AWARD NUMBER: W81XWH-18-1-0312

TITLE: Biomarkers for predicting response to immune checkpoint blockers

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CONTRACTING ORGANIZATION: University of Texas Southwestern Medical Center

REPORT DATE: JULY 2020

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
JULY 2020	Annual	1 July 2019 – 30 June 2020

4. TITLE AND SUBTITLE
Biomarkers for predicting response to immune checkpoint blockers

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-18-1-0312

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
UT Southwestern Medical Center
5323 Harry Hines Blvd.
Dallas, TX 75390-7208

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Among currently available treatments for non-small cell lung carcinoma (NSCLC), immune checkpoint inhibitors (ICI) have produced the most significant improvement in survival rates. However, this best treatment benefits only a minority of treated cases (10-20%), with >80% of patients primarily resistant to this therapy. Our subject is to understand resistance mechanisms to immunotherapy with immune checkpoint inhibitor and to identify biomarkers distinguishing responders vs. non-responders. In this funding period, we examined whether NSCLC patients express soluble DC-HIL (sDC-HIL) protein in the blood and whether high blood levels of sDC-HIL correlate with poor response to ICI therapy. Blood sDC-HIL at pretreatment (week 0) was measured and analyzed for correlation with cancer progression. Responders to ICI therapy displayed sDC-HIL at levels no different from healthy donors. By contrast, non-responders had significantly higher sDC-HIL levels ($p<0.0001$ by Mann-Whitney U test). Among non-responders, the % change in sDC-HIL levels in the first 6 weeks after treatment correlated significantly with % change in tumor size ($p<0.00001$). These results indicate that blood sDC-HIL levels strongly associate with cancer progression and poor outcomes. Our studies demonstrate DC-HIL's negative influence on ICI therapy, highlighting its potential as a blood biomarker to predict treatment responsiveness and clinical benefit.

15. SUBJECT TERMS
Biomarker, Cancer immunotherapy, Immune checkpoint inhibitors, Myeloid-derived suppressor cells, non-small cell lung carcinoma, Resistance, Soluble DC-HIL receptor, and T-cells.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	12	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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PROGRESS REPORT. LUNG CANCER RESEARCH PROGRAM DoD Award W81XWH-18-1-0312

Funding period: July 1, 2019 to June 30, 2020

Title: Biomarkers for predicting response to immune checkpoint blockers

PI: Kiyoshi Ariizumi, PhD, Dermatology, and Co-PI: David E. Gerber, MD, Hematology-Oncology, at Harold C. Simmons Comprehensive Cancer Center.

1. INTRODUCTION:

Among currently available treatments for non-small cell lung carcinoma (NSCLC), immune checkpoint inhibitors (ICI; e.g., anti-PD1/PDL1 mAb) have produced the most significant improvement in survival rates. However, this best treatment benefits only a minority of treated cases (10-20%), with >80% of patients primarily resistant to this therapy. Our subject is to understand resistance mechanisms to immunotherapy with immune checkpoint inhibitor and to identify biomarkers distinguishing responders vs. non-responders. Since the new immune checkpoint, termed DC-HIL, is divergent from the PD1/PDL1 checkpoint pathway in expression and inhibitory mechanisms, we hypothesize that the DC-HIL pathway is the key mechanism for resistance to ICI therapy.

2. KEYWORDS:

cancer immunotherapy; DC-HIL; immune checkpoint; immunosuppression; monoclonal antibodies (mAb); myeloid-derived suppressor cells (MDSC); non-small cell lung carcinoma (NSCLC); resistance; and T cells.

3. ACOMPLISHMENTS:

Major goals of the project:

Under Aim 1

1. Determine whether blood levels of DC-HIL^{+}MDSC before treatment discriminate between responders and non-responders to anti-PD1/PDL1 mAb via FACS.

2. Examine fluctuation in DC-HIL expression among cancer patients

3. Determine blood sDC-HIL level of lung cancer patients.

4. Evaluate effect of DC-HIL blockade on the suppressed T-cell response

5. Assay expression of DC-HIL and PDL1 in cancer tissues.

Aim 2. Identify MDSC genes that underlie responsiveness/resistance to anti-PD1/PDL1 therapy.

Accomplishments:

1. Blood levels of DC-HIL^{+}MDSC before treatment discriminate between responders and non-responders to anti-PD1/PDL1 mAb.

a. Major activities: We are recruiting metastatic NSCLC patients who are registered to receive ICI therapy. Blood samples are collected from these patients at week 0 (before treatment), 12 and 24 weeks after the first injection of ICI mAb. Note: (1) at Weeks 12 and 24, our Co-PI David Gerber M.D. and

his colleagues measure the tumor size and evaluate response to ICI therapy; and (2) these time points' samples were used for experiments in the 2nd goal (see below). Response is evaluated using RECEIST and categorized to complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD). For assay of blood DC-HIL$^+$MDSC levels, peripheral leucocytes are separated from erythrocytes by differential sedimentation in a dextran/saline solution and subjected to flow-cytometry analysis to determine % HLA-DR$^{no/lo}$ CD14$^+$ MDSC among total peripheral leukocytes and % DC-HIL (or PDL1)-positivity among total MDSC. We analyzed correlation between blood DC-HIL$^+$ MDSC levels at Week (baseline) and response to ICI.

b. Specific objectives: To examine: (1) whether expansion of MDSC population in the blood of NSCLC patients at Week 0 associates with poor response to ICI therapy; and (2) whether high blood levels of DC-HIL$^+$ MDSC subset at pretreatment correlate with PD disease type.

c. Significant results or key outcomes: As reported in the last year, we had serious problem with recruiting patients who are registered for ICI monotherapy: The standard of care for advanced NSCLC has been changed to combination treatment with Chemotherapy: ICI monotherapy is available only for patients who have high expression of tumor-PDL1 (<5% of total patients), which are a minor population among advanced NSCLC patients. Indeed, we found only 2 patients between September and December 2018.

We have faced another serious problem. Since March 2019, we have collected blood samples from NSCLC patients who have been treated with IC/Chemotherapy. Just one year later, as of March 17, Dr. W. P. Andrew Lee, the Executive Vice President and Dean at UT Southwestern Medical School, requests clinical researchers not to take live samples from patients for prevention of COID-19 pandemic. Thus, we were unable to collect samples until July 17, 2020. We just resumed collecting samples (from August 4, 2020). Actually there was no progress for this goal.

d. Other achievements: The new standard of care is a combination treatment of ICI and chemotherapy, including (1) Pembrolizumab (anti-PD1), Carboplatin, and Pemetrexed and (2) Pembrolizumab, Cisplatnin, and Pemetrexed. Because our previous studies showed that chemotherapy drugs may have no effect on DC-HIL expression on MDSC,[1] we assume no influence on DC-HIL expression and no difference if any in DC-HIL expression between these chemotherapy drugs. Again, we are collecting samples.

2. Fluctuation of DC-HIL expression by MDSC:

a. Major activities: We are collecting blood samples of foregoing patients on Week 0, 12 and 24 during ICI/chemotherapy treatment. These samples are centrifuged to separate the cell fraction and the supernatant (plasma), followed by flow-cytometry analysis of MDSC and by ELISA for quantification of soluble DC-HIL (sDC-HIL) (**Goal #3**).

b. Specific objectives: To examine whether % DC-HIL$^+$ MDSC among PBMC alters during ICI/chemotherapy and, if so, determine the range of fluctuations in the blood levels.

c. Significant results or key outcomes: As described above, we had almost no progress in this goal. Nothing to report.

d. Other achievements: Nothing to report.

3. Blood sDC-HIL level

a. Major activities: Plasma samples at Week 0, 12, and 24 are assayed by ELISA for sDC-HIL concentration (ng/ml) and analyzed for association with tumor response.

b. Specific objectives: To examine whether blood sDC-HIL levels at Week 0 (baseline) associate with tumor response to ICI/chemotherapy.

c. Significant results or key outcomes: Again, our serum collection is consisted of small number of patients (n=11) not enough to perform statistical analysis. Thus, we have nothing to report about the original goal.

d. Other achievements: Instead, we quantified sDC-HIL levels in the plasma collection that was prepared by Dr. Gerber group. This collection comprises plasma samples of 76 NSCLC patients at Week 0 after ICI monotherapy.[2] Blood sDC-HIL was detected at pretreatment (week 0, ≤25 ng/ml) and the levels did not correlate with baseline tumor size (**Fig. 1A**). We then examined correlation of baseline sDC-HIL levels and tumor response (**Fig. 1B**); characterizing cases as having progressive disease (PD, n=33), stable disease (SD, n=38) or partial response (PR, n=5) at the first evaluation (6 or 12 weeks after treatment). Responders (SD and PR) displayed sDC-HIL at levels no different from healthy donors (2.9 ± 0.5, p=0.9). By contrast, non-responders (PD) had significantly higher sDC-HIL levels (p<0.0001 by Mann-Whitney U test) at all time-points examined (**Fig. 1C**). Among non-responders, the % change in sDC-HIL levels in the first 6 weeks after treatment correlated significantly with % change in tumor size (**Fig. 1D**, p<0.00001). These results indicate that blood sDC-HIL levels strongly associate with *de-novo* poor response.

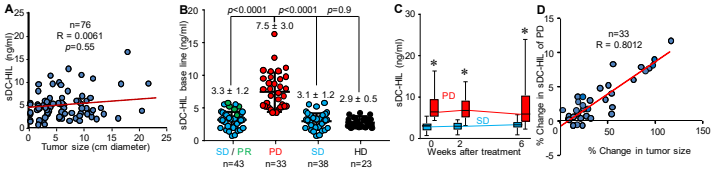

Fig. 1. Expression of sDC-HIL in the blood of advanced NSCLC patients during treatment with ICI. **A,** sDC-HIL blood levels of NSCLC patients at baseline are plotted against initial tumor size (sum of tumor cm diameter) (n=76). **B,** NSCLC patients were evaluated for response to ICI at Week ~12 after the first cycle and sorted into non-responders (PD) and responders (SD and PR). Blood sDC-HIL levels (mean ± SD) at baseline are compared between these 2 cohorts. SD and healthy donors (HD) are separately compared to each other. P values are shown using Mann-Whitney U test. **C,** Kinetics of blood sDC-HIL levels between PD and SD cohorts is shown at Weeks 0, 2, and 6 after ICI treatment. *p<0.0001. **D,** % change in sDC-HIL levels of PD patients (n=33) is plotted against % change in tumor size between Weeks 0 and 6. Pearson Correlation Coefficient R = 0.8012, p=0.00001.

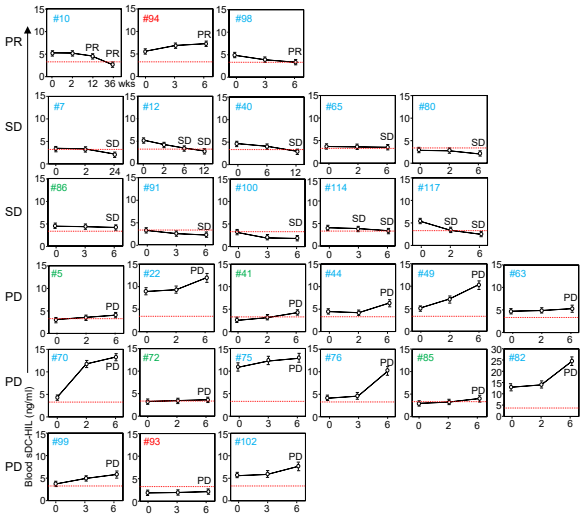

Fig. 2. Fluctuations in blood sDC-HIL expression following ICI treatment. Blood sDC-HIL levels (ng/ml) in 28 cases are shown at indicated time points after ICI treatment. Red dashed lines indicate mean value (3.3 ng/ml) of responders (SD and PR). Cases are sorted into PR, SD and PD: Cases (n=21) highlighted in blue display negative correlation of sDC-HIL levels and tumor response; cases (n=5) in green exhibit an opposite but weak correlation; and cases (n=2) in red show positive relationship.

We then examined fluctuations in sDC-HIL during treatment **(Fig. 2)**. In 28 cases for which sDC-HIL levels were available for more than 3 time-points (weeks 0, 2 and 6), we analyzed individual kinetics. Among non-responders, 14 of 15 cases showed increasing or persistently elevated levels (>3.3 ng/ml, the median of SD/PR groups), while one case had low levels at all the time (<3.3 ng/ml). For responders, 12 of 13 cases had decreasing or persistently low levels, with one PR patient #94 that showed increasing levels. We focused on patients (n=13) plotted in the zone of 4-6 ng/ml sDC-HIL overlapping between SD/PR and PD groups **(Fig. 1B).** PR patients #10 and #98 expressed 5.1 ng/ml sDC-HIL at baseline was higher than 3.3 ng/ml. This measure gradually fell at later time-points, during which time the treatment response was characterized as PR. Patient #70 (representative of 6 patients) had a baseline measure of 4.3 ng/ml that rose to 11-13 ng/ml at weeks 2 and 6; this case's treatment response was judged PD. In the case of patient #117 (representative of 5 patients), the baseline value of 5.8 ng/ml went down to 3.3 and 2.3 ng/ml and this treatment response was SD. We tested for significant differences in slope among the three groups using a mixed effect model. The estimated slopes and their 95% confidence intervals are: PD: slope=0.468, 95% CI: 0.273, 0.662. PR: slope= -0.145, 95% CI: -0.579, 0.289. SD: slope= -0.203, 95% CI: -0.441, 0.035. There was significant difference in slope between SD and PD (p=0.001). There was no significant change in sDC-HIL levels of healthy donors in 2 weeks. These cases illustrate fluctuations in blood sDC-HIL levels during the treatment, with considerable variation in magnitude and direction of the changes.

4. Recovery of T-cell function by DC-HIL blockade

This has been completed.

5. Tumor-DC-HIL and -PDL1 expression

a. Major activities: Archive cancer tissues of recruited NSCLC patients are immunohistochemistry-stained for expression of DC-HIL or PDL1 (as a reference) in cancer cells. Pathologist counts positive staining among lung cancer cells in a single blind manner.

b. Specific objectives: To examine whether expression of tumor-DC-HIL correlates with blood sDC-HIL levels and whether this tumor-DC-HIL expression associates with poor response to ICI therapy.

c. Significant results or key outcomes: We just resumed collecting samples. Nothing to report.

d. Other achievements: Instead, we probed for which cell types as likely producers of sDC-HIL in NSCLC patients **(Fig. 3)**. To examine DC-HIL expression on tumor cells, we used 3 NSCLC lines established from cancer biopsies. Among these lines, only H1957 NSCLC line expressed DC-HIL on the cell surface; it was greater than SK-MEL-28 melanoma cell line **(Fig. 3A)**, but lower levels of DC-HIL mRNA **(Fig. 3B)**. By contrast, all cells expressed PDL1 on the surface constitutively. Despite high levels of surface expression, H1957 line did not produce sDC-HIL in the culture supernatant even when the cell expressed ADAM10 mRNA. For leukocytes, PBMC from NSCLC patients (n=3) were fluorescently stained with anti-HLA-DR and anti-CD14 Ab and sorted into 4 fractions; Fr. 1 (HLA-DR$^{no/lo}$ CD14^{+} cells); Fr. 2 (HLA-DR$^{med/hi}$ CD14^{+}); Fr. 3 (HLA-DR$^{med/hi}$ CD14^{neg}); and Fr. 4 (HLA-DR$^{no/lo}$ CD14^{neg}). Each fraction was assayed for DC-HIL and PDL1 expression (Fig. 3C). Among these fractions, HLA-DR$^{no/lo}$ MDSC and HLA-DR$^{med/hi}$ CD14^{+} cells expressed highest levels of DC-HIL (ΔMFI: 91 and 62, respectively), with no expression in the other fractions. We then examined sDC-HIL production by these leukocytes. Because monocytic MDSC are a minuscule fraction, PBMC were sorted into CD14^{+} monocytes, CD15^{+} granulocytes, and the other (CD14^{neg} CD15^{neg}, which contain B- and T-cells). DC-HIL mRNA was highest in CD14 monocytes, with no expression by CD15 granular cells (which contain granulocytic MDSC) nor by other leukocytes **(Fig. 3D)**. DC-HIL^{+} cells also expressed ADAM10 mRNA and secreted sDC-HIL into culture (54-113 pg/ml). Thus CD14 monocytes, but not tumor cells nor granulocytes, may be the primary source of sDC-HIL.

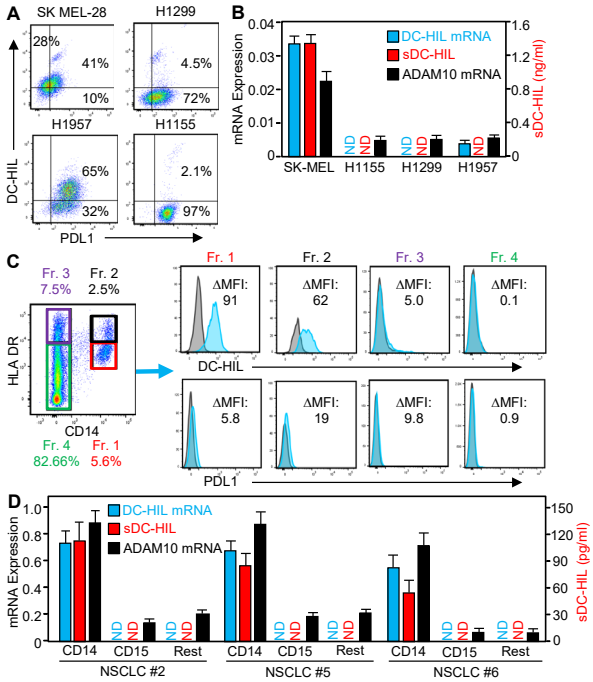

Fig. 3. Expression of DC-HIL by lung cancer cells versus PBMC isolated from metastatic NSCLC patients. A, Three NSCLC cell lines and SK-MEL-28 melanoma (as reference) were examined by flow-cytometry for expression of DC-HIL vs. PDL1 receptors. Expression is shown by % positivity. **B,** These tumor cells were assayed by qRT-PCR for mRNA expression of DC-HIL (blue bars) and ADAM10 (black bars) relative to GAPDH and determined for secretion of sDC-HIL (red bars with the right y-axis) in the culture. ND means not determined. **C**, PBMC of metastatic NSCLC patients were fluorescently stained with Ab to HLA-DR and CD14, and sorted into four fractions; Fr. 1 (HLA-DRno/lo CD14+ cells); Fr. 2 (HLA-DRmed/hi CD14+); Fr. 3 (HLA-DRmed/hi CD14neg); and Fr. 4 (HLA-DRno/lo CD14neg). Each fraction was determined for expression of DC-HIL and PDL1 receptors. Data shown are representative of 5 NSCLC patients. **D**, PBMC from blood of NSCLC patients (n=3) were sorted into 3 fractions (CD14^{+}, CD15^{+} and others) and quantified for DC-HIL and ADAM10 mRNA and sDC-HIL protein.

6. Identify MDSC genes that underlie resistance to anti-PD1/PDL1 therapy.

a. Major activities: Using cell sorters, MDSC are purified from fresh blood samples of NSCLC patients at 0, 12 and 24 weeks after ICI therapy. From purified MDSC, total RNA is extracted and converted to cDNA, and subjected to RNA-Seq gene expression analysis.

b. Specific objectives: To study alterations in transcriptome of MDSC during ICI therapy and find MDSC signature genes that associate with poor response to ICI therapy.

c. Significant results or key outcomes: We have purified total RNA from MDSC of 9 NSCLC patients and 5 healthy controls. Yield of RNA (100 ng) was high in MDSC fraction of healthy donors, but extremely low (>0.1 ng) in the same fraction in NSCLC patients. This yield is not enough to perform regular RNA-seq analysis.

d. Other achievements: We are now establishing PCR amplification of initial RNA followed by RNA-seq analysis.

What opportunities for training and professional development has the project provided?

"Nothing to Report."

How were the results disseminated to communities of interest?

Publication:

DC-HIL/Gpnmb is a Negative Regulator of Tumor Response to Immune Checkpoint Inhibitors

Jin-Sung Chung,[1] Vijay Ramani,[1] Masato Kobayashi,[1,§] Farjana Fattah,[2]
Vinita Popat,[2] Song Zhang,[3] Ponciano D. Cruz, Jr.,[1] David E. Gerber,[2] and Kiyoshi Ariizumi[1,*]
Departments of Dermatology,[1] Hematology Oncology,[2] and Population Data Sciences,[3] The University of Texas Southwestern Medical Center, and Dermatology Section (Medical Service), [§]Current addresses: Departments of Veterinary Clinical Pathology and Veterinary Science, Nippon Veterinary and Life Science University, Tokyo, Japan

What do you plan to do during the next reporting period to accomplish the goals?

We extended this project with no extra cost for one year. In the year, we will focus on correlation of blood sDC-HIL levels with tumor response of NSCLC patients. Since we showed significant correlation of high sDC-HIL levels with *de novo* resistance, we are particularly interested in the potential of sDC-HIL as a biomarker for acquired resistance. We will also overcome technical problem in obtaining RNA amounts enough for RNA-Seq or will establish the new method of PCR amplification/RNS-Seq analysis.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The most important finding in our studies is to show the high potential of blood sDC-HIL levels to be a biomarker to predict response to ICI therapy (**Fig. 2**). If our studies demonstrate the high accuracy to identify good (or poor) responders to ICI therapy before treatment, this biomarker allows customization of management of cancer patients: Doctors can treat patients who will benefit most to this expensive ICI immunotherapy. Conversely, this sDC-HIL biomarker is likely to also sort resistant patients at the outset, and thus sparing them from unnecessary cost and adverse risks, and saving time and effort for alternative treatment modalities.

What was the impact on other disciplines?

Our research is strongly relevant to understanding: (1) predictive and prognostic markers for distinguishing responders and nonresponders to anti-PD1/PDL1 mAb (the current industry standard among immune checkpoint blockers in the treatment of metastatic cancers); and (2) susceptibility or resistance to anti-PD1/PDL1 immunotherapy. Furthermore, our research will also elevate the potential of anti-DC-HIL therapy not only for patients resistant to anti-PD1/PDL1 mAb, but also fortify the basis for using it as monotherapy or in combination immunotherapy.

What was the impact on technology transfer?
If the high potency of anti-DC-HIL mAb to restore the suppressed IFN-γ T cell response by MDSC is appreciated with significant number of patients, this DC-HIL inhibitor may be useful for treating advanced NSCLC patients. If so, we will humanize this 3D5 anti-DC-HIL mAb clone (mouse IgG), using IgG genetic engineering technology, which is necessary for clinical trials to treat lung cancer.

What was the impact on society beyond science and technology?
If our hypothesis that DC-HIL is a biomarker to predict response to ICI therapy is proven, this marker could have the potential to change the way doctors select treatment for lung cancer patients. Thus, our studies may contribute to "Precise Cancer Immunotherapy".

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change.
We had a minor change in our project. As mentioned above, a standard care of ICI monotherapy has modified by combining with chemotherapy. The monotherapy is now only available for patients with high tumor expression of PDL1. Since UT Southwestern now permitted us to take clinical samples from patients, we are now collecting blood samples from patients treated with IC/Chemotherapy combination.

Actual or anticipated problems or delays and actions or plans to resolve them.
Because we showed that chemotherapy does not alter DC-HIL expression in MSDC,[1] we anticipate not having difficulties to perform our studies. Ideas to solve other problems are discussed above.

Changes that had a significant impact on expenditures
No significant impact on expenditures.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.
Nothing to report.

6. PRODUCTS:

Publications, conference papers, and presentations.
We submitted our abstracts to American Society of Clinical Oncology (ASCO) 2019 annual meeting.
Authors: J-S Chung, V Ramani, M Kobayashi, V. Popat, PD Cruz Jr, DE Gerber, K Ariizumi.
Title: Soluble DC-HIL/Gpnmb blood levels during anti-PD1/PDL1 therapy of advanced non-small cell lung carcinoma (NSCLC).

Journal publications.

DC-HIL/Gpnmb is a Negative Regulator of Tumor Response to Immune Checkpoint Inhibitors
Jin-Sung Chung,[1] Vijay Ramani,[1] Masato Kobayashi,[1,§] Farjana Fattah,[2]
Vinita Popat,[2] Song Zhang,[3] Ponciano D. Cruz, Jr.,[1] David E. Gerber,[2] and Kiyoshi Ariizumi[1,*]

Books or other non-periodical, one-time publications.
Nothing to Report.

Other publications, conference papers, and presentations.
Nothing to Report.

Website(s) or other Internet site(s):
Nothing to Report.

Technologies or techniques.
Nothing to Report.

Inventions, patent applications, and/or licenses.
Nothing to Report.

Other Products.
Data or databases: We have made flow-cytometry data of peripheral leukocytes from lung cancer patients (n=11).
Biospecimen collections: We collected plasma (1 ml/patient) from lung cancer patients (n=11) at three time points: baseline, 12 and 24 weeks post-treatment.

Not applicable for all other categories: Audio or video products; Software; Models; Educational aids or curricula; Instruments or equipment; Research material; Electrical interventions; and New business creation.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

What individuals have worked on the project?
No change.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to report.

What other organizations were involved as partners?
Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS.

COLLABORATIVE AWARDS:
Not applicable.

QUAD CHARTS:
Not applicable.

9. APPENDICES:

Reprint of published paper:

DC-HIL/Gpnmb is a negative regulator of tumor response to immune checkpoint inhibitor.s
Jin-Sung Chung, Vijay Ramani, Masato Kobayashi, Farjana Fattah, Vinita Popat, Song Zhang, Ponciano D. Cruz Jr, David E. Gerber, and Kiyoshi Ariizumi
Clin Cancer Res 15; 1449-1459. 2020. PMID: 31822499.

References:

1. Kobayashi, M.*, et al.* Blocking Monocytic Myeloid-Derived Suppressor Cell Function via Anti-DC-HIL/GPNMB Antibody Restores the In Vitro Integrity of T Cells from Cancer Patients. *Clinical cancer research : an official journal of the American Association for Cancer Research* **25**, 828-838 (2019).
2. Khan, S.*, et al.* Immune dysregulation in cancer patients developing immune-related adverse events. *British journal of cancer* **120**, 63-68 (2019).