Systemic Interferon-γ Increases MHC Class I Expression and T-cell Infiltration in Cold Tumors: Results of a Phase 0 Clinical Trial

Shihong Zhang1, Karan Kohli1, R. Graeme Black1, Lu Yao1, Sydney M. Spadinger1, Qianchuan He2, Venu G. Pillarisetty3, Lee D. Cranmer1,4, Brian A. Van Tine5, Cassian Yee6, Robert H. Pierce1, Stanley R. Riddell1,4, Robin L. Jones7, and Seth M. Pollack1,4

Abstract

Interferon-γ (IFNγ) has been studied as a cancer treatment with limited evidence of clinical benefit. However, it could play a role in cancer immunotherapy combination treatments. Despite high expression of immunogenic cancer-testis antigens, synovial sarcoma (SS) and myxoid/round cell liposarcoma (MRCL) have a cold tumor microenvironment (TME), with few infiltrating T cells and low expression of major histocompatibility complex class I (MHC-I). We hypothesized that IFNγ treatment could drive inflammation in a cold TME, facilitating further immunotherapy. We conducted a phase 0 clinical trial treating 8 SS or MRCL patients with weekly systemic IFNγ. We performed pre- and posttreatment biopsies. IFNγ changed the SS and MRCL TME, inducing tumor-surface MHC-I expression and significant T-cell infiltration (P < 0.05). Gene-expression analysis suggested increased tumor antigen presentation and less exhausted phenotypes of the tumor-infiltrating T cells. Newly emergent antigen-specific humoral and/or T-cell responses were found in 3 of 7 evaluable patients. However, increased expression of PD-L1 was observed on tumor-infiltrating myeloid cells and in some cases tumor cells. These findings suggest that systemic IFNγ used to convert SS and MRCL into “hot” tumors will work in concert with anti-PD-1 therapy to provide patient benefit.

Introduction

Interferon-γ (IFNγ) is an FDA-approved cytokine therapy, used as a standard-of-care treatment for osteopetrosis and chronic granulomatous disease. In the 1990s, IFNγ was evaluated as a single-agent cancer immunotherapy in several prospective randomized trials (1–3). These trials found no benefit in cancer and thus IFNγ was largely written off as an ineffective treatment. However, these studies were done prior to the modern era of immunotherapy. The failure of IFNγ to demonstrate efficacy may have been partly a result of checkpoint upregulation, particularly PD-L1 (4–6).

IFNγ can enhance expression of surface major histocompatibility complex (MHC) molecules and increase the processing and presentation of tumor-specific antigens (?), thus facilitating T-cell recognition and cytotoxicity (8–11). However, even though IFNγ is one of the best-studied cytokines in animal models and has undergone extensive clinical testing, little is known regarding the impact of IFNγ on the immune cells of the human tumor microenvironment (TME). Most of the clinical studies focus on the efficacy of IFNγ, although one study shows increased MHC expression on tumor cells in fine-needle aspirates from melanoma patients treated with IFNγ (12). Interest in IFNγ has resurfaced as investigators have uncovered possible synergy between IFNγ and PD-1 inhibitors (for example, see clinical trials NCT02614456, NCT03063632 on clinicaltrials.gov). Therefore, understanding the molecular mode of action of IFNγ may be key for cancer immunotherapy development.

Our group has been interested in developing immunotherapy for two immunologically quiet (also called “cold”) tumors (13): synovial sarcoma (SS) and myxoid/round cell liposarcoma (MRCL). These malignancies are translocation driven and have a low mutational burden but highly express cancer-testis antigens, e.g., NY-ESO-1, PRAME, and MAGE families (14, 15). Encouraging results have been observed in trials using NY-ESO-1–specific vaccines (16) and TCR-transduced T-cell therapies but the response rate and durability need to be increased (17, 18). One obstacle is that SS and MRCL have low MHC class I (MHC-I) expression (19) and few infiltrating T cells, both of which likely help the tumor evade adoptive T-cell therapy and other immunotherapies. Although a number of injectable agents can inflame the TME in a single tumor site, we hypothesized that systemic, subcutaneous IFNγ could induce widespread...
tumor inflammation potentially allowing for more effective immunotherapy combinations. Here we describe results of a pilot phase 0 trial, with the primary objective of determining whether systemic weekly subcutaneous IFNγ treatment would increase MHC-I expression in SS and MRCL patients.

Materials and Methods

Clinical protocol and sample collection

Informed written consent in accordance with the Declaration of Helsinki was obtained from all subjects prior to enrollment in an IRB-approved protocol (NCT01957709); eligibility criteria are listed in Supplementary Table S1. Patients generally received 4 weekly injections of IFNγ 100 μg/m²; however, to accommodate patient schedules as few as 2 injections were allowed. Core needle biopsies were performed prior to starting IFNγ and 1 to 3 days after their last dose. Toxicity data were tabulated using CTCAE v4.0. The primary objective was to determine whether IFNγ would increase MHC-I expression. Secondary objectives were to determine whether IFNγ increased MHC-II expression and to examine the changes seen in the immune infiltrates of SS and MRCL tumors.

Patient sample preparation and flow cytometry

Core needle biopsy specimens were either formalin-fixed paraffin-embedded (FFPE) or placed in RPMI-1640 media for other studies. To prepare single-cell suspensions, tumorspecimens were cut into 1 to 2 mm fragments, incubated in an enzymatic cocktail containing Type IV collagenase, DNase, and hyaluronidase for 30 minutes at 37°C, then pressed through a 70-μm mesh cell strainer. Flow cytometry was performed on the same day as biopsy collection using a multicolor panel. Detailed staining panel is listed in Supplementary Table S2. Stained samples were analyzed on a BD FACSAria II. CD45+ tumor cells, CD8+ T cells, and CD4+ T cells were sorted separately from live single cells. HLA-ABC and PD-L1 gates made on the tumor cells were determined by T-cell gates as positive and negative controls, respectively. Statistical and PD-L1 gates made on the tumor cells were determined by T-value subtracted by pretreatment log2 value generated by limma. Fold change preranked lists were generated with posttreatment log2 values above 9-fold over background, and the background is lower quartile of all serum data after Gnjatic and colleagues (21). Because antibodies usually take about 4 weeks to be detected after the host is exposed to a certain antigen, we only tested sera when it was available at least 4 weeks posttreatment for this analysis.

Serum tumor antigen–specific antibody measurement

Serum antibodies specific for tumor antigens were measured by Serametrix. The detailed antigen list is shown in Supplementary Fig. S1. Highly positive responses shown in the Results are defined as >9-fold over background, and the background is lower quartile of all serum data after Gnjatic and colleagues (21). Because antibodies usually take about 4 weeks to be detected after the host is exposed to a certain antigen, we only tested sera when it was available at least 4 weeks posttreatment for this analysis.

Multiplex immunohistochemistry (mHIC) sample processing and staining procedure

Staining was performed on 4-μm thick FFPE sections by using automated staining. After deparaffinization, slides were treated with antigen retrieval (AR) buffer (Diva Decloaker from BioCare Medical or Leica Bond Epitope Retrieval Solution 2) and heated for 15 minutes at 95 to 100°C. Slides were allowed to cool in the AR buffer for 15 minutes at room temperature and were then rinsed with deionized water and 1 × Tris-buffered saline with Tween-20. Endogenous peroxidase was blocked using 3% hydrogen peroxide. Protein stabilization and background reduction was done using intellIPATH Background Punisher. Slides were then incubated for 1 hour with primary antibodies against HLA-ABC (clone EMR8-5), PD-L1 (clone RBF-PDL1) followed by the secondary antibody (PerkinElmer OPAL Polymyrr HRP Ms Plus Rb) application for 30 minutes and the application of the tertiary TSA-amplification reagent (PerkinElmer OPAL fluor) for 10 minutes. Antigen stripping was performed either by heating with Leica Bond Epitope Retrieval Solution 2 or with Biocare medical denaturation reagent at room temperature. Slides were imaged with either Leica Aperio FL Immunofluorescence slide scanner or Leica SP8 confocal microscope.

Results

Patient demographics and clinical data

Eight patients (mean age 51, range, 24–68) were treated with 2 to 4 doses of IFNγ weekly 100 μg/m². Patients’ information is listed in Table 1. Seven were evaluable for both pre- and posttreatment biopsy samples; patient # 3 refused to undergo a posttreatment biopsy. Six patients had SS and two had MRCL. Two patients received less than the full 4-week course in order to
accommodate their schedules, as was permitted on this phase 0 trial. Following his treatment, on examination of ultrasound studies, it was found that patient #7 had his pretreatment biopsy taken very close to an area that had been radiated several weeks prior, where acute radiation-related inflammation was likely occurring. Although data from this patient were not censured, this was considered during data interpretation as their results differed from other patients. No patients had unexpected grade 3 or higher toxicity due to treatment and none discontinued treatment due to toxicity. All toxicity resolved completely or to grade 1 by 72 hours after injection. A list of all adverse events can be found in Supplementary Table S3.

Tumor cell antigen processing and presentation

Tumor single-cell suspension from both pre- and posttreatment time points were prepared and analyzed for surface MHC-I and -II molecules from all 7 evaluable patients. Overall, the percentage of MHC-I\(^+\) cells in the CD45\(^-\) fraction was increased in posttreatment samples (1.39%–92.2%, median 26.6%) compared with pretreatment (0.131%–51.6%, median 8.91%). Major (>30%) and moderate (15%–30%) increases in MHC-I\(^+\) were observed in 4 out 7 patients (#2, #4, #6, and #8). Although patients #1 and #5 had only minor increases in HLA-ABC (<15% increase), their tumor cells were negative for HLA-ABC pretreatment, and HLA-ABC\(^+\) populations became detectable posttreatment (Fig. 1A and B), suggesting that although the increases were small in magnitude they may be clinically relevant. Patient #7, whose pretreatment biopsy may have been influenced by receipt of radiotherapy shortly before biopsy, was the only patient in whom HLA-ABC decreased posttreatment. The intensity of HLA-ABC expression also increased by mIHC in patients with available FFPE samples (Fig. 1C). These data suggest that tumor cells express HLA-ABC more abundantly and intensely after IFNg treatment. In contrast, the MHC-II molecule HLA-DR did not change significantly on SS or MRCL tumor cells.

Gene-expression profiling was performed on sorted tumor cells from the biopsy samples of patients #6, #7, and #8. GSEA, with the KEGG antigen processing and presentation gene set, was adopted for this assessment. GSEA results were consistent with

Table 1. Patient information and treatment history

<table>
<thead>
<tr>
<th>ID</th>
<th>Subtype</th>
<th>Sex</th>
<th>Age</th>
<th>Treatment duration</th>
<th>Prior treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MRCL</td>
<td>M</td>
<td>68</td>
<td>4 weeks</td>
<td>Surgery, radiation, chemo, LV305</td>
</tr>
<tr>
<td>2</td>
<td>SS</td>
<td>F</td>
<td>60</td>
<td>2 weeks</td>
<td>Surgery, radiation, chemo, LV305</td>
</tr>
<tr>
<td>3</td>
<td>SS</td>
<td>M</td>
<td>50</td>
<td>4 weeks</td>
<td>Chemo, LV305</td>
</tr>
<tr>
<td>4</td>
<td>SS</td>
<td>M</td>
<td>67</td>
<td>4 weeks</td>
<td>Surgery, chemo, radiation</td>
</tr>
<tr>
<td>5</td>
<td>MRCL</td>
<td>M</td>
<td>49</td>
<td>4 weeks</td>
<td>Surgery, chemo, radiation, LV305</td>
</tr>
<tr>
<td>6</td>
<td>SS</td>
<td>F</td>
<td>38</td>
<td>3 weeks</td>
<td>Radiation, surgery, LV305, atezolizumab</td>
</tr>
<tr>
<td>7</td>
<td>SS</td>
<td>M</td>
<td>54</td>
<td>4 weeks</td>
<td>Surgery, radiation, chemo</td>
</tr>
<tr>
<td>8</td>
<td>SS</td>
<td>F</td>
<td>24</td>
<td>4 weeks</td>
<td>Radiation, surgery, chemo</td>
</tr>
</tbody>
</table>

NOTE: LV305 is an NY-ESO-1 vaccine that patients received as part of a clinical trial. Some patients received monoclonal antibody treatments as part of their chemotherapy, including olaratumab (anti-PDGFR\(\alpha\)), or on a clinical trial of MorAb-004 (anti-endosialin).
the tumor-surface HLA-ABC expression. Patient #6, who had a 41% increase of tumor-surface HLA-ABC expression, also had significantly higher antigen processing and presentation capability after treatment, whereas little change was observed in patient #8 who had moderate increase in HLA-ABC (17%) and patient #7 had decreased antigen processing and presentation (Fig. 1D; Supplementary Fig. S2). This effect was also seen in other genes related to antigen presentation; log2 expression of TAP1 in patient #6 pretreatment increased from 3.77 to 6.87. Gene-expression profiling confirmed expression of commonly found tumor antigens (Supplementary Fig. S3). The major responder patient #6 also had a more apoptotic tumor gene-expression profile (Supplementary Fig. S4), suggesting the possibility of immune-mediated tumor cell apoptosis.

**Change in T-cell infiltration and phenotype**

Pretreatment, low frequencies of tumor-infiltrating T cells (% CD45\(^+\)CD3\(^+\) in live cells from single-cell suspensions) were observed. All but one patient had fewer than 0.5% of their pretreatment tumor comprised of T cells (0.015%–1.05%, median 0.14%). T-cell percentages increased by >0.5% in 5 patients posttreatment, with a max of 3.39% (0.074%–3.39%, median 0.82%; P = 0.03; Fig 2A). Except for patient #1, all subjects had CD8\(^+\) T-cell biased infiltration, and IFN\(\gamma\) did not change the dominance of CD8\(^+\) or CD4\(^+\) T-cell infiltrates (Supplementary Fig. S5). T-cell infiltration was correlated with the tumor-surface HLA-ABC expression in our cohort (\(r^2 = 0.59, P = 0.001\); Fig 2B), suggesting the increase of T-cell infiltration may have been driven by the tumor HLA-ABC expression.

**Figure 2.** Increase of T-cell infiltration and functionality after IFN\(\gamma\) treatment. A, Percentages of CD45\(^+\)CD3\(^+\) cells out of all live cells from the analyzed tumor single-cell suspension from all 7 patients tested. T cells significantly increased (P = 0.03). Statistical analysis was performed with Wilcoxon signed-rank test. B, Correlation of T-cell infiltration and tumor-surface HLA-ABC expression. Closed and open dots represent samples from pretreatment and posttreatment, respectively. C, GSEA analysis of patients #6 and #8 CD8\(^+\) TIL reveals a more effector phenotype after treatment, comparing with a more exhausted phenotype before treatment. D, Individual plots of serum CXCL10, CCL2, and IL16 concentrations. E, ICS shows patient #1 had an increased NY-ESO-1-specific T-cell response posttreatment (right) compared with pretreatment (middle). These plots were gated on CD4\(^+\) single live cells and are representative of results from 3 independent experiments done on different PBMC aliquots from the same samples. F, Numbers of tumor antigens that are recognized by serum antibodies in 6 patients who received IFN\(\gamma\) treatment for 4 weeks.
Gene-expression profiling of CD8+ TIL phenotypes was available from patients #6 and #8. GSEA analysis using previously defined gene sets (22) revealed that in both subjects, pretreatment CD8+ TIL exhibited an exhausted phenotype and demonstrated a significant conversion toward an effector phenotype after IFNγ treatment (P < 0.001 for patient #6; P < 0.01 for patient #8, Fig. 2C). These results suggest that IFNγ treatment either reversed TIL exhausted state or, more likely, induced infiltration of nonexhausted T cells.

Serum cytokine change
A total of 79 cytokines, chemokines, and other molecules were measured in sera (Supplementary Fig. S6). CXCL10, also known as interferon-inducible protein 10 (IP10), was significantly increased among all patients, demonstrating the systemic response from subcutaneous injections (Fig. 2D). For individuals, CXCL10 concentration changes were consistent with the tumor-surface HLA-ABC expression and antigen presentation changes. In addition, increased CCL2 and decreased IL16 expression was observed after treatment, which, like CXCL10, have chemotactic roles and may be related to immune cell migration and infiltration into the tumors.

Induction of tumor-specific immune responses
We next compared antigen-specific T cells for NY-ESO-1, MAGE-A4, and PRAME in peripheral blood (Supplementary Fig. S3). T-cell responses to cancer-testis antigens are difficult to detect in the blood of SS and MRCL patients without extensive culture and stimulation (23). Nevertheless, NY-ESO-1–specific T-cell responses developed following IFNγ treatment in patient #1 (Fig. 2E). Before treatment, as indicated by ICS, no NY-ESO-1–specific response was detectable from patient #1. After treatment, an IFNγTNFα+CD4+ T-cell population was seen upon NY-ESO-1 peptide pool stimulation. Patient #1 had predominantly CD4+ T-cell infiltration, suggesting that CD4+ T-cell help may be important for immunity against SS. Serum antibodies specific to 29 tumor antigens were measured from the pre- and posttreatment sera. Three patients (#1, #5, and #8) developed humoral responses against more tumor antigens following treatment, with an average of 4 (pre-) antigen specificities increasing to 14 (posttreatment; Supplementary Fig. S1, and Fig. 2F).

Increase of tumor and myeloid cell PD-L1 expression
We next evaluated PD-L1 expression on both tumor cells and tumor-infiltrating myeloid cells. Based on flow cytometry, 2 out of the 7 patients had increases of PD-L1+ tumor cell percentages. Before treatment, both patients #2 and #8 had <5% PD-L1+ tumor cells, whereas after treatment, PD-L1+ increased to 64% and 15%, respectively (Fig. 3A and B). An increased percentage of PD-L1+ cells was found on posttreatment tumor-infiltrating myeloid cells in all patients analyzed. Compared with pretreatment (0.694%–97.1%, median 34.6%), PD-L1+ myeloid cell numbers increased significantly after IFNγ treatment (33.6%–100%, median 55.8%. P = 0.03; Fig. 3C and D). This increased PD-L1 expression on tumor cells and myeloid cells in the tumor may function to regulate the newly activated T cells in the TME.
Discussion
In this report, we present the results of a phase 0 trial using weekly IFNα in patients with SS and MRCL. Although the analysis of these patients was limited by a small sample size, we did observe that, although SS and MRCL have cold TMEs, expression of HLA-ABC and T-cell infiltration can increase after IFNα treatment. IFNα is used in two FDA-approved indications and multiple large randomized studies. We provide here detailed analyses of the impact of IFNα on human tumors focusing on the effect of IFNα on the TME of cold solid tumors that generally have a low mutation burden.

Although PD-L1 is an imperfect biomarker, its expression has been correlated with response to PD-1 blockade. IFNα can induce tumor PD-L1 expression in laboratory models of different cancer types, including melanoma, gastric, and ovarian cancers (4–6). Here we show this induction clinically, in two tumors (SS and MRCL) that generally lack strong PD-L1 expression in the untreated setting (19). PD-L1 is consistently upregulated on tumor-infiltrating myeloid cells in response to inflammation, which may also suppress T-cell functions and is more important than PD-L1 expression on tumor cells in some models (24, 25). We also analyzed other potential immune-escape mechanisms followed by IFNα treatment, including impairment of IFNα pathway and upregulation of other immune inhibitory molecules. We saw no significant changes, possibly due to the small number of patients, but these theoretical mechanisms should be kept in mind in future studies.

In summary, our results demonstrate that the cold TME of SS and MRCL was malleable and could be altered to facilitate immunotherapy. Based on these data, a cohort of patients is being added to the multicenter Cancer Immunotherapy Networks (CITN) trial CITN-13 (NCT03063632) to test combining IFNα with pembrolizumab for SS patients.

Disclosure of Potential Conflicts of Interest
V.G. Pillarisetty reports receiving a commercial research grant from Merck and is a consultant/advisory board member for the same. L.D. Cranmer reports receiving a commercial research grant from Eli Lilly, Tracfon, AADi, and Exelixis and is a consultant/advisory board member for Blueprint and Regeneron. B.A. Van Tine reports receiving a commercial research grant from Merck, Pfizer, and Tracfon, has received honoraria from the speakers bureau of Caris, Janseen, and Lilly, is a consultant/advisory board member for Epizyme, Lilly, Cytrix, Janssen, Immune Design, Daichi Sankyo, Plexicon, and Adaptimmune, and has received an expert testimony from Lilly. C. Yee has ownership interest (including stock, patents, etc.) in Immatics US, is a consultant/advisory board member for Immatics US and Berkeley Lights. S. Riddell reports receiving a commercial research grant from, has ownership interest (including stock, patents, etc.) in, and is a consultant/advisory board member for Juno Therapeutics, a Celgene company. R.L. Jones is a consultant/advisory board member for Adapimmune, Blueprint, Pharmamart, Tracfon, Clinigen, Eisa, Epizyme, Daichi, Deciphera, Innunmedesign, Lilly, and Merck. S.M. Pollack has received honoraria from Seattle Genetics, Bayer, Tempus, Daichi Sankyo, Blueprint and grants and research funding from Merck, EMD Sereno, Incyte, Presage, Janssen, Oncosor, Juno Therapeutics. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: S. Zhang, C. Yee, R.L. Jones, S.M. Pollack
Development of methodology: S. Zhang, K. Kohli, C. Yee, R.H. Pierce, R.L. Jones, S.M. Pollack
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Kohli, R.G. Black, S.M. Spadinger, L.D. Cranmer, B.A. Van Tine, R.H. Pierce, R.L. Jones, S.M. Pollack
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Zhang, K. Kohli, L. Yao, L.D. Cranmer, B.A. Van Tine, R.H. Pierce, R.L. Jones, S.M. Pollack
Writing, review, and/or revision of the manuscript: S. Zhang, K. Kohli, R.G. Black, S.M. Spadinger, Q. He, V.G. Pillarisetty, L.D. Cranmer, B.A. Van Tine, S. Riddell, R.L. Jones, S.M. Pollack
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Zhang, R.L. Jones
Study supervision: B.A. Van Tine, R.L. Jones, S.M. Pollack

Acknowledgments
Interferon-γ was provided for the study by Horizon Pharmaceutical. Support for the trial was also provided by The Gilman Sarcoma Foundation and NIH-NCI R23CA175167.

Received January 3, 2019; revised March 9, 2019; accepted June 3, 2019; published first June 6, 2019.

References


Infiltration in Cold Tumors: Results of a Phase 0 Clinical Trial

Shihong Zhang, Karan Kohli, R. Graeme Black, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/2326-6066.CIR-18-0940

Supplementary Material
Access the most recent supplemental material at:
http://cancerimmunolres.aacrjournals.org/content/suppl/2019/06/05/2326-6066.CIR-18-0940.DC1

Cited articles
This article cites 25 articles, 5 of which you can access for free at:
http://cancerimmunolres.aacrjournals.org/content/7/8/1237.full#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
http://cancerimmunolres.aacrjournals.org/content/7/8/1237.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerimmunolres.aacrjournals.org/content/7/8/1237.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.