 genes with the greatest significant fold change.

| Gene | fold change | *P* |
|---|---|---|
| **After limb infusion** | | |
| TREM1 | 12.49 | 0.023 |
| CCL3L1 | 10.59 | 0.009 |
| CCL3 | 10.36 | 0.013 |
| TNFRSF10C | 9.27 | 0.004 |
| CXCL3 | 8.9 | 0.042 |
| TREM2 | 8.09 | 0.003 |
| CXCR2 | 7.72 | 0.030 |
| CCL18 | 7.59 | 0.046 |
| MARCO | 7.43 | 0.002 |
| CCL23 | 7.31 | 0.016 |
| CXCL2 | 7.17 | 0.029 |
| S100A12 | 7.05 | 0.047 |
| SLC11A1 | 7.04 | 0.027 |
| CXCL1 | 6.96 | 0.039 |
| S100A8 | 6.9 | 0.038 |
| LILRA5 | 5.7 | 0.025 |
| C9 | 5.28 | 0.006 |
| CCL4 | 5.03 | 0.027 |
| PLAUR | 5.03 | 0.041 |
| IRGM | 4.84 | 0.006 |
| CCRL2 | 4.84 | 0.017 |
| IL21R | 4.82 | 0.016 |
| IL3RA | 4.79 | 0.001 |
| CD70 | 4.77 | 0.010 |
| ITGAX | 4.76 | 0.032 |
| **After limb infusion and ipilimumab** | | |
| CHIT1 | 17.27 | 0.001 |
| CCL18 | 8.09 | 0.001 |
| GZMA | 7.35 | 0.001 |
| CXCR6 | 7.23 | 0.000 |
| LY9 | 6.8 | 0.002 |
| KLRC2 | 6.68 | 0.000 |
| GZMH | 6.39 | 0.001 |
| GZMK | 6.34 | 0.003 |
| CCL26 | 6.25 | 0.004 |
| CCL3 | 6.1 | 0.010 |
| TREM2 | 6.01 | 0.003 |

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

| Gene | fold change | *P* |
|---|---|---|
| CCL3L1 | 5.95 | 0.009 |
| PDCD1 | 5.73 | 0.011 |
| SH2D1A | 5.4 | 0.005 |
| KLRK1 | 5.39 | 0.001 |
| CD3G | 5.27 | 0.006 |
| CD2 | 5.26 | 0.009 |
| NCR1 | 4.97 | 0.001 |
| IFNG | 4.9 | 0.011 |
| KLRG1 | 4.89 | 0.005 |
| IL18RAP | 4.88 | 0.006 |
| CXCR3 | 4.88 | 0.019 |
| CCL5 | 4.87 | 0.003 |
| CD27 | 4.82 | 0.015 |
| ICOS | 4.81 | 0.010 |