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Pilot trial of the hu14.18-IL2 immunocytokine in patients with completely resectable recurrent stage III or stage IV melanoma

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Author contributions

Mark R. Albertini: study conception and design, protocol chairman, laboratory and clinical data analysis and interpretation, drafted manuscript; Richard K. Yang: histology data analysis and interpretation; Erik A. Ranheim: histology data analysis and interpretation; Jacquelyn A. Hank: laboratory co-chair, immunologic monitoring data collection, analysis and interpretation; Cindy L. Zuleger: immunologic monitoring data analysis, manuscript table and figure preparation; Sharon Weber: surgical team; Heather Neuman: surgical team; Greg Hartig: surgical team; Tracey Weigel: surgical team; David Mahvi: surgical team; Mary Beth Henry: patient care; Renae Quale: clinical data analysis; Thomas McFarland: patient care; Jacek Gan: processing of clinical samples, immunologic monitoring data collection, analysis and interpretation; Lakeesha Carmichael: statistical data analysis; KyungMann Kim: protocol statistician, statistical design of the study, statistical data analysis; Hans Loibner: laboratory and clinical data interpretation; Stephen D. Gillies: creator of hu14.18-IL2, laboratory and clinical data interpretation; Paul M. Sondel: study conception and design, principal investigator for NIH grant supporting the study, laboratory and clinical data analysis and interpretation. All authors reviewed and had opportunity to edit the manuscript, and all authors approved the manuscript.

Compliance with Ethical Standards

Conflict of Interest

The authors have the following financial or other conflicts of interests to disclose related to this publication: Dr. Hans Loibner is CEO for Apeiron Biologics AG, and Apeiron Biologics AG has ownership of the hu14.18-IL2 immunocytokine used in this study. The remaining authors reported no financial or other conflicts of interest to disclose related to this publication.

Ethical approval and ethical standards

The UW Human Subjects Committee and the FDA approved the study ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study?term=NCT00590824&rank=1) identifier: NCT00590824; IND 12220). All procedures performed in studies involving human participants were in accordance with ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Written informed consent was obtained from all individual participants included in the study and all individual participants registered with the Biostatistics Registration Desk prior to treatment.

Note on Previous Publication

Preliminary findings from this study were previously presented as a poster presentation at the 2014 Annual Meeting of the American Society of Clinical Oncology (ASCO 2014), Chicago, IL, USA, May 30-June 3, 2014 [1]. Final statistical analysis of data from this study were previously presented as a poster presentation at the 31st Annual Meeting of the Society for Immunotherapy of Cancer (SITC 2016), National Harbor, MD, USA, November 9–13, 2016 [2].

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Abstract

Phase I testing of the hu14.18-IL2 immunocytokine (IC) in melanoma patients showed immune activation, reversible toxicities, and a maximal tolerated dose of 7.5 mg/m²/day. Preclinical data in IC-treated tumor bearing mice with low tumor burden documented striking antitumor effects. Patients with completely resectable recurrent stage III or stage IV melanoma were scheduled to receive 3 courses of IC at 6 mg/m²/d i.v. on days 1, 2 and 3 of each 28-day course. Patients were randomized to complete surgical resection either following neoadjuvant (Group A) or prior to adjuvant (Group B) IC course 1. Primary objectives were to: 1) evaluate histological evidence of anti-tumor activity and 2) evaluate recurrence-free survival (RFS) and OS. Twenty melanoma patients were randomized to Group A (11 patients) or B (9 patients). Two Group B patients did not receive IC due to persistent disease following surgery. Six of 18 IC-treated patients remained free of recurrence, with a median RFS of 5.7 months (95% confidence interval (CI): 1.8-not reached). The 24-month RFS rate was 38.9% (95% CI: 17.5–60.0%). The median followup of surviving patients was 50.0 months (range: 31.8–70.4). The 24-month OS rate was 65.0% (95% CI: 40.3–81.5%). Toxicities were similar to those previously reported. Exploratory tumorinfiltrating lymphocyte (TIL) analyses suggest prognostic value of TILs from Group A patients. Prolonged tumor-free survival was seen in some melanoma patients at high risk for recurrence who were treated with IC.

Précis:

This study translates preclinical murine data suggesting enhanced antitumor activity in the setting of minimal residual disease of hu14.18-IL2, a monoclonal antibody reactive with GD2 disialoganglioside linked to interleukin-2, to melanoma patients.

Keywords

Immunotherapy; Immunocytokine; Melanoma; Anti-ganglioside antibody; Interleukin-2

Introduction

Immunotherapeutic strategies for melanoma have evolved over the past twenty-five years from systemic activation of the immune system via administration of high-dose IL-2 to molecularly targeted vaccines, adoptive immunotherapy, antitumor monoclonal antibodies and several antibodies that help activate the immune system via “checkpoint blockade” [3–6]. One of the potential benefits of immunotherapy is the ability to destroy not only grossly visible disease but also micrometastases, which have been a significant barrier to achieving long-term benefit from locally-driven modalities such as surgery and radiation, as well as a challenge for cytotoxic chemotherapy. Often these sites of micrometastatic disease are able to withstand initial therapeutic efforts only to recur at a later date with significant morbidity and mortality. An ideal therapy would be one that is well-tolerated, tumor-specific, and able to manage both macro- and microscopic sites of disease.

The hu14.18-IL2 IC is a humanized mAb that is covalently linked to two molecules of IL-2 at the Fc region [7, 8]. The hu14.18 mAb recognizes GD2, a disialoganglioside that is a cell membrane molecule found in tumors of neuroectodermal origin (including melanoma, neuroblastoma, and certain sarcomas) while relatively rare in normal tissues (mostly cerebellum and peripheral nerves) [9–11]. This IC has been studied extensively *in vitro* and in preclinical models, including both melanoma and neuroblastoma [12–16], and has undergone both Phase I and II testing in adults with melanoma and children with neuroblastoma [17–20]. In mice, the antitumor effect of hu14.18-IL2 against melanoma and neuroblastoma can involve T cells and NK cells [12, 16, 21]. Mice with smaller tumors or with minimal residual disease show the best responses to IC [16]. Hu14.18-IL2 given i.v. to adults with melanoma or children with neuroblastoma was generally well-tolerated and was able to produce demonstrable immune activation *ex vivo* and *in vivo* [1820]. However, i.v. hu14.18-IL2 had minimal antitumor effects for patients with measurable disease in Phase II trials: 0 responses of 15 patients with neuroblastoma; and 1 transient partial response (PR) and 0 complete response (CR) of 14 patients with melanoma in our Phase II trials [17, 20]. In contrast, and consistent with our preclinical data, patients with evaluable but non-bulky, non-measurable disease (neuroblastoma patients with disease evaluable only by ¹²³MIBG scintigraphy or by bone marrow histology) showed reproducible antitumor activity: 5 of 24 evaluable patients with CR [20]. We now evaluate hu14.18-IL2 in advanced melanoma patients who achieved a complete response through surgery.

Materials and Methods

Patients:

Twenty patients with advanced melanoma participated in this trial [University of Wisconsin (UW) Carbone Cancer Center Protocol CO05601]. Initially this protocol was designed to evaluate the effect of hu14.18-IL2 and of cilengitide, alone and in combination, for patients with stage III or stage IV melanoma thought to have completely resectable disease. The protocol opened in the spring of 2008, with patients randomized to one of four groups. Based on toxicities seen in one patient who received both cilengitide alone (course 1) and hu14.18-IL2 in combination with cilengitide (course 2), enrollment was suspended after the initial 4 patients, including 3 patients treated with cilengitide. The protocol was then

amended to drop the treatment groups with cilengitide and proceed with enrollment and randomization using the treatment groups containing only hu14.18-IL2. The 3 patients who also received cilengitide are excluded from the study analyses (toxicity, outcome and lab data) in this report.

All 20 patients had recurrent stage III (i.e., recurrent regional metastasis), or stage IV (i.e., any distant metastasis) melanoma for which surgical resection would be clinically recommended, with biopsy proven (current or previous) stage III or stage IV disease (staging was according to the 7th edition of the American Joint Committee on Cancer [AJCC] Cancer Staging Manual). Patients needed to have disease that had not yet been completely excised and involved three or fewer sites. “Clustered” nodal, subcutaneous, or cutaneous lesions that could be removed in a single surgical excision were scored as one site. Patients could have: 1) stage III melanoma with recurrence after prior surgery, with or without subsequent adjuvant therapy, or 2) stage IV melanoma (cutaneous, ocular, mucosal, or unknown primary) without any prior systemic therapy for stage IV disease. Patients needed to have an Eastern Cooperative Oncology Group (ECOG) Performance Status (PS) of 0–1 and adequate end organ function. Patients with active or inactive brain metastases were ineligible.

Hu14.18-IL2

The hu14.18-IL2 (APN301) IC was supplied collaboratively by the National Cancer Institute Biological Resources Branch (NCI-BRB, Frederick, MD) via a cooperative research and development agreement, currently held by Apeiron Biologics (Vienna, Austria). Preclinical evaluation has shown that 1 mg of hu14.18-IL2 contains approximately 3×10^6 units of IL-2 (based on a proliferative assay with IL-2 responsive Tf-1 beta cells) and approximately 0.8 mg of the hu14.18 mAb [13].

Study Design

Eligible patients were enrolled and randomized into two Groups. The design was for patients in Group A to receive surgical resection after the 1st course of neoadjuvant hu14.18-IL2 and then to receive two subsequent courses of adjuvant hu14.18-IL2 following their surgical resection. Patients in Group B were to receive their surgical resection prior to the 1st of 3 courses of adjuvant hu14.18-IL2. Patients were randomized to Group A or B in a 1:1 ratio using permuted blocks of size 4, stratified by type of surgical resection (extensive vs. non-extensive). The treatment schema for each study Group is shown in Supplementary Fig. S1. Patients were scheduled to receive hu14.18-IL2 on days 1, 2, and 3 of each course of therapy as a 4-hour continuous i.v. infusion at a daily dose of 6 mg/m²/day. Patients were scheduled to receive a total of three 28-day courses of treatment at the same dose level provided that there was no dose limiting toxicity (DLT) or significant non-DLT toxicity. The protocol allowed for two dose modifications for DLT or significant non-DLT toxicity: the first a 50% dose reduction to 3 mg/m²/day, and the second an additional 50% dose reduction to 1.5 mg/m²/day. Missed doses were not made up, and all dose reductions were permanent.

Surgical Considerations

All patients were assessed by a surgical oncologist and needed to have completely resectable disease in 3 or fewer sites. In the event that the pathologic margins demonstrated microscopic residual disease, patients could remain on protocol provided that the disease evaluation following course 2 demonstrated no radiographically or clinically evident disease. Patients with stage IV disease could have visceral disease, cutaneous disease, and/or subcutaneous disease. Surgical resection was considered “extensive” if it required thoracotomy, laparotomy or excision of deep soft tissue metastases in three anatomic locations. Surgical resection was considered “non-extensive” if it involved only cutaneous/subcutaneous excisions and/or excision of deep soft tissue disease in only one or two locations.

Toxicity Grading and Dose Modifications

DLT was defined as Grade 3 or 4 toxicity, using the NCI Common Terminology Criteria Adverse Events (CTCAE), version 3.0, except for selected, previously reported toxicities representing known side effects of IL-2 and hu14.18-IL2 therapy that are generally transient and well controlled clinically [17, 18]. Those previously reported toxicities, if seen, were graded and recorded but not used as criteria for determining DLTs as they were expected, based on observations of hu14.18-IL2 given to 33 adults with melanoma, administered by the same schedule at similar doses to that being tested in this trial [18]. All Grade 4 toxicities (except for transient lymphopenia) were considered DLT and required stopping treatment with hu14.18-IL2. For any occurrence of DLT, treatment with protocol therapy was stopped. Treatment could not be resumed with dose modification until after the toxicity resolved to meet original eligibility criteria.

Immunologic Monitoring

Peripheral blood samples were obtained from all patients to measure C-reactive protein (CRP) levels and to determine lymphocyte counts. The CRP assay and lymphocyte counts were performed by the UW Hospitals and Clinics (UWHC) clinical lab. Serum ELISA assays determined the peak hu14.18-IL2 serum levels achieved with each i.v. infusion during the 3 courses of hu14.18-IL2, as previously described [1, 18]. Serum obtained following therapy was also assayed for the generation of an antibody response to the hu14.18-IL2 immunocytokine, as previously described [19]. Serum soluble IL-2 receptor (sIL-2R) alpha levels were determined using the Human CD25/IL-2 R alpha DuoSet ELISA Kit (R&D Systems, Minneapolis, MN, catalog number DY223), following manufacturer’s instructions.

Processing and Histological Analyses of Tumor Biopsies

Each patient’s surgical resection specimen (obtained following neoadjuvant IC course 1 for Group A and before adjuvant IC course 1 for Group B) was processed by UWHC Surgical Pathology. Representative sections of each patient’s tumor were coded and given blindly to our board-certified anatomic pathologist (author Dr. Erik A. Ranheim) for diagnosis confirmation and TIL assessment on H&E slides. Quantitation of TILs percentage was performed using the following equation: Quantitation of TILs percentage (%) = (Cross-sectional area of TILs / Area of tumor) × 100. TILs were tightly defined as truly

“infiltrating” by applying the following parameters: (1) Lymphocytes need to be imbedded between tumor cells, (2) Exclude lymphocytes at the tumor invasive margin, (3) Exclude lymphocytes touching vasculature, (4) Exclude lymphocytes touching other lymphocytes, (5) Exclude plasma cells, macrophages, or granulocytes. Immunohistochemical staining for CD3, CD4, CD8, CD20, CD56, and CD68 was performed using a Ventana system (Roche, Tucson, Arizona) on paraffin embedded sections to confirm the identity of the TILs. Assessment of GD2 expression was performed on frozen sections of the tumor biopsies using the mouse anti-GD2 mAb clone 14.G2a (Santa Cruz Biotechnology, Dallas, TX). Following overnight incubation in 1% rabbit serum and washing, a biotinylated rabbit anti-mouse secondary Ab was applied for 90 minutes, followed by an avidin-biotin complex (ABC, Vector Laboratories, Burlingame, CA). Slides were developed with 3,3diaminobenzidine (DAB, Vector Laboratories) for 4.5 minutes, counterstained with Mayer’s hematoxylin (Sigma, St. Louis, MO) for 90 seconds, washed in running tap water for 5 minutes, and mounted.

Statistical Analysis

The primary objectives were: 1) Evaluate the histological evidence of antitumor activity (apoptosis/necrosis) of hu14.18-IL2 in subjects with advanced melanoma who have achieved a CR through surgical resection of all known metastatic disease approximately 1 week following the first course of neoadjuvant treatment with hu14.18-IL2, and 2) Evaluate RFS and OS following treatment with neoadjuvant and/or adjuvant hu14.18-IL2 in subjects with biopsyproven recurrent stage III or stage IV melanoma who have achieved a CR through surgical resection of all known metastatic disease. RFS was defined as the number of days from the day of evaluation following course 2 of IC treatment to the day the subject experienced an event of recurrence or death, whichever occurred first. Patients who did not experience an event of recurrence or death at the time of analysis were censored at the date of the last evaluation for recurrence. OS was defined as the number of days from randomization to the date of the patient’s death. Patients who did not experience an event of death at the time of analysis were censored at the date of the last follow up. RFS and OS were analyzed using the Kaplan-Meier (KM) methodology for both Groups combined. RFS and OS were compared between the GD2 Groups using log-rank tests. Continuous variables were summarized using median and ranges, and categorical variables were summarized using frequencies and percentages. The change in CRP and lymphocyte counts from baseline were secondary endpoints that were evaluated using a Wilcoxon signed rank test for within Group comparisons and a Wilcoxon rank sum test for between Group comparisons. The TIL data analysis and graphs were generated and significance tests were performed using Prism 7, version 7.02, software (GraphPad). Due to the exploratory nature of the statistical inference for the TIL data, no adjustments were made for multiple testing. The immune activation graphs were generated using R, version 3.5.0, and the ggplot2 package, version 2.2.1 [22].

Results

Patient Characteristics

A total of 20 eligible patients were enrolled between March 28, 2008 and May 4, 2012. Of these 20 patients, 11 were randomized to Group A and nine to Group B. Two patients in Group B were not treated with adjuvant hu14.18-IL2: one patient was taken off protocol after surgery due to extensive disease identified at that time, and the second patient was taken off protocol after surgery due to findings noted on the PET/CT scan prior to the first adjuvant IC course. Subsequently these two patients were excluded from the RFS analysis but included in the OS analysis.

The baseline characteristics of the 20 patients randomized to Group A or B are provided in Table 1. Thirteen of the 20 patients were men, the median age was 46 years (range, 21–69 years), and all patients were ECOG performance status of 0 or 1. Fourteen of the patients received prior systemic adjuvant therapy.

Treatment Summary

The treatment summary of patients is shown in Table 2. Six of the 20 patients had an “extensive surgical resection”, and 14 of the 20 patients had a non-extensive surgical resection. Two of the patients (sequence numbers 12 and 15) did not proceed to receive hu14.18-IL2 as each patient had disease that could not be completely resected with surgery. Ten of the 18 IC-treated patients received all planned hu14.18-IL2 infusions without treatment modifications. Hu14.18-IL2-associated toxicities that required treatment modifications included: hypotension (3 patients); syncopal episode (1 patient); elevated AST (2 patients); elevated bilirubin (1 patient); elevated creatinine with decreased urine output (1 patient); and pain (1 patient). One of the three patients (sequence number 10) who experienced hypotension at 6 mg/m² also experienced recurrent hypotension during subsequent courses requiring dose modification at 3 mg/m² and at 1.5 mg/m². One of the 3 patients (sequence number 18) who had a dose modification due to hypotension experienced that toxicity at an hu14.18-IL2 dose which had been reduced to 3 mg/m² due to pain after course 1.

The anticipated IL-2 constitutional symptoms (fever, chills, fatigue, pruritis, and myalgias) were seen in the majority of patients treated with hu14.18-IL2. Many patients also had transient Grade 1 and 2 laboratory changes that were similar to those reported in our prior phase I and phase II studies of hu14.18-IL2 [17, 18]. Only Grade 3 and 4 adverse events (AEs) as well as AEs requiring dose modification were collected in this study, and a summary of the collected clinical and laboratory AEs is provided as supplementary data (Supplementary Table S1). Overall, the AEs seen in this study were similar to previously described AEs with hu14.18-IL2 [17, 18]. Intravenous fluids were used for transient hypotension for 5 patients (occurrences in 10 courses). Eight patients (occurrences during 12 doses) received intravenous opioids for management of pain associated with infusion of hu14.18-IL2. The hu14.18-IL2 associated toxicities experienced by patients in this study were reversible.

Immunologic Monitoring

In vitro studies were performed to monitor various aspects of the immune response to hu14.18IL2. As previously reported, lymphopenia was seen on day 3 of each course and followed by lymphocytosis (data not shown) [17, 18]. The change in lymphocyte count rose from baseline to course 2, day 1 for Groups A and B ($p<0.001$ and $p=0.016$ respectively), as noted previously. We also monitored values of CRP over time as a measure of immune activation (data not shown). There was a significant difference in the change in CRP values from baseline to course 1, day 3 for Group A ($p=0.002$) and Group B ($p=0.016$). Serum ELISA assays showed that the trends in peak IC levels were consistent across the three courses of therapy with low baseline levels followed by sharply increased levels 4 hours post-IC infusion (Figure 1a). Dose reduction modifications were required during the study for patients in both groups (Table 2). IC dose was reduced 50% for three patients in Group A beginning at course 2, day 1, and for one of these three patients, the IC dose was further reduced 75% in course 3. IC dose was reduced 50% for two patients in Group B beginning at course 2, day 1, and for a third patient in Group B the IC dose was reduced 50% for the last dose of course 3 (course 3, day 3). Peak IC levels detected were generally lower following dose modifications as compared to course 1 levels on an individual patient level (i.e., before dose reduction) (data not shown). All 18 IC-treated patients developed antibodies to the hu14.18-IL2 immunocytokine that could be measured in a bridging ELISA assay at some point during the course of treatment (Supplementary Fig. S2). In 17 of the 18 IC-treated patients, the highest level of bridging activity was not at the end of therapy (i.e., course 3, day 3) and the level of anti-IC antibody did not interfere with the detection of the IC with subsequent infusions. The exception was a Group A patient (sequence number 20) where there was a substantial level of bridging activity at the end of course 2 which was maintained during course 3 and significantly limited the detection of the IC in the serum during course 3. Bridging activity is measured in OD units and serial dilution of patient 20's serum resulted in corresponding reductions of the OD such that at a dilution of 1/125, the anti-IC level was at background levels (data not shown).

The level of sIL-2R alpha is an indication of immune activation and Fig 1b indicates a significant increase in the sIL-2R alpha during the 3 courses of therapy. This increase was seen in patient 20 even in the presence of anti-IC antibodies which limited the detection of the IC in the serum. The increases in sIL-2R alpha levels detected over the course of this study recapitulated the levels of CRP detected (data not shown).

Histology of Tumor Biopsies

A wide range of histologic patterns of lymphoid inflammation were evident, even prior to treatment (Supplementary Fig. S3). Twelve patients had evaluable tumor samples for GD2 analysis. Frozen sections of patient tumor biopsies were stained by IHC to evaluate GD2 expression. Tumors fell into three categories, near complete GD2 staining of tumor cells in a membrane and granular cytoplasmic pattern, partial GD2 staining in 10–40% of tumor cells, or predominantly absent GD2 staining (Supplementary Fig. S4). The GD2 results were summarized in terms of positive (GD2 expression high or low/moderate; $n=6$) and negative (GD2 expression undetectable; $n=6$) for analysis of RFS and OS (see following section).

Efficacy

The KM estimate of the survival distribution for RFS is shown in Figure 2; the median RFS was 5.73 months [95% CI: 1.80-not reached (months)]. There was no difference in RFS by GD2 status (Supplementary Fig. S5). The KM estimate of the survival distribution for OS is given in Figure 3; the median OS was 61.57 months [95% CI: 13.67-not reached (months)]. There was no difference in OS by GD2 status (Supplementary Fig. S6).

Tumor Infiltrating Lymphocyte Analyses

Quantitation of TIL from resected tumors from the 18 IC-treated patients revealed a median TIL percentage of 5% (of all cells). This was used as the cut-off to categorize low versus high TIL (Fig. 4a-4b). Of the patients who received neoadjuvant hu14.18-IL2 prior to surgical resection (n=11) (Group A), those whose tumors had high TILs post-IC neoadjuvant therapy (>5%, n=6) had increased RFS (median = 43.2 months) compared to patients whose tumors had low TILs post-IC neoadjuvant therapy (0–5%, n=5, median=2.33 months, p=0.0121) (Fig. 4c). Also, Group A, patients whose tumors had high TILs (>5%, n=6) had a non-significant trend toward increased OS (median not yet reached) compared to patients whose tumors had low TILs (0–5%, n=5, median=22.2 months, p=0.0842) (Fig. 4d). These comparisons were not significant in patients who received adjuvant hu14.18-IL2 after surgical resection (Group B); there was no significant difference in RFS or OS for the Group B patients with high TILs vs. low TILs (Fig. 4c4d).

Discussion

Our data show that surgery combined with administration of 3 courses of the hu14.18-IL2 immunocytokine at an i.v. dose of 6 mg/m²/d on days 1, 2 and 3 of each 28-day course can result in prolonged tumor-free survival in some melanoma patients at high risk for recurrence and death. There was no difference in RFS or OS by GD2 status in the 12 patients with evaluable tumor samples for GD2 analysis. The RFS and OS endpoints, along with histological analysis of resected melanoma for anti-tumor activity mediated by hu14.18-IL2, were primary endpoints of this study. The current data are mature, as the median follow-up of surviving patients is over 4 years (range: 31.8–70.4 months). The patients in this study had a very high risk of relapse, as eligibility required patients to have recurrent stage III (i.e., recurrent regional metastasis), or stage IV (i.e., any distant metastasis) melanoma for which surgical resection would be clinically recommended. All 20 patients had biopsy confirmation (current or previous) of stage III or stage IV disease. Fourteen of the 20 patients had prior systemic adjuvant therapy, and 7 of the 20 patients had stage IV disease.

There have been major recent advances in systemic adjuvant therapy for patients with resected high-risk melanoma [23–25]. These advances include checkpoint inhibitor immunotherapies, including drugs that target PD-1 or CTLA-4, and drugs that target the MAPK pathway (BRAF and MEK inhibitor combination). Findings from these recently reported studies provide a perspective to interpret our data. The use of adjuvant ipilimumab resulted in a significantly higher rate of 5-year survival than placebo (65.4% vs. 54.4%) for patients with resected stage III melanoma [23]. The use of adjuvant nivolumab resulted in a

significantly higher 12-month RFS rate than ipilimumab (70.5% vs 60.8%) for patients with resected stage IIIB, IIIC, or IV melanoma [25]. Previous systemic therapy for melanoma was not allowed. The use of adjuvant dabrafenib plus trametinib resulted in a significantly higher 3-year RFS rate than placebo (58% vs 39%) for patients with resected stage III melanoma [24]. Previous systemic therapy for melanoma was not allowed.

The higher baseline recurrence risk and the smaller sample size of our study does not allow direct comparison of RFS and OS rates with patient subsets from the recent large prospective adjuvant melanoma studies[23–25]. However, the 24-month RFS rate was 38.9% (95% CI: 17.5–60.0%) and the 24-month OS rate was 65.0% (95% CI: 40.3–81.5%) for the 20 high-risk patients in our study. A prospective randomized study would be needed to determine whether surgery combined with administration of 3 courses of the hu14.18-IL2 immunocytokine given intravenously, alone or in combination with immune checkpoint blockade, improves RFS or OS compared to any of the currently approved adjuvant therapies. A trial design of neoadjuvant hu14.18-IL2 to induce a lymphocytic infiltration into tumors, followed by adjuvant anti-PD1 therapy after surgery, is suggested for subsequent investigation.

The hu14.18-IL2 dose for this trial was 6 mg/m² and was one dose level below the maximum tolerated dose of 7.5 mg/m² established in our phase I trial [18]. This dose and schedule (6 mg/m²/day for three consecutive days) was previously shown to result in immune activation and to be well tolerated with reversible toxicities in our prior phase II study of 14 patients with measurable metastatic melanoma [17]. In previous studies, we have shown that some patients develop antibodies to the hu14.18-IL2 immunocytokine that can be measured in a bridging ELISA assay [26]. We found that all 18 IC-treated patients developed a bridging antibody at some point during the course of treatment (Supplementary Fig. S2), but this anti-IC antibody did not appear to affect serum levels of IC in 17 of those 18 patients. Importantly, immune activation was achieved in the patients in this study as evidenced by rebound lymphocytosis, elevated CRP, and sIL-2R alpha. The spectrum of toxicities experienced by patients in this study were similar to those previously reported for melanoma patients receiving this same IC dose and schedule and were readily managed and were reversible [17].

The current study was designed to allow for histological analysis of resected melanoma for antitumor activity mediated by hu14.18-IL2, as patients in Group A received their surgical resection after the first course of neoadjuvant hu14.18-IL2 and patients in Group B received their surgical resection prior to the first course of adjuvant hu14.18-IL2. We only collected and evaluated the tumors obtained at surgical resection, additional biopsies of different sites or at different times were not obtained. While the histological analysis identified inflammation and tumor necrosis in many tumor samples, the variability in immune infiltrate between anatomic sites was substantial and did not allow for an instructive comparison of inflammation and tumor necrosis between Groups. In the future, we plan to prospectively collect biopsies before and after hu14.18-IL2 from the same tumor sites in each patient.

We proceeded with exploratory analyses of TIL to evaluate whether quantification of TIL would provide a biomarker of response for patients treated with IC. Quantification of TIL in

the resected tumors did not provide prognostic information for patients who underwent surgical resection prior to receiving adjuvant hu14.18-IL2 (Group B). In contrast, patients who received neoadjuvant hu14.18-IL2 prior to surgical resection (Group A) and had tumors with high TILs had increased RFS compared to Group A patients whose tumors had low TILs (Fig. 4c). In addition, patients whose tumors had high TILs (6–100%, n=6) following treatment with neoadjuvant hu14.18-IL2 had a trend towards increased OS compared to patients whose tumors had low TILs following hu14.18-IL2. We interpret this to suggest that patients who are unable to mount a TIL infiltrate following neoadjuvant IC therapy appear to be less likely to respond clinically and vice versa. With the caveat of low sample size, the degree of TIL infiltration present prior to adjuvant IC therapy (i.e., in Group B patients) did not appear to be predictive of IC responsiveness. In our preclinical testing in mice bearing the syngeneic B78 melanoma, we have seen that the combination of low dose radiation therapy followed by intratumoral administration of IC causes a potent infiltration of T cells into tumors and dramatically potentiates the response of relatively large murine melanomas to anti-CTLA-4 treatment [21]. Clinical testing of this combination is being initiated.

In conclusion, patients with resectable recurrent stage III or stage IV melanoma can experience prolonged tumor-free survival following surgery and 3 courses of IC. We suggest prospective testing of early post-treatment TIL as a biomarker of response for immunotherapy that includes IL2. Additional investigations are needed to determine whether hu14.18-IL2, either alone or as a combination therapy, can improve upon current adjuvant treatment options for patients with resected high-risk melanoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AEs	adverse events
AJCC	American Joint Committee on Cancer
AST	aspartate transaminase

CI	confidence interval
CR	complete response
CRP	C-reactive protein
CTCAE	Common Terminology Criteria Adverse Events
DLT	dose limiting toxicity
ECOG	Eastern Cooperative Oncology Group
Fc	region of antibody
GD2	ganglioside expressed by tumor cells
IC	immunocytokine
KM	Kaplan-Meier
NCI	National Cancer Institute
PR	partial response
PS	performance status
RFS	recurrence-free survival
sIL-2R	soluble IL-2 receptor
TIL	tumor-infiltrating lymphocyte
UW	University of Wisconsin
UWHC	UW Hospitals and Clinics

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Fig. 1. Immune activation following IC administration. IC levels and sIL-2R alpha levels were measured in Group A (filled circles) and Group B (open circles) patients. **a** Peak serum IC levels measured pre-treatment and 4 hours post-treatment for each course. Results are shown as individual data points and mean ng/ml $\pm$ SEM. The number of patients analyzed per Group at each timepoint is presented. **b** sIL-2R alpha levels measured pre-treatment, day 4, day 8 and day 25 post-treatment for each course. Results are shown as individual data points

and mean pg/ml $\pm$ SEM. The number of patients analyzed per Group at each timepoint is presented.

Fig. 2. Recurrence-free Survival. The estimate of the survival distribution for RFS is shown. The median RFS was 5.73 months [95% CI: 1.80-not reached (months)].

Fig. 3. Overall Survival. The estimate of the survival distribution for OS is shown. The median OS was 61.57 months [95% CI: 13.67-not reached (months)].

Fig. 4.

Tumor Infiltrating Lymphocytes in hu14.18-IL2 Treated Tumors Prognosticates Improved Relapse Free Survival (RFS). **a** H&E section of example of melanoma with low TIL percentage (400x). Lymphocytes truly infiltrating between tumor cells are scant. **b** H&E section of example of melanoma with high TIL percentage (400x). Tumor infiltrating lymphocytes are located histologically between tumor cells. **c** Relapse free survival of patients stratified by treatment group and TIL percentage. **d** Overall survival of patients stratified by treatment group and TIL percentage.

Table 1.

Summary of Demographics and Baseline Characteristics

Patient Characteristic	Category	Treatment Group		
		A (11 pts) ^a	B (9 pts) ^a	Overall (20 pts) ^a
Age, median (range)		46 (21–65)	52 (38–69)	46 (21–69)
ECOG PS ^b	0	9 (82)	7 (78)	16 (80)
	1	2 (18)	2 (22)	4 (20)
Gender ^b	Female	5 (45)	2 (22)	7 (35)
	Male	6 (55)	7 (78)	13 (65)
Disease extent ^b	Extensive	4 (36)	2 (22)	6 (30)
	Non-extensive	7 (64)	7 (78)	14 (70)
Disease stage ^{b,c}	III	7 (64)	6 (67)	13 (65)
	IV	4 (36)	3 (33)	7 (35)
Histology ^b	Cutaneous Melanoma	10 (91)	8 (89)	18 (90)
	Unknown Melanoma Primary	1 (9)	0	1 (5)
	Subungual Melanoma	0	1 (11)	1 (5)
Prior therapy ^b	Granulocyte-Macrophage Colony Stimulating Factor	1 (9)	0	1 (5)
	Chemotherapy multiple agent	0	1 (11)	1 (5)
	Interferon alpha-2b	7 (64)	5 (56)	12 (60)
	No prior therapy	1 (9)	0	1 (5)
	Radiation therapy	1 (9)	2 (22)	3 (15)
	Surgery	10 (91)	9 (100)	19 (95)

^a A total of twenty patients (pts) were randomized, 11 to Arm A and 9 to B.

^b Values in parentheses represent percentages, except for the age range in the top line.

^c Sites of metastasis include lung nodules (3 patients); connective, subcutaneous, and other soft tissues including head and neck sites (12 patients); and lymph nodes (6 patients). One patient had metastases both to a lung nodule and a head and neck site.

Table 2.**Treatment Summary**

Sequence No.	Group	Surgical Site(s)	Treatment Modifications ^a
Extensive surgical resection			
5	A	Left auricle, left facial nerve, left pre-tracheal node, mastoidectomy	None
13	A	Lung nodule	None
14	B	Lung nodule, right temporal	None
17	B	Peri-aortic lymph node	C3D2 dose held (hypotension). C3D3 dose reduced 50%.
19	A	Right upper lobe nodule	None
21	A	Mediastinal node	None
Non-extensive surgical resection			
2	B	Left chest wall nodules, left axillary soft tissue mass	C1D3 held (syncopal episode evening of C1D2). Subsequent cycles given at 50% dose reduction.
6	B	Right supraclavicular area	C1D3 held (grade 3 AST). Subsequent cycles given at a 50% dose reduction.
7	B	Right chest wall nodule	None
8	A	Left scapula soft tissue lesion	C3D3 held (elevated creatinine & low urine output).
9	A	Rectus femoris lesion	None
10	A	Left inguinal nodule	Cycle 2 50% dose reduced (hypotension after C1D3). C2D3 held (hypotension after C2D2). C3 given at a 75% overall dose reduction. C3D3 held (hypotension).
11	A	Left inguinal nodule	C1D3 held (grade 3 AST). C2 & C3 dose reduced 50%.
12	B	Right inguinal nodule	No treatment after surgery due to extensive disease.
15	B	Left occipital and temporal scalp	No treatment after surgery due to PET/CT findings.
16	B	Right inguinal nodule	None
18	A	Left breast mass	C2 50% dose reduced (grade 3 pain after C1). C3D2 dose held (hypotension). C3D3 dose reduced 75%.
20	A	Right axillary nodule	None
22	B	Left upper arm node	C1D3 held (grade 3 bilirubin). C2 & C3 dose reduced 50%
23	A	Right shin	None

^aCourse = "C", Day = "D" (*i.e.*, Course 1 Day 2 = C1D2)