Document Coversheet

Study Title:

Phase I/IIa Clinical Trial Evaluating the Safety and Efficacy of Rintatolimod Combined with IFNα2b (Bioferon®) to Enhance the Effectiveness of Pembrolizumab in Patients with Metastatic Triple Negative Breast Cancer

Institution/Site:	Roswell Park Comprehensive Cancer Center
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Study # I 3010822

This Study has Local Clarification(s) inserted.
Please see below and refer to the documents behind this title page.

Protocol Version: **Version 7 dated 07AUG2025**

Local Clarification #1: PI transfer form dated 22APR2024, resolved

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Phase I/IIa Clinical Trial Evaluating the Safety and Efficacy of Rintatolimod Combined with IFNα2b (Bioferon®) to Enhance the Effectiveness of Pembrolizumab in Patients with Metastatic Triple Negative Breast Cancer

PROTOCOL NUMBER:

I-3010822

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Confidentiality Statement

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1 OBJECTIVES

1.1 Primary Objectives

- To evaluate the safety profile of modified CKM (celecoxib, rintatolimod and interferon alpha-2b) regimen (replacement of INTRON® A, using alternative source of IFNα2b) combined with pembrolizumab therapy in metastatic triple negative breast cancer patients.

1.2 Secondary Objectives

- To evaluate the efficacy of the CKM in combination with pembrolizumab in patients with metastatic triple negative breast cancer (mTNBC) as compared to historic outcomes of pembrolizumab and other anti-PD1/PD-L1 therapies alone, as determined by secondary measures of efficacy including:
    - progression-free survival (PFS)
    - overall survival (OS)
    - overall response rate (ORR) to the combination therapy using iRECIST
    - disease control rate (DCR) using iRECIST

1.3 Exploratory Objectives

- Examine the immune analysis profile of CKM and pembrolizumab combination:
    - Examine the relationship of infiltrating CD4+ and CD8+ T cells and other immune and genetic markers, and their associated PD-1, CD45RA or CD45RO levels
    - Correlate PD-L1 expression within both neoplastic and nonneoplastic stromal elements of the tumor microenvironment to PFS, OS, ORR and AEs.
    - Correlate Immune Panel results with ORR, PFS, OS and AEs.
- Comparison of response assessment criteria for a prospective analysis.
    - RECIST 1.1 response assessment (Appendix F)
    - iRECIST response assessment (Appendix G)

2 BACKGROUND

2.1 Triple Negative Breast Cancer

Triple-negative breast cancer (TNBC) refers to a subgroup of breast carcinomas that do not express the estrogen receptor (ER), progesterone receptor (PR), and exhibit normal expression of the human epidermal growth factor receptor type 2 (HER2). Due to a lack of well-defined molecular targets, chemotherapy is the standard of care treatment for TNBC. The triple-negative phenotype is found in approximately 10-17% of all breast cancers, and is associated with a poor prognosis, characterized by early relapse and a significantly shorter survival following recurrence compared with non-triple-negative cancers. (1-6) Data suggest that the triple-negative phenotype is an independent predictor of distant metastasis and cause-specific survival. (7) In the metastatic setting, patients with TNBC have a median survival of approximately 13 months, and short duration of response to treatment (median duration of response equals 12 weeks for first-line

treatment; 9 weeks for second-line; and 4 weeks for third-line). (8) Thus, there is an unmet clinical need for more effective treatments in this group of patients.

2.2 Immune checkpoint inhibitors in the treatment of metastatic TNBC

There is one Phase I trial that reported results for (anti-CTLA4 antibody) in combination with exemestane in ER positive metastatic breast cancer. The tremelimumab study included 26 patients and the best overall response was stable disease for > 12 weeks in 11 patients (42%). (9)

The KEYNOTE-012 trial (NCT01848834) included 32 patients with a median age of 50 years to assess the safety and efficacy of the anti-PD-L1 antibody MK-3475 (pembrolizumab 10mg/kg every 2 weeks) in metastatic TNBC that showed > 1% PD-L1 positivity by immunohistochemistry using the 22C3 antibody. One hundred and eleven patients were screened for PD-L1 expression and 59% had PD-L1 positive tumors. The most common drug-related adverse events were mostly Grade 1-2 and included myalgia, headache and fatigue. Potentially immune-related adverse events were rare and included rash (4.5%), influenza-like symptoms (3%), pruritis (2.2%), eczema (2.2%), vitiligo (2.2%), hypothyroidism (2.2%), pyrexia (1.5%), cough (1.5%), and colitis (<1%). The rate of grade 3 or greater toxicities were 15.6%. The safety and adverse effect profile of MK-3475 has been established in over 800 patients who were treated in various trials and many patients received treatment over 1 year in the metastatic setting due to prolonged stable disease in melanoma and lung cancer. In the 27 patients who were evaluable for assessment, the overall response rate was 18.5%, the median time to response was 18 weeks and the median duration of response was not reached at the time. (10)

The second Phase I trial (NCT01375842) tested the efficacy and safety of the anti-PDL1 antibody atezolizumab (MPDL3280A) and required >5% PD-L1 positivity by immunohistochemistry using the SP142 antibody. Sixty-nine percent of patients who were screened, tested positive for PD-L1 expression. Three dose levels were evaluated including 15mg/kg, 20mg/kg and a 1200 mg fixed dose. Twenty-one patients were evaluable for efficacy, and 19% objective response rate was observed. The 24-week PFS rate was 27%. Fifty-four patients were evaluable for toxicity: most adverse events were < Grade 2, but 11% had treatment-related > Grade 3 adverse events.

Expansion cohort (initially restricted only to PD1 positive but eventually opened to all patients with TNBC) enrolled total 115 patients. Overall response rate was 26% first line and 11% 2nd line and beyond. In this trial the PD1 positive subgroup had a higher response rate of 17% as compared to PD1 negative subgroup which had response rate of 8%. The responders had durable responses. There were no unexpected toxicities. (11)

KEYNOTE-086 trial is a phase II trial of single agent pembrolizumab in TNBC. 170 patients were enrolled, and overall response rate was 4.7%. Interestingly, there was no difference between PD1 positive and negative tumors. The overall disease control rate was 7.6%. The subgroup that had significantly improved response rate was patients who received the treatment as first line. Toxicities were in line with previously described in other diseases. (12)

More importantly, both studies have shown a very impressive early OS (data from NCT01375842) with 100% responders being alive, 69% of patients with SD alive at 1 year (no data for 2 years yet) and 33% and 11% being alive at 1 and 2 years for patients that had no clinical benefit. KEYNOTE-86 survival data is less mature but the early trends are very similar.

Durvalumab and tremelimumab were studied in metastatic breast cancer (MBC, NCT02536794). The trial did not meet its preselected criteria to proceed to second stage in unselected MBC

population. The overall response rate in 18 evaluable patients was 17%. In 7 patients with TNBC, the response rate was 43% and in 11 estrogen receptor positive MBC patients the response rate was 0%. The rate of grade 3 (no grade 4 reported) toxicities were reported in 5/18 (27%) patients.(13)

2.3 Pre-clinical Studies with Chemokine Modulation

Intratumoral (i.t.) accumulation of IFNα-producing CD8+ cytotoxic T cells (CTLs) in tumor microenvironment (TME) and local expression of PD-1 (by CTLs) and PD-L1 and PD-L1 (by cancer cells, tumor stroma and inflammatory infiltrate) predict patients' benefit from PD-1/PD-L1 blockade (14-19). Prompted by poor responsiveness to PD-1 blockade (16-18, 20-22) of many common cancers, including metastatic breast cancer, we pursued modulation of TME as a strategy (23) **to improve the therapeutic effectiveness of PD-1 blockade in breast cancer patients**.

Figure 1

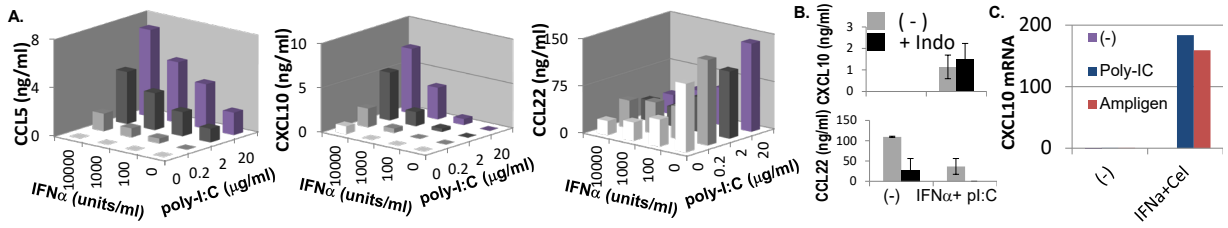


Fig. 1. Development of combinatorial adjuvants for selective induction of CTL-attracting chemokines: A. Opposite regulation of poly-IC-induced effector-type vs. suppressive chemokines by IFNα. Macrophages were used as the optimal source of CCL5 and CCL22, and fibroblasts were used as the source of CXCL10 (*see* ***Ref.***[1] *for experimental details*). **B.** COX-2 blockade selectively enhances the production of a *Teff*-attractant (CCL5) but suppresses the production of *Treg*-attracting CCL22. Chemokine levels in 48h supernatants. (ELISA). **C.** back-to back comparison of CXCL10 induction by CKM involving poly-I:C and Ampligen (Taqman). Similar data was obtained in case of CCL2 and CXCL22.

The chemokine receptors CXCR3 and CCR5 are typically used by CTLs to enter inflamed tissues. High tumor production of CCL5/RANTES (ligand for CCR5) and CXCL9/MIG, CXCL10/IP10, and CXCL11/ITAC (three known ligands for CXCR3) is associated with high CTL infiltration in different cancer types (24-26). Our previous correlative and mechanistic studies in other cancer types (27-29) showed the critical roles of intratumoral production of CCL5, CXCL9, and CXCL10 in local infiltration with CD8+GrB+ CTLs, while CCL22 was found responsible for the attraction of FOXP3+ Tregs.

Combinatorial adjuvants: Synergistic and selective induction of CTL-attracting chemokines and suppression of Treg attractants: In an attempt to correct the deficit in local production of CXCR3 and CCR5 ligands seen in many cancer lesions, we compared the ability of toll like receptor (TLR) ligands and cytokines to induce different classes of chemokines. Although TLR3 ligands, such as poly-I:C were relatively ineffective when used alone and induced a significant production of Treg-attracting CCL22 (MDC; CCR4 ligand; Fig. 1), the combination TLR3-L with IFNα (and especially with IFNα and COX2 blockers), resulted in a strong synergy in selective induction of CXCR3 and CCR5 ligands, with concomitant suppression of CCL22 (Figure 1).

Studies completed within our NCI-funded program, 1P01CA132714: "Directing Tumor-Specific T Cells to Tumors," identified TLR3 ligands, poly-I:C (double-stranded RNA; a molecular mimic of viral infections and activator TLR3 and RIG-I/MDA5 helicases) and its derivative, **rintatolimod, a selective TLR3 ligand,** which does not activate helicases and shows improved pattern of TME activation (30) with enhanced safety profile for systemic (i.v.) use, as preferred adjuvants which preferentially induce the CTL-/Th1/NK cells-attracting chemokines (27-32) and **direct selective CTL accumulation in TMEs** (27-30). Furthermore, the combination of rintatolimod (or poly-I:C) with IFNα and COX2 blockers (to enhance TLR3 signals and abrogate negative aspects of inflammation) are **10- to 100-fold more effective** than each of these factors alone in inducing CTL attractants, and **eliminates Treg attraction** (27-30). Such combinatorial chemokine-modulating regimen (**CKM**) **acts in tumor-selective fashion**, avoiding activation of healthy tissues (27), and abrogates the heterogeneity of different tumor lesions (27), **converting all "cold" tumors** into inflamed ones (27-30).

Our mouse data shows **safety of CKM combined with PD-1 blockade** and their strong **therapeutic synergy** in inducing long-term survival (>150 days) of mice with PD-1-resistant, epithelial tumors (Figure 2).

Figure 2

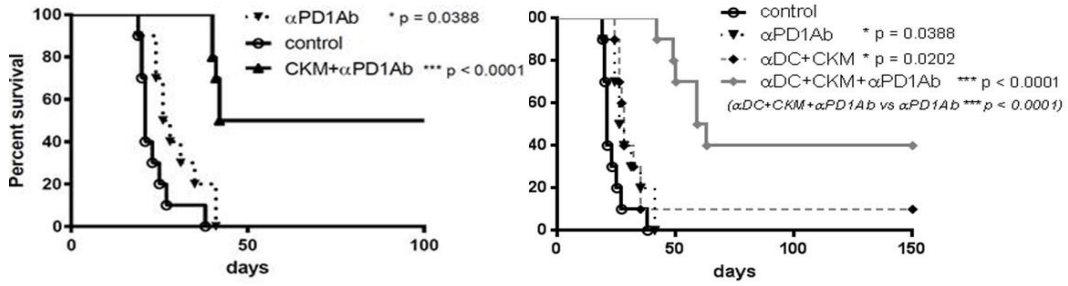


Fig. 2. Safety and effectiveness to CKM+αPD1 therapy in nominally PD1-resistant mouse tumors. B6 mice (10/ group) were injected i.p. with 10^6 MC38 cells (d 0). On days 3, 6, 9, mice received αPD-1 mAb (Rmp1-14, BioXCell, 90 μg/dose). CKM (rintatolimod, IFNα, celecoxib) was given twice. *Right only*: Mice also received two s.c. doses of $3x10^5$ αDC1s loaded with killed MC38 cells (days 7, d). αPD-1 with rintatolimod alone showed partial therapeutic effect (*not shown*)

2.4 Clinical Studies with Chemokine Modulation

Early data from our clinical trials of CKM in CRC: NCT01545141, i.v.; and ovarian cancer (NCT02432378; i.p.) demonstrated safety of rintatolimod, administered alone or with IFNα, and local efficacy in the TME of patients receiving systemic or local CKM (chemokines, immune markers, and PD-1, PD-L, PD-L2; Figure **3**).

Figure 3

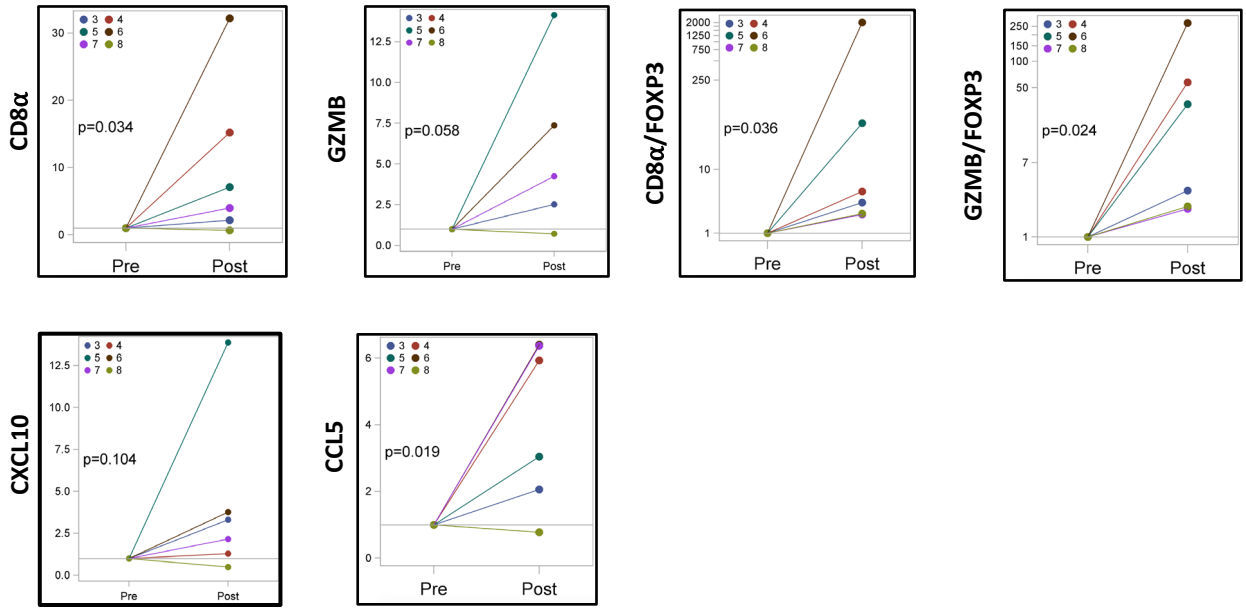


Figure 3. Normalized mRNA transcript measured using TaqMan analysis in pre- and post-CKM biopsies. Consistent increases in CTL markers and CTL-attracting chemokines in the post-CKM tumor biopsies observer on the completed study I 62218 (NCT03599453) performed under IND 145344, which uses INTRON®-A as a component of the CKM.

We observed uniform increase of immune markers upon treatment: CD8α mRNA (6.1-fold; p-0.034), GZMB mRNA (3.5-fold; p=0.058), ratios of CD8α/ FOXP3 and GZMB/FOXP3 (5.7-fold; p=0.036, and 7.6-fold; p=0.024 respectively), thus successfully meeting the endpoint. In addition, we also observed an increase in CTL attractants CXCL10 (2.6-fold; p=0.104) and CCL5 (3.3-fold; p=0.019). In contrast, Treg marker FOXP3 or Treg attractants CCL22 or CXCL12 were not enhanced.

2.5 Rationale for Using Chemokine Modulation to Enhance the Effectiveness of anti-PD1 therapy (Pembrolizumab)

As stated above **rintatolimod, a selective TLR3 ligand,** which does not activate helicases and shows improved pattern of TME activation (see **Appendix K**) (30) with enhanced safety profile for systemic (i.v.) use, as preferred adjuvants which preferentially induce the CTL-/Th1/NK cells-attracting chemokines (27-32) and **direct selective CTL accumulation in TMEs** (27-30). Furthermore, the combination of rintatolimod (or poly-I:C) with IFNα and COX2 blockers (to enhance TLR3 signals and abrogate negative aspects of inflammation) are **10- to 100-fold more effective** than each of these factors alone in inducing CTL attractants, and **eliminates Treg attraction** (27-30). The CME combination is able to change "cold" tumors into "hot" tumors which are then able to respond to anti-PD1 treatment such as pembrolizumab.

In our completed pilot study I 62218 (NCT03599453) Chemokine Modulation Therapy and Pembrolizumab in Treating Participants with Metastatic Triple-Negative Breast Cancer. PI: Shipra Gandhi (P. Kalinski: Laboratory Lead) performed under IND 145344 , we observed a good safety profile and the ability of the tested CKM regimen to enhance intratumoral expression of CTL

marker CD8α (predetermined primary endpoint of efficacy) s and CTL attracting chemokines (33) (See **Appendix J** for the poster reporting the safety and efficacy data at AACR 2022).

2.6 Rationale for the Proposed Phase I/II Evaluation.

The development of the CKM regimen has been jeopardized by Merck's decision to discontinue the production of INTRON®-A, one of the three components of the CKM (in addition to rintatolimod and celecoxib). The goal of the current trial is to obtain safety data of alternate (non-U.S. licensed) IFN α-2b product (Bioferon®, from Argentinian supplied by Biosidus S.A.) used at the same 20 MU/M^2 dose as the INTRON®-A dose used in the trials conducted under IND 145344, and (for safety reason, as requested by the CDER during our recent type C meeting) at the initial lower dose of 10 MU/m^2. Bioferon® is not FDA-approved and is considered an investigational new drug in the United States. Following the testing of the lower dose of IFNα-2b (CKM on 3 days within week 1, and 3 days within week 2) use prior to implementation of pembrolizumab (starting on week 3), we will further test the CKM involving alternate (non-U.S. licensed) IFNα-2b product (first at 10 MU/M^2 and then at 20 MU/M^2) in a modified regimen, with the initial dose of pembrolizumab being administered on week 1 of the protocol, rather than week 3. The resulting safety data, and expected pilot efficacy data, will be used to design larger trials evaluating the clinical effectiveness of the combination of CKM and PD1 blockade.

The use of Bioferon to replace INTRON A, discussed with the FDA in type C meeting held in June 2022, was FDA approved on October 14, 2022 (IND 163681 - held by Roswell Park).

We plan to use this CKM/Pembrolizumab regimen in the frontline setting for all TNBC patients independent of their PDL1 status, based on: a) The documented ability of CKM to promote attraction of PD1+ CTLs to tumors, as observed in our preclinical mouse models and the recently completed clinical study I-62218, b) The ability of CKM to directly enhance the PDL1/2 expression on target cells and to promote local attraction of IFNγ-producing CTLs which can enhance PD-L1 (and PD-L2) expression indirectly, due to local IFNγ production, c) Strong synergy between the CKM and PD1 blockade observed by us in multiple preclinical nominally PD1-resistant mouse models of breast in other cancers (**Figure 2** and Basse, Kalinski et al, manuscript in preparation), d) Very low toxicity of the proposed regimen (recently completed clinical study I-62218; (Gandhi) and, e) Short duration of the experimental treatment, allowing a rapid transition to chemotherapy in case of lack of efficacy in the individual patients.

3 INCLUSION AND EXCLUSION CRITERIA

NOTE: For blood chemistry labs, Roswell Park clinical blood chemistries are performed on plasma unless otherwise indicated.

3.1 Inclusion Criteria:

To be included in this study, participants must meet the following criteria:

1 Age ≥ 18 years of age.

2 Have pathologically confirmed diagnosis of PDL-1-negative or PDL1 positive unresectable or metastatic TNBC with no curative treatment options.

3 Have been informed of other treatment options.

4 Patient has lesion that can be biopsied and is willing to undergo the procedure as part of the protocol. **Note:** For Cohort 1 and Cohort 2: Patient with accessible tumor will be offered *optional* pre-treatment and post-treatment biopsies. Biopsies are *mandatory* for Cohort 3.

5 Have an ECOG performance status of ≤ 1. Refer to Appendix A.

6 Participants of child-bearing potential must agree to use adequate contraceptive methods (e.g., hormonal or barrier method of birth control; abstinence) prior to study entry. Should a woman become pregnant or suspect she is pregnant while she or her partner is participating in this study, she should inform her treating physician immediately.

7 Ability to swallow and retain oral medication.

8 Have measurable disease per RECIST 1.1 criteria present.

9 Any line of therapy allowed, radiologically confirmed progression.

10 No cancer-directed therapy for at least 3 weeks prior to study treatment (bone-directed therapies are allowed).

11 Must have adequate organ and marrow function present as defined below:

- Platelets ≥ 100,000/μL
- Hemoglobin ≥ 9.0 g/dL
- Absolute Neutrophil Count (ANC) ≥ 1500/μL
- Total bilirubin ≤ institutional upper limit of normal (ULN)
- AST (SGOT) and ALT (SGPT) ≤ 2.5 X institutional ULN
- Creatinine < ULN or, creatinine clearance ≥ 50 mL/min per Cockcroft-Gault Equation for patients with creatinine levels greater than ULN (refer to Appendix D).

12 Participant must understand the investigational nature of this study and sign an Independent Ethics Committee/Institutional Review Board approved written informed consent form prior to receiving any study related procedure.

Please refer to Appendix B for the Investigator Study Eligibility Verification Form: Inclusion Criteria.

3.2 Exclusion Criteria:

Participants will be excluded from the study for the following:

1 Patients currently treated with systemic immunosuppressive agents, including steroids (greater than equivalent of 10 mg daily of prednisone), are ineligible until 3 weeks after removal from immunosuppressive treatment. (Inhaled steroids are allowed.)

2 Patients with active autoimmune disease or history of transplantation.

3 Pregnant or nursing female participants.

4 Unwilling or unable to follow protocol requirements.

5 Patients with known serious mood disorders. (Major depression diagnosis is an exclusion. Other stable mood disorders on stable therapy for > 6 months or not requiring therapy may be allowed after consultation with the Principal Investigator.)

6 Cardiac risk factors including:

- Patients experiencing cardiac event(s) (acute coronary syndrome, myocardial infarction, or ischemia) within 3 months of signing consent. While our published clinical studies involving short-term CKM (34, 35) did not indicate increased risk of cardiac events, the CKM can induce flu-like symptoms, providing justification for its avoidance in patients with recent cardiac events.
- Patients with a New York Heart Association classification of III or IV (Appendix E)
- Patients with a history of stroke.

7 History of upper gastrointestinal ulceration, upper gastrointestinal bleeding, or upper gastrointestinal perforation within the past 3 years.

8 Prior allergic reaction or hypersensitivity to NSAIDs or any drugs administered on protocol.

9 Any condition which in the Investigator's opinion deems the participant an unsuitable candidate to receive study drug.

10 Any patients with a positive Antinuclear Antibodies test will be excluded from study.

11 Has a known history of Human Immunodeficiency Virus (HIV) infection.

12 Concurrent active Hepatitis B (defined as HBsAg positive and/or detectable HBV DNA) and Hepatitis C virus (defined as anti-HCV Ab positive and detectable HCV RNA) infection. Note: Hepatitis B and C screening tests are not required unless known history of HBV and HCV infection.

Please refer to Appendix C for the Investigator Study Eligibility Verification Form: Exclusion Criteria.

3.3 Special Populations

The following special populations will be excluded from this study:

- Cognitively impaired adults/adults with impaired decision-making capacity
- Individuals who are not yet adults (infants, children, teenagers)
- Pregnant women
- Prisoners

3.4 Inclusion of Women and Minorities

Both men and women and members of all races and ethnic groups are eligible for this study.

4 LOCAL AND STUDY-WIDE NUMBER OF SUBJECTS

A maximum of 12-15 evaluable patients from Roswell Park will be enrolled.

5 LOCAL AND STUDY-WIDE RECRUITMENT METHODS

5.1 Recruitment

Potential participants will be identified and recruited during scheduled visits to Roswell Park. Non-investigator Roswell Park physicians and community physicians may also refer potential subjects to the investigator for evaluation. There will be no "cold calling." Informed consent will be obtained on all subjects by the investigator prior to all study specific procedures (including screening procedures). No subject will be entered into this clinical trial without having a signed written consent form.

6 MULTI-SITE RESEARCH

Not applicable: This is a single-site study.

7 STUDY TIMELINES

In the absence of disease progression, unacceptable toxicity or withdrawal from study, it is anticipated that the patients will be on active protocol treatment for approximately 8 weeks (CKM treatment #1 & #2, followed by 4 cycles of pembrolizumab. Pembrolizumab will be introduced at the end of the first cycle of the CKM in cohort 2 and cohort 3, while in cohort 1 it will be delayed by one CKM cycle to immediately follow CKM cycle #2).

After completion of 4 cycles/doses of pembrolizumab, subsequent treatment will be off-study per the treating clinician. To capture all possible delayed immune-related adverse events, patients will be followed 90 days past last immunotherapy (patients, who in consultation with their clinician will be continued on pembrolizumab after cycle/dose 4, will be included in continued follow-up for immune mediated toxicities).

Follow-up for scans (i.e., CT of chest, abdomen and pelvis done as SOC, i.e., every 3 months following cycle/dose #4 of Pembrolizumab) using iRECIST will continue until patient has progressive disease or until pembrolizumab is discontinued. In addition, tissue from resected tumor (and margin) harvested within standard care from these patients may be requested for correlative analysis for potential identification of biomarkers that may be related to clinical outcome.

Accrual is expected to take up to 3 years.

8 STUDY ENDPOINTS

8.1 Primary Endpoint

- Overall safety profile characterized by type, frequency, severity (according to CTCAE version 5.0), timing, seriousness, and relationship to study treatment

8.2 Secondary Endpoints

- Progression-free survival (PFS)
- Overall response rate (ORR) to the combination therapy per iRECIST criteria

- Overall survival (OS)
- Disease control rate (DCR) per iRECIST

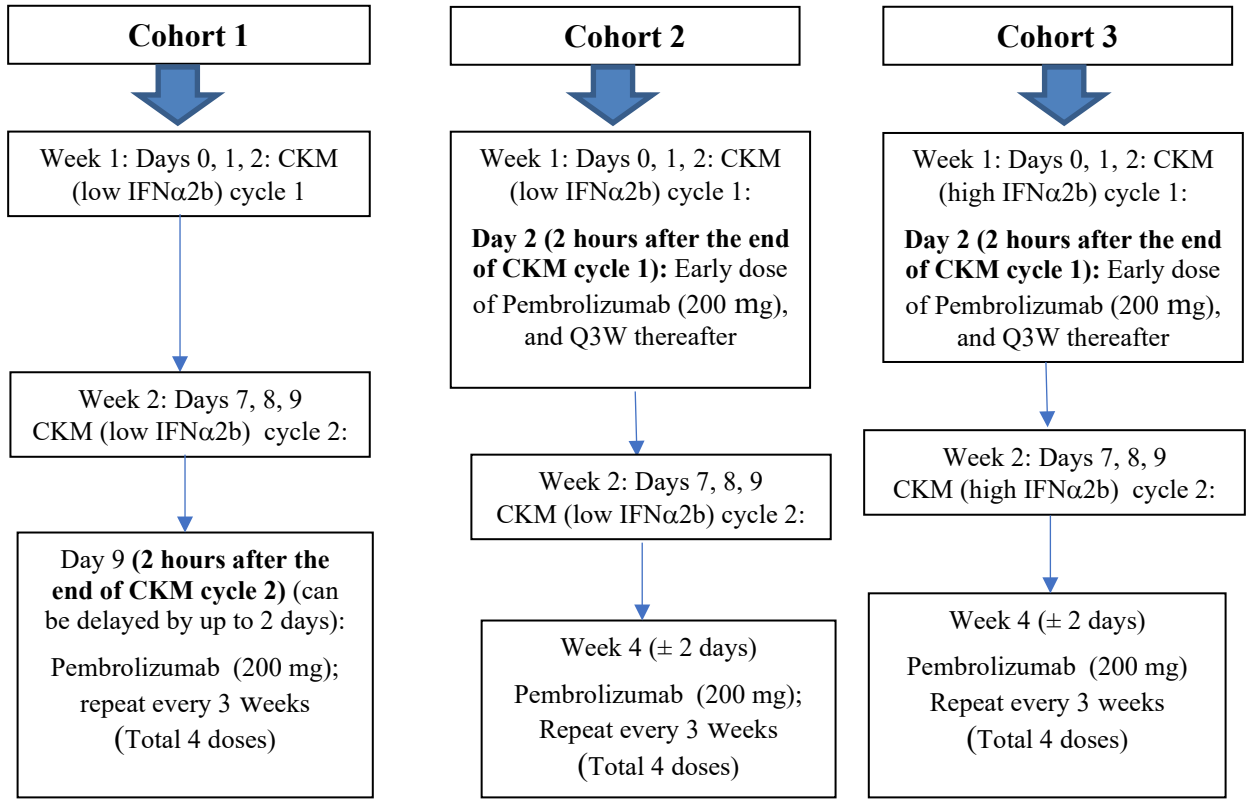
8.3 Exploratory Objectives

- Examine the immune analysis profile of CKM and pembrolizumab combination:
    - Examine the relationship of infiltrating CD4+ and CD8+ T cells and other immune and genetic markers, and their associated PD-1, CD45RA or CD45RO levels
    - Correlate PD-L1 expression within both neoplastic and nonneoplastic stromal elements of the tumor microenvironment to PFS, OS, ORR and AEs.
    - Correlate Immune Panel results with ORR, PFS, OS and AEs.
- Comparison of response assessment criteria for a prospective analysis.
    - RECIST 1.1 response assessment (Appendix F)
    - iRECIST response assessment (Appendix G)

9 DESIGN

This is an open-label, non-randomized, single center, dose escalation Phase I/IIa study of modified CKM regimen (replacement of INTRON® A, using alternative source of IFNα2b) combined with pembrolizumab therapy in metastatic breast cancer patients. The study schema is depicted in Figure 4.

Figure 4 Study Schema



Biopsies can be performed within 1 week before the start of CKM treatment and within 24 hours (can be delayed to 3 days in case of logistic or compliance issues) after completion of the 2nd cycle of the CKM treatment. Biopsies are ***optional*** for Cohort 1 and Cohort 2 and ***mandatory*** for Cohort 3.

10 TREATMENT

Treatment is intended for an outpatient setting. However, at the investigator's/physician's discretion, the participant may receive treatment as an inpatient, if deemed necessary. On-study visit procedures and assessments are allowed a window of ± 1 day unless otherwise noted. No staggering between starting patients within a cohort is necessary.

10.1 Dosing and Administration

COHORT #1 (n=3):

- Week 1 (days 0, 1, 2 of CKM cycle 1): Rintatolimod 200 mg IV + Celecoxib 200 mg* + IFN 10 million units/m^2 IV over 30 minutes
- Week 2 (days 7,8,9 of CKM cycle 2): same treatment as Week 1
- Day 9 (± 2 days): Start of Pembrolizumab (200 mg every 3 weeks).
    - Note: CKM must be given within 14 days prior to the start of Pembrolizumab.

COHORT #2 (n=3-6):

- Week 1 (days 0, 1, 2 of CKM cycle 1): Rintatolimod 200 mg IV + Celecoxib 200 mg* + IFN 10 million units/m^2 IV over 30 minutes
    - Week 1 Day 2 (2 hours following the completion of CKM cycle #1) : Early dose #1 of Pembrolizumab (200 mg) and thereafter every 3 weeks.
- Week 2 (days 7,8,9 of CKM cycle 2): same treatment as Week 1
- Week 4: Start of Pembrolizumab (200 mg every 3 weeks). CKM must be given within 14 days prior to the start of Pembrolizumab.

COHORT #3 (n=3):

- Week 1 (days 0, 1, 2 of CKM cycle 1): rintatolimod 200 mg IV + celecoxib 200 mg* + IFN 20 million units/m^2 IV over 30 minutes
    - Week 1 Day 2 (2 hours following the completion of CKM cycle #1): Early dose of Pembrolizumab (200 mg) and thereafter, every 3 weeks
- Week 2 (days 7,8,9 of CKM cycle 2): same treatment as Week 1
- Week 4: Start of Pembrolizumab (200 mg every 3 weeks). CKM must be given within 14 days prior to the start of Pembrolizumab.

***Note:** Participant will be administered 200 mg Celecoxib (orally) in the Clinical Trail Unit (morning dose), and will be instructed to take a second dose of Celecoxib (200 mg orally) at home at approximately 12 hours following the initial dose (evening dose).

COHORT #3 Expansion Cohort (n=3): If 1/3 responses, then expand to enroll another 3 patients for a total of 6 patients. Note: If cohort 3 dosing is not tolerated, then 3 more patients will be enrolled at the cohort 2 dose level and declared as the MTD.

10.1.1 Chemokine Modulating (CKM) Regimen

The patient will be given CKM treatment #1: Week 1 (dosing days 0, 1 and 2: ± 1 day for each treatment day) and CKM treatment #2: Week 2 (dosing days 7, 8 and 9 (± 1 day) for each treatment day).

For Cohort 1: CKM must be given within 14 days prior to the start of Pembrolizumab.

For Cohort 2 and Cohort 3: an additional early dose of pembrolizumab will be administered on day 2 (following completion of CKM cycle #1) and following doses will be given at 3-week intervals.

Each patient with accessible tumor will be offered *optional* pre-treatment (before initiation of CKM regimen treatment #1) and post-treatment (after CKM treatment #2 and prior to receiving first dose of pembrolizumab) biopsies. Biopsies are mandatory for Cohort 3 and optional for Cohort 1 and Cohort 2.

On each CKM dosing day, the patient will be given the following medications in this order:

Pre-treatment:

- Pre-treatment: 500 mL Normal saline IV over 60 minutes

- Pre-meds: Acetaminophen (Tylenol) 650 mg by mouth x 1 dose; Prochlorperazine (Compazine) 10 mg by mouth x 1 dose – administered 30 minutes (±5 minutes) after starting pre-treatment hydration, the CKM treatment to begin 30 minutes (±5 minutes) after that, at completion of IV normal saline

Study treatment:

- Celecoxib: 200 mg orally, administered along with pre-meds
- Interferon α-2b:
  - **Cohort 1 and Cohort 2:** 10 million units/m^2 IV over 30 minutes
  - **Cohort 3:** 20 million units/m^2 IV over 30 minutes
- Rintatolimod: 200 mg IV, initial administration should begin at a slow rate of infusion (approximately 20 cc/hour) and increase to 40 cc/hour after 30 minutes. Tubing should be flushed with 30 to 50 mL of normal saline solution upon completion. Administration will be followed by 1 hour of observation and vital signs at 30 and 60 minutes post infusion (± 5 minutes).

Note: Participants will be instructed to take a second dose of Celecoxib (200 mg orally) at home at approximately 12 hours following the initial dose.

Pre-treatment normal saline and pre-meds only, excluding CKM regimen and pembrolizumab, listed above are recommendations and may be modified based upon institutional SOC requirements and at the discretion of the investigator.

10.1.2 Pembrolizumab

Pembrolizumab will be administered in accordance with institutional guidelines: 200 mg, IV, Q3W).

Pembrolizumab infusion solution must be administered intravenously over 30 minutes through an intravenous line containing a sterile, non-pyrogenic, low-protein binding 0.2 micron to 5 micron in-line or add-on filter. Do not co-administer other drugs through the same infusion line.

10.2 Definition of Dose-Limiting Toxicity

Participants will be monitored for DLTs. The following events will be considered a DLT:

- The DLT period is defined from the first dose of CKM (day 0) through 3 weeks following 1st dose of combination CKM + pembrolizumab therapy.
- The toxicity has been determined by the investigator to be possibly, probably or definitely related to celecoxib, rintatolimod, interferon-α2b or pembrolizumab.
- Any death not clearly due to the underlying disease or extraneous causes.
- If greater than or equal to 2 out of 5 patients experience a treatment delay of > 21 days, the study will be suspended and the Roswell Park Data Safety Monitoring Committee will review the study and will make recommendations that include but not limited to; (a) continuation of the study, (b) modifications to the design, (c) suspension of, or (d) termination of the study.

- Subsequent Enrollment for cohort 2 and 3 will only proceed after the DLT period has been completed for the previous cohort of patients.

Table 1 DLT Definition

Toxicity	Dose Limiting Toxicity
Hematology	• CTCAE grade 4 neutropenia lasting more than 7 consecutive days despite growth factor support • CTCAE grade 3 thrombocytopenia lasting more than 7 consecutive days and grade 4 thrombocytopenia • ≥CTCAE grade 3 thrombocytopenia with bleeding • CTCAE grade 3 or 4 febrile neutropenia • CTCAE grade 3 infection with/ without neutropenia
Cardiac	• Cardiac toxicity ≥ CTCAE grade 2 • Clinical signs of cardiac disease, such as unstable angina or myocardial infarction
Gastro-intestinal	• ≥ CTCAE grade 3 vomiting ≥48 hours despite optimal anti-emetic therapy • ≥ CTCAE grade 3 diarrhea ≥48 hours despite optimal anti-diarrhea treatment • ≥ CTCAE grade 3 mucositis
Hepato-biliary	• CTCAE grade 2 total bilirubin for more than 7 consecutive days • CTCAE grade 3 or higher total bilirubin • ≥ CTCAE grade 3 ALT/AST for > 4 consecutive days • CTCAE grade 4 ALT or AST • Grade 4 alkaline phosphatase >7 consecutive days • Any AST or ALT >3 x ULN and concurrent total bilirubin > 2 x ULN without initial findings of cholestasis (elevated alkaline phosphatase)
Renal	• ≥ CTCAE grade ≥3 creatinine
Infusion Related Reaction	• ≥ CTCAE grade 3
Non-hematologic events	• ≥ CTCAE grade 3, except for the exclusions noted below:
Any immune-related reaction	• ≥ CTCAE grade 3 • ≥ CTCAE grade 1 fever lasting more than 7 consecutive days
Neurological	• ≥ CTCAE Grade 3 neurotoxicity
Exceptions to DLT criteria	• Grade 3 alopecia • < 5 days of CTCAE grade 3 fatigue

Dose escalation will proceed according to **Section 19.1**.

Participants who do not receive 5 (out of 6) doses of the CKM regimen due to non-disease and non-treatment related reasons may remain on study but will be considered as "non-evaluable for the primary outcome" and will be replaced.

10.3 Dose Modifications and Treatment Delays

10.3.1 CKM Regimen: Dose Modifications and Treatment Delays

The following dose modification rules will be used with respect to potential toxicity. Toxicity will be assessed according to the NCI Common Terminology Criteria for Adverse Events Version 5.0 (CTCAE v5.0).

10.3.1.1 Celecoxib Hematologic and Non-Hematologic Toxicities

Considered to be possibly, probably, or definitely related to Celecoxib (Interferon α-2b and rintatolimod will be continued). Celecoxib will be discontinued for any attributable Grade ≥3 toxicity.

10.3.1.2 Interferon α-2b and/or Rintatolimod Hematologic and Non-hematologic toxicities

Considered to be possibly, probably, or definitely related to Interferon α-2b and rintatolimod:

Transient (no longer than 5 days) lymphopenia or neutropenia is expected post interferon or rintatolimod (and their combination) therefore this does not require any dose adjustment and is not a DLT.

Interferon α-2b

- **Hematologic Toxicities (except transient lymphopenia or neutropenia no longer than 5 days)**

For Grade 3 toxicity:

- If < 7 days to resolution (transfusions are allowed): Treatment to continue if toxicity is resolved to ≤ Grade 1 or baseline) at a dose reduction of 33%. The dose can be re-escalated if toxicity remains at ≤ Grade 1 or baseline.
- If > 7 days to resolution: Treatment to continue if toxicity is resolved to ≤ Grade 1 or baseline at the next study visit at a dose reduction of 33%. The dose cannot be re-escalated.
- 2nd incidence: Treatment to continue if toxicity is resolved to ≤ Grade 1 or baseline at the next study visit at a dose reduction of 33% (total 66% reduction if the dose was not re-escalated). The dose cannot be re-escalated.

For Grade 4 toxicity

- If < 7 days to resolution (transfusions are allowed): Treatment to continue if toxicity is resolved to ≤ Grade 1 or baseline at the next study visit at a dose reduction of 33%. The dose cannot be re-escalated.
- If > 7 days to resolution: Discontinue

- **Non-Hematologic toxicities**

For Grade 3 toxicity:

- 1st episode: Treatment to continue if toxicity is resolved to ≤ Grade 1 or baseline at a dose reduction of 33%. The dose can be re-escalated if toxicity remains at ≤ Grade 1 or baseline.
- 2nd episode: Treatment to continue if toxicity is resolved to ≤ Grade 1 or baseline at the next study visit at a dose reduction of 33% (total 66% reduction if the dose was not re-escalated). The dose cannot be re-escalated.

For Grade 4 toxicity:

- 1st episode: Treatment to continue if toxicity is resolved to ≤ Grade 1 or baseline at the next study visit at a dose reduction of 33%. The dose cannot be re-escalated.
    - 2nd episode: Off study, patient will be followed until the event resolves to ≤ Grade 1 or baseline study (patient may receive pembrolizumab off of the protocol at treating physician's discretion).

Rintatolimod

- **Hematologic Toxicities (except lymphopenia):**

For Grade 3 toxicity:

- If < 7 days to resolution (transfusions are allowed): Treatment to continue if toxicity is resolved to ≤ Grade 1 or baseline) at a dose reduction of 33%. The dose can be re-escalated if toxicity remains at ≤ Grade 1 or baseline.
- If > 7 days to resolution: Treatment to continue if toxicity is resolved to ≤ Grade 1 or baseline at the next study visit at a dose reduction of 33%. The dose cannot be re-escalated.
- 2nd incidence: Treatment to continue if toxicity is resolved to ≤ Grade 1 or baseline at the next study visit at a dose reduction of 33% (total 66% reduction if the dose was not re-escalated). The dose cannot be re-escalated.

For Grade 4 toxicity:

- If < 7 days to resolution (transfusions are allowed): Treatment to continue if toxicity is resolved to ≤ Grade 1 or baseline at the next study visit at a dose reduction of 33%. The dose cannot be re-escalated.
- If > 7 days to resolution: Discontinue

- **Non-Hematologic toxicities**

For Grade 3 toxicity:

- 1st episode: Treatment to continue if toxicity is resolved to ≤ Grade 1 or baseline at a dose reduction of 20%. The dose can be re-escalated if toxicity remains at ≤ Grade 1 or baseline.
- 2nd episode: Treatment to continue if toxicity is resolved to ≤ Grade 1 or baseline at a dose reduction of 20% (total dose reduction 20% if dose was not re-escalated). The dose can be re-escalated if toxicity remains at ≤ Grade 1 or baseline.

For Grade 4 toxicity:

- 1st episode: Treatment to continue if toxicity is resolved to ≤ Grade 1 or baseline at a dose reduction of 20% (total dose reduction 20% if dose was not re-escalated). The dose can be re-escalated if toxicity remains at ≤ Grade 1 or baseline.
- 2nd episode: Off study (patient may receive pembrolizumab off of the protocol at treating physician's discretion)

10.3.2 Toxicity Management

The toxicity of high dose interferon (20 million units/ m^2/d) has been established by Kirkwood et al. in a number of trials. Most notable was the E1684 trial (36) where HDI (20 million units/m^2/d) was administered daily for 5 days x 4weeks. In that trial (n=143), grade 3 toxicities were 67%, grade 4 toxicities were 9% (mainly constitutional and neurologic), and there were 2 treatment related mortalities (Grade 5) due to hepatoxicity. The proportion of Grade 3 and 4 toxicities in that trial were 48.2% for constitutional toxicities (defined as 'worst grade of any constitutional toxicity, including fever, chills, flu-like symptoms, fatigue, malaise, and diaphoresis), and 66% for non-constitutional toxicities (23.8% for myelosuppression, 13.9% for hepatotoxicity, 28% for neurological toxicity).

While the combination of HDI and rintatolimod may lead to synergistic additive side effects, we will monitor subject toxicities for unexpected increases in constitutional symptoms, myelosuppression, hepatotoxicity, and neurological toxicity. Moreover, the presence of celecoxib may improve the side effect profile. Our strategy is to continuously monitor treatment related occurrences of constitutional symptoms (including fever, chills, flu-like symptoms, fatigue, malaise, or diaphoresis) and more importantly immune related toxicities after initiation of pembrolizumab. We will suspend the trial if there is evidence indicating that these toxicities are occurring at a greater frequency than would be expected from interferon treatment alone during pre-treatment phase and from pembrolizumab treatment in treatment phase. The decision to move forward will them be made by EPCT safety review committee with appropriate changes to the protocol if appropriate.

These are suggestions for specific toxicities and may be modified for institutional guidelines and treating physician preference.

- **Gastrointestinal Toxicity:**
  - Nausea and/or vomiting should be controlled with adequate antiemetic therapy. Prophylactic antiemetic therapy can be used at the discretion of the investigator/sub-investigator. Subjects are encouraged to take plenty of oral fluids.
  - Diarrhea should be managed with appropriate antidiarrheal therapy. Subjects should be encouraged to take plenty of oral fluids. If symptoms do not decrease to Grade 1 or less with adequate antidiarrheal therapy, all protocol drugs should be held until resolved to < Grade 1.
- **Pain/Fever/Chills**
  - For chills and/or fever or mild local pain, acetaminophen will be utilized at the discretion of the investigator/sub-investigator or designee.

- **Hypersensitivity Reactions**

Caution: Subjects who had a mild to moderate hypersensitivity reaction have been successfully re-challenged, but careful attention to prophylaxis and bedside monitoring of vital signs is recommended. Hypersensitivity reactions to Interferon α-2b and/or rintatolimod will be managed as follows:

  - Mild symptoms (e.g., mild flushing, rash, pruritus): Complete infusion. Supervise at bedside. No treatment required.
  - Moderate symptoms (e.g., moderate rash, flushing, mild dyspnea, chest discomfort): Stop infusion. Give intravenous diphenhydramine 50 mg and intravenous famotidine 2 mg. Resume infusion after recovery of symptoms at a 66% slower rate, then, if no further symptoms, rate can be titrated up every 5-10 min. to initial rate until infusion is complete. If symptoms recur, at lowest infusion rate stop the infusion and no further Interferon α-2b administered on that day. If symptoms recur after the rate has been increased, stop the infusion, treat reaction as per institutional guidelines, consider dexamethasone 20 mg IV and restart at 66% slower rate (over 1 hour) without uptitration. Future doses pre-treatment modifications and rate modification after discussion with the investigator. Record toxicity.
  - Severe life-threatening symptoms (e.g., hypotension requiring pressor therapy, angioedema, respiratory distress requiring bronchodilation therapy, generalized urticaria): Stop infusion. Treat as per institutional guidelines. Subject should be removed from further protocol therapy. Report as serious adverse event.

10.3.3 Criteria for Replacement

Participants who do not receive 5 (out of 6) doses of the CKM regimen due to non-disease and non-treatment related reasons may remain on study but will be considered as “non-evaluable for the primary outcome” and will be replaced.

10.4 Pembrolizumab: Dose Modifications and Treatment Delays

Withhold pembrolizumab for any of the following:

- Grade 2 pneumonitis
- Grade 2 or 3 colitis
- Grade 3 or 4 endocrinopathies
- Grade 2 nephritis
- Grade 3 severe skin reactions or suspected Stevens-Johnson syndrome (SJS) or toxic epidermal necrolysis (TEN)
- Aspartate aminotransferase (AST) or alanine aminotransferase (ALT) greater than 3 and up to 5 times upper limit of normal (ULN) or total bilirubin greater than 1.5 and up to 3 times ULN
- Any other severe or Grade 3 treatment-related adverse reaction

- Platelets < 100,000
- Per clinician judgement, pembrolizumab can be held for any grade 2 or higher adverse events determined to be related to the CKM treatment, until events have resolved to a grade 1.

Resume pembrolizumab in patients whose adverse reactions recover to Grade 0-1. If dose has to be held for longer than 1 week, it will be considered a missed dose and treatment to restart at next scheduled cycle.

Permanently discontinue pembrolizumab for any of the following, deemed at least possibly related to pembrolizumab treatment, reasons:

- Any life-threatening adverse reaction (excluding endocrinopathies controlled with hormone replacement therapy)
- Grade 3 or 4 pneumonitis or recurrent pneumonitis of Grade 2 severity
- Grade 3 or 4 nephritis
- Grade 4 severe skin reactions or confirmed SJS or TEN
- AST or ALT greater than 5 times ULN or total bilirubin greater than 3 times ULN
    - For patients with liver metastasis who begin treatment with Grade 2 AST or ALT, if AST or ALT increases by greater than or equal to 50% relative to baseline and lasts for at least 1 week
- Grade 3 or 4 infusion-related reactions
- Inability to reduce corticosteroid dose to 10 mg or less of prednisone or equivalent per day within 12 weeks
- Persistent Grade 2 or 3 adverse reactions (excluding endocrinopathies controlled with hormone replacement therapy) that do not recover to Grade 0-1 within 12 weeks after last dose of pembrolizumab.
- Any severe or Grade 3 treatment-related adverse reaction that recurs.
- Grade 2, 3 or 4 myocarditis.

10.5 General Concomitant Medication and Supportive Care

Additional cancer directed chemo-, immune-, and radiotherapies are not permitted during the active treatment period of this trial. Palliative radiotherapy may be permitted for symptomatic control of pain from bone metastases following discussion with the Principal Investigator, provided that the radiotherapy does not affect target lesions and it does not reach the criteria of PD per RECIST 1.1 criteria.

10.6 Duration of Treatment

Participants may receive up to 4 cycles/doses of pembrolizumab on protocol. Subsequent therapy off-study will be per the clinician’s discretion. If there is evidence of clinical benefit from 4 cycles/doses of pembrolizumab, the patient’s clinician can choose to obtain approval to continue pembrolizumab through the normal Roswell Park breast cancer clinic procedures.

10.7 Compliance

On days that the CKM regimen is given, the morning dose of celecoxib will be administered in the Clinical Research Center (or other clinical area) and documented by the nurse on the Medication Administration Record. The evening dose of celecoxib will be self-administered at home and documented by the participant on the patient diary. The participant will be asked to bring the diary with him/her to each clinical visit. Refer to Appendix H.

11 PROCEDURES INVOLVED

The study-specific assessments are outlined in Appendix I (Study Calendar). Unless otherwise stated on the study calendar, Baseline and/or Screening assessments must be performed within **21** days prior to the first dose of investigational product. Any results falling outside of the reference ranges may be repeated at the discretion of the investigator.

Unless otherwise defined in the written protocol text, all procedures/assessments will be conducted in accordance with Roswell Park Clinical Trials Office Standard Operating Procedures.

Eligibility of each participant will be established prior to start of treatment.

Informed consent ***MUST*** be completed prior to receiving any study related procedures.

11.1 Post-Treatment Follow-Up Evaluations

To capture all possible delayed immune-related adverse events, follow-up safety evaluations will occur at least monthly for 90 days after last dose of study drug or until resolution of any drug-related toxicity (telephone contact is acceptable). Patients, who in consultation with their clinician that will continue on pembrolizumab after cycle/dose #4, will be included in the 90-day follow-up for immune mediated toxicities.

- Concomitant medications: List any ongoing medications with dose changes, as applicable.
- Adverse events
- Follow-up for scans (i.e., CT of chest, abdomen and pelvis done as SOC) using iRECIST 1.1 will continue until patient has progressive disease or until pembrolizumab is discontinued.
- Tissue from resected tumor (and margin) harvested within standard care from patients may be requested for correlative analysis for potential identification of biomarkers that may be related to clinical outcome.

11.2 Long-Term Follow-Up Evaluations

After 90 day post treatment follow-up visit, participants will be followed every 6 months for 2 years to assess survival status. Participants who are unavailable for follow-up evaluations should be classified as lost to follow-up for 1 of the following reasons:

- Lost to follow-up: For a participant to be considered lost to follow-up, the investigator must make two separate attempts to re-establish contact with the participant. The attempts to re-establish participant contact must be documented (e.g., certified letter).
- Death: Date and cause of death will be recorded for those participants who die within 30 days after last dose of study drug (telephone verification is acceptable).

11.3 Correlative Studies

Blood samples (60 cc) will be collected at the time points specified on the study calendar (Appendix I). At each time point, (1) 10 mL red top tube and (5) 10 mL sodium heparin green top tubes will be collected. Collection tubes are to be labeled with the participant's MR number, participant's initials, participant's study number, time of collection, and protocol day. Specimens will be sent at room temperature to the attention of the Clinical Research Laboratory Services (Pneumatic Station 19) where they will get accessioned for tracking in the source document. Once specimen receipt has been documented, the specimens will be sent at ambient temperature to pneumatic station 641 (located in CCC LOB-4th floor). The Kokolus Laboratory will be notified via telephone *and* e-mail (all contacts to be copied with each e-mail- see contact information below) prior to sample shipment and, the samples will be held in CSPO until a response is received acknowledging that personnel are available to procure the samples from the pneumatic tube station. Samples will be processed in the Kokolus Laboratory for future analysis.

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Note: All investigator or analyzing research laboratories housing research samples need to maintain current **Temperature Logs** and study-specific **Sample Tracking and Shipping Logs**. The Principal Investigator/Laboratory Manager **must** ensure that the stated lab(s) have a process in place to document the receipt/processing/storage/shipping of study-related samples/specimens. This is required for both observational and interventional clinical studies collecting clinical samples.

11.4 Fresh Biopsy Samples

A fresh biopsy may be obtained at the following time points:

- At Baseline Visit: Optional for Cohort 1 and Cohort 2 – Mandatory for Cohort 3. Biopsies can be performed within 1 week before the start of CKM treatment.
- After completion of CKM pre-treatment regimen #2. (preferably same lesion if safe to perform): Optional for Cohort 1 and Cohort 2 – Mandatory for Cohort 3. Biopsies can be performed within 24 hours (can be delayed to 3 days in case of logistic or compliance issues) after completion of the 2nd cycle of the CKM treatment.

Biopsies are ***optional*** for Cohort 1 and Cohort 2 and ***mandatory*** for Cohort 3.

At each biopsy collection time point, 5 total cores may be obtained, including at least 4 tumor cores and 1 additional core of surrounding tissue (to enhance the stringency of our assays). Three cores of tumor and 1 core of surrounding tissue will be placed in Dulbecco's Phosphate-buffered

saline, and 1 core of tumor will be placed in formalin, and processed to FFPE block per institute standard.

If core biopsy cannot be performed due to size/location of tumor(s), a punch biopsy or other clinically indicated biopsy may be performed as per institute policy. Enough tissue should be collected from the biopsies such that 5 cores, as described above, can be obtained from the specimen.

Label the samples with study-specific subject ID number, clinical study number, protocol time point, and protocol day. Phosphate-Buffered Saline will be supplied by Dr. Kokolus's lab and samples will be kept at ambient temperature. Samples in Dulbecco's Phosphate-Buffered Saline will be sent to Dr. Kokolus's lab upon completion of processing (preferably ASAP within 2 hours of collection, but within 4 hours of collection is allowed). Once the remaining core has been processed to FFPE block, it will be delivered to Dr. Kokolus's Laboratory.

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Note: All investigator or analyzing research laboratories housing research samples need to maintain current **Temperature Logs** and study-specific **Sample Tracking and Shipping Logs**. The Principal Investigator/Laboratory Manager **must** ensure that the stated lab(s) have a process in place to document the receipt/processing/storage/shipping of study-related samples/specimens. This is required for both observational and interventional clinical studies collecting clinical samples.

12 WITHDRAWAL OF SUBJECTS

12.1 Treatment Discontinuation

Upon treatment discontinuation all end of treatment evaluations and tests will be conducted. All participants who discontinue due to an AE must be followed until the event resolves or stabilizes. Appropriate medical care should be provided until signs and symptoms have abated, stabilized, or until abnormal laboratory findings have returned to acceptable or pre-study limits. The final status of the AE will be reported in the participant's medical records and the appropriate eCRF.

Reasons for treatment discontinuation should be classified as follows:

- Death
- Progressive disease
- Toxicity; treatment related or unrelated
- Investigator judgment

  - The Investigator may discontinue a participant if, in his/her judgment, it is in the best interest of the participant to do so
- Noncompliance
- Participant voluntary withdrawal
  - A participant may withdraw from the study at any time, for any reason. If a participant discontinues treatment, an attempt should be made to obtain information regarding the reason for withdrawal.
- Sponsor decision

13 RISKS TO SUBJECTS

13.1 Celecoxib

Prolonged use of celecoxib may cause dyspepsia, headaches (including migraines) and borderline elevated liver function tests (which could indicate liver damage). Recently, information from three long-term studies of celecoxib has become available. In the first study, a cancer prevention study, an increased risk of heart attacks, strokes, and/or deaths resulting from heart or blood vessel disease was reported among people taking celecoxib. Approximately 1 in 100 subjects enrolled in this study receiving the placebo treatment had one of these serious events. In contrast, between 2 and 3 in 100 subjects taking celecoxib (between 400 and 800 mg daily) had one of these serious events. Another clinical cancer prevention study found no increased risks in subjects taking celecoxib 400 mg daily. The third study, an Alzheimer's disease prevention study, did not find increased risks with celecoxib. Dosing has been suspended in all three of these studies based on the findings of the first cancer prevention study. As a result, the FDA is now evaluating the possibility that celecoxib increases the risk of heart attack, stroke, and or death resulting from heart or blood vessel disease. Known infrequent side effects of celecoxib include nausea and/or vomiting, diarrhea, flatulence, abdominal and stomach pain, bleeding ulcer, upper respiratory tract infection, pharyngitis, rhinitis, sinusitis, peripheral edema, back pain, dizziness, insomnia, and skin rash. Rare risks include sudden death (unexpected or instant death that occurs within minutes or hours from any cause other than violence), vasculitis, hepatitis, liver failure, kidney failure, blood dyscrasia, hypoglycemia, hyponatremia, viral meningitis, severe allergic reaction, visual changes, and transient ischemic attack. Warnings/Precautions: Stomach problems may be more likely to occur if patients drink alcoholic beverages while taking this medicine. Taking two or more of the nonsteroidal anti-inflammatory drugs together on a regular basis may increase the chance of unwanted effects. Also, taking acetaminophen, aspirin or other salicylates, or ketorolac (e.g., Toradol) regularly while taking a nonsteroidal anti-inflammatory drug may increase the chance of unwanted effects. The risk will depend on how much of each medicine is taken every day and how long the medicines are taken together. Therefore, patients should not take acetaminophen or aspirin or other salicylates or ketorolac (e.g., Toradol). Clinical studies with celecoxib have identified potentially significant interactions with fluconazole and lithium. Experience with nonsteroidal anti-inflammatory drugs (NSAIDs) suggests the potential for interactions with furosemide and ACE inhibitors. Celecoxib is contraindicated in patients with known hypersensitivity to celecoxib. Celecoxib should not be given to patients who have demonstrated allergic-type reactions to sulfonamides. Celecoxib should not be given to patients who have experienced asthma, urticaria, or allergic-type reactions after taking aspirin or other NSAIDs. Severe, rarely fatal, anaphylactic-like reactions to NSAIDs have been reported in such patients.

For more risk information reference the Investigator's Brochure or package insert.

13.2 Interferon-α2b

General Symptoms: Most patients suffer from flu-like symptoms, such as asthenia, fever, chills, anorexia, myalgia, headache, arthralgia, and sweat. Simultaneous administration of acetaminophen allows the normal relief or elimination of these acute side effects, which tend to decrease as time progresses, either reducing the dose or maintaining the same treatment. Compliance with schedule can cause lethargy, weakness and asthenia.

Digestive Tract: Around two thirds of the patients suffer from anorexia and approximately a half, nausea. Less frequently vomiting, taste disorders, mouth dryness, weight loss, diarrhea or mild to moderate abdominal pain were observed. Rarely, constipation, flatulence, hypermotility and epigastric pain were reported. Exceptionally, cases of reactivation of peptic ulcer and mild gastrointestinal bleeding have been reported.

Liver Function Disorders: Mainly, ALT increase and also alkaline phosphatase have been described, LDH and bilirubin rise may occur though generally a dose adjustment was not required. In patients with hepatitis B, increase in transaminase values can be interpreted as HBeAg seroconversion marker.

Central Nervous System: Dizziness, vertigo, visual disorders, decrease of mental status, memory loss, depression, somnolence, confusion, nervousness, and sleep disturbances are not frequent. In rare occasions, intense somnolence, coma, cerebral ictus and transient impotence have occurred.

Peripheral Nervous System: Occasionally, paresthesia, numbness, neuropathy and tremor have been described.

Respiratory and Cardiovascular Systems: Secondary effects were observed in approximately one fifth of patients with cancer which included, generally transient episodes of hypotension or hypertension, edema, cyanosis, arrhythmia, palpitations and chest pain. Rarely, patients have reported cough or mild dyspnea. Isolated cases of pulmonary edema, congestive heart failure, cardiorespiratory arrest, myocardial infarction have been observed. Cardiovascular adverse reactions were infrequent in patients with hepatitis.

Skin, mucous membrane and appendages: Rarely, exanthemas, labial herpes exacerbation, pruritus, dry skin and mucoses, rhinorrhea and epistaxis have been described. Up to one fifth of patients developed mild to moderate alopecia, but it was reversible once treatment was discontinued.

Urinary Tract: Rarely, renal function is affected. Electrolytic alterations (generally related to anorexia or dehydration), proteinuria and sediment cell count increase have been observed. Exceptionally, urea, creatinine and uric acid increase has been noted.

Hematopoietic System: One third to more than a half of patients have experienced transient leukopenia, but on few occasions, the dose had to be reduced. In non-myelosuppressive patients, thrombocytopenia was less frequent and rarely hemoglobin and hematocrit decrease occurred. In non-myelosuppressed patients, thrombocytopenia and hemoglobin decrease were more frequent. Generally, severe hematologic alterations were normalized to pre-treatment levels, within 7 to 10 days after discontinuing interferon alpha 2 treatment.

Other side effects: In almost half of patients, hypocalcemia has been detected without consequences. Around one third of cancer patients have shown a rise in glycemia values. In some isolated cases, neutralizing antibodies of interferon alpha 2 have been detected. No clinical consequences derived from their presence have been reported up to date. In some disease (cancer, systemic lupus erythematosus, herpes zoster), spontaneous antibodies against human leukocyte interferon in patients that never received exogenous interferon have been detected. Local reactions in the site of injection have been described. In Rhesus monkeys treated with doses much higher than the clinically recommended ones, transient irregularities in the menstrual cycle have been observed (prolonged menstrual period). The significance of these findings for human beings has not been assessed.

Drug Interactions: Since interferons alpha alter cell metabolism, there is a possibility that BIOFERON® modified the activity of other drugs. A trial recruiting a small number of patients has demonstrated that interferon alpha 2 affects specific microsomic enzymatic systems, but the clinical significance of this finding is not yet known. Interferons alpha can exert an influence upon the oxidative metabolic process. This fact should be taken into account before prescribing a concomitant treatment with drugs that are metabolized by this route. However, there is no specific information regarding this subject. BIOFERON® can affect the central nervous system and therefore, an interaction is possible when drugs that affect CNS are used. Interferons can enhance neurotoxic, hematotoxic or cardiotoxic effects of other medications previously or concomitantly administered.

13.3 Rintatolimod

Clinical experience with rintatolimod totals over 800 patients with more than 400 patients receiving Rintatolimod for at least six (6) months, greater than 200 patients for one (1) year, over 50 patients up to two (2) years, and with 20 or more patients over two (2) years at doses as high as 1200 mg i.v. twice weekly. No evidence of dose-limiting organ toxicity, including hematologic, liver, or renal toxicity, has been observed.

Adverse events related to infusion such as mild flu-like symptoms, transient headache, fever, myalgia, arthralgia, and fatigue/malaise which were seen, usually occur during the initial weeks of treatment and tend to subside on repeated administration. These side events were seen in Chronic Fatigue Syndrome patients, cancer patients, chronic hepatitis B infected patients and individuals infected with HIV at doses of 200 and 400 mg and higher. Patients that experience these minor side effects can continue on Rintatolimod and as noted, these signs and symptoms typically subside after several weeks of continued treatment. Specific symptoms of note include a flushing reaction, characterized by at least one occurrence of erythema of the face, neck and chest, which has been observed in approximately 10% of patients treated in various studies. Usually, the flushing is both mild and transient and disappears with repeated dosing. Occasionally, it can be accompanied by a tightness of the chest, tachycardia, anxiety, shortness of breath, subjective reports of "feeling hot," diaphoresis, and nausea. The reaction is usually infusion-rate dependent and may generally be controlled by slowing the infusion rate. An antihistamine (diphenhydramine hydrochloride) can be helpful in controlling and reducing the response in the occasional patient for whom the symptom persists. Other less frequently occurring adverse effects include nausea, diarrhea, itching, urticaria, bronchospasm, transient hypotension, photophobia, rash, bradycardia and transient visual disturbances. A severe unexpected local reaction to extravasation of rintatolimod (Ampligen®, Poly I:Poly $C_{12}U$) at the infusion site in the dorsum of the left hand was reported in a Chronic

Fatigue Syndrome patient with chilblains. Several patients experienced liver enzyme level elevations while receiving Rintatolimod associated with chronic dosing over many weeks.

Rintatolimod has been dosed in combination with interferon-α in investigator-initiated studies under investigator IND applications during the period between December 1985 and April 1994. A total of 24 patients received combination treatments. Clinical conditions included renal cell carcinoma, chronic myelogenous leukemia, melanoma, and ovarian cancer. Rintatolimod was given as an IV infusion at a dose of 300 mg twice weekly. The starting dose was sometimes as low as 1-10 mg. The interferons were administered at a dose of 3 million units daily, with some doses of 0.75 million units at the low side and up to 6 million units at the higher side.

The therapy with rintatolimod in combination with interferon-α was generally well tolerated without evidence of dose-limiting or cumulative toxicities. The most frequent adverse reactions were considered minor in severity and duration. Most of them were flu-like symptoms such as chills, cold feeling, fatigue, decreased appetite, fever, and muscular aches. Also, shortness of breath has been seen as well as hypotension, nausea, anemia, dyspnea, numbness, itching, and blurred vision. These adverse events were judged possibly related to the condition of the patients, but also possibly related to the administration of rintatolimod or interferon. In some cases worsening of the patient's condition has been seen due to tumor progression, but in general favorable clinical patterns were observed in these patients with advanced disease. Such combinations were not intended to be immune modulating and were evaluated in the context of clinical cancer care.

For more risk information reference the Investigator's Brochure or package insert.

13.4 Pembrolizumab

The most common adverse reactions (reported in ≥ 20% of patients) were fatigue, musculoskeletal pain, decreased appetite, pruritis, diarrhea, nausea, rash, pyrexia, cough, dyspnea, and constipation. For a complete list of adverse reactions, warnings and precautions please refer to the package insert.

14 POTENTIAL BENEFITS TO SUBJECTS

Pembrolizumab has modest activity in patients with metastatic TNBC. It is currently not FDA approved for this indication as a monotherapy. In limited studies, patients who responded to checkpoint inhibitors had much better clinical outcomes then patients who did not respond. All patients would gain access to treatment with pembrolizumab for 4 cycles/doses.

In addition, we hope that pre-treatment with CKM will improve immune infiltration and increase possibility of receiving clinical benefit from pembrolizumab.

15 DATA AND SPECIMEN BANKING

All samples for correlative analysis will be sent to Dr. Kokolus's Laboratory for processing (CCC-502F). Samples will be used for planned study assays as well as for future analysis for other yet to be identified biomarkers that may be related to the clinical outcome of the study population, and novel ways to enhance their effectiveness. In addition to on-trial blood and tissue biopsies (See Appendix I: Study Calendar), such studies may also involve any unused portions of the resected tumor (and margin) harvested within standard care from these patients. Any clinical data that is associated with the samples, will be stored on a secure server in the Department of Medicine, will

be accessible only by the PI, Co-Investigators and PI designated data manager and, will be password protected. All computer entry and networking programs will be done using PIDs only. Any clinical data and/or specimens for future research will require verification of an IRB-approved protocol and will be de-identified before being released.

Note: All investigator or analyzing research laboratories housing research samples need to maintain current Temperature Logs and study-specific Sample Tracking and Shipping Logs. The Principal Investigator/Laboratory Manager must ensure that the stated lab(s) have a process in place to document the receipt/processing/storage/shipping of study-related samples/specimens. This is required for both observational and interventional clinical studies collecting clinical samples.

16 MEASUREMENT OF EFFECT

Solid Tumors

Response and progression will be evaluated in this study using RECIST 1.1 criteria. Please refer to Appendix F.

For the purposes of this study, patients should be re-evaluated for response after 4 cycles/doses of pembrolizumab.

Follow-up for scans (i.e., CT of chest, abdomen and pelvis done as SOC, i.e., every 3 months following Cycle/Dose #4 of pembrolizumab) using iRECIST (Appendix G) will continue until patient has progressive disease or until pembrolizumab is discontinued.

17 SAFETY EVALUATION

17.1 Adverse Events

An adverse event or adverse experience (AE) is any untoward medical occurrence associated with the use of a drug in humans, whether or not considered drug related. Therefore, an AE can be ANY unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medicinal (investigational) product, whether or not considered related to the medicinal (investigational) product (attribution of ‘unrelated,’ ‘unlikely,’ ‘possible,’ ‘probable,’ or ‘definite’).

An AE is considered “unexpected” if it is not listed in the investigator brochure or is not listed at the specificity or severity that has been observed; or if an investigator brochure is not required or available, is not consistent with the risk information described in the general investigational plan in other study-related documents.

17.2 Diagnosis Versus Signs and Symptoms

If known, a diagnosis should be recorded on the CRF rather than individual signs and symptoms (e.g., record only liver failure or hepatitis rather than jaundice, asterixis, and elevated transaminases). However, if a constellation of signs and/or symptoms cannot be clinically characterized as a single diagnosis or syndrome at the time of reporting, each individual event should be recorded as an AE or SAE on the CRF. If a diagnosis is subsequently established, it should be reported as follow-up information.

17.3 Adverse Events Occurring Secondary to Other Events

In general, AEs occurring secondary to other events (e.g., cascade events or clinical sequelae) should be identified by their primary cause. For example, if severe diarrhea is known to have resulted in dehydration, it is sufficient to record only diarrhea as an AE or SAE on the CRF. However, clinically significant AEs occurring secondary to an initiating event that are separated in time should be recorded as independent events on the CRF. For example, if a severe gastrointestinal hemorrhage leads to renal failure, both events should be recorded separately on the CRF.

17.4 Abnormal Laboratory Values

All clinically significant lab abnormalities will be recorded as AEs or SAEs on the CRF (e.g., abnormalities that require study drug dose modification, discontinuation of study treatment, more frequent follow-up assessments, further diagnostic investigation, etc.).

If the clinically significant laboratory abnormality is a sign of a disease or syndrome (e.g., alkaline phosphatase and bilirubin 5 x the upper limit of normal associated with cholecystitis), only the diagnosis (e.g., cholecystitis) needs to be recorded on the Adverse Event CRF.

If the clinically significant laboratory abnormality is not a sign of a disease or syndrome, the abnormality itself should be recorded as an AE or SAE on the CRF. If the laboratory abnormality can be characterized by a precise clinical term, the clinical term should be recorded as the AE or SAE. For example, an elevated blood potassium level of 7 mEq/L should be recorded as "hyperkalemia."

Observations of the same clinically significant laboratory abnormality from visit to visit should not be repeatedly recorded as AEs or SAEs on the CRF, unless their severity, seriousness, or etiology changes.

17.5 Preexisting Medical Conditions (Baseline Conditions)

A preexisting medical condition should be recorded as an AE or SAE only if the frequency, severity, or character of the condition worsens during the study. When recording such events on an Adverse Event CRF, it is important to convey the concept that the preexisting condition has changed by including applicable descriptors (e.g., "more frequent headaches").

17.6 Grading and Reporting Adverse Events

17.6.1 Grading and Relationship to Drug

The descriptions and grading scales found in the CTEP Version 5 of the NCI Common Terminology Criteria for Adverse Events (CTCAE) will be utilized for AE reporting. CTEP Version 5.0 of the CTCAE is identified and located at:

http://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm.

AEs not covered by specific terminology listed should be reported with common medical terminology, and documented according to the grading scales provided in the CTCAE Version 5. The relationship of event to study drug will be documented by the Investigator as follows:

Unrelated: The event is clearly related to other factors such as the participant's clinical state, other therapeutic interventions or concomitant drugs administered to the participant.

Unlikely: The event is doubtfully related to investigational agent(s). The event was most likely related to other factors such as the participant's clinical state, other therapeutic interventions, or concomitant drugs.

Possible: The event follows a reasonable temporal sequence from the time of drug administration, but could have been produced by other factors such as the participant's clinical state, other therapeutic interventions or concomitant drugs.

Probable: The event follows a reasonable temporal sequence from the time of drug administration, and follows a known response pattern to the study drug. The event cannot be reasonably explained by other factors such as the participant's clinical state, therapeutic interventions or concomitant drugs.

Definite: The event follows a reasonable temporal sequence from the time of drug administration, follows a known response pattern to the study drug, cannot be reasonably explained by other factors such as the participant's condition, therapeutic interventions or concomitant drugs; AND occurs immediately following study drug administration, improves upon stopping the drug, or reappears on re-exposure.

17.6.2 Reporting Adverse Events

Routine AEs occurring between the start date of intervention until 30 days after the last intervention, or until the event has resolved, the study participant is lost to follow-up, the start of a new treatment, or until the study investigator assesses the event(s) as stable or irreversible, will be reported. New information will be reported after it is received.

Guidelines for Routine Adverse Event Reporting for Phase 1 Studies (Regardless of Expectedness)

Attribution	Grade 1	Grade 2	Grade 3	Grade 4
Unrelated	X	X	X	X
Unlikely	X	X	X	X
Possible	X	X	X	X
Probable	X	X	X	X
Definite	X	X	X	X

Guidelines for Routine Adverse Event Reporting for Pilot, Phase 2, and Phase 3 Studies (Regardless of Expectedness)

Attribution	Grade 1	Grade 2	Grade 3	Grade 4
Unrelated			X	X
Unlikely			X	X
Possible	X	X	X	X
Probable	X	X	X	X
Definite	X	X	X	X

Adverse Events of Special Interest

All grade 2 toxicities that in investigator opinion are at least possibly related to CKM or pembrolizumab that are deemed immune-related will also be reported in the EDC system.

17.7 Serious Adverse Events

A serious adverse event (SAE) is any adverse event (experience) that in the opinion of either the investigator or sponsor results in ANY of the following:

- Death.
- A life-threatening adverse event (experience). Any AE that places a participant or participants, in the view of the Investigator or sponsor, at immediate risk of death from the reaction as it occurred. It does NOT include an AE that, had it occurred in a more severe form, might have caused death.
- Inpatient hospitalization or prolongation of existing hospitalization (for > 24 hours).
- A persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions.
- A congenital anomaly or birth defect.
- Important Medical Event (IME) that, based upon medical judgment, may jeopardize the participant and may require medical or surgical intervention to prevent one of the outcomes listed above.

17.7.1 Reporting Serious Adverse Events

All new SAEs occurring from the date the participant signs the study consent until 90 days after last dose of pembrolizumab will be reported (even if pembrolizumab is continued after the 4 cycles/doses). The Roswell Park SAE Source Form is to be completed with all available information, including a brief narrative describing the SAE and any other relevant information.

SAEs occurring after the 90-day follow-up period that the investigator determines to be possibly, probably or definitely related to the study intervention should be reported.

SAEs identified as an Unanticipated Problem by the Investigator must be reported. Please refer to **Section 17.9** for details on reporting Unanticipated Problems.

17.8 Follow-Up for Serious Adverse Events

All related SAEs should be followed to their resolution, until the study participant is lost to follow-up, the start of a new treatment, or until the study investigator assesses the event(s) as stable or irreversible. New information will be reported when it is received.

17.9 Unanticipated Problems

An Unanticipated Problem (UP) is any incident, experience, or outcome that meets all of the following criteria:

- Unexpected (in terms of nature, severity, or frequency) given:

  - The research procedures that are described in the study-related documents, including study deviations, as well as issues related to compromise of participant privacy or confidentiality of data.
  - The characteristics of the participant population being studied.

- Related or possibly related to participation in the research (possibly related means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research).
- Suggests that the research places participants or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized and if in relation to an AE is also deemed **Serious** per **Section 17.3.**

17.9.1 Reporting Unanticipated Problems:

The Reportable New Information (RNI) Form will be submitted to the CTO Quality Assurance (QA) Office within 1 business day of becoming aware of the Unanticipated Problem. After review, CTO QA Office will submit the RNI to the IRB.

When becoming aware of new information about an Unanticipated Problem, submit the updated information to the CTO QA Office with an updated Reportable New Information Form. The site Investigator or designated research personnel will report all unanticipated problems to the IRB in accordance with their local institutional guidelines.

17.10 FDA Reporting

Roswell Park is the IND holder for this study (IND 163681).

When Roswell Park is the IND holder the following describes the FDA reporting requirements by timeline for AEs and new safety findings that meet the criteria outlined below:

Within 7 Calendar Days

Any adverse event that meets ALL the following criteria:

- Related or possibly related to the use of the study drug;
- Unexpected; and
- Fatal or life-threatening.

Within 15 Calendar Days

Any adverse event that meets ALL the following criteria:

- Related or possibly related to the use of the study drug;
- Unexpected; and
- Serious but not fatal or life-threatening;

Or, meets ANY of the following criteria:

- A previous adverse event that is not initially deemed reportable but is later found to fit the criteria for reporting (report within 15 days from when event was deemed reportable).

- Any findings from other studies, including epidemiological studies, pooled analysis of multiple studies, or other clinical studies conducted with the study drug that suggest a significant risk in humans exposed to the drug.
- Any findings from animal or in vitro testing that suggest a significant risk for human participants including reports of mutagenicity, teratogenicity, or carcinogenicity or reports of significant organ toxicity at or near the expected human exposure.
- Any clinically important increase in the rate of occurrence of a serious, related or possibly related adverse event over that listed in the protocol or investigator brochure.

Sponsors are also required to identify in IND safety reports, all previous reports concerning similar adverse events and to analyze the significance of the current event in the light of the previous reports.

Reporting Process

The principal investigator or designee will complete and submit a FDA Form 3500A MedWatch for any event that meets the above criteria. Forms will be submitted to the CTO QA Office via email to CTO-QA@RoswellPark.org.

18 DATA MANAGEMENT AND CONFIDENTIALITY

18.1 Data Collection

Full build studies are managed by Roswell Park Clinical Data Management for analysis by Roswell Park Biostatisticians. All electronic case report form (eCRF) data are captured for these studies.

Clinical Data Management activities are performed using CTMS and EDC systems that enable the collection, cleaning and viewing of clinical trial data. CTO Clinical Data manages, designs and develops the study-specific database. Once the database design is approved by the Investigator, Statistician, and Clinical Research Coordinator, the database is put into Production and data entry can begin. Data can be entered and changed only by those with the rights to do so into the eCRFs.

18.2 Maintenance of Study Documents

Essential documents will be retained per Roswell Park's policy for 6 years from the study termination date. These documents could be retained for a longer period, however, if required by the applicable local regulatory requirements or by an agreement with Roswell Park.

18.3 Revisions to the Protocol

Roswell Park may make such changes to the protocol as it deems necessary for safety reasons or as may be required by the U.S. FDA or other regulatory agencies. Revisions will be submitted to the IRB/ERC for written approval before implementation.

18.4 Termination of the Study

It is agreed that, for reasonable cause, either the Roswell Park Investigators or the Sponsor, may terminate this study, provided a written notice is submitted within the time period provided for in the Clinical Trial Agreement. In addition, Roswell Park may terminate the study at any time upon

immediate notice if it believes termination is necessary for the safety of participants enrolled in the study.

18.5 HIPAA Authorization

All information/PHI provided to the Investigator by Roswell Park including preclinical data, protocols, CRFs, and verbal and written information, will be kept strictly confidential and confined to the clinical personnel involved in conducting this study, and no disclosure shall be made except in accordance with any right of publication granted to the Investigator. This information may be related in confidence to the IRB/ERC or other committee functioning in a similar capacity. No report or information about the study will be provided to anyone not involved in the study without consent of Roswell Park except if required by law.

The Roswell Park SOPs: Informed Consent Process for Research (HRP-090) and Written Documentation of Consent (HRP-091) will be followed.

Refer to section 25.0 “Provisions to Protect the Privacy Interests of Subjects”.

19 STATISTICAL PLAN

19.1 Primary Analysis

The primary objective is to evaluate the safety of the CKM in combination with CKM treatment.

A modified 3+3 design will be utilized. Initially, cohort 1 will enroll n=3 DLT evaluable subjects. If 2 or more DLTs are observed, then the study will be suspended for safety. If 1 or fewer DLTs are observed, then n=3 DLT evaluable patients will be enrolled in cohort 2. If 2 or more DLTs are observed in cohort 1, then the study will be suspended for safety. In cohort 2, if 1 DLT is observed, then an additional n=3 subjects will be enrolled in cohort 2. If 1 or fewer out of 6 or 0 out of 3 subjects experience a DLT in cohort 2, then n=6 DLT evaluable subjects will be enrolled in cohort 3. If, at any time, 2 DLTs are experienced in cohort 3, enrollment will be suspended. If 1 or fewer DLTs are observed in cohort 3, then it will be considered safe and the recommended Phase 2 dose.

The DLTs will be summarized by cohort using frequencies and relative frequencies.

Design Performance: The performance of this design is summarized in the table below. We consider several different scenarios for the true DLT rates within each cohort.

Scenario	True DLT rates	Chance of stopping due to DLTs in:			Chance of Cohort 3 Being Identified as Safe
		Cohort 1	Cohort 2	Cohort 3	
1 (all unsafe)	Cohort 1: 0.35 Cohort 2: 0.45 Cohort 3: 0.55	28.1%	55.0%	14.7%	2.1%
2 (all safe)	Cohort 1: 0.05 Cohort 2: 0.15 Cohort 3: 0.25	0.7%	18.6%	32.3%	48.4%
3 (all safe)	Cohort 1: 0.05 Cohort 2: 0.15 Cohort 3: 0.20	0.7%	18.6%	23.6%	57.3%
4 (low tox)	Cohort 1: 0.05 Cohort 2: 0.10 Cohort 3: 0.15	0.7%	9.3%	16.8%	73.2%
5 (3 unsafe)	Cohort 1: 0.10 Cohort 2: 0.20 Cohort 3: 0.40	2.7%	28.0%	47.9%	21.3%
6 (3 borderline)	Cohort 1: 0.10 Cohort 2: 0.20 Cohort 3: 0.30	2.7%	28.0%	35.1%	34.1%

19.2 Secondary Analyses

The secondary objectives/endpoints are analyzed as objective response rate (ORR; per iRECIST criteria), disease control rate (DCR), progression-free survival (PFS), and overall survival (OS).

The response data will be summarized using frequencies and relative frequencies, while estimates of the ORR and DCR will be obtained with 90% confidence intervals obtained using Jeffrey's prior method.

The survival outcomes (OS and PFS) will be summarized using standard Kaplan-Meier methods, with estimates of the median survival obtained with 90% confidence intervals. OS is defined as the time from the start of post-CKM therapy until death due to any cause or last follow-up. PFS is defined as the time from the start of post-CKM therapy until disease progression, death due to disease, or last follow-up.

19.3 Exploratory Analyses

The CD8+ cells counts will be summarized by pre/post-CKM using the mean, median, and standard deviation; and graphically using dot-plots. The change in CD8+ cell counts will be summarized similarly. The change in CD8+ cells will be evaluated using a two-sided permutation paired t-test at an overall $\alpha = 0.1$.

Detectable Effect Size: We assume that the mean pre-treatment CD8+ cell count is approximately 800 with a coefficient of variation of 0.75; which is similar to data observed in prostate cancer (37). Assuming a pre to post treatment correlation of 0.5 then if the post-treatment mean CD8+ cell count at least triples relative to pre-treatment, then a two-sided paired t-test has power >0.99 to detect a difference.

Of additional interest is whether patients achieve a three-fold increase in CD8+ cells in tumor TME from pre- to post-CKM. This will be summarized using frequencies and relative frequencies, with a 90% confidence interval about the three-fold increase rate obtained using Jeffrey's prior method. Correlative studies will be evaluated using descriptive statistics.

19.4 Safety Analysis

AEs, SAEs, and toxicities during CKM will be summarized by attribution (overall and related/unrelated to treatment) and grade using frequencies and relative frequencies. The safety data will be reviewed regularly by the research team (to include the Roswell Park Early Phase Committee and study PI) to discuss possible safety concerns and actions regarding study suspension or continuation.

19.5 Sample Size Determination

This is a study of a modified CKM regimen (replacement of the previously used INTRON-A® with an alternate (non-U.S. licensed) IFNα2β product) in patients with mTNBC, which evaluates safety and efficacy to inform on future study designs/development. The sample size calculations are based on the primary analysis, which evaluates safety.

For safety, if an AE has an expected incidence rate of 10%, then we have a 46.9% chance of observing such an AE. Additional scenarios are given below:

AE Rate	0.05	0.10	0.20	0.30	0.40	0.50
Chance of Observing at least 1 AE (n=3)	14.3%	27.1%	48.8%	65.7%	78.4%	87.5%
Chance of Observing at least 1 AE (n=6)	26.5%	46.9%	73.8%	88.2%	95.3%	98.4%

Additionally, performance of the study design relative to evaluating safety across cohorts is provided in Section 19.1.

20 PROVISIONS TO MONITOR THE DATA TO ENSURE THE SAFETY OF SUBJECTS

All Roswell Park Phase I studies are reviewed at the scheduled Early Phase Clinical Trial Committee meetings.

The Roswell Park Data Safety Monitoring Committee will assess the progress of the study, the safety data, and critical efficacy endpoints (Phase I studies will be reviewed quarterly; Phase II, III and pilot investigator-initiated studies will be reviewed semi-annually). The DSMC will review the study annually and will make recommendations that include but not limited to; (a) continuation of the study, (b) modifications to the design, (c) suspension of, or (d) or termination of the study.

21 VULNERABLE POPULATIONS

Not applicable.

22 COMMUNITY-BASED PARTICIPATORY RESEARCH

Not applicable.

23 SHARING OF RESULTS WITH SUBJECTS

Individual response data is shared with the participant as a part of their clinical care.

24 SETTING

Potential subjects with metastatic breast cancer will be identified and recruited during scheduled visits to Roswell Park. Potential subjects, who are referred by their Roswell Park or community physicians, will be scheduled with an investigator in the clinic for evaluation. All participant-related research procedures will be conducted at Roswell Park.

25 PROVISIONS TO PROTECT THE PRIVACY INTERESTS OF SUBJECTS

Any data, specimens, forms, reports, video recordings, and other records that leave the site will be identified only by a participant identification number (Participant ID, PID) to maintain confidentiality. All records will be kept in a limited access environment. All computer entry and networking programs will be done using PIDs only. Information will not be released without written authorization of the participant.

26 RESOURCES AVAILABLE

Pawel Kalinski, MD, PhD. is Professor, Department of Obstetrics, Gynecology & Reproductive Sciences University of Pittsburgh School of Medicine and Vice Chair of Translational Research Development in Women's Health Magee-Women's Research Institute. Dr. Kalinski will receive de-identified data related to patients enrolled for the purpose of writing papers for publication. Dr. Kalinski may work with Dr. Kokolus and the Roswell Park lab team in review/interpretation of data. At this time, the trial is closed to accrual. The role of the external collaborator is to review aggregate data summary reports that have been deidentified and they will never have access to identifiable data. The following types of analyzed data will be provided: Bulk RNA sequencing

data (collected vis 10X Genomics- GSR), Gene expression data (collected via Visium- GSR), Flow cytometry data (collected via Cytek- FIASR), Proteomic data (collected via Luminex- FIASR), and Spatial biology data (collected via PhenoCycler- FIASR). The Roswell Park legal department will ensure proper sharing agreements are in place with the University of Pittsburgh School of Medicine. Local IRB approval will be obtained from the University of Pittsburgh School of Medicine.

27 PRIOR APPROVALS

Not applicable.

28 COMPENSATION FOR RESEARCH-RELATED INJURY

If the subject believes they have been injured as a direct result of their participation in this research study, they will be advised to notify the Roswell Park Patient Advocate at (716) 845-1365 or the Study Doctor at (716) 845-1486.

Medical diagnosis and treatment for the injury will be offered, and a determination will be made regarding appropriate billing for the diagnosis and treatment of the injury. A financial counselor (716-845-4782) will be able to provide an explanation of coverage and to answer questions the subject may have regarding study related billing.

The subject is not prevented from seeking to collect compensation for injury related to malpractice, fault, or blame on the part of those involved in the research.

29 ECONOMIC BURDEN TO SUBJECTS

The participants will not be subject to any economic burden.

30 CONSENT PROCESS

This study will not be initiated until the protocol and informed consent document(s) have been reviewed and approved by a properly constituted Institutional Review Board (IRB) or Independent Ethics Committee (IEC). Each participant (or legal guardian) shall read, understand, and sign an instrument of informed consent prior to performance of any study-specific procedure. It is the responsibility of the investigator to ensure that the participant is made aware of the investigational nature of the treatment and that informed consent is given.

The Investigator is responsible for the retention of the participant log and participant records; although personal information may be reviewed by authorized persons, that information will be treated as strictly confidential and will not be made publicly available. The investigator is also responsible for obtaining participant authorization to access medical records and other applicable study specific information according to Health Insurance Portability and Accountability Act regulations (where applicable).

This study will be conducted in compliance with all applicable laws and regulations of the state and/or country and institution where the participant is treated. The clinical trial should be conducted in accordance with the ethical principles embodied in the Belmont Report: Ethical Principles and Guidelines for the Protection of Human Subjects of Research, consistent with good clinical practice and the applicable regulatory requirements and according to the guidelines in this protocol, including attached appendices.

Informed consent will be obtained according to SOP: Informed Consent Process for Research (HRP-090).

31 PROCESS TO DOCUMENT CONSENT IN WRITING

The Investigator (or IRB specified designee) is responsible for obtaining written consent from each participant in accordance with GCP guidelines using the approved informed consent form, before any study specific procedures (including screening procedures) are performed. The informed consent form acknowledges all information that must be given to the participant according to applicable GCP guidelines, including the purpose and nature of the study, the expected efficacy and possible side effects of the treatment(s), and specifying that refusal to participate will not influence further options for therapy. Any additional information that is applicable to the study must also be included. Additional national or institutionally mandated requirements for informed consent must also be adhered to. The participant should also be made aware that by signing the consent form, processing of sensitive clinical trial data and transfer to other countries for further processing is allowed.

The Investigator shall provide a copy of the signed consent form to the participant and the signed original shall be maintained in the Investigator File. A copy of the signed consent form must be filed in the participant file. At any stage, the participant may withdraw from the study and such a decision will not affect any further treatment options.

SOP: Written Documentation of Consent (HRP-091) will be followed.

32 DRUGS OR DEVICES

Roswell Park will hold the IND for this study (IND 163681).

32.1 Celecoxib

A sulfa non-steroidal anti-inflammatory drug (NSAID) used in the treatment of osteoarthritis, rheumatoid arthritis, acute pain, painful menstruation, and menstrual symptoms, and to reduce numbers of colon and rectum polyps in patients with familial adenomatous polyposis.

32.1.1 Other Names

Celebrex, Celebra or Onsenal (commercially available).

32.1.2 Formulation and packaging

Celecoxib as capsules in the following dosages: 100 mg and 200 mg.

32.1.3 Drug Shipment

Celecoxib will be provided by Roswell Park (purchased through grant funding).

32.1.4 Drug administration

Celecoxib 200 mg will be administered orally twice a day approximately 12 hours apart during each of both CKM courses. The morning dose will be given to the participant at the same time as the other pre-medications for the CKM regimen. The evening dose of Celecoxib will be self-

administered by the participant at home and documented on the patient diary. Refer to Appendix H.

32.1.5 Drug Storage and accountability

The Investigator or designate will be responsible for ensuring that the investigational product is securely maintained in a locked, limited access facility in accordance with the applicable regulatory requirements.

Store Celecoxib at room temperature (20-25 °C) away from light and moisture. Brief storage between 59-86 °F (15-30 °C) is permitted.

Drug storage temperature will be maintained and recorded, as applicable.

32.2 Interferon α-2b (Bioferon®)

32.2.1 Active Substance and Source

Interferon α-2b (BIOFERON®) is supplied in a single-dose vial containing sterile lyophilized powder. Each vial contains 10 million lnternational Units (MIU) of recombinant human interferon alfa 2b, glycine, dibasic sodium phosphate dodecahydrated, monobasic sodium phosphate anhydrous and human albumin. For its administration, lyophilized powder should be reconstituted by adding 1mL of sterile water for injection. Bioferon® is not FDA-approved and is considered an investigational new drug in the United States.

32.2.2 Drug Shipment

Interferon α-2b (Bioferon®) will be provided by Biosidus S.A. and shipped to the Roswell Park IDS.

The date of receipt and the amount of drug received will be documented. Drug shipment records will be retained by the investigational pharmacist or designee.

How supplied: Vial containing 10 million Units r-Hu-IFN α-2b freeze dried powder. Each package contains 1 solvent ampule with 1 mL sterile WFI.

32.2.3 Preparation

The lyophilized product is reconstituted as directed by the manufacturer. Investigational Drug Service Pharmacy (IDS) will prepare and dispense.

10 million units/mL, lyophilized powder, which must be reconstituted prior to administration.

- Vial size: 10 million units/vial
- Diluent: Interferon α-2b (Bioferon®) should be reconstituted with 1 mL sterile water to reach a final concentration of 10:1. IV dose should be diluted in sodium chloride 0.9%/100 mL given over 30 minutes. The final concentration of interferon α-2b should not be less than 10 million units/100 mL.

Once reconstituted, mix the suspension with gentle rotation movements, do not shake vigorously.

For IV injection, it is recommended that Interferon α-2b be administered as a solution of no less than 10 million units/100 mL to minimize adsorption of the drug to glass and plastic containers.

32.2.4 Storage and Stability

The Investigator or designate will be responsible for ensuring that the investigational product is securely maintained in a locked, limited-access facility, as specified and in accordance with the applicable regulatory requirements.

Interferon α-2b (BIOFERON®) should be kept refrigerated (2 - 8 °C). Do not freeze. Do not freeze the ampoule containing water for injection since it can leak. Once the Interferon α-2b is reconstituted, it should be used within the following 24 hours, storing the solution refrigerated (2 - 8ºC) and following strict aseptic conditions during powder reconstitution.

Vials containing reconstituted solution should be used within two hours if they are kept at room temperature, or within 24 hours if they are stored at 2 - 8ºC.

This preparation does not contain preservative agents. Therefore, it is advisable not to extract more than one dose from the vial to prevent contamination. Do not use this medication after the expiry date printed in its container.

Drug storage temperature will be maintained and recorded, as applicable.

32.2.5 Drug Administration

Interferon α-2b (10 million units/M^2 or 20 million units/M^2) will be administered intravenously over 30 minutes during CKM treatment #1 (days 0, 1 and 2) and CKM treatment #2 (days 7, 8, and 9).

32.2.6 Concomitant Medications

Interactions between Interferon α-2b and other drugs have not been fully evaluated. Caution should be exercised when administering Interferon α-2b therapy in combination with other potentially myelosuppressive agents such as zidovudine. Concomitant use of Interferon α-2b and theophylline decreases theophylline clearance, resulting in a 100% increase in theophylline levels. Concomitant Interferon α-2b and REBETOL® (Ribavirin) use is contraindicated.

32.3 Rintatolimod (poly IC analog)

A substituted double stranded polyribonucleic acid (polyI: poly$C_{12}U$), rintatolimod preserves activity of PolyI:C with a much improved systemic toxicity profile. The product has been studied extensively for use as a vaccine adjuvant and for its direct antiviral activity, as well in several cancer studies as a monotherapy, but most extensively in chronic fatigue syndrome (CFS).

32.3.1 Other Names

poly$IC_{12}U$, Ampligen®, poly I: poly$C_{12}U$; Polyinosinic: polycytidylic-polyuridylic acid; polyriboinosinic/polyribocytidylic (uridylic) acid

32.3.2 Formulation and Packaging

Rintatolimod is supplied as a liquid solution in glass bottles containing 200 mg (100 mg in case of toxicity) per 80 mL. Rintatolimod is a colorless solution containing 2.5 mg/mL in physiological

salts (0.15 M NaCl, 0.01 M phosphate, 0.001 M Mg++). The product does not contain preservatives or antioxidants.

32.3.3 Drug Shipment

Rintatolimod will be provided by AIM ImmunoTech and shipped to the participating site.

The date of receipt and the amount of drug received will be documented. Drug shipment records will be retained by the investigational pharmacist or designee.

32.3.4 Preparing and Dispensing

A vial of rintatolimod is suitable for direct IV infusion. IDS will prepare and dispense. Each vial should be taken from the refrigerator and allowed to equilibrate to room temperature.

32.3.5 Drug Administration

Rintatolimod 200 mg will be administered by intravenous infusion after Interferon α-2b during CKM treatment #1 (on days 0, 1 and 2) and during CKM treatment #2 (on days 7, 8, and 9). Additional details on the procedures for receiving, storing and using rintatolimod (Ampligen®) can be found in a separate document entitled "Procedures for Receiving, Storing, and Using Ampligen® (Poly I:Poly $C_{12}U$) Liquid Solution."

The initial administration should begin at a slow rate of infusion (approximately 20 cc/ hour) and increase to 40 cc/ hour after 30 minutes. Tubing should be flushed with 30 to 50 mL of normal saline solution upon completion. It is recommended that vented micro drip (60 gtts/mL) tubing, a 1.2 micron in-line filter and volume controller and/or infusion pump be utilized for the administration of Ampligen®.

32.3.6 Handling and Disposal

The Investigator or designee will be responsible for dispensing and accounting for all investigational drug provided by AIM ImmunoTech exercising accepted medical and pharmaceutical practices. Study drugs must be handled as cytotoxic agents and appropriate precautions taken per the institution's environmentally safe handling procedures. All investigational drugs will be dispensed in accordance with the Investigator's prescription or written order.

All products dispensed will be recorded on a product accountability record. Records of product lot numbers and dates received will be entered on a product accountability form. This record will be reviewed by the Sponsor's staff or representative during periodic monitoring visits. It is the Investigator's responsibility to ensure that an accurate record of investigational drug issued and returned is maintained.

Used vials (excess drug) will be destroyed according to standard practices after properly accounting for the dispensing. Partially used vials of study drug will not be re-used for other participants.

Under no circumstances will the Investigator supply investigational drug to a third party or allow the investigational drug to be used in a manner other than as directed by this protocol.

In regards to drug receipt, accountability and storage, SOP IDS-601 will be followed.

32.4 Pembrolizumab

Pembrolizumab is commercially available. Four doses of pembrolizumab will be paid for by the study. Patients, who in consultation with their physician, are going to be continued on pembrolizumab off study after Dose #4: pembrolizumab will be obtained via standard procedure for obtaining pembrolizumab and insurance coverage/ authorization currently in place at the Roswell Park Comprehensive Cancer Center breast medicine clinic.

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34 APPENDICES/SUPPLEMENTS

Appendix A ECOG Performance Status Scores

Description	Status
Fully active, able to carry on all pre-disease performance without restriction.	0
Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light housework, office work.	1
Ambulatory and capable of all self-care but unable to carry out any work activities.	2
Capable of only limited self-care, confined to bed or chair more than 50% of waking hours.	3
Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair.	4
Dead	5

Appendix B INVESTIGATOR STUDY ELIGIBILITY VERIFICATION FORM: INCLUSION CRITERIA

Participant Name: _____

Medical Record No.: _____

Title: Phase I/IIa Clinical Trial Evaluating the Safety and Efficacy of Rintatolimod Combined with IFN α-2b (Bioferon®) to Enhance the Effectiveness of Pembrolizumab in Patients with Metastatic Triple Negative Breast Cancer

INCLUSION CRITERIA				
Yes	**No**	**N/A**	**All answers must be "Yes" or "N/A" for participant enrollment.**	**Date**
☐	☐	☐	1. Age ≥ 18 years of age.	
☐	☐	☐	2. Have pathologically confirmed diagnosis of unresectable or metastatic PDL-1-negative or PDL1 positive TNBC with no curative treatment options.	
☐	☐	☐	3. Have been informed of other treatment options.	
☐	☐	☐	4. Patient has lesion that can be biopsied and is willing to undergo the procedure as part of the protocol. **Note:** For Cohort 1 and Cohort 2: Patient with accessible tumor will be offered *optional* pre-treatment and post-treatment biopsies. Biopsies are *mandatory* for Cohort 3.	
☐	☐	☐	5. Have an ECOG performance status of ≤ 1. Refer to Appendix A.	
☐	☐	☐	6. Participants of child-bearing potential must agree to use adequate contraceptive methods (e.g., hormonal or barrier method of birth control; abstinence) prior to study entry. Should a woman become pregnant or suspect she is pregnant while she or her partner is participating in this study, she should inform her treating physician immediately.	
☐	☐	☐	7. Ability to swallow and retain oral medication.	
☐	☐	☐	8. Have measurable disease per RECIST 1.1 criteria present.	
☐	☐	☐	9. Any line of therapy allowed, radiologically confirmed progression.	
☐	☐	☐	10. No cancer-directed therapy for at least 3 weeks prior to study treatment (bone directed therapies are allowed).	
☐	☐	☐	11. Must have adequate organ and marrow function present as defined below: -Platelets ≥ 100,000/μL -Hemoglobin ≥ 9.0 g/dL -Absolute Neutrophil Count (ANC) ≥ 1500/μL -Total bilirubin ≤ institutional upper limit of normal (ULN) -AST (SGOT) and ALT (SGPT) ≤ 2.5 X institutional ULN -Creatinine < ULN or, Creatinine clearance ≥ 50 mL/min per Cockcroft-Gault Equation for patients with creatinine levels greater than ULN-refer to Appendix D.	

INCLUSION CRITERIA				
Yes	**No**	**N/A**	**All answers must be "Yes" or "N/A" for participant enrollment.**	**Date**
☐	☐	☐	12. Participant must understand the investigational nature of this study and sign an Independent Ethics Committee/Institutional Review Board approved written informed consent form prior to receiving any study related procedure.	

Investigator Signature: _____ Date: _____

Printed Name of Investigator: _____

Appendix C INVESTIGATOR STUDY ELIGIBILITY VERIFICATION FORM: EXCLUSION CRITERIA

Participant Name: _____

Medical Record No.: _____

Title: Phase I/IIa Clinical Trial Evaluating the Safety and Efficacy of Rintatolimod Combined with IFN α-2b (Bioferon®) to Enhance the Effectiveness of Pembrolizumab in Patients with Metastatic Triple Negative Breast Cancer

EXCLUSION CRITERIA				
Yes	**No**	**N/A**	**All answers must be "No" or "N/A" for participant enrollment.**	**Date**
☐	☐	☐	1. Patients currently treated with systemic immunosuppressive agents, including steroids (> than equivalent of 10 mg daily of prednisone), are ineligible until 3 weeks after removal from immunosuppressive treatment. (inhaled steroids are allowed).	
☐	☐	☐	2. Patients with active autoimmune disease or history of transplantation.	
☐	☐	☐	3. Pregnant or nursing female participants.	
☐	☐	☐	4. Unwilling or unable to follow protocol requirements.	
☐	☐	☐	5. Patients with known serious mood disorders. (Major depression diagnosis is an exclusion. Other stable mood disorders on stable therapy for > 6 months may be allowed after consultation with PI).	
☐	☐	☐	6. Cardiac risk factors including: • Patients experiencing cardiac event(s) (acute coronary syndrome, myocardial infarction, or ischemia) within 3 months of signing consent. While our published clinical studies involving short-term CKM (34, 35) did not indicate increased risk of cardiac events, the CKM can induce flu-like symptoms, providing justification for its avoidance in patients with recent cardiac events. • Patients with a New York Heart Association classification of III or IV (Appendix E). • Patients with a history of stroke.	
☐	☐	☐	7. History of upper gastrointestinal ulceration, upper gastrointestinal bleeding, or upper gastrointestinal perforation within the past 3 years.	
☐	☐	☐	8. Prior allergic reaction or hypersensitivity to NSAIDs or any drugs administered on protocol.	
☐	☐	☐	9. Any condition which in the Investigator's opinion deems the participant an unsuitable candidate to receive study drug.	
☐	☐	☐	10. Any patients with a positive Antinuclear Antibodies test will be excluded from study	
☐	☐	☐	11. Has a known history of Human Immunodeficiency Virus (HIV) infection.	
☐	☐	☐	12. Concurrent active Hepatitis B (defined as HBsAg positive and/or detectable HBV DNA) and Hepatitis C virus (defined as anti-HCV Ab positive and detectable HCV RNA) infection. Note: Hepatitis B and C	

EXCLUSION CRITERIA				
Yes	**No**	**N/A**	**All answers must be "No" or "N/A" for participant enrollment.**	**Date**
			screening tests are not required unless known history of HBV and HCV infection.	

Participant meets all entry criteria: ☐ **Yes** ☐ **No**
If "NO", do not enroll participant in study.

Investigator Signature: _____ Date: _____

Printed Name of Investigator: _____

Appendix D Cockcroft-Gault Equation

Cockcroft-Gault Equation*

Men: CrCl = [(140-YR) x IBW] / (PCr x 72)

Women: CrCl = 0.85 x [(140-YR) x IBW] / (PCr x 72)

Where:

CrCl is creatine clearance (mL/min)

IBW is ideal body weight (kg)

PCr is plasma creatinine (mg/dL)

YR is age (years)

NOTE: For blood Chemistry labs, Roswell Park clinical blood chemistries are performed on plasma unless otherwise indicated.

Appendix E NYHA CLASSIFICATION

NEW YORK HEART ASSOCIATION CLASSIFICATION OF CARDIAC DISEASE

Class	Functional Capacity	Objective Assessment
I	Patients with cardiac disease but without resulting limitations of physical activity. Ordinary physical activity does not cause undue fatigue, palpitation, dyspnea, or anginal pain.	No objective evidence of cardiovascular disease.
II	Patients with cardiac disease resulting in slight limitation of physical activity. They are comfortable at rest. Ordinary physical activity results in fatigue, palpitation, dyspnea, or anginal pain.	Objective evidence of minimal cardiovascular disease.
III	Patients with cardiac disease resulting in marked limitation of physical activity. They are comfortable at rest. Less than ordinary activity causes fatigue, palpitation, dyspnea, or anginal pain.	Objective evidence of moderately severe cardiovascular disease.
IV	Patients with cardiac disease resulting in inability to carry on any physical activity without discomfort. Symptoms of heart failure or the anginal syndrome may be present even at rest. If any physical activity is undertaken, discomfort is increased.	Objective evidence of severe cardiovascular disease.

Appendix F RECIST 1.1

Objective Tumor Response

All protocol-defined imaging studies must be performed at the investigative site or sponsor-approved facility using protocol-defined parameters. The same method of assessment and the same technique should be used to characterize each identified and reported lesion at baseline and during follow-up. RECIST 1.1 will be used to assess objective tumor response to determine baseline eligibility.

Target Lesions

All measurable lesions up to a maximum of 2 lesions per organ and 5 lesions in total, representative of all involved organs, will be identified as target lesions and recorded and measured at baseline. Target lesions will be selected on the basis of their size. Lesions with the longest diameter (short axis for lymph nodes) and are ≥ 10 mm (CT and MRI), ≥ 15 mm lymph nodes, > 20 mm CXR and are for accurate repetitive measurements (either by imaging techniques or clinically) will be chosen. A sum of the longest diameter (short axis for lymph nodes) of all target lesions will be calculated and reported as the baseline sum diameters. This will be used as reference to further characterize the objective tumor response of the measurable dimension of the disease.

Complete Response (CR): Disappearance of all target lesions. Any lymph nodes must have a reduction in short axis to < 10 mm. Changes in tumor measurements must be confirmed by repeat studies performed no less than 6 weeks after the criteria for response are first met.

Partial Response (PR): At least a 30% decrease in the sum of diameters of target lesions, taking as reference the baseline sum diameters. Changes in tumor measurements must be confirmed by repeat studies performed no less than 6 weeks after the criteria for response are first met.

Progressive Disease (PD): At least a 20% increase in the sum of diameters of target lesions, taking as references the smallest sum on study (this includes the baseline sum if that is the smallest on study). In addition to the relative increase of 20%, the sum must also demonstrate an absolute increase of at least 5 mm. The appearance of one or more new lesions is also considered progression.

Stable Disease (SD): Neither a sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as reference the smallest sum diameter while on study. Participants having a documented response with no confirmation of the response will be listed with stable disease.

Non-Target Lesions

All other small lesions (longest diameter < 10 mm or lymph nodes ≥ 10 mm to < 15 mm short axis) and non-measurable lesions (i.e., leptomeningeal disease, ascites, pleural or pericardial effusion, inflammatory breast disease, lymphangitic involvement of skin or lung, blastic bone lesions, or abdominal masses / abdominal organomegaly identified by physical exam that is not measurable by imaging) should be identified as non-target lesions and indicated as present in the source documents at baseline. The general location will also be documented on the images drawing a regularly-shaped Region of Interest. Measurements of the non-target lesions will not be performed, but the presence or absence of each should be noted throughout follow-up and evaluation.

Complete Response: Disappearance of all non-target lesions and normalization of tumor marker level, if applicable. All lymph nodes must be non-pathological in size (< 10 mm short axis).

Non-Complete Response/Non-Progressive Disease: Persistence of 1 or more non-target lesion(s) and/or maintenance of tumor marker level above the upper limits of normal.

Progressive Disease: Appearance of 1 or more new lesions or the unequivocal progression of existing non-target lesions. Although a clear progression of non-target lesions is exceptional, in such circumstances, the opinion of the treating physician should prevail and the progression status should be confirmed at a later time.

Cytology and Histology

These techniques can be used to differentiate between partial responses (PR) and complete responses (CR) in rare cases (e.g., residual lesions in tumor types, such as germ cell tumors), where known residual benign tumors can remain).

The cytological confirmation of the neoplastic origin of any effusion that appears or worsens during treatment when the measurable tumor has met criteria for response or stable disease is mandatory to differentiate between response or stable disease (an effusion may be a side effect of the treatment) and progressive disease.

Evaluation of Response

Will be performed after 4 doses of pembrolizumab.

To determine time point response, refer to tables below:

Time Point Response Criteria: target (± non-target lesion)

Target Lesions	Non-Target Lesions	New Lesions	Overall Response
CR	CR	No	CR
CR	Non-CR/Non-PD	No	PR
CR	Not evaluated	No	PR
PR	Non-PD or not all evaluated	No	PR
SD	Non-PD or not all evaluated	No	SD
Not all evaluated	Non-PD	No	NE
PD	Any	Yes or No	PD
Any	PD	Yes or No	PD
Any	Any	Yes	PD

Time Point Response Criteria: non-target disease only

Non-Target Lesions	New Lesions	Overall Response
CR	No	CR
Non-CR/non-PD	No	Non-CR/non-PD[1]
Not all evaluated	No	NE
Unequivocal PD	Yes or No	PD
Any	Yes	PD

1 Non-CR/non-PD is preferred over SD for non-target disease since SD is used as endpoint for assessment of efficacy in trials so to assign this category when no lesions can be measured is not advised.

The best overall response is the best response recorded from the start of study treatment until the end of treatment or disease progression/recurrence, whichever occurs earlier, taking into account any requirement for confirmation. In general, the participant's best response assignment will depend on the achievement of both measurement and confirmation criteria and will be determined by combining the participant's status of target lesions, non-target lesions, and new lesions.

Symptomatic Deterioration: Participants with global deterioration of health status requiring discontinuation of treatment without objective evidence of disease progression at that time, and not related to study treatment or other medical conditions should be reported as progressive disease due to "symptomatic deterioration." Every effort should be made to document objective progression even after discontinuation of treatment due to symptomatic deterioration. Symptomatic deterioration that may lead to discontinuation of treatment include, but is not limited to, symptoms such as:

Weight loss > 10% of body weight.

Worsening of disease-related symptoms (e.g., worsening dyspnea, increasing pain/increasing requirement for narcotic analgesics).

Decline in performance status of > 1 level on ECOG scale.

Confirmation Measurement

A confirmatory assessment is required no less than 6 weeks after a PR or CR is deemed.

Guidelines for Evaluation of Measurable Disease

All measurements should be taken and recorded in metric notation. All baseline evaluations should be performed as closely as possible to the beginning of treatment and never more than 4 weeks before the beginning of the treatment.

The same method of assessment and the same technique should be used to characterize each identified and reported lesion at baseline and during follow-up. Imaging-based evaluation is preferred to evaluation by clinical examination unless the lesion(s) being followed cannot be imaged but are assessable by clinical exam.

Clinical Lesions: Clinical lesions will only be considered measurable when they are superficial (e.g., skin nodules and palpable lymph nodes) and ≥ 10 mm diameter as assessed using calipers (e.g., skin nodules). In the case of skin lesions, documentation by color photography, including a ruler to estimate the size of the lesion, is recommended.

Chest x-ray: Lesions on chest x-ray are acceptable as measurable lesions when they are clearly defined and surrounded by aerated lung. However, CT is preferable.

Conventional CT and MRI: This guideline has defined measurability of lesions on CT scan based on the assumption that CT slice thickness is 5 mm or less. If CT scans have slice thickness greater than 5 mm, the minimum size for a measurable lesion should be twice the slice thickness. MRI is also acceptable in certain situations (e.g., for body scans).

Use of MRI remains a complex issue. MRI has excellent contrast, spatial, and temporal resolution; however, there are many image acquisition variables involved in MRI, which greatly impact image quality, lesion conspicuity, and measurement. Furthermore, the availability of MRI is variable globally. As with CT, if an MRI is performed, the technical specifications of the scanning sequences used should be optimized for the evaluation of the type and site of disease. Furthermore,

as with CT, the modality used at follow-up should be the same as was used at baseline and the lesions should be measured/assessed on the same pulse sequence. It is beyond the scope of the RECIST guidelines to prescribe specific MRI pulse sequence parameters for all scanners, body parts, and diseases. Ideally, the same type of scanner should be used and the image acquisition protocol should be followed as closely as possible to prior scans. Body scans should be performed with breath-hold scanning techniques, if possible.

Ultrasound: Ultrasound is not useful in assessment of lesion size and should not be used as a method of measurement. Ultrasound examinations cannot be reproduced in their entirety for independent review at a later date and because they are operator dependent, it cannot be guaranteed that the same technique and measurements will be taken from one assessment to the next. If new lesions are identified by ultrasound in the course of the study, confirmation by CT or MRI is advised. If there is concern about radiation exposure at CT, MRI may be used instead of CT in selected instances.

Endoscopy, Laparoscopy: The utilization of these techniques for objective tumor evaluation is not advised. However, such techniques may be useful to confirm complete pathological response when biopsies are obtained or to determine relapse in trials where recurrence following complete response (CR) or surgical resection is an endpoint.

Tumor Markers: Tumor markers alone cannot be used to assess response. If markers are initially above the upper normal limit, they must normalize for a participant to be considered in complete clinical response.

Cytology, Histology: These techniques can be used to differentiate between partial responses (PR) and complete responses (CR) in rare cases (e.g., residual lesions in tumor types, such as germ cell tumors, where known residual benign tumors can remain).

The cytological confirmation of the neoplastic origin of any effusion that appears or worsens during treatment when the measurable tumor has met criteria for response or stable disease is mandatory to differentiate between response or stable disease (an effusion may be a side effect of the treatment) and progressive disease.

FDG-PET: While FDG-PET response assessments need additional study, it is sometimes reasonable to incorporate the use of FDG-PET scanning to complement CT scanning in assessment of progression (particularly possible 'new' disease). New lesions on the basis of FDG PET imaging can be identified according to the following algorithm:

Negative FDG-PET at baseline, with a positive FDG-PET at follow-up is a sign of PD based on a new lesion.

No FDG-PET at baseline and a positive FDG-PET at follow-up: If the positive FDG-PET at follow-up corresponds to a new site of disease confirmed by CT, this is PD. If the positive FDG-PET at follow-up is not confirmed as a new site of disease on CT, additional follow-up CT scans are needed to determine if there is truly progression occurring at that site (if so, the date of PD will be the date of the initial abnormal FDG-PET scan). If the positive FDG-PET at follow-up corresponds to a pre-existing site of disease on CT that is not progressing on the basis of the anatomic images, this is not PD.

FDG-PET may be used to upgrade a response to a CR in a manner similar to a biopsy in cases where a residual radiographic abnormality is thought to represent fibrosis or scarring. The use of FDG-PET in this circumstance should be prospectively described in the protocol and supported by disease-specific medical literature for the indication. However, it must

be acknowledged that both approaches may lead to false positive CR due to limitations of FDG-PET and biopsy resolution/sensitivity.

Note: A ‘positive’ FDG-PET scan lesion means one which is FDG avid with an uptake greater than twice that of the surrounding tissue on the attenuation corrected image.

Appendix G Immune-Related Response Criteria (iRECIST)

Tumor response and disease progression will be assessed using the Immune-Related response Criteria (iRESIST) as described by Seymour et al, 2017*.

iRECIST Response Assessment

Overall response will also be assessed using iRECIST. Immunotherapeutics may result in infiltration of immune cells leading to transient increase in the size in malignant lesions, or undetectable lesions becoming detectable. The criteria are identical to those of RECIST 1.1 in many respects but have been adapted to account for instances where an increase in tumour burden, or the appearance of new lesions, does not reflect true tumour progression.

Key differences are described below. All responses defined using iRECIST criteria are designated with a prefix. iRECIST time-point and best overall responses will be recorded separately.

Confirming Progression

Unlike RECIST 1.1, iRECIST requires the confirmation of progression and uses the terms iUPD (unconfirmed progression) and iCPD (confirmed progression). Confirmatory scans should be performed at least 4 weeks, but no longer than 8 weeks after iUPD.

iCPD is confirmed if further increase in tumour burden, compared to the last assessment, is seen as evidenced by one or more of the following:

- Continued increase in tumour burden (from iUPD) where RECIST 1.1 definitions of progression had been met (from nadir) in target, non-target disease or new lesions
    - Progression in target disease worsens with an increase of at least 5 mm in the absolute value of the sum
    - Continued unequivocal progression in non-target disease with an increase in tumour burden
    - Increase in size of previously identified new lesion (s) (an increase of at least 5 mm in the absolute value of the sum of those considered to be target new lesions) or additional new lesions.
- RECIST 1.1 criteria are met in lesions types (target or non-target or new lesions) where progression was not previously identified, including the appearance of additional new lesions.

If iUPD is not confirmed at the next assessment, then the appropriate response will be assigned (iUPD if the criteria are still met, but no worsening, or iSD, iPR or iCR if those criteria are met compared to baseline). As can be seen in Table B, the prior documentation of iUPD does not preclude assigning iCR, iPR, or iSD in subsequent time-point assessments or as best overall response (BOR) providing that iCPD is not documented at the next assessment after iUPD.

New lesions

New lesions should be assessed and measured as they appear using RECIST 1.1 criteria (maximum of 5 lesions, no more than 2 per site, at least 10 mm in long axis (or 15 mm in short axis for nodal

lesions) and recorded as New Lesions-Target (NLT) and New Lesion-Non-Target (NLNT) to allow clear differentiation from baseline target and non-target lesions.

New lesions may either meet the criteria of NLT or NLNT to drive iUPD (or iCPD). However, the measurements of target lesions should NOT be included in the sum of measures of original target lesions identified at baseline. Rather, these measurements will be collected on a separate table in the case record form.

PD is confirmed in the New Lesion category if the next imaging assessment, conducted at least 4 weeks (but not more than 8 weeks) after iUPD confirms further progression from iUPD with either an increase of at least 5 mm in the absolute value of the sum of NLT OR an increase (but not necessarily unequivocal increase) in the size of NLNT lesions OR the appearance of additional new lesions.

The following tables describe the time-point response (Table A) and best overall response (Table B) assessment when using iRECIST.

Target Lesions*	Non-Target Lesions*	New Lesions*	Time Point Response	
			No prior iUPD**	Prior iUPD**; ***
iCR	iCR	No	iCR	iCR
iCR	Non-iCR/Non-	No	iPR	iPR
iPR	Non-iCR/Non-	No	iPR	iPR
iSD	Non-iCR/Non-	No	iSD	iSD
iUPD with no change OR decrease from last TP	iUPD with no change OR decrease from last TP	Yes	NA	NLs confirms iCPD if NLs were previously identified and increase in size (≥5 mm in SOM for NLT or any increase for NLNT) or number. If no change in NLs (size or number) from last TP, remains iUPD
iSD	iUPD	No	iUPD	Remains iUPD unless iCPD confirmed based in further increase in size of NT disease (need not meet RECIST 1.1 criteria for unequivocal PD)
iUPD	Non-iCR/Non-iUPD	No	iUPD	Remains iUPD unless iCPD confirmed based on: o further increase in SOM of at least 5 mm, otherwise remains iUPD
iUPD	iUPD	No	iUPD	Remains iUPD unless iCPD confirmed based on further increase in: o previously identified T lesion iUPD SOM ≥5 mm and / or o NT lesion iUPD (prior assessment - need not be unequivocal PD)
iUPD	iUPD	Yes	iUPD	Remains iUPD unless iCPD confirmed based on further increase in: o previously identified T lesion iUPD ≥5 mm and / or o previously identified NT lesion- iUPD (need not be unequivocal) and /or o size or number of new lesions previously identified
Non-iUPD/PD	Non-iUPD/PD	Yes	iUPD	Remains iUPD unless iCPD confirmed based on: o increase in size or number of new lesions previously identified
* Using RECIST 1.1 principles. If no PSPD occurs, RECIST 1.1 and iRECIST categories for CR, PR and SD would be the same. ** in any lesion category. *** previously identified in assessment immediately prior to this TP.				

All patients will have their iBOR from the start of study treatment until the end of treatment classified as outlined below.

Table B: iRECIST Best Overall Response (iBOR)

TPR1	TPR2	TPR3	TPR4	TPR5	iBOR
iCR	iCR, iPR, iUPD, NE	iCR, iPR, iUPD, NE	iUPD	iCPD	iCR
iUPD	iPR, iSD, NE	iCR	iCR, iPR, iSD, iUPD, NE	iCR, iPR, iSD, iUPD, iCPD, NE	iCR
iUPD	iPR	iPR, iSD, iUPD, NE	iPR, iSD, iUPD, NE,	iPR, iSD, iUPD, NE, iCPD	iPR
iUPD	iSD, NE	PR	iPR, iSD, iUPD, NE	iPR, iSD, iUPD, iCPD, NE	iPR
iUPD	iSD	iSD, iUPD, NE	iSD, iUPD, iCPD, NE	iSD, iUPD, ICPD, NE	iSD
iUPD	iCPD	Anything	Anything	Anything	iCPD
iUPD	iUPD	iCPD	Anything	Anything	iCPD
iUPD	NE	NE	NE	NE	iUPD

- Table assumes a randomized study where confirmation of CR or PR is not required.
- NE = not evaluable that cycle.
- Designation “I” for BOR can be used to indicate prior iUPD to aid in data interpretation.

Methods of Measurement

Refer to protocol Section 16.

For additional information, refer to the following article and online supplemental information:
Seymour L, Bogaerts J, Perrone A, Ford R, Schwartz LH, Mandrekar S, Lin NU, Litiere S, Dancey J, Chen A, Hodi FS, Therasse P, Hoekstra OS, Shankar LK, Wolchok JD, Ballinger M, Caramella C, de Vries EG, group Rw. iRECIST: guidelines for response criteria for use in trials testing immunotherapeutics. Lancet Oncol. 2017;18(3):e143-e52. doi: 10.1016/S1470-2045(17)30074-8. PubMed PMID: 28271869.
(38)

Appendix H Celecoxib Patient Diary

Protocol # _____ Patient Name: _____
Drug Name: Celecoxib

Med. Record #: ___ ___ ___ ___ ___ ___

Study Medication Calendar

You are participating in a study that requires Celecoxib 200 mg to be given twice daily, approximately 12 hours apart on certain days. Your morning dose of Celecoxib will be given to you along with your pre-medications while you are in clinic. Please complete this calendar to document your evening doses of Celecoxib. Your nurse will let you know what time you receive your morning dose of Celecoxib so you can take your evening dose approximately 12 hours after.

CKM Regimen #_____:

Date			
Time that morning dose was taken (to be completed by the nurse in clinic)			
Evening Dose			
Time			
Patient's Initials			

Please remember to bring this calendar and your pill bottle (including any unused pills) with you to your next study appointment.

Coordinator's Use Only

$$\%\ Compliance = \left(\frac{Number\ of\ Pills\ Dispensed - Number\ of\ Pills\ Returned}{Number\ of\ Pills\ Scheduled}\right) \times 100$$

$$\_\_\_\%\ Compliance = \left(\frac{\quad}{\quad}\right) \times 100$$

Subject's Signature : _____ Date : _____

CRC's Signature : _____ Date : _____

Investigator's Signature: _____ Date: _____

Appendix I Study Calendar

Evaluation[1]	Baseline[2]	CKM cycle #1 (Day 0, 1, 2) ± 1 day	CKM cycle #2 (Day 7, 8, 9) ± 1 day	Pembrolizumab (200 mg Q3W)					End of Protocol Treatment[15] (+ 7 days)	Follow-up[16]
				Cycle 2 Day1 (*Pembrolizumab Dose #2*) (± 3 days)	Cycle 2 Day 8 (± 1 days)	Cycle 2 Day 15 (± 1 days)	Cycle 3 Day 1 (*Pembrolizumab Dose #3*) (±2 days)	Cycle 4 Day 1 (*Pembrolizumab Dose #4*) (±2 days)		
Written Informed Consent	X[3]									
Recording of Concomitant Medications	X[4]	X	X	X	X	X	X	X	X	X
Recording of Adverse Events		X	X	X	X	X	X	X	X	X
Survival Follow-up										X
Clinical Assessments										
Medical & Surgical History	X									

Evaluation[1]	Baseline[2]	CKM cycle #1 (Day 0, 1, 2) ± 1 day	CKM cycle #2 (Day 7, 8, 9) ± 1 day	Pembrolizumab (200 mg Q3W)					End of Protocol Treatment[15] (+ 7 days)	Follow-up[16]
				Cycle 2 Day1 (*Pembrolizumab Dose #2*) (± 3 days)	Cycle 2 Day 8 (± 1 days)	Cycle 2 Day 15 (± 1 days)	Cycle 3 Day 1 (*Pembrolizumab Dose #3*) (±2 days)	Cycle 4 Day 1 (*Pembrolizumab Dose #4*) (±2 days)		
Physical Examination including height and weight[5]	X	X[11]	X[12]	X			X	X	X	
Vital Signs[6]	X	X[6]	X[6]	X	X	X	X	X	X	
ECOG Performance Status	X	X	X	X			X		X	
Laboratory Procedures										
Hematology[7]	X	X[11]	X[12]	X	X	X	X	X	X	
Chemistry[8]	X	X[11]	X[12]	X	X	X	X	X	X	
Thyroid studies	X[18]						X[18]		X[18]	
Serum hCG	X[9]									
Troponin I level[13]	X			X	X	X				
CA 15-3 level	X									
Blood (60cc) for in vitro assays	X[17]	X[17]	X[17]	X[17]			X[17]			
Antinuclear antibodies	X									

Evaluation[1]	Baseline[2]	CKM cycle #1 (Day 0, 1, 2) ± 1 day	CKM cycle #2 (Day 7, 8, 9) ± 1 day	Pembrolizumab (200 mg Q3W)					End of Protocol Treatment[15] (+ 7 days)	Follow-up[16]
				Cycle 2 Day1 (*Pembrolizumab Dose #2*) (± 3 days)	Cycle 2 Day 8 (± 1 days)	Cycle 2 Day 15 (± 1 days)	Cycle 3 Day 1 (*Pembrolizumab Dose #3*) (±2 days)	Cycle 4 Day 1 (*Pembrolizumab Dose #4*) (±2 days)		
Imaging/ Other Procedures										
12-Lead ECG[13]	X			X	X	X				
Biopsy[14]	X		X[14]							X[14a]
Tumor/Disease Assessment (CT and/or MRI)	X[10]								X[10]	X[20]
Study Drug Administration										
CKM regimen administration		X[19]	X							
Pembrolizumab		X[21]	X[21a]	X[22]			X	X		

1. Assessments to be performed at the times stipulated in the table and as clinically required in the management of the participant.
2. To be performed within 21 days prior to study treatment unless otherwise stated.
3. Written informed consent must be obtained prior to receiving any study related procedure.
4. Medications ongoing, or discontinued, within 1 week prior to first dose of study treatment.

5. Physical exam to include assessment of Head/Ears/Eyes/Nose/Throat (HEENT), Skin, Chest/Lungs, Heart, Neck/Lymph nodes, Abdomen, Extremities and weight. Height required at baseline only.

6. Vital Signs include blood pressure, pulse, temperature and respiratory rate. On days that Rintatolimod is given, vital signs will be obtained pre-treatment, 30 and 60 minutes post Rintatolimod (± 5 minutes)

7. CBC with automated differential

8. CMP

9. Within 7 days of first dose of study drug in women of child-bearing potential.

10. Baseline disease assessment within 31 days prior to first dose of study drug is acceptable. Repeat disease assessment after 4 cycles/doses of pembrolizumab at End of Protocol treatment visit or sooner. Both RECIST 1.1 and iRECIST will be documented during the trial. RECIST 1.1 will be used to determine baseline eligibility. iRECIST will be used to evaluate Response and to determine Disease Progression.

11. Assessments to be performed on day 0 visit and as clinically indicated on days 1 and 2.

12. Assessments to be performed on day 7 visit and as clinically indicated on days 8 and 9.

13. A single 12-lead ECG and Troponin I level to be performed at screening, Cycle 2 Day 1, Cycle 2 Day 8, Cycle 2 Day 15 and then only as clinically indicated thereafter. On dosing days, ECG and Troponin I level to be completed prior to dosing.

14. Biopsies can be performed within 1 week before the start of CKM treatment and within 24 hours (can be delayed to 3 days in case of logistic or compliance issues) after completion of the 2nd cycle of the CKM treatment. Biopsies are optional for Cohort 1 and Cohort 2 and mandatory for Cohort 3.

14 a. In addition to on-trial tissue biopsies, any tissue from resected tumor (and margin) harvested within standard care may be requested for correlative analysis for potential identification of biomarkers that may be related to clinical outcome.

15. End of protocol treatment (end of 4 doses of pembrolizumab, + 7 day window). Any additional treatment with pembrolizumab will be considered as treatments given during follow-up and any further drug will be from commercial supplies.

16. To capture all possible delayed immune-related adverse events, follow-up safety assessments will occur at least monthly for 90 days after the last dose of study drug or until resolution of any drug related toxicity (telephone contact is acceptable). Patients, who in consultation with their clinician will be continued on pembrolizumab after Dose #4, will be included in the 90 day follow-up for immune mediated toxicities.). Thereafter patients will be contacted by phone every 6 months up to 2 years to assess survival status.

17. Blood for *in vitro* assays will be obtained at the following time points: Screening visit, on day 0 prior to the start of CKM regimen; 2 hours post completion of Rintatolimod on day 2; prior to the start of CKM regimen on day 7; pre-dose on day 1 of Pembrolizumab Dose 2 & Dose 3; and at time of disease progression.

18. TSH, Free T4, T3 will be done at baseline. TSH will be obtained pre-dose on Pembrolizumab Cycle 3 Dose #3. TSH, Free T4, T3 will be obtained at end of treatment.

19. Each CKM dosing day will have a window of ± 1 day. All doses of CKM must be given within 14 days of initiation of Pembrolizumab Cycle 1 Day 1.

20. CT of chest, abdomen and pelvis will be done as per SOC for patients continuing on pembrolizumab after the 4 on-study doses. RECIST 1.1 and iRECIST scans will be requested for all patients until they have disease progression or until Pembrolizumab is discontinued. Window of assessment is ± 5 days.

21. Cohort 2 and Cohort 3 only: Early dose of Pembrolizumab 200 mg IV on CKM Cycle 1 Day 2 (Cohort 1 Pembrolizumab start on day 9 at 2 hours after completion of CKM) is pembrolizumab Dose #1, and Q3W thereafter for a total of 4 doses.

21a. Cohort 1 only: CKM Cycle 2 Day 9 **(2 hours after the end of CKM cycle 2)** - can be delayed by up to 2 days. Pembrolizumab (200 mg) – is pembrolizumab Dose #1; and Q3W thereafter for a total of 4 doses.

22. Pembrolizumab will be administered at the following timepoints for Cohort 1, Cohort 2, and Cohort3:

- Cohort 1: Day9 **(2 hours after the end of CKM cycle 2)** - can be delayed by up to 2 days. Pembrolizumab (200 mg); and Q3W thereafter for a total of 4 doses.
- Cohort 2: **(2 hours after the end of CKM cycle 1):** Early dose of Pembrolizumab (200 mg), and Q3W thereafter for a total of 4 doses.
- Cohort 3: **(2 hours after the end of CKM cycle 1):** Early dose of Pembrolizumab (200 mg), and Q3W thereafter for a total of 4 doses.

Appendix J AACR 2022 Poster and Safety Data

ROSWELL PARK COMPREHENSIVE CANCER CENTER

Systemic Rintatolimod and Interferon-α2b Selectively Reprogram Local Tumor Microenvironments in Patients with Metastatic Triple Negative Breast Cancer for Enhanced Influx of Cytotoxic T-lymphocytes but not Regulatory T-cells

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Abstract No. CT145

Background

- Clinical effectiveness of immune therapies depends on the presence of cytotoxic T-lymphocytes (CTLs) in the tumor microenvironment (TME).
- Our preclinical data showed synergy between TLR3 ligands and interferon-α (IFN-α) in selectively reprogramming the TME, but not healthy tissues, to enhance CTL attraction and reduce Treg attraction, providing rationale for their systemic application to enhance local CTL densities in "cold" tumors.
- The study NCT03599453 evaluated the safety of systemic chemokine modulating regimen (CKM) composed of i.v. rintatolimod (Ampligen; selective TLR3 ligand) and IFN-α, and its ability to promote local CTL influx to metastatic triple negative breast cancer (mTNBC) lesions.

Methods

- Inclusion criteria included histologically confirmed, surgically unresectable mTNBC, Eastern Cooperative Oncology Group performance status (ECOG PS) ≤ 1, measurable disease per RECIST v1.1, no cancer directed therapy for at least 3 weeks prior to study treatment, biopsy-accessible lesion, adequate bone marrow reserve and hepatic and renal function.
- Exclusion criteria included prior receipt of anti-PD-1/PD-L1 treatment, immunosuppressive conditions, autoimmune disease, serious mood disorders, history of gastrointestinal ulceration, hypersensitivity to NSAIDs.
- Six evaluable patients (33-75 years) with mTNBC received 6 doses of rintatolimod (200 mg i.v.), IFN-α2b (INTRON-A; 20MU/m2 i.v.) and COX-2 inhibitor (celecoxib; 2 x 200 mg, p.o.) over 2 weeks, with tumor biopsies obtained before (within 6 days) and after (within 5 days) CKM. All pts received follow-up pembrolizumab (200 mg, i.v, Q3 weeks). Fig. 1 shows the schema of the clinical trial.
- The primary endpoint was the change in the CTL marker CD8α in the TME with a planned interim analysis after 3 patients (α=0.03) and final analysis after 6 patients (α=0.084).
- The secondary endpoints included assessment of overall response rate (ORR) to combination treatment per RECIST v1.1, progression-free survival (PFS), overall survival (OS) and disease-control rate (DCR).
- Correlative studies analyzed additional markers of CTLs, regulatory T-cells (T_{regs}), and CTL- and T_{reg}-attracting chemokines in the TME and blood.

Results

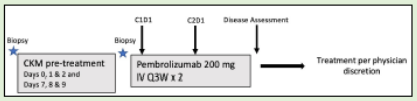


Figure 1. Study design schema with pre-conditioning chemokine modulation regimen (CKM) followed by pembrolizumab. Stars indicate the time of scheduled tumor biopsies.

Table 1. Baseline demographic characteristics of the population. Eight patients were enrolled on the study, of which six patients received pre- and post-treatment biopsy and were considered evaluable for the primary endpoint.

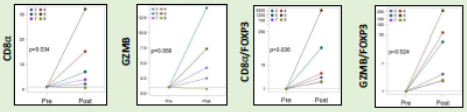
Table 1. Baseline characteristics

Characteristics	Total No. (%)
No. of patients	8 (100%)
Age in years, median (Min/Max)	52.9 (32.8-74.5)
Race	
Caucasian	7 (87.5%)
African-American	1(12.5%)
ECOG PS	
0	7 (87.5%)
1	1 (12.5%)
*Best Response	
Stable disease	3 (37.5%)
Progressive disease	5 (62.5%)
Lines of prior systemic treatment in the metastatic setting	
First line	6 (75%)
Second line	2 (25%)
PD-L1 >=1% (Ventana SP142)	5 (62.5%)

Abbreviations: ECOG PS, Eastern Cooperative Oncology Group performance status; PD-L1, programmed cell death ligand 1.
* Response calculated per RECIST v1.1

- We observed uniform increase of immune markers upon treatment: CD8α mRNA (6.1-fold; p-0.034), GZMB mRNA (3.5-fold; p=0.058), ratios of CD8α/ FOXP3 and GZMB/FOXP3 (5.7-fold; p=0.036, and 7.6-fold; p=0.024 respectively), thus successfully meeting the endpoint (Fig. 2).
- In addition, we also observed an increase in CTL attractants CXCL10 (2.6-fold; p=0.104) and CCL5 (3.3-fold; p=0.019). In contrast, Treg marker FOXP3 or Treg attractants CCL22 or CXCL12 were not enhanced (Fig.2).

Figure 2. Local efficacy of systemic CKM: mRNA transcripts in pre-versus post-CKM tumor biopsies, measured using Taqman (normalized for HPRT1).



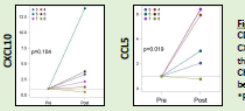
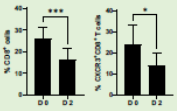


Figure 3. Decrease in CD8⁺T-cells and CXCR3⁺CD8⁺T-cells in the circulation after CKM treatment between days 0 and 2. *P<0.05, ***P<0.005



- The above TME changes were accompanied by transient decreases in circulating CD3⁺CD8⁺CTLs (but not Tregs), selectively affecting the cells expressing CXCR3 (receptor for CXCL10), but not CCR4 or CXCR4 (receptors for CCL22 and CXCL12) (Fig. 3).

Results (cont.)

- Three patients had stable disease lasting 2.4, 2.5 and 3.8 months, as of data cut off September 1, 2021 (Fig. 4 and 5).

Figure 4. Waterfall plot

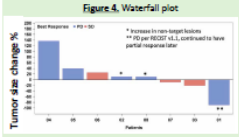
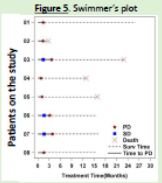


Figure 5. Swimmer's plot



- An additional patient (non-evaluable) had a partial response (breast auto-amputation) (Fig. 6) with massive tumor necrosis (Fig.7) in the post-CKM biopsy.

Figure 6. Serial CT scan showing breast tumor auto-amputation

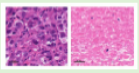


Figure 7. Post-CKM tumor biopsy showing tumor necrosis

Conclusion

- The study successfully met the predetermined primary endpoint (increase in CD8α in TME).
- This proof-of-concept study shows that short-term systemic CKM followed by pembrolizumab is safe and selectively enhances local CTL infiltration in the TME, providing rationale for concurrent CKM and PD1 blockade in prospective phase II studies.

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Safety Data

Adverse Events reported for I-62218: Pilot Open Label Clinical Trial Evaluating the Safety and Efficacy of Chemokine Modulation to Enhance the Effectiveness of Pembrolizumab in Patients with Metastatic Triple Negative Breast Cancer

		Total (N(%))				
		Total(N=8)				
System Organ Class	Adverse event	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Blood and lymphatic system disorders	Immune thrombocytopenic purpura	0(0.0)	0(0.0)	1(12.5)	0(0.0)	0(0.0)
Eye disorders	Eye disorder	1(12.5)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
Gastrointestinal disorders	Mouth hemorrhage	0(0.0)	1(12.5)	0(0.0)	0(0.0)	0(0.0)
	Nausea	1(12.5)	3(37.5)	0(0.0)	0(0.0)	0(0.0)
	Vomiting	1(12.5)	1(12.5)	0(0.0)	0(0.0)	0(0.0)
General disorders and administration site conditions	Chills	6(75.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
	Fatigue	2(25.0)	2(25.0)	1(12.5)	0(0.0)	0(0.0)
	Malaise	0(0.0)	0(0.0)	1(12.5)	0(0.0)	0(0.0)
	Pain	1(12.5)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
	Pyrexia	1(12.5)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
Investigations	Alanine aminotransferase increased	3(37.5)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
	Aspartate aminotransferase increased	3(37.5)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
	Blood alkaline phosphatase increased	0(0.0)	1(12.5)	0(0.0)	0(0.0)	0(0.0)
	Lymphocyte count decreased	0(0.0)	1(12.5)	0(0.0)	0(0.0)	0(0.0)
	Neutrophil count decreased	0(0.0)	0(0.0)	1(12.5)	0(0.0)	0(0.0)
	Platelet count decreased	0(0.0)	0(0.0)	0(0.0)	1(12.5)	0(0.0)
	White blood cell count decreased	0(0.0)	1(12.5)	0(0.0)	0(0.0)	0(0.0)
Metabolism and nutrition disorders	Decreased appetite	0(0.0)	1(12.5)	0(0.0)	0(0.0)	0(0.0)
Musculoskeletal and connective tissue disorders	Myalgia	1(12.5)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
Nervous system disorders	Dizziness	1(12.5)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
	Headache	1(12.5)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
	Taste disorder	0(0.0)	1(12.5)	0(0.0)	0(0.0)	0(0.0)
Respiratory, thoracic and mediastinal disorders	Dyspnea	0(0.0)	0(0.0)	1(12.5)	0(0.0)	0(0.0)
	Hypoxia	0(0.0)	0(0.0)	1(12.5)	0(0.0)	0(0.0)
	Pneumonitis	0(0.0)	0(0.0)	1(12.5)	0(0.0)	0(0.0)

		Total (N(%))				
		Total(N=8)				
System Organ Class	Adverse event	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
	Night sweats	1(12.5)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
	Petechiae	0(0.0)	1(12.5)	0(0.0)	0(0.0)	0(0.0)
	Pruritus	2(25.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
Skin and subcutaneous tissue disorders	Rash	2(25.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
	Hot flashes	3(37.5)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
Vascular disorders	Hypotension	1(12.5)	0(0.0)	0(0.0)	0(0.0)	0(0.0)

SUMMARY:

No novel toxicities of the follow up pembrolizumab therapy was observed in any of the eight patients. The most common treatment-related adverse events of any grade (≤ 3) were chills (75%), fatigue (62.5%), nausea (50%), hot flashes (37.5%), ALT increase (37.5%) and AST increase. One event of grade 3 pneumonitis resulted in hospitalization and was described as definitely related to pembrolizumab. One event of non-clinically significant grade 3 neutropenia was also observed, possibly related to combination of rintatolimod and interferon. The only other serious adverse event attributed to the combination was grade 3 immune thrombocytopenic purpura which was treated with steroids, IVIG and rituximab and thereafter resolved.

Appendix K

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Tumor Biology and Immunology

Cancer Research

Helicase-Driven Activation of NFκB-COX2 Pathway Mediates the Immunosuppressive Component of dsRNA-Driven Inflammation in the Human Tumor Microenvironment

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Abstract

Presence of cytotoxic $CD8^+$ T cells (CTL) in tumor microenvironments (TME) is critical for the effectiveness of immune therapies and patients' outcome, whereas regulatory T(reg) cells promote cancer progression. Immune adjuvants, including double-stranded (ds)RNAs, which signal via Toll-like receptor-3 (TLR3) and helicase (RIG-I/MDA5) pathways, all induce intratumoral production of CTL-attractants, but also Treg attractants and suppressive factors, raising the question of whether induction of these opposing groups of immune mediators can be separated. Here, we use human tumor explant cultures and cell culture models to show that the (ds) RNA Sendai Virus (SeV), poly-I:C, and rintatolimod (poly-I:C_{12}U) all activate the TLR3 pathway involving TRAF3 and IRF3, and induce IFNα, ISG-60, and CXCL10 to promote CTL chemotaxis to *ex vivo*–treated tumors. However, in contrast with SeV and poly I:C, rintatolimod did not activate the MAVS/helicase pathway, thus avoiding NFκB– and TNFα-dependent induction of COX2, COX2/PGE2-dependent induction of IDO, IL10, CCL22, and CXCL12, and eliminating Treg attraction. Induction of CTL-attractants by either poly I:C or rintatolimod was further enhanced by exogenous IFNα (enhancer of TLR3 expression), whereas COX2 inhibition enhanced the response to poly-I:C only. Our data identify the helicase/NFκB/TNFα/COX2 axis as the key suppressive pathway of dsRNA signaling in human TME and suggest that selective targeting of TLR3 or elimination of NFκB/TNFα/COX2-driven suppression may allow for selective enhancement of type-1 immunity.

Significance: This study characterizes two different poly-I:C-induced signaling pathways in their induction of immunostimulatory and suppressive factors and suggests improved ways to reprogram the TME to enhance the antitumor efficacy of immunotherapies. *Cancer Res; 78(15); 4292–302. ©2018 AACR.*

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Introduction

The impact of local inflammation on tumor progression is determined by its character. Presence of $CD8^+$ cytotoxic T-cells (CTL) in tumor microenvironments (TME) predicts prolonged survival of patients with multiple types of tumors (1, 2) and effectiveness of different forms of immunotherapies, including PD-1/PD-L1 blockade (3, 4). In contrast, intratumoral accumulation of regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC) predicts resistance to treatments and accelerated cancer progression (2, 5, 6). Because these different immune subsets are attracted by separate sets of chemokines, significant efforts have been dedicated to selective enhancement of intratumoral production of CTL attracting chemokines (such as CXCL9/MIG, CXCL10/IP10 CXCL11/I-TAC and CCL5/RANTES; ref. 7) while suppressing MDSC- and Treg-attracting chemokines (such as CCL22/MDC or CXCL12/SDF-1; refs. 8, 9).

Toll-like receptor (TLR) ligands have been widely used as adjuvants to enhance systemic immunization and to promote CTL entry into tumor tissues. Ligands for TLR3 (double-stranded RNA; dsRNA; such as poly-I:C and its analogs), TLR4 (LPS and its variant, MPLA), TLR7/8 (imiquimod) or TLR9 have been evaluated extensively in clinical trials (10, 11).

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The ability of dsRNA (and other TLR ligands) to simultaneously induce CTL attractants and Treg-attracting chemokines and other tumor-promoting factors (11–13) raises the question of whether these functionally different factors are induced by separate signaling pathways, which can be dissociated to selectively enhance the desirable aspects of inflammation. dsRNA is known to trigger TLR3, which, through TIR domain-containing adaptor TRIF (14, 15), activates TRAF3 (16) and induces nuclear translocation of IRF3, leading to the transcription of type 1 interferons (17, 18). Concomitant activation of TRAF6 by TRIF through RIP-1 leads to nuclear translocation of NFκB and induction of additional pro-inflammatory cytokines such as TNFα (19). In addition to the TLR3/TRIF pathway, dsRNA can also activate the helicases RIG-I and MDA5 present in the cytosol (19, 20). Helicases interact with the mitochondrial anti-viral signaling protein (MAVS) and activate both, type-1 interferon pathway (21) and the NFκB pathway (22, 23). Although NFκB activation can promote the induction of type-1 interferons and the development of type-1 inflammation (24, 25), its intratumoral activation is typically associated with enhanced cancer cell proliferation, resistance to apoptosis, and cancer progression (26, 27).

The induction of NFκB and the associated induction of TNFα in response to either poly-I:C (28) or selective TLR3 ligand rintatolimod (poly-I:C12U; ref. 29), is strongly elevated in mice and other rodent species, compared with humans (28, 29), making mouse and rabbit studies poor models to predict toxicity and other undesirable effects of adjuvants involving different forms of dsRNA (29). Guided by the above considerations, we evaluated the role of different dsRNA-induced signaling pathways in the induction of immunostimulatory and suppressive factors and tested the feasibility of their separation to enhance the pattern of response in complete human TME.

Materials and Methods

Study design and patients

Following informed, written consent, specimens (ovarian tumor, $n = 14$, see Table 1) were collected during primary debulking surgeries for ovarian cancer under the University of Pittsburgh Institutional Review Board–approved protocol UPCI 07-058 (Prognostic Marker: Acquisition of Blood Samples and Tissue for Research Purposes (Gyn-Onc # 22-096). At least three biological replicates were performed for each experiment with the exact numbers specified in figure legends. All studies have been performed in accordance with the Declaration of Helsinki and other institutional and federal guidelines.

Table 1. Clinical and pathology data

Clinicopathological data	Patients ($n = 14$)
	n (%)
Age, y	
≤61	7 (50)
>61	7 (50)
(range, 36–79)	
Stage	
I	8 (57)
II	0 (0)
III	5 (36)
IV	1 (7)
Histological grade	
Well	1 (7)
Moderate	2 (14.3)
Poor	9 (64.3)
Not reported	2
Histology	
Serous	7 (50)
Endometrioid	3 (21.4)
Mucinous	2 (14.3)
Clear cell	2 (14.3)

Tissue explant culture system

The *ex vivo* whole-tissue culture system, developed in our laboratory at the University of Pittsburgh and which allows us to avoid cell activation during tumor dissociation, has been described in our previous publication (30). In brief, using a 4-mm biopsy punch knife, the cubes of tumor were prepared and placed in antibiotic containing medium for 24 hours, in the absence or presence of the indicated factors and their combinations (Supplementary Fig. S1). The differentially treated tumor issues were harvested for mRNA extraction and culture supernatants were harvested for ELISA and chemotaxis assays (30).

Toll-like receptor ligands and modulators of their biologic activity

All TLR-ligands involved in this study were used at the previously established optimal concentrations, which all induced comparable levels of CXCL10 (Supplementary Figs. S2–S4). dsRNA, poly-I:C, a synthetic dsRNA with one strand composed of an inosine polymer and the other-cytidine polymer, known to activate both TLR3 and cytosolic helicases was obtained from (Sigma Aldrich; 20 μg/mL). Rintatolimod (poly-I:C12U), a modified dsRNA through an uracil mismatch at every 12th base position of the C-strand, which is a selective TLR3 ligand with a shorter half-life and lack of helicase-activating function (31, 32) was a gift from Hemispherx Bio and used at 125 μg/mL. Our titration experiments showed that at the concentration of 125 μg/mL of rintatolimod induced the same levels of CXCL10 expression (mRNA and protein levels) as poly-I:C (20 μg/mL) was visible (Supplementary Fig. S2). When indicated, the tissues were treated with IFNα (Life Technologies; 10,000 U/mL) and/or COX1/2 inhibitor indomethacin (Sigma Aldrich; 50 μmol/L). In some experiments, LPS (Sigma-Aldrich; 250 ng/mL), FSL-1 (Invivogen, 100 ng/mL), imiquimod (Sigma-Aldrich, 10 μg/mL), MPLA (Invivogen; 1 μg/mL) or inhibitory reagents eg. Indomethacin (Sigma-Aldrich, 50 μmol/L), Celecoxib (Biovision; 10 mmol/L), TNFα receptor inhibitor (TNFαRI; R&D Systems, 1 μg/mL) or an inhibitor of the NFκB pathway (BAY, BAY11-7082; Sigma-Aldrich; 10 μmol/L) were used.

Generation of macrophages

PBMCs were isolated from whole-blood samples of healthy donors through lymphocyte separation medium (Ficoll), as described (12, 33). Monocytes were isolated as light fraction of PBMCs through Percoll (GE Healthcare Life Sciences) density gradient centrifugation and cultured for 6 days in 24-well plates in IMDM complete medium, in the presence of GM-CSF (1,000 U/mL). On day 6, the cells were stimulated as indicated above (see: Tissue explant culture system) for 30 minutes, 1, 4, or 24 hours depending on the experiment. After harvesting, the supernatants were used for protein assays and the cells were lysed in RLT buffer for mRNA extraction, used for Western blot analysis or evaluated by immunofluorescence microscopy and image stream, as indicated.

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Culture of fibroblasts

Human adult dermal fibroblasts (Thermo Fisher, C0135C) were cultured until confluent status was reached. After harvesting, the cells were reseeded at 2 × 105 cells/well in 24-well plates and treated with IFNα 10,000 U/mL), poly-I:C (20 μg/mL) or rintatolimod (125 μg/mL), LPS (250 ng/mL), FSL-1 (100 ng/mL), Imiquimod (10 μg/mL), MPLA (1 μg/mL) or indomethacin (50 μmol/L) for 24 hours. Supernatants were analyzed for chemokine production and cell lysates were used for mRNA expression analysis.

Generation of $CD8^+$ effector T cells

Naïve $CD8^+$ T cells were extracted from whole blood of healthy individuals, as the heavy fraction of PBMCs (via Ficoll), followed by the positive separation of $CD8^+$ T cells using microbeads (Miltenyi). The $CD8^+$ T cells were cocultured with DCs loaded with SEB (1 ng/mL) for 6 days, as described previously (12). On day 6, cells were counted and adjusted to the desired density for chemotaxis assays (12).

TaqMan analysis of immune gene expression

Tumor biopsies or macrophages were taken to extract RNA using the RNeasy Kit (Qiagen). The RNA was used for cDNA synthesis and 25 ng of cDNA was used for mRNA expression analysis by TaqMan on the StepOnePlus system (Applied Biosystems). Commercially-available TaqMan primers (Thermo Fisher Scientific Life Technologies) were purchased to evaluate local expression of the key chemokines involved in the attraction of the effector cells (CCL5 and CXCL10), Treg (CCL22), and MDSCs (CXCL12). Furthermore, expression of EP4, COX2, TNFα, IFNβ, IDO, and IL10 was measured. The expression of each gene was normalized for the HPRT1 housekeeping gene.

Analysis of chemokines in *ex vivo* tumor explant cultures and cell cultures

Culture supernatants were analyzed by ELISA for the presence of the key chemokines involved in the attraction of the effector cells, Treg and MDSCs as previously described (13). Briefly, ELISA plates were coated with primary antibody at 1 μg/mL overnight at room temperature. Next, plates were washed and blocked with 4% BSA in PBS for 3 hours. Samples were loaded (50 μl/well) and after a 1-hour incubation the biotinylated secondary antibody was added (0.5 μg/mL). For detection, Streptavidin-HRP conjugate (Thermo Fisher Scientific Pierce Biotechnology Inc.) was used for 30 minutes. After addition of TMP substrate (Thermo Fisher Scientific Pierce), reaction was stopped with 2% H_2SO_4 solution. Absorbance, at 450 nm, was measured on Wallace 1420 Victor 2 microplate Reader (PerkinElmer). The antibodies were purchased from R&D Systems.

Chemotaxis assay

Chemotaxis assays were performed in 24 Transwell plates with a 5-μm pore-size polycarbonate filter (#CLS3421; SIGMA). All media and supernatants were brought to room temperature before usage. The lower chamber contained 500 μL of tumor-supernatants and the upper chamber 2 × 105 of either freshly isolated $CD4^+$ T cells or *ex vivo*-expanded $CD8^+$ T effector cells in 200 μL of IMDM for 1.5 hours at 37°C. The migrated cells in the lower chamber were harvested and stained for either CD8 or CD4 and FoxP3 and measured by flow cytometry (Accuri C6, BD Biosciences; ref. 12). The number of migrated cells per condition was subtracted by the number of cells migrated towards plain media only.

Western blot

Antibody against IκBα was purchased from the Cell Signaling Technology (#9242); the antibody against α-tubulin from the Santa Cruz Biotechnology (Santa Cruz Biotechnology) and ISG60 has been described before (34). Monocyte-derived macrophages were treated as indicated and whole cell lysates were re-suspended in self-made lysis buffer (20 mmol/L HEPES pH 7.4, 1% Triton-X 100, 150 mmol/L NaCl, 1.5 mmol/L MgCl2, 12.5 mmol/L β-glycerophosphate, 2 mmol/L EGTA, 10 mmol/L NaF, 2 mmol/L DTT, 1 mmol/L Na3VO4, 1 mmol/L PMSF plus 1x protease inhibitors). Equal amounts of protein were immunoblotted with IκBα antibody or ISG 60 and a-tubulin antibody. The blots were quantitated by densitometry using the ImageJ software (NIH, Bethesda). The integrated pixel value was determined for each band by multiplying the image intensity by the band area after having subtracted the mean background value.

Image flow analysis

Imaging flow analysis for detection of NFκB nuclear translocation was performed with Amnis Imaging Flow Cytometers (Millipore Sigma) and analyzed with IDEAS Software (Millipore Sigma). Macrophage cultures were left untreated or treated with poly-I:C or rintatolimod at the indicated concentrations and stained with the Amnis NFκB Translocation Kit (ACS10000, Millipore, Sigma) according to the manufacturer's protocol.

Immunofluorescence

Macrophages were seeded at a density of 10^5 cells on rat tail collagen-type 1 coated coverslips. After a period of >6 hours, attached cells were treated with rintatolimod or poly-I:C for 1, 4, and 24 hours. Untreated cultures served as a negative control. As positive control, macrophages were infected with Sendai virus (SeV; 80 HAU/mL) for 18 hours. Following treatments, cells were fixed in 3.33% PFA for 20 minutes at room temperature. After four washes with PBS cells were permeabilized with 0.1% Triton X. Cells, incubated with primary antibodies (MAVS: Bethyl Lab # A300-782A, 1:100; RIP-1 BD # 610459 1:250, TRAF3: Thermo Fisher #PA5-29091, 1:100; IRF3 provided by Dr. S. Sarkar, University of Pittsburgh, 1:500) overnight at 4°C, washed and incubated with a secondary antibody for one hour at room temperature (1:500, Alexa Fluor 488). Cells were then incubated with Hoechst (1:1,000) for 5 minutes and mounted using Prolong gold solution (Thermo Fisher Scientific Invitrogen, # P10144). Imaging was performed using the Carl Zeiss LSM 880 confocal microscope at fixed settings across treatments.

Phospho-CREB activation

Macrophages were differentiated as described above. After treatment with PGE_2 (Sigma, 10^{-6} mol/L) or PGE_2 in combination with IFNα, macrophages were harvested and fixed using 4% paraformaldehyde for 15 minutes at room temperature. After washing with PBS, cells were permeabilized by adding ice cold Methanol to a final concentration of 90% for 30 minutes. After two washing steps, blocking was performed with whole mouse IgG (10 μg/mL) for 15 minutes at room temperature. The phospho-CREB antibody (#9198, Cell Signaling Technology) was

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added and incubated for 1 hour at room temperature. After two washing steps, cells were incubated with fluorochrome conjugated secondary ab (Alexa Fluor 647, #4414, Cell Signaling Technology) for 30 minutes at room temperature in the dark. After another two washing steps, phosphor-CREB activation was measured by flow cytometry (Accuri C6, BD Biosciences).

Mouse studies

Female 6- to 8-week-old C57BL/6 mice were purchased from The Jackson Laboratory and maintained in the Roswell Park Animal Facility, under Institutional Animal Care and Use Committee (IACUC)-approved protocols. Bone marrow was isolated from the femur and tibia of C57BL/6 and cultured in the presence of GM-CSF, as described previously (35). On day 6 to 7, bone marrow–derived macrophages were activated overnight with the indicated concentrations of poly(I:C) or rintatolimod, in the absence or presence of celecoxib, as indicated, before RNA extraction and RT-PCR (TaqMan) analysis, as described previously (36).

Statistical analysis

All data points represent biological replicates. All statistical analyses were conducted using GraphPad Prism 5 software. Multiple treatment conditions of the same tumor sample or culture sample were compared with each other using the nonparametric Wilcoxon matched paired signed-rank. $P < 0.05$ was considered significant.

Results

Different forms of dsRNA differ in their ability to induce Treg/MDSC-attracting chemokines and suppressive factors in human TME despite being similarly effective in promoting CTL attraction.

To evaluate the impact of immune adjuvants on complete human microenvironments, we used our previously developed tumor explant model, which includes all elements of TME (Supplementary Fig. S1; ref. 12). As shown in Fig. 1A, fresh biopsies of human ovarian cancer exposed to either poly-I:C or rintatolimod (poly-I:C12U, a selective TLR3 ligand with a shorter systemic half-life and lacking a cytosolic helicase-activating function (31, 32), induced similar levels of the desirable CXCL10. Strikingly, however, only poly-I:C, but not rintatolimod, induced CCL22 or CXCL12 at either mRNA expression level and protein secretion in cancer tissue explants and in macrophage and fibroblast cultures (Fig. 1A; Supplementary Figs. S2 and S3A–S3D), independently on the concentration used (Supplementary Fig. S2). CCL5 production showed an intermediate pattern of regulation, being induced by both forms of dsRNA, but at higher levels by poly-I:C (Supplementary Fig. S3A–S3B).

These differences were reflected by enhanced ability of rintatolimod to promote selective attraction of CTLs, with concomitant reduction in Treg attraction, compared with poly-I:C (Fig. 1B).

Analysis of cancer-associated suppressive factors COX2 (rate-limiting mediator of PGE_2 synthesis), IDO and IL10, further showed the selective ability of poly-I:C, but not rintatolimod, to induce tumor-associated suppressive factors in the tumor biopsies and macrophage cultures (Fig. 1C). The dual character of poly-I:C-driven inflammation was shared by multiple additional TLR ligands, which all induced COX2, IDO, and CCL22, in addition to CXCL10 (Supplementary Fig. S4).

Activation of NFκB- versus type-1 IFN pathway reflects the respective activation of the helicase- versus TLR3-mediated dsRNA recognition.

To investigate the mechanisms underlying different patterns of responses to poly-I:C versus rintatolimod, we compared the induction of IFNβ and ISG60 (representatives of type-1 interferon pathway) versus NFκB (as a mediator of the TRAF6- and helicase-mediated pathways of dsRNA recognition).

As shown in Fig. 2A–D, poly-I:C and rintatolimod were both equally potent inducers of IFNβ and ISG60 in macrophage cultures, suggesting their similar ability to activate IRF3. This was in sharp contrast to the activation of the NFκB system, which was driven exclusively by poly-I:C, but not rintatolimod as shown by its inability to degrade IκB, the blocker of nuclear translocation and activation of NFκB (37).

As shown in Fig. 2D, degradation of IκB was evident after 1 hour of stimulation by poly-I:C, with its re-synthesis and re-appearance observed at 4 hours and return to baseline levels at 24 hours. As expected, no degradation by rintatolimod is apparent at any time point. Image flow analysis demonstrated that nuclear translocation of NFκB is selectively induced by poly-I:C, but not by rintatolimod (Fig. 2E; the similarity score reflects colocalization between 7-AAD nuclear stain and NFκB stain). In contrast to the untreated cells and rintatolimod-treated cells, which showed no colocalization of NFκB and the nuclear marker, the poly-I:C–treated cells showed strong colocalization between nuclear stain and NFκB stain.

To directly evaluate the impact of both forms of dsRNA on two upstream NFκB–activating pathways of dsRNA recognition, RIP-1 (TLR3-dependent pathway) and MAVS (cytoplasmic helicase-dependent pathway), we performed immunofluorescence analysis of the upstream regulatory proteins, MAVS and RIP-1. As shown in Fig. 2F, poly-I:C treatment activated both MAVS and RIP-1, similar to the SeV, whereas no activation of either MAVS or RIP-1 was observed after rintatolimod exposure. In sharp contrast, both poly-I:C and rintatolimod promoted similar induction of TRAF3 and IRF3 nuclear translocation, the key mediators of the TLR3/TRAF pathway of type-1 IFN induction.

Positive feedback between TNFα to NFκB is involved in the dsRNA-triggered TNFα and COX2 activation

TNFα is a major pro-inflammatory cytokine, which both activates NFκB and is induced itself as a result of NFκB activation (38). TNFα is also a potent inducer of COX2 (38), the key mediator of PGE_2 synthesis (39), which coordinates intratumoral production of multiple other suppressive factors and Treg/MDSC attractants (9, 33, 39, 40). To investigate the sequence of events and the role of the interplay between TNFα, NFκB and COX2 during the induction of antitumor- and tumor-promoting aspects of dsRNA signaling, we investigated the regulation of TNFα and NFκB by poly-I:C or rintatolimod, and the impact of their blockade upon the induction of each other and COX2.

As shown in Fig. 3A, poly-I:C-treated macrophages showed significantly higher levels of TNFα expression compared with untreated- or rintatolimod-treated cells (which showed similar levels of TNFα). To test whether endogenous TNFα and activated NFκB are needed for poly-I:C-driven COX2 induction we performed inhibitory assays using BAY11-7082, an NFκB inhibitor and soluble TNFαRI, as a competitive TNFα receptor antagonist. Suppression of COX2 was observed when combining poly-I:C

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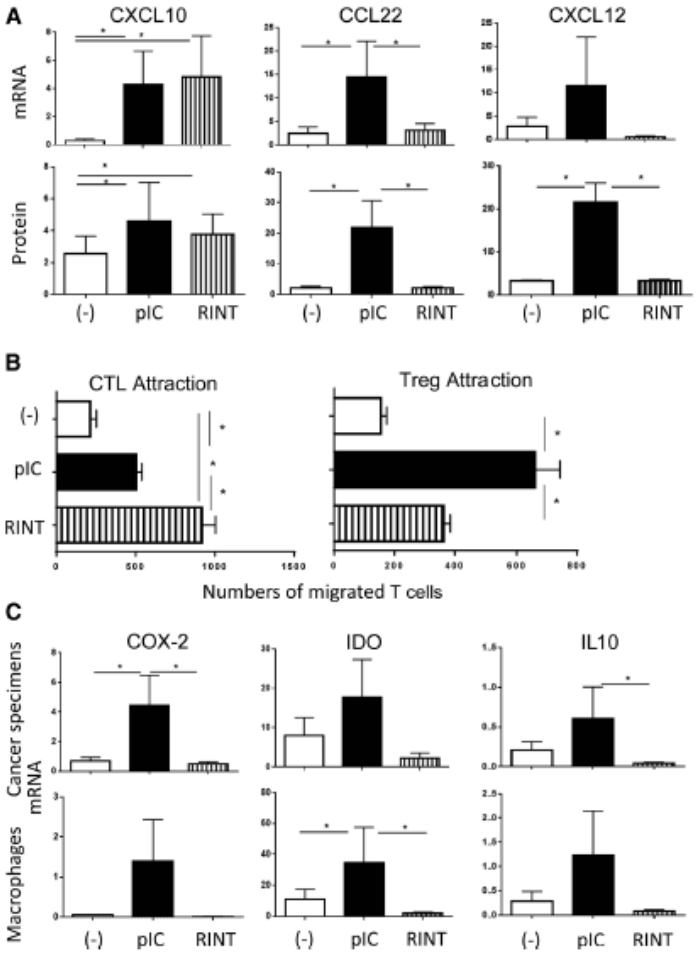

Figure 1.
Poly-I:C and rintatolimod both induce CXCL10 and promote CTL attraction to cancer tissues, but differ in their ability to promote Treg attraction and production of suppressive factors. **A,** The effects of poly-I:C (pIC) and rintatolimod (RINT) on the individual chemokines' gene expression (relative to HPRT1) and protein secretion (ng/mL) in *ex vivo*–cultured ovarian cancer explants ($n = 8$ patients). Note the comparable induction of CXCL10 by both TLR3 ligands, but selective induction of CCL22 and CXCL12 by poly-I:C. **B,** poly-I:C–treated tumor explants show selectively reduced attraction of CTLs and elevated Treg attraction, compared with the rintatolimod-treated tumors. Data from *ex vivo* migration assays using either DC-sensitized $CD8^+$ CTLs ($n = 9$; ref. 50) or $CD4^+$ T cells (Treg identified in post-migration fraction as $CD4^+/FoxP3^+$ cells; $n = 5$) in the upper chamber and differentially treated supernatants of cancer specimens ($n =$ supernatants from tumors of 9 patients for CTL migration and $n = 5$ patients for Treg migration) in the lower chamber. **C,** Induction of COX2- and COX2-dependent suppressive factors (9, 33, 40) by the two forms of dsRNA in ovarian cancer specimen ($n = 6$ patients) and in monocyte-derived macrophages ($n = 7$) is a selective feature of poly-I:C, but not rintatolimod. Results (mRNA levels, normalized for HPRT) are mean ± SEM. *, $P < 0.05$ (Wilcoxon signed-rank test).

with either one of these inhibitors, indicating that both NFκB and TNFα are involved in COX2 induction (Fig. 3B).

Interestingly, blocking either TNFα or NFκB, each suppressed COX2, CCL22, IL10 and TNFα expression (Fig. 3B), indicating that dsRNA-triggered TNFα induction involves NFκB and the existence of a poly-I:C-NFκB–TNFα-dependent feed forward loop. In support of the direct TNFα-independent ability of poly-I:C to activate NFκB within minutes of dsRNA exposure, IκB degradation occurred within 30 minutes of either poly-I:C or TNFα treatment, and could not be prevented by TNFα blockade (Fig. 3C), although such blockade clearly inhibited the poly-I:C–induced expression of COX2, as well as IL10 and CCL22, again indicating the existence of a feed forward loop (Fig. 3B). Cumulatively, these data indicate that dsRNA triggers initial NFκB activity in a TNFα-independent fashion, but that NFκB-dependent TNFα induction is critically needed for the optimal COX2 induction and COX2–dependent induction of suppressive factors and Treg and MDSC attractants (Fig. 3; Supplementary Fig. S3).

Blockers of PGE_2 synthesis and signaling selectively eliminate undesirable aspects of poly-I:C–induced tissue response

Because exogenous IFNα enhances the expression of TLR3 (41, 42), but, paradoxically, suppresses the poly-I:C–driven induction of CCL22 (12, 13, 30), we tested whether it can affect the differences between the suppressive aspects of poly-I:C and

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rintatolimod-driven inflammation in human ovarian cancer TME. As shown in Fig. 4A, the addition of IFNα, partially eliminated the induction of CCL22 by poly-I:C, but did not abrogate the differences between the two forms of dsRNA. Because IFNα suppressed the expression of EP4, the main receptor-mediating suppressive effects of PGE_2 (9, 33, 39, 40), blocking the PGE_2-induced CREB phosphorylation (Supplementary Fig. S5A–S5B), and we previously observed that COX2-driven PGE_2 production orchestrates the production of the MDSC/Treg attractant CXCL12 and multiple other suppressive factors in OvCa TME (9, 33, 40), we tested whether the overall differences in the patterns of tumor–tissue responses to poly-I:C versus rintatolimod can be abolished by the addition of a COX1/2 inhibitor, indomethacin. Indeed, the combination of IFNα and indomethacin reduced the levels of poly-I:C-induced CCL22 induction down to the baseline (and to the levels observed with rintatolimod; Fig. 4A), with similar effects being observed when combining indomethacin with poly-I:C versus rintatolimod, in the absence of IFNα (Supplementary Fig. S3D).

These observations were further supported by the functional studies, showing that PGE_2 suppression reduced the levels of Treg attraction to poly-I:C–treated tumors, abrogating the functional differences between these two variants of dsRNA (Fig. 4B).

In accordance with the mobilization of the suppressive COX2/PGE_2 system as the central differentiating element between the adjuvant activity of different forms of dsRNA, the poly-I:C–dependent induction of COX2, IL10, IDO, and CXCL12 induction in cancer specimens, macrophage cultures and fibroblast cultures, was suppressed by addition of IFNα and indomethacin, down to the baseline levels observed with rintatolimod (Fig. 4C). COX2 dependence of these effects was confirmed by the observations that both, indomethacin and celecoxib (selective COX2 inhibitor) suppressed the undesirable effects of poly-I:C signaling (Supplementary Fig. S5C).

Discussion

The ability of dsRNA and other TLR ligands to promote the induction and effector phases of cytotoxic type-1 responses, mediated by CTLs, Th1 and NK cells, is critical to their ability to act as "danger signals" alerting the immune system to infections and facilitating the clearance of the relevant pathogens, and has been used in the development of immune stimulants in cancer immunotherapy.

Unexpectedly, our current data demonstrate that whereas two forms of dsRNA, poly-I:C and a selective TLR3 ligand, rintatolimod, are both similarly effective in inducing the intratumoral production of type-1 interferon and type-1 interferon-dependent CTL attractant CXCL10, only poly-I:C promoted the expression of the MDSC/Treg attractants CXCL12 and CCL22 and amplified the production of multiple tumor-associated suppressive factors, in human isolated macrophages and fibroblasts and with *ex vivo*-treated explants of whole ovarian cancer tumor tissues. Selective ability of poly-I:C to activate the helicase pathway of dsRNA recognition led to NFκB–dependent induction of TNFα and COX2 and the resulting mobilization of Treg/MDSC-attracting chemokines and suppressive factors, while limiting the effectiveness of poly-I:C as inducer of CXCL10 and CTL attraction. Because multiple other TLRs (which signal through MyD88) are known to activate the NFκB/TNFα pathway (43), and all induce COX2 and additional suppressive factors (Supplementary Fig. S4), our current data suggest that selective targeting of these factors (rather than NFκB) can be achieved without inhibiting the IFN pathway and may be used to enhance the selectivity and antitumor potency of multiple TLR–based adjuvants.

Although only poly-I:C, but not rintatolimod, required COX2 inhibition for its optimal effectiveness, both forms of dsRNA benefited from their combination with IFNα, consistent with the ability of IFNα to enhance the expression of TLR3 (41, 42), in addition to suppressing the key receptor mediating suppressive effects of PGE2, EP4. Interestingly, not only CXCL10, but particularly CCL5, which is NFκB and type-1 IFN dependent and may contribute to CTL attraction (12, 44), benefitted from the combination of rintatolimod with exogenous IFNα (Supplementary Fig. S3C), raising the possibility that IFNα may facilitate the ability of TLR3 to induce partial, helicase-independent, NFκB activation.

The mechanism of the interplay between type-1 IFNs and PGE_2 in the regulation of the inflammatory responses to dsRNA and other TLR-Ls is a subject of our continued analyses. Previous reports demonstrated that IFN enhances the expression of TLR3 (41, 42), whereas PGE_2 blocks the induction of endogenous type-1 IFNs (45). Our preliminary data indicate that IFNα blocks the expression of the PGE_2 receptor EP4, and the PGE_2-induced phospho-CREB activation (Supplementary Fig. S5A–S5B). These observations suggest that in the absence of exogenous IFNα, PGE_2, which is present at significant levels in OvCa TME at baseline (9, 33) and further amplified by the poly-I:C-driven activation of the NFκB/COX2/PGE_2 system (the current data), limits the levels of endogenous IFNβ, making its induction suboptimal, and favoring the suppressive aspects of inflammation.

Interestingly, we observed that TNFα, which similarly effective as poly-I:C in inducing NFκB and that endogenous TNFα production, was needed for the optimal mobilization of the COX2/PGE_2 pathway in poly-I:C-activated cells. These observations and the ability of both NFκB-blockade and TNFα blockade to suppress COX2 induction indicate the scenario where the early poly-I:C–driven NFκB activation is needed for the initial induction of endogenous TNFα, leading to the establishment of a prolonged feed forward loop between poly-I:C, NFκB, and TNFα and the resulting induction of COX2, representing the negative component of poly-I:C signaling (Supplementary Fig. S6). Such feed forward loop is consistent with lack of complete abrogation of the poly-I:C–induced IκB degradation by TNFαRI, since TNFαRI blocks only the TNFα-related part of the loop.

Previous reports have shown that while poly-I:C activates both TLR3 and cytoplasmic helicases (MDA5 and RIG-1), leading to activation of MAVS and NFκB (46, 47), rintatolimod selectively activates only the TLR3 pathway (31, 32). Our current data show that the optimal induction of type-1 IFNs, type-1 IFN-dependent CXCL10 and CTL attraction, requires neither the cytoplasmic helicase pathway nor the TLR3- induced RIP-1–mediated NFκB pathway, demonstrating a separation of the type-1–inducing versus suppressive pathways of signaling of dsRNA.

Although NFκB activation can amplify type-1 inflammation (26, 27, 48), the dominant aspects of its activation in TMEs are enhanced cancer cell proliferation, resistance to spontaneous and treatment-induced apoptosis, as well as tumor-associated neo-angiogenesis and metastatic spread (26, 27). The possibility to induce type-1 inflammation in cancer tissues without concomitant activation of NFκB and the resulting induction of COX2, one of the orchestrators of tumor-promoting inflammatory response,

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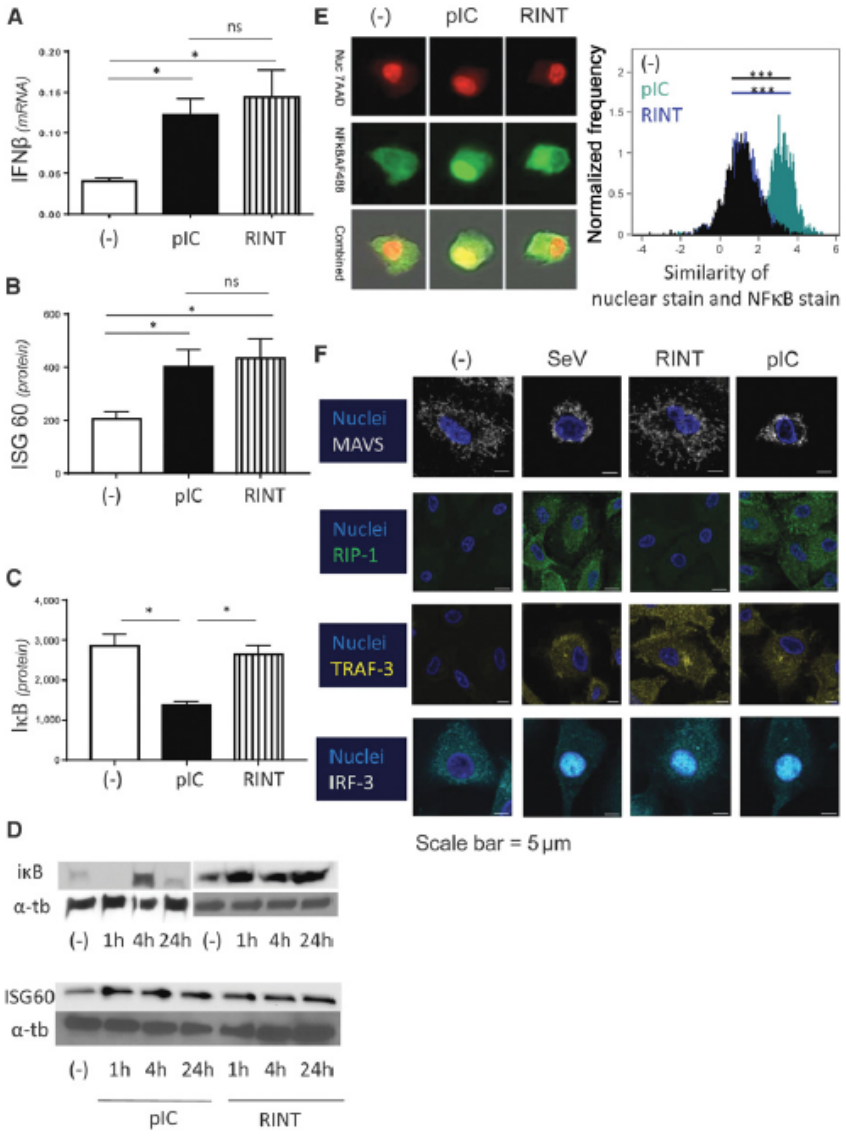

Figure 2.

Poly-IC and rintatolimod both activate the IFN pathway of dsRNA recognition, but only poly-IC activates the helicase/MAVS- and RIPK-1-dependent pathways of dsRNA recognition and activates NFκB. **A** and **B**, Poly-IC (pIC) and rintatolimod (RINT) promote similar induction of IFNβ and ISG60 in human macrophages. **A**, IFNβ mRNA levels ($n = 4$). **B**, Similar induction of ISG60 protein ($n = 4$). Results are presented as the mean values ± SEM of four Western blots measured by densitometry. **C**, IκB degradation is only visible by poly-IC treatment ($n = 3$). (*Continued on the following page.*)

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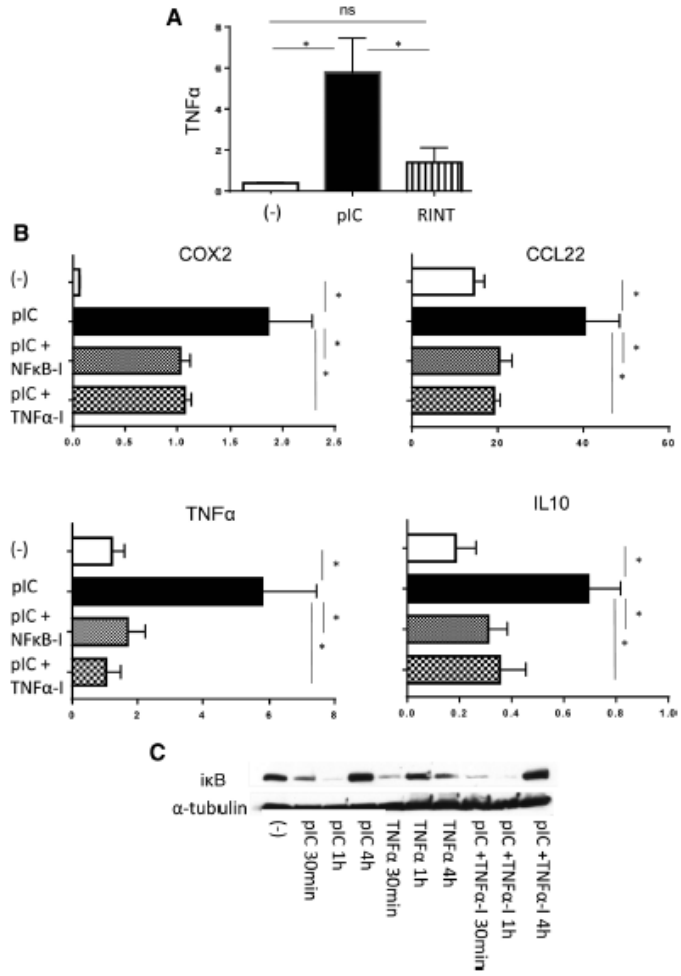

Figure 3.

Effective COX2 induction depends on the positive feedback between poly-IC–induced NFκB activation and endogenous TNFα. **A**, Effective TNFα induction in human macrophages exposed to poly-IC (pIC) but not rintatolimod (RINT; $n = 6$). **B**, Poly-IC–induced expression of TNFα and COX2 (as well as COX2-dependent suppressive factors CCL22 and IL10) is suppressed by NFκB inhibitor BAY11-7082 (NFκB-I) or TNFα receptor inhibitor TNFαRI (TNFα-I) in poly-IC–stimulated human macrophages ($n = 6$; mRNA levels, normalized for HPRT). **C**, Poly-IC triggers IκB degradation (30 minutes and 1 hours) in macrophages, whereas the TNFα-induced degradation is faster and is compensated already at 1 hour. Note that the IκB degradation triggered by Poly-IC cannot be prevented by TNRαRI. Results are mean ± SEM. *, $P < 0.05$ (Wilcoxon signed-rank test).

(*Continued.*) IκB degradation measured after 1 hour treatment. Results are presented as the mean values ± SEM of three Western blots measured by densitometry. **D**, Representative Western blot results of ISG60 and IκB analysis; α-tubulin (α-tb) served as a positive control. **E**, Unique induction of NFκB nuclear translocation in response to poly-IC, but not rintatolimod (Amnis image flow). Human macrophages were treated with poly-IC or rintatolimod, as indicated. Nuclear translocation of NFκB (and cytoplasmic presence of NFκB) was evaluated after 30–40 minutes. Colocalization of nuclear stain and NFκB stain was analyzed using the similarity score. A high similarity score between nuclear stain and NFκB stain (high colocalization of both stains) is seen in the poly-IC–treated group, whereas the untreated and rintatolimod-treated cells show low similarity scores (the nuclear stain and the NFκB stain do not overlap), demonstrating lack of nuclear NFκB translocation. **F**, Different patterns of activation of helicase- and TLR3-related signaling pathways by different forms of dsRNA. Human cultured macrophages were treated with rintatolimod or poly-IC or were infected with SeV (80 HAU/ml) for 20 hours. Note the selective activation of MAVS (helicase-pathway) and RIP-I (TLR3 adapter responsible for TLR3-dependent activation of NFκB) after treatment with poly-IC and SeV but not rintatolimod. In contrast, TRAF3, TLR3-dependent activator of type-I IFN pathway, is similarly activated by rintatolimod, poly-IC, and SeV, but not in untreated cells. Similarly, nuclear translocation of IRF3, the type-I IFN pathway–induced transcription factor, is observed after activation with poly-IC, rintatolimod or SeV but not in the control cells ($n = 3$). Results are shown as mean ± SEM. *, $P < 0.05$ (Wilcoxon signed-rank test).

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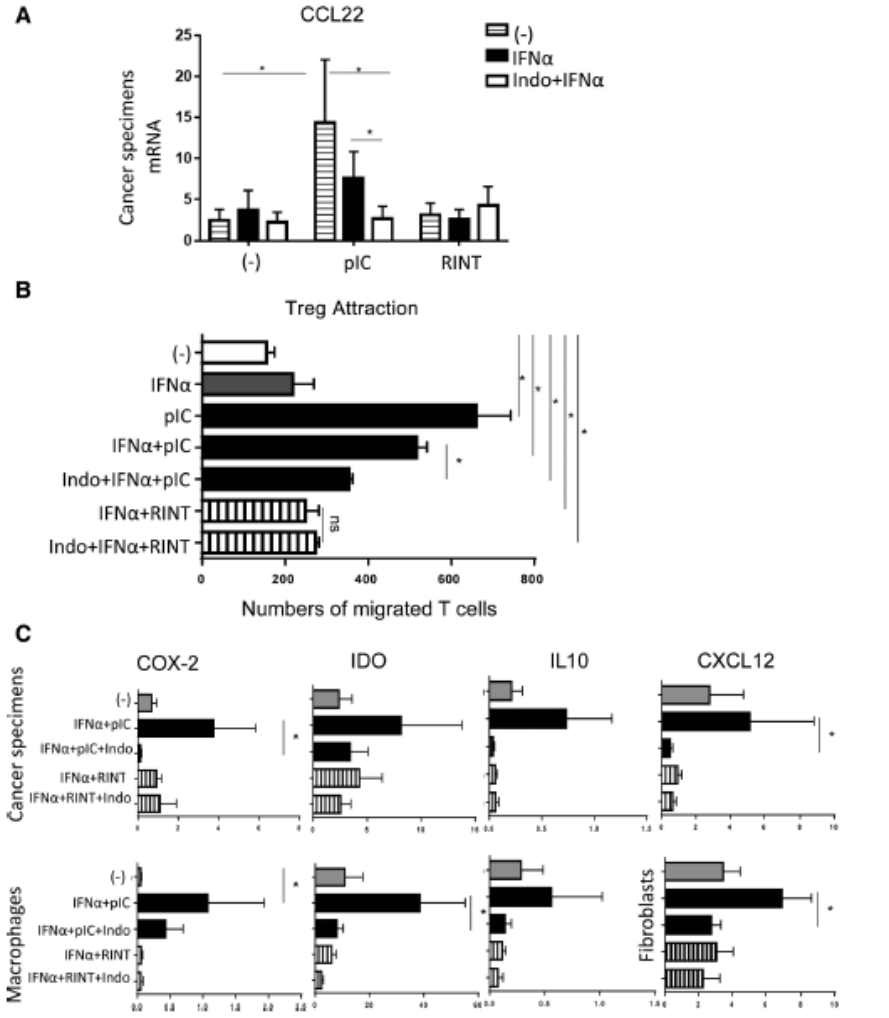

Figure 4.

Combination of IFNα and indomethacin counteracts the induction of suppressive factors by poly-IC. **A,** Undesirable CCL22 induction by poly-IC (pIC) is partially suppressed by IFNα (which suppresses EP4; see Supplementary Fig. S5A) and abrogated by the combination of IFNα and indomethacin, down to the baseline levels observed with rintatolimod (RINT). Data from tumor samples from $n = 8$ patients. **B,** IFNα alone and the combination of IFNα and indomethacin suppress the ability of poly-IC to promote Treg migration to the treated tumors ($n = 5$). **C,** Combination of IFNα and indomethacin (Indo) abrogates the induction of COX2- and COX2-dependent suppressive factors IDO, IL10 in poly-IC–treated cancer specimen ($n = 6$), and macrophage cultures ($n = 7$). Because CXCL12 is not produced by human macrophages, human fibroblast cultures were used to evaluate CXCL12 induction ($n = 5$).
Results (mRNA levels normalized for HPRT) are mean ± SEM. *, $P < 0.05$ (Wilcoxon signed-rank test).

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may facilitate the development of new treatments avoiding multiple levels of the negative aspects of tumor-associated inflammation.

Our follow-up work evaluates the impact of different forms of dsRNA (and other TLR-Ls, which all, with the exception of rintatolimod, promoted substantial induction of COX2, IL10 and CCL22 in human cells; see Supplementary Fig. S4) upon the accumulation of immune cells in mouse tumor tissues and antitumor effectiveness of immune checkpoint blockade, cancer vaccines and adoptive T-cell therapies. Because COX2 has been recently shown to direct mouse TMEs toward preferential production of tumor-promoting chemokines and cytokines, limiting the effectiveness of immune therapies (49), we expect that COX2 blockade will be uniformly advantageous to the antitumor impact of TLR ligands in mouse models.

However, murine cells do not distinguish between different forms of dsRNA with regard to NFκB activation and TNFα induction (28, 29) and produce TNFα also in response to rintatolimod injection, resulting in its enhanced toxicity in rodents compared with primates (29). Because the differential induction of COX2 and CCL22 in the human cells responding to poly-IC versus rintatolimod results from the differential NFκB activation and TNFα induction (current results), lack of such differences in rodent cells may complicate the use of mouse models to evaluate the TLR3-selective and nonselective dsRNAs with regard to Treg attraction. Indeed, our preliminary mouse data indicate that mouse macrophages behave similarly to human cells with regard to poly-IC– or rintatolimod-induced CXCL10 production, but, in sharp in contrast to human macrophages and whole tumor explants from patients with cancer, they elevate CCL22 production both in response to poly-I:C and to rintatolimod, in a COX2–independent manner (Supplementary Fig. S7A–S7C).

In summary, our current data demonstrate the molecular separation of the immunostimulatory and suppressive pathways of dsRNA recognition in human cancer tissues and the feasibility of their separate modulation to improve the selectivity of inflammation induced by dsRNA adjuvants. We show that the TLR3-driven activation of type-1 IFN pathway and intratumoral induction of CTL attractants by dsRNA, can proceed in the absence of the helicase/MAVS or TLR3/RIPK1–driven activation of the NFκB/TNFα pathway and the resulting induction of COX2– and COX2–dependent suppressive factors and Treg/MDSC-attracting chemokines. Our data help to understand the interplay between the immunostimulatory and suppressive functions of immune adjuvants and facilitate the development of improved therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

This work involves commercially available reagents and materials, with the exception of rintatolimod, which was obtained under an MTA from Hemispherx, Bio and the tumor samples from patients with ovarian cancer.

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