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**IL-15 by continuous i.v. infusion to adult patients with solid tumors in a Phase I trial induced dramatic NK cell subset expansion**

Kevin C. Conlon1, E. Lake Potter2, Stefania Pittaluga3, Chyi-Chia Richard Lee3, Milos D. Miljkovic1, Thomas A. Fleisher4, Sigrid Dubois1, Bonita R. Bryant1, Michael N. Petrus1, Liyanage P. Perera1, Jennifer Hsu1, William D. Figg Sr5, Cody J. Peer5, Joanna H. Shih6, Jason L. Yovandich7, Stephen P. Creekmore7, Mario Roederer2 and Thomas A. Waldmann1, 8

**Supplementary Data**

**Supplementary Methods and Materials**

**Patients and Methods**

The primary objective of this trial was to define the safety, toxicity profile and maximum tolerated dose (MTD) for this 10-day (240 hour) continuous intravenous (CIV) treatment using a standard 3 + 3 phase 1 dose-escalation design. Secondary objectives were determination of rhIL-15 pharmacokinetics (PK) for this treatment and characterization of changes in lymphocyte populations, inflammatory cytokine production and assessment of antitumor response.

These patients were age ≥ 18 years, had histologically confirmed metastatic solid tumors, ECOG performance status 0 or 1, DLCO/VA and FEV1 > 50% predicted, absolute neutrophil count >1,500/mcL, platelets >100,000/mcL, total bilirubin within normal institutional limits, AST /ALT ≤ 2.5 upper limit of normal (ULN), serum creatinine ≤ 1.5 x ULN, absence of active CNS metastases, no history of clinically significant autoimmune disease or hematopoietic malignancy, no history of severe asthma, did not require use of systemic corticosteroid treatment or inhaled steroids, no evidence of clinically active infection, no history of or serology positive for HIV or hepatitis B or C or HTLV-1, no clinically significant congestive (NYHA class II or greater) heart disease. Pregnant female patients were excluded, patients must be more than 4 weeks from their most recent treatment, 6 weeks for nitrosourea/mitomycin, 8 weeks from anti-CTLA-4 or anti-PD1), more than 2 weeks from radiation therapy, have recovered from previous treatment, not be receiving any other investigational treatment and be able to give informed consent.

Dose escalation was to proceed unless dose-limiting toxicity (DLT) occurred in ≥ 2 out of 3 or 6 patients treated in one of the dose levels during the first treatment cycle. In the event that ≥ 2 patients in any dosing cohort experienced DLTs dose escalation would be halted and the next prior level considered the MTD. The NCI Common Toxicity Criteria version 4 (CTCv4) was used to assess adverse events (AEs). This was a single institution, open-label, nonrandomized 3 + 3 design phase I dose-escalation study. Groups of 3 to 6 subjects received CIV rhIL-15 at doses of 0.1, 0.25, 0.5, 1, 2 and 4 mcg/kg/day for 10 days provided that the DLT has not been observed. DTL was defined as follows: Any grade 3 or 4 toxicity deemed possibly, probably or definitely related to study drug by the PI during the first cycle of treatment with the following exceptions:t

**Hematologic Exceptions:**

* Asymptomatic grade 3 or 4 lymphocytopenia unless there are clinical signs of significant infection (persistent fevers, labile blood pressure, localized complaints or finding on physical exam, hypoxia or organ dysfunction).
* Grade 3 granulocytopenia unless there are clinical signs indicating a significant infection as listed above.
* Grade 3 leukocytosis (WBC >100,000/mm3) in the absence of leukostasis.

**Nonhematologic exceptions:**

* Transient (< 24 hours) grade 3 hypoalbuminemia, hypokalemia, hypomagnesemia, hypophosphatemia that respond to medical intervention.
* Nonsustained (< 7 days) grade 3 liver function tests (ALT, AST, alkaline phosphatase, total or direct bilirubin) abnormalities in the absence of clinical signs of hepatic dysfunction (lethargy, confusion, anorexia, pruritis, tremor).

Dose escalation proceeded in cohorts of 3 to 6 patients. Patients were not begun on treatment at the next higher dose level unless all patients treated at the previous level reached day 21 of the protocol, recovered from any clinical or laboratory toxicities and would be able to initiate another cycle of treatment unless restaging has demonstrated progression of their disease.

Patients receiving the 10-day treatment schedule without evidence of ongoing response after any 2 consecutive cycles of treatment discontinued rhIL-15. Patients manifesting an ongoing response defined as >15% decrease in sum of marker lesions and/or improvement or disappearance of some non-measurable lesions and/or >10% decrease in tumor markers received additional cycles. Cycles 1 and 2 were 42 days in length but all subsequent cycles were 28 days in length. Toxicities of only the first cycle were considered in selecting the MTD/RP2D.

Patients could continue rhIL-15 if there was ongoing evidence of clinical activity or for 2 additional cycles beyond when a radiographic complete response (CR) was observed. Patients with stable disease (SD) after 2 cycles of treatment were followed for response until disease progression was documented or they chose to start another treatment. Patients were given nonsteroidal anti-inflammatory drugs (NSAIDs) or other antipyretics, analgesics, antiemetics, anti-diarrheals and meperidine for fevers, myalgias, nausea/vomiting, diarrhea and rigors initially on a PRN basis.

**Pharmacokinetic Procedures:**

The rhIL-15 concentrations in serum were assessed using a human IL-15-specific ELISA kit from R&D Systems (Minneapolis, MN) according to manufacturer’s directions.

Thorough PK analysis of serum IL-15 levels was performed during cycle 1 treatment on serum samples obtained just prior to the first dose, then at 10 minutes, 1, 2, 4, 8, 12, approximately 24 and 48 hours after injection, once daily days 7 through 10. Day 11 at the end of the infusion 10 minutes, 30 minutes, 1, 2, 4, and 24 hours after completion of treatment with an assay lower limits of quantitation (LLOQ) of 30 pg/mL.

The maximum serum concentration (Cmax), average steady-state concentration during infusion (Css), and area under the curve to the last time point where IL-15 could be measured (AUClast) were determined. The inferred area under the curve value was calculated by extrapolation by dividing the last measurable drug concentration by the rate constant at the terminal phase. All statistical analyses were carried out using NCSS 2044 software (<http://www.ncss.com>). P < .05 was considered to be statistically significant in paired t tests.

**Hematology, Clinical Chemistry and Fluorescence-Activated Cell-Sorting Analyses**

Hematologic tests performed after infusion included WBC, RBC, hemoglobin, hematocrit, mean corpuscular volume, platelet counts, and percent and absolute numbers of peripheral blood lymphocytes, monocytes, neutrophils, and eosinophils. Bone marrow aspirates were obtained on day 8. The following clinical chemistry tests were also performed: serum Na, K, Cl, glucose, phosphorus, alkaline phosphatase, ALT, AST, total and direct bilirubin, BUN, creatinine, total protein, albumin, troponin-T, cholesterol, triglycerides, magnesium, amylase, lactate dehydrogenase, and uric acid. The following coagulation tests were performed: prothrombin time, partial thromboplastin time, and fibrinogen. Assays for soluble serum IL-2 receptor alpha and IL-18 concentrations were assessed on these samples using enzyme-linked immunosorbent assay (ELISA) procedures. Plasma IL-6, IL-8, IL-10, IL-12 interferon gamma, IL-1β, and tumor necrosis factor alpha concentrations were performed using a mesoscale method (1).

The serum concentrations of PD-1, PD-L1 and PD-L2 of patients receiving 2 mcg/kg/day IL-15 by CIV were assayed using Invitrogen (Carlsbad, California) PD-1 ELISA Kit Cat No. BMS2214 and R&D Systems PD-L1 ELISA Kit Cat. No. DB7H10 respectively. The PD-L2 serum concentrations were performed using Affymetrix eBioscience Human PD-L2 ELISA Kit Cat. No. BMS2215. The assays were performed according to manufacturer’s instructions.

**Flow Cytometry**

Polychromatic flow cytometry analysis was performed, as described previously, on heparinized blood obtained pre-infusion and at various times after the first infusion, with analysis of multiple lymphocyte populations including naïve, central memory, and effector memory CD4 and CD8 T- cells; B cells, NK cells, T-regulatory cells, gamma delta T cells, and monocytes (Supplementary Figure 7). Proliferating cells were detected by the analysis of intracellular Ki-67 on all cellular subsets. Conjugated antibodies were purchased from BD Biosciences (San Jose, CA), BioLegend (San Diego, CA), eBioscience (San Diego, CA), Beckman Coulter (Brea, CA), or ThermoFisher Scientific (Waltham, MA) (Supplementary Table 3). Unconjugated antibodies were purchased from BD Pharmingen and conjugated with either Alexa Fluor 594 at 51 μg dye/mg antibody (for anti-perforin) or an in-house tandem of Cy5PE at 400 uL dye/mg antibody for (anti-HLA-DR).

Frozen peripheral blood mononuclear cells were thawed in RPMI 1640 media containing 10% heat inactivated fetal bovine serum and 1% penicillin-streptomycin-glutamate using Thawsome adaptors, extensively washed, and stained immediately with LIVE/DEAD viability dye (Invitrogen) in PBS at room temperature for 20 minutes. Cells were washed and stained with a panel of monoclonal antibodies at room temperature for 20 minutes. For the analysis of intracellular molecules (Ki-67, granzyme B, perforin), cells were fixed and permeabilized with eBioscience Foxp3/Transcription Factor buffer according to the manufacturer’s instructions and subsequently incubated with a panel of monoclonal antibodies at room temperature for 30 minutes, washed and stored PBS. Samples were immediately analyzed after staining on a BD FACSymphony A5.

Flow cytometric data were compensated and analyzed with FlowJo software (version 9.9.6; <http://www.flowjo.com>). Data were analyzed and presented with JMP software (version 13.0) and SPICE software (version 6.0).

**ELISA Method for Detection of Host Production of Antibody Directed Toward Infused rhIL-15**

Serum samples were analyzed for the *in vivo* production of antibodies to rhIL-15 at days 14, 21, 28 and 42 after initial IL-15 infusion using an ELISA method developed in our laboratory (1). This two-arm capture ELISA procedure achieved a limit of quantitation of 156 ng/mL in undiluted serum and 470 ng/mL when test sample was diluted at a ratio of 1:3. The study material, rhIL-15, was used as the antigenic ligand. rhIL-15 100μL at a concentration of

100 ng/mL was added to wells of a 96-well microliter plate; the plate was sealed and incubated at 37oC for 3 hours. The plates were then washed three times with PBS using a plate washer. To eliminate any remaining active plate sites, PBS 300 μL/3% bovine serum albumin blocking buffer was added to all wells, and the plate was incubated at 37oC for 1 hour, followed by washing as described. A commercial affinity purified goat antihuman IL-15 (R&D Catalog No. AF315) was used to form a standard curve for antibody quantitation by serially diluting the antibody from 100 to 9.8 ng/mL. Test serum samples were diluted at a ratio of 1:3 and added concomitantly to the appropriate IL-15-coated wells, along with positive and negative controls. After overnight incubation at 4oC, the plates were washed three times with PBS, followed immediately by addition of 100 μL of biotinylated rhIL-15 at a final concentration determined by prior optimal titration. The plates were sealed and incubated for 2 hours at 37oC and then washed three times with PBST using a plate washer. 100 μL of streptavidin-alkaline phosphatase diluted in PBS plus 1% bovine serum albumin, according to manufacturer’s instructions, were added to each well and the plate was incubated for 2 hours at 37oC followed by three washes with PBS. Substrate p-nitrophenyl phosphate, freshly dissolved in diethanolamine buffer, was added to all wells for color development. The plate was incubated at 37oC for 1 hour and the resultant color was detected at 405-nm absorbance using the SpectraMax Plus 384.

1. Conlon KC, Lugli E, Welles HC, Rosenberg SA, Fojo AT, Morris JC, et al: Redistribution, hyperproliferation, activation of natural killer cells and CD8 T cells, and cytokine production during first-in-human clinical trial of recombinant human interleukin-15 in patients with cancer. J Clin Oncol 2015; **33**:74-8.
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