Research Article

Intralesional Treatment of Stage III Metastatic Melanoma Patients with L19–IL2 Results in Sustained Clinical and Systemic Immunologic Responses


Abstract

L19–IL2 is a recombinant protein comprising the cytokine IL2 fused to the single-chain monoclonal antibody L19. In previous studies, intralesional injection with IL2 has shown efficacy for the locoregional treatment of cutaneous/subcutaneous metastases in patients with advanced melanoma. The objectives of this study were to investigate whether (i) intralesional delivery of a targeted form of IL2 would yield similar results, with reduction of injection frequency and treatment duration; and (ii) systemic immune responses were induced by the local treatment. Patients with stage IIIB/IIIC melanoma and cutaneous/subcutaneous injectable metastases received weekly intratumoral injections of L19–IL2 at a maximum dose of 10 MIU/week for 4 consecutive weeks. Tumor response was evaluated 12 weeks after the first treatment. Twenty-four of 25 patients were evaluable for therapy-induced responses. A complete response (CR) by modified immune-related response criteria (irRC) of all treated metastases was achieved in 6 patients (25%), with long-lasting responses in most cases (5 patients for ≥24 months). Objective responses were documented in 53.9% of all index lesions [44.4% CR and 9.5% partial responses (by irRC)], and 36.5% of these remained stable, while 9.5% progressed. Toxicity was comparable with that of free IL2, and no serious adverse events were recorded. A significant temporary increase of peripheral regulatory T cells and natural killer cells, sustained increase of absolute CD4+ lymphocytes, and decrease of myeloid-derived suppressor cells were observed upon treatment. Finally, we recorded encouraging data about the progression time to distant metastases and overall survival. Cancer Immunol Res 2(7): 668–78. © 2014 AACR.

Introduction

Advanced cutaneous melanoma has a poor prognosis, with a median survival time of approximately 8 months (1) and about 46,000 deaths per year worldwide (2). Stage III of the disease is characterized by the presence of metastases, limited to an anatomic region between primary melanoma and the next proximal lymph node (LN) basin. It includes patients with LN and skin or subcutaneous metastasis (satellite and in-transit metastases). Patients with locoregional satellite or in-transit metastases are classified as N2c or N3 depending on the absence or presence of concurrent or prior LN metastasis. According to the current American Joint Committee on Cancer (AJCC) classification, N2c patients are aligned to the clinical stage IIIB in case of a nonulcerated primary melanoma, while all other patients are classified as stage IIIC (3). Patients with distant soft-tissue metastasis are classified as stage IV M1a if serum lactate dehydrogenase (LDH) is within the normal range and other visceral metastases are absent.

Surgery is the first therapeutic option in these patients and is performed with a curative intention in most cases. In contrast, options are few and of limited efficacy if surgery is not feasible due to unresectable disease or continuous recurrences despite repeated surgery. Until 2011, in addition to radiotherapy (4) and isolated limb perfusion (ILP; ref. 5), most of these patients received systemic therapy with dacarbazine with palliative intention. All of these treatments are characterized by low efficacy. High-dose treatment with IL2 was approved by the U.S. Food and Drug Administration (FDA) in 1998 but, due to its inherent toxicity, it found application only in a limited number of young patients with excellent performance status (6). The situation improved after FDA approval of ipilimumab and vemurafenib, but the treatment-related survival benefit is restricted to a minority of patients and long-term survival is still rare.
Intralesional therapy for satellite/in-transit melanoma metastases has been proposed as an attractive therapeutic avenue with distinctive advantages over other approaches (7). Concentrations of the administered drug that can be reached within the tumor are higher than those for systemic administration, resulting in a beneficial therapeutic effect. Conversely, low systemic concentrations of the drug lead to reduced toxicity. In contrast to surgery, diffuse metastatic spread distributed over large anatomic regions can be treated and potential indirect beneficial systemic effects may result. Several cytokines (IFNα, IFNβ, GM-CSF) have been used for intralesional treatment of metastatic melanoma with variable results (8–12).

Different groups, including ours, have reported the promising response of melanoma metastases to intralesional injection of IL2 (13–17). To improve this approach, we now considered the use of antibody-based pharmacodelivery strategies. Cytokines of interest are expressed as fusion proteins with antibody fragments, which allow the preferential accumulation of the payload at the site of disease while sparing normal organs (18). We reasoned that a targeted form of IL2 might reside in the metastatic lesion longer than the untargeted form, thereby allowing a reduction in administration frequency and treatment duration.

L19–IL2 is an immunocytokine, i.e., a recombinant fusion protein of the monoclonal single-chain variable fragment (scFv) of antibody L19 and cytokine IL2. L19 recognizes the alternatively spliced extra-domain B (EDB) of fibronectin (FN), a marker of angiogenesis (19). EDB-containing FN is present in the neovasculature of most solid tumors and hematologic malignancies (19) but absent from almost all healthy adult tissues (except tissues of the female reproductive cycle). Therefore, L19–IL2 represents a targeted form of IL2, which is able to accumulate at sites where angiogenesis occurs and the EDB-FN antigen is expressed.

L19–IL2 has already been studied in many preclinical models, and in patients, in phase I monotherapy studies of renal cell carcinoma (20) or in combination with dacarbazine in metastatic melanoma (21), with encouraging results.

Here, we describe the results of a phase II clinical trial based on the intralesional administration of L19–IL2 in patients with stage IIIB and IIIC metastatic melanoma.

Materials and Methods

Patients

This multicenter study (ClinicalTrials.gov identifier: NCT01253096) included patients from three clinical centers [University Medical Center (Tübingen, Germany), Hannover Medical School (Hannover, Germany), and Universitätsklinik Graz (Graz, Austria)] with approvals from the local ethic committees and national authorities. The study was conducted in accordance with the Declaration of Helsinki; all patients were included after written informed consent. Patient inclusion criteria comprised histopathologically proven malignant melanoma, presence of measurable and injectable soft-tissue metastases in clinical stage IIIB or IIIC, male or female gender, with age ≥18 years, either without or after one line of prior systemic treatment of metastatic disease, Eastern Cooperative Oncology Group (ECOG) performance status <2, LDH <2 × the upper limit of normal, and a life expectancy of ≥12 weeks. Patients with evidence of visceral or brain metastases at screening or severe cardiac disease (New York Heart Association grade >2) or having undergone antitumor therapy within 4 weeks of the administration of study treatment (except minor surgery), and pregnant or lactating women were excluded. Concurrent treatment of metastatic melanoma was not allowed.

Study design and treatment

The trial was an open-label, nonrandomized prospective phase II study. Ten million international units of IL2 equivalents (Mio IU) of L19–IL2 (Darleukin; Philogen S.p.A.) was dissolved in 6 mL glucose 5% supplemented with 0.2% human serum albumin. Four milligrams of L19–IL2 corresponds to 24 Mio IU of IL2 equivalents.

The treatment schedule was once weekly on an outpatient basis. L19–IL2 was injected intratumorally using 30-gauge needles for superficial injections and 27-gauge needles for deep injections, and the total daily dose was divided between all injectable lesions. All lesions present at screening were injected; a detailed list of lesions with their size and location is provided in Supplementary Table S1. Sonography was used to guide injections of deep soft-tissue metastases. Patients received analgesic treatment with acetaminophen or metamizole 1 hour before treatment with L19–IL2 and received 0.5 to 1.0 g of acetaminophen 5 or 10 hours after injection, as needed.

Tumor assessment was performed 2 weeks before the start of treatment (baseline) and at week 12. An additional tumor assessment was performed at week 6 after the first injection to check that no visceral metastases had developed under treatment. End-of-study (EoS) assessment was performed at week 16 and follow-up for recurrence and survival every 6 weeks thereafter. Adverse events were graded according to the Common Toxicity Criteria (version 3).

Response evaluation

The primary endpoint was the rate of patients with complete responses (CR) at day 85, according to both immune-related response criteria (irRC; ref. 22) and Response Evaluation Criteria In Solid Tumors (RECIST) version 1.1 (23) modified as described below.

All lesions present at baseline were classified as index/nonindex lesions (irRC) or as target/nontarget lesions (RECIST). A lesion qualified as an index/target lesion by being measurable as judged by the treating investigator. Lesion diameters of ≥5 mm × 5 mm (irRC) or ≥5 mm (RECIST) were defined as cutoff points for measurability. Besides the lower cutoff size for RECIST (with respect to the cutoff size of ≥1.0 cm recommended in the original guideline; ref. 23), the assessment was not limited to five skin metastases, but included all the measurable injected lesions.

At later tumor assessments, the change in tumor burden included the measurement of index and new measurable lesions, but all lesions (index, nonindex, new unmeasurable, and measurable lesions) were included in the overall modified...
irRC response assessment (22). For modified RECIST, the change in tumor burden was based on the target lesions, but new lesions and nontarget lesions were likewise considered for defining the overall response (23).

In addition, all treated metastases (index and nonindex lesions) were evaluated individually for response. Index lesions were measured at day 85 and EoS and classified as CR, partial response (PR), stable disease (SD), or progressive disease (PD) with respect to baseline. Response of injected nonindex lesions, which were almost exclusively <5 mm in size at baseline, was classified only as either CR (complete disappearance) or non-CR at day 85 and EoS.

Subcutaneous metastases were evaluated by sonography or CT scan. Whenever possible, in doubtful cases (e.g., if there was residual pigmentation), biopsies were taken for histopathologic confirmation of response.

**Analysis of immune cell subsets**

For translational side studies, blood was drawn at baseline, 1 week after the last application of L19–IL2 (day 29), and at tumor assessment (day 85). Peripheral blood mononuclear cells (PBMC) were immediately isolated from fresh blood by Ficol/hypaque density gradient centrifugation and cryopreserved until use.

After thawing, different populations of immune cells were evaluated using multicolor flow cytometry. For the analysis of CD4+ and CD8+ T cells, myeloid-derived suppressor cells (MDSC), regulatory T cells (Treg), and natural killer (NK) cells, Fc receptors were initially blocked with Gamunex (human immunoglobulin; Bayer), and dead cells were labeled for exclusion with ethidium monoazide (EMA; Invitrogen). All statistical analyses were conducted using the SPSS software package Version 21 (IBM Inc.). Differences in mean cell frequencies between different time points were calculated with the two-tailed paired t test. Differences in the proportion of patients with antigen-specific T cells and in response rates were calculated using a two-tailed Fisher exact test. For Kaplan–Meier analyses, follow-up time for the calculation of survival was defined from the date of first treatment either to the date of the last follow-up or to the date of recurrence [recurrence-free survival (RFS)], diagnosis of the first distant metastasis [distant metastasis-free survival (DMFS)] or death [overall survival (OS)], respectively. Disease-specific OS probabilities have been calculated, and only deaths due to melanoma have been considered, whereas deaths due to other causes were regarded as censored events. Estimates of cumulative survival probabilities according to Kaplan–Meier were described together with 95% confidence intervals and compared using log-rank tests. Throughout the analysis, P values of less than 0.05 were regarded as statistically significant.

**Results**

**Patients and treatments**

Enrolment started on June 30, 2010, and was completed by August 23, 2012. The baseline data from the 25 patients are summarized in Table 1. The median patient age was 68.4 years (range, 36.5–90.5 years). Most patients were treated in stage IIIA (21), and 4 patients were treated in stage IIIB. All metastases were accessible and injected in all patients.

Apart from surgical resection of the primary tumor, 17 patients (68%) had undergone surgery for locoregional recurrences. Thirteen of 17 patients had undergone two or more
previous surgeries, with the last surgery having been carried out <8 weeks before the start of L19–IL2 therapy in 5 of the patients. In 4 patients, limb perfusion or radiotherapy had also been performed.

Previous systemic treatments with IFN-α (12 patients), chemotherapy (3 patients), or both modalities subsequently (1 patient) had been applied before L19–IL2 treatment was initiated. The median number of metastases treated per patient was 5.5 (range, 1–118).

### Toxicity

All 25 patients were included in the analysis of toxicity. The treatment was generally well tolerated, with mostly grade 1 and 2 toxicities recorded. In 76% of patients, intratumoral L19–IL2 therapy caused an inflammatory injection site reaction (local swelling and erythema) limited to the tumor tissue, reaching in a few cases to grade 3, followed by a selective tumor necrosis that generally did not affect the surrounding normal tissue. Injection pain was present (only one grade 3 case), manageable by the application of a local anesthetic cream and oral metamizole. Twenty percent of patients experienced low-grade fever, easily controlled by acetaminophen. Transient fatigue (<8 hours) was reported by 25% of patients (with one grade 3), and pruritus (16% of patients) and edema (24% of patients) were also fairly common. These symptoms were usually mild and of short duration. A summary of frequent adverse events is presented in Supplementary Fig. S1. Adverse events that were observed in <10% of patients but that were at least possibly related to the treatment included chills, headache, pyrosis, and dry oral mucosa. One patient presented with an enlarged groin LN, and one had eczema. No adverse effect recorded was considered serious.

### Clinical responses to L19–IL2 treatment

According to the modified irRC, 6 of 24 evaluable patients (25%) presented an overall irCR at the end of treatment. Twenty-five percent of patients presented overall irPR, while the disease remained stable in another 29.2% of patients (irSD). Finally, 20.8% of patients showed a PD (irPD).

Detailed overall tumor response in each patient according to both modified irRC and RECIST is summarized in Supplementary Table S1. We also evaluated the response at the level of individual metastases. In total, 514 separately treated metastases, (63 index lesions and 451 nonindex lesions) could be evaluated for local tumor response (Table 2). Analysis of the results

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**Table 1. Patients’ characteristics**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number of patients (n = 25)</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>14</td>
<td>56</td>
</tr>
<tr>
<td>Women</td>
<td>11</td>
<td>44</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>IIIB</td>
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<td>16</td>
</tr>
<tr>
<td>IIBC</td>
<td>21</td>
<td>84</td>
</tr>
<tr>
<td>Site of treated metastases</td>
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<td></td>
</tr>
<tr>
<td>Cutaneous</td>
<td>14</td>
<td>56</td>
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<tr>
<td>Subcutaneous</td>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td>Both</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Total number of treated metastases</td>
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<tr>
<td>&lt;20</td>
<td>17</td>
<td>68</td>
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<tr>
<td>≥20</td>
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<td>32</td>
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<tr>
<td>Surgery</td>
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</tr>
<tr>
<td>Limb perfusion</td>
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<td>16</td>
</tr>
<tr>
<td>Radiotherapy</td>
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<td>16</td>
</tr>
<tr>
<td>Adjuvant IFNα</td>
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<td>48</td>
</tr>
<tr>
<td>Systemic chemotherapy</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>Intraleisional IL2</td>
<td>4</td>
<td>16</td>
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</table>

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**Table 2. Locoregional tumor response (modified irRC)**

<table>
<thead>
<tr>
<th>Response, index lesions</th>
<th>N (%)</th>
<th>Response, nonindex lesions</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>irCR</td>
<td>28 (44.4)</td>
<td>CR</td>
<td>205 (45.4)</td>
</tr>
<tr>
<td>irPR</td>
<td>6 (9.5)</td>
<td>Non-CR</td>
<td>246 (54.6)</td>
</tr>
<tr>
<td>irSD</td>
<td>23 (36.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>irPD</td>
<td>6 (9.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>63 (99.9)</td>
<td></td>
<td>451 (100)</td>
</tr>
</tbody>
</table>

irCR, complete response (disappearance of any evidence of vital tumor).

irPR, partial response (a ≥50% decrease in the product of the two largest perpendicular diameters (LPD) of the lesion compared with baseline).

irSD, stable disease (a variation in LPDs, which was neither sufficient to qualify the lesion as irPR nor as irPD).

irPD, progressive disease (a ≥25% decrease in the product of LPDs of the lesion compared with baseline).

CR, complete response (disappearance of any evidence of vital tumor).

Non-CR, non-complete response.
revealed that 53.9% of all index lesions exhibited an objective response (44.4% irCR and 9.5% irPR), 36.5% of index lesions remained stable, and 9.5% of index lesions progressed. When the analysis was extended to nonindex lesions (n = 451), a CR (disappearance) was observed in 205 lesions (45.4%), while another 246 lesions showed a non-CR (54.6%).

Figure 1 illustrates the clinical course of 5 patients with complete responses (Fig. 1A–C), partial responses (Fig. 1D), or SD (Fig. 1E). Further details are reported in the Supplementary Data.

**Progression-free and overall survival**

To evaluate the progression and OS of this cohort of patients, data were collected during the study and beyond the 12-month follow-up period.

In 20 of 24 evaluable patients, disease progressed after the start of L19–IL2 treatment. The first recurrence site was locoregional (new cutaneous/subcutaneous metastases) in all 20 patients, who progressed further in the course of disease. Figure 2A shows Kaplan–Meier curves reporting the progression-free survival (PFS) data for progression to new cutaneous/subcutaneous metastases (skin), LN metastases, or distant metastases (systemic), respectively.

In 19 of 24 patients, a locoregional recurrence to either cutaneous/subcutaneous metastases or LN metastases was observed within 1 year from the first treatment. The median time to locoregional progression was 93 days (range, 41–864 days). However, in 5 patients, no further lesion appeared for 2 years or longer.

Among the 19 patients who showed recurrence to locoregional sites (cutaneous/subcutaneous and LN), 9 patients progressed further to systemic disease. The median progression time to systemic disease was 331 days (range, 67–960 days). However, 15 patients remained free of distant metastases for time intervals ranging from 130 to 1,015 days.

Figure 2B shows the OS data for the 24 evaluable patients. Three patients died, with one each at 278, 616, and 966 days after the first treatment. The other 21 patients had survived at the time point of the last follow-up for periods ranging from 130 to 1,048 days. The median survival time is 905 days.

**Analysis of systemic immune responses**

Altogether, 45 blood samples from 17 patients were available for the evaluation of systemic immune responses. Complete results are presented in Table 3. A significant increase in the frequency of CD4+ CD25hi Fox-P3hi Tregs was observed in 15 of 16 evaluable patients after 4 weeks of treatment compared with baseline (mean frequency, 12.1% vs. 8.5%; P < 0.001). After stopping treatment, frequencies returned to baseline values at day 85 (Fig. 3A). There was a decrease of CD14++CD11b+ HLA-DR++/low MDSCs from 11.7% at baseline to 8.7% at day 29 and 7.6% at day 85 (Fig. 3B). Both the differences between baseline and end of treatment and between baseline and day 85 were statistically significant with P values of 0.001 and 0.002, respectively. No change in frequency was observed for NK, CD4+ T cells, and CD8+ T cells. In contrast with the frequency, the absolute numbers of NK cells (P = 0.005; Fig. 3C) and CD8+ T cells (P = 0.007) were increased at day 29 and decreased thereafter to baseline values. The absolute lymphocyte count increased until day 29, mainly driven by an increase of CD4+ T cells, and remained elevated several weeks after day 85 as compared with baseline (P = 0.009; Fig. 3D).

The proportions of patients with detectable antigen-specific T cells were 47.1% at baseline, 29.4% at day 29, and 27.3% at day 85 for Melan-A–specific T cells and 52.9%, 58.8%, and 45.5% for NY-ESO-1–specific T cells. None of these changes was statistically significant. The de novo appearance of specific T cells between baseline and day 29 was observed in 3 patients for Melan-A– and 2 for NY-ESO-1–specific T cells, but conversely some patients also lost a preexistent baseline T-cell reactivity at day 29 (6 patients for Melan-A– and 1 for NY-ESO-1–specific T cells). No change in reactivity was observed among the remaining patients.

Changes in the frequency of NY-ESO-1–, Melan-A–, and influenza-specific T cells upon treatment were analyzed.
separately for CD8\(^+\) and CD4\(^+\) T cells and for each of six cytokines (Supplementary Table S2). A significant decrease during therapy was observed for the proportion of IFN\(\gamma\)\(^+\)CD4\(^+\) T cells. A nonsignificant tendency was observed for a decrease in the frequency of IFN\(\gamma\)\(^+\)CD8\(^+\), TNF\(\alpha\)\(^+\)CD8\(^+\), and TNF\(\alpha\)\(^+\)CD4\(^+\) T cells (all \(P > 0.100\)) upon stimulation with NY-ESO-1 peptides. Upon stimulation with Melan-A peptides, we again observed a tendency for decreasing levels of IFN\(\gamma\)\(^+\)CD4\(^+\) and TNF\(\alpha\)\(^+\)CD4\(^+\) T cells (\(P > 0.100\)) and no change in the CD8\(^+\) Melan-A–specific cells.

All changes were temporary, as no significant differences in the frequency of antigen-specific T cells were observed in a comparison of baseline and day 85. No changes in the frequency of influenza-specific T cells were observed between the different time points.

No significant predictive or surrogate markers for clinical response could be identified among all the analyzed immune-cell parameters.

Discussion

To the best of our knowledge, this is the first study to address the use of an IL2-based immunocytokine for the intralesional treatment of satellite or in-transit metastases in patients with melanoma. The treatment was generally well tolerated, with few adverse events, limited in grade and similar in nature to those recorded for untargeted IL2.

In terms of efficacy, the objective response rate recorded in this study was lower than that shown by untargeted IL2. This difference deserves some comment. In previous studies using untargeted IL2, we reported CRs of injected metastases, according to adapted RECIST criteria, in more than 60% of patients (14, 15), which is significantly higher than the CR rate of 25% according to modified irRC/RECIST observed here. In our opinion, this difference does not reflect a lower efficacy of L19–IL2 compared with untargeted IL2, but is more likely related to differences in the patient populations and trial designs. First, the two studies that used untargeted IL2 were carried out in patients whose disease stage was evenly distributed among stages IIIB, IIIC, and IV (24, 25, and 23 patients, respectively). The results of the follow-up study (32) clearly show that patients who benefited the most, in terms of OS, were those in stage IIIB at the screening visit (86.8% 5-year survival rate), as opposed to patients in stage IIIC, who only showed a 31.4% 5-year survival rate. This last value was close to that recorded for stage IV M1a patients (16.7% 5-year survival rate). In the present study, most patients (21 of 25) were in stage IIIC at screening and only 4 were at stage IIIB. This might explain the lower percentage of patients who, in our study, could be classified as CR at the EoS visit. Second, there are differences between the studies with untargeted IL2 and the present study in the treatment regimen and schedule. In IL2-based studies, the treatment schedule was two to three times weekly with a maximum daily dose of 16 MIU IL2 or variable, according to the individual tumor burden of patients (14, 15).
Importantly, in these trials, new lesions arising during treatment were also injected, and treatment continued until all lesions, including the new ones, finally regressed. Therefore, the overall length of treatment varied between 1 and 57 weeks (median, 7.5 weeks). In the present study, L19 antibody would ensure a longer residence time of L19 IL2 concentrations are required for efficacy; and (ii) the antibody would ensure a longer residence time of L19 IL2 in the lesions, therefore allowing a reduced number of injections as compared with IL2. A standardized schedule with fewer injections and shorter treatment duration would reduce injection pain and discomfort for patients and facilitate logistics. The rate of objective responses recorded in the two sets of studies is of comparable magnitude in any case, with differences possibly attributable to different distribution of patients among the disease stages. An analysis of PFS for the patients enrolled in the present study shows that, although most patients relapsed within 1 year to new in-transit/satellite metastases, progression to proximal LN and to distal systemic disease occurred in a relatively low number of patients and was much slower. In 2010, Romano and colleagues (33) published a retrospective study based on clinical records of patients with stage III melanoma with no evidence of disease (NED) seen at Memorial Sloan-Kettering Cancer Center (New York, NY) between 1992 and 2004, who ultimately relapsed to locoregional, nodal, or systemic disease. Among relapsing patients, 340 had adequate follow-up to be evaluable for all parameters. In that study, similar to our study, most NED stage IIIC patients (~80%) who relapsed to locoregional disease did so in less than 1 year. However, the data also show that progression to nodal disease of NED stage IIIC patients occurred even faster (90% of patients who progressed did so after 1 year), and that progression to systemic disease was observed in virtually all patients who had relapsed after 2 years of follow-up.

### Table 3. Analysis of systemic immune responses

<table>
<thead>
<tr>
<th>Cell subset</th>
<th>Definition/phenotype</th>
<th>Reference</th>
<th>Baseline (mean) N = 16</th>
<th>End of treatment (mean) N = 17</th>
<th>Follow-up (mean) N = 11</th>
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<tr>
<td>Absolute WBC count</td>
<td>Blood count</td>
<td>μL</td>
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<td>7,009&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6,438</td>
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<tr>
<td>Absolute lymphocyte count</td>
<td>Blood count</td>
<td>μL</td>
<td>1.476</td>
<td>2,005&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1,937&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Frequency lymphocytes</td>
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<td>1,040&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>Lymphocytes</td>
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<td>Frequency CD8&lt;sup&gt;+&lt;/sup&gt; lymphocytes</td>
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<td>Lymphocytes</td>
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<tr>
<td>Frequency NK cells</td>
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<tr>
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<tr>
<td>MDSCs</td>
<td>CD14&lt;sup&gt;+&lt;/sup&gt;CD11b&lt;sup&gt;+&lt;/sup&gt;HLA-DR&lt;sup&gt;-&lt;/sup&gt;/C0&lt;sup&gt;low&lt;/sup&gt;</td>
<td>PBMCs</td>
<td>11.7%</td>
<td>8.7%&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.6%&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

NOTE: Statistically significantly changed values are highlighted by bold letters.

Abbreviation: WBC, white blood cells.

<sup>a</sup>P < 0.001 compared with baseline.

<sup>b</sup>P < 0.001 compared with end of treatment.

<sup>c</sup>P < 0.01 compared with baseline.

<sup>d</sup>P < 0.05 compared with baseline.
Our study indicates that only 17% (4 of 24) and 25% (6 of 24) of patients had progressed to nodal or systemic disease at 1 or 2 years after the first intralesional treatment with L19–IL2, respectively. Although obtained in a much smaller cohort of patients, our data suggest that the control of in-transit/satellite metastases, obtained through intralesional injection of L19–IL2, translates into a slower diffusion rate of the disease to distant sites.

In terms of OS, 3 of the 24 patients died during the follow-up period at 278, 616, and 966 days after first treatment, respectively. Our data indicate that 48% of patients survived >2 years from the date of first treatment, with another 44% having been monitored only for shorter times. Although we find these data encouraging, it is still too early to compare our statistics with historical data (3), which show that 3-year survival for patients with stage IIIC melanoma is approximately 50%.

Translational side studies in 17 patients revealed that intra-tumoral treatment resulted in significant frequency changes of immune-cell populations in the peripheral blood. Besides a temporary increase in Tregs during treatment, observed in almost all patients, we observed a sustained decrease of MDSCs. These cells have been described to possess clinically relevant immunosuppressive functions, at least in malignant melanoma (28). Filipazzi and colleagues analyzed patients with stage II/III melanoma with NED and found a trend for better disease-free survival in patients with low amounts of CD14+.11b.HLA-DR++MDSCs compared with those with a high frequency of these cells ($P = 0.08$), but their patient cohort ($n = 33$) was very small (34). Walter and colleagues reported a strong association of this particular MDSC population with negative outcome and survival in patients with renal cell cancer after multipeptide vaccination (27). The decrease of MDSC frequency might, therefore, partially explain the beneficial mode of action of intratumoral L19–IL2, represented by what seems to be a favorable DMFS rate as compared with that for historical controls. On the other hand, a transient increase of Tregs was observed after IL2 therapies (35, 36). A direct interaction between IL2 used in a therapeutic intention and Tregs can be assumed because of the high expression levels of the $\alpha$-chain of the IL2 receptor (CD25) on Tregs. Nevertheless, the clinical impact and the functional state of these treatment-induced Tregs and of circulating Tregs in patients with melanoma in general remain unclear (reviewed in ref. 37). In contrast with MDSCs, no prognostic role of Tregs was observed in what is thus far the largest study of patients with advanced
melanoma published recently (38). Moreover, a sustained increase in the frequency and absolute count of lymphocytes (mainly of the CD4+ T cells) and the temporary increase of NK cell count was observed here, as previously reported for IL2-based treatments (20). The analysis of NY-ESO-1– and Melan-A–specific T cells, for which a strong prognostic impact was previously demonstrated by our group in stage IV patients (31), was inconclusive in this trial. At day 29, fewer patients had detectable Melan-A–specific T cells compared with baseline, with a tendency to decrease in frequency for these cells. Frequency and detection rate returned to levels similar to baseline after the treatment was stopped. These observations have to be interpreted with caution because they are based on a small sample size and were not confirmed by statistical testing. A significant decrease of the CD4+IFNy– T-cell level was observed after stimulation with NY-ESO-1 peptides in a comparison of day 29 with baseline, but, in contrast, NY-ESO-1–specific T cells were detectable in general in one more patient at day 29. Even if a decrease of melanoma-specific T-cell responses upon L19–IL2 treatment was postulated (which cannot be demonstrated from data presented here), the biologic significance of such a potential temporary decrease in the peripheral blood would remain unclear. The decrease in the frequency of NY-ESO-1– or Melan-A–specific CD4+ cells can be partly attributed to the significant absolute increase of CD4+ cell counts. Moreover, it might be a correlate of T-cell trafficking from the circulation to metastatic tissue in the periphery during active therapy and would be in agreement with the observation that tumor sites become strongly infiltrated by immune cells before rejection.

The assessment of response to intraläsional treatment in melanoma is very challenging, as it is not always possible to decide whether a lesion has completely disappeared or not. This is true for both cutaneous lesions, which after treatment may leave behind hyperpigmented regions of difficult classification (see, e.g., Fig. 2E), and subcutaneous lesions, as sonography or CT may still generate abnormal signals in lesions devoid of tumor cells (39), where heavy lymphocytic infiltration has occurred or necrotic/fibrotic areas have developed. This suggests that the existing response evaluation guidelines are not optimal and raises the need for the scientific community to consider new, ad hoc evaluation criteria and study endpoints for this pathology and treatment modality.

In this study, in addition to evaluating objective response, we also analyzed response separately for each individual injected lesion present at baseline. New lesions were neither injected in the trial nor evaluated in this particular assessment. At EoS, tumor burden of index/target lesions was compared with that present at baseline and used to classify the patient as CR, PR, SD, or PD. Response of nonindex/nontarget lesions, which were almost exclusively smaller than 5 mm in size and therefore difficult to assess with regard to the exact size, was only classified as either CR (complete disappearance), which was confirmed by histopathology in equivocal cases, or as non-CR at day 85 and EoS. We believe that, even in the presence of the described difficulties in unambiguously assessing responses, this additional analysis limited to injected baseline lesions gives a fair representation of the efficacy of L19–IL2 for intraläsional treatment of patients with stage III melanoma. However, it seems that DMFS (i.e., time to stage IV) is a more solid and clinically meaningful endpoint for comparing long-term efficacy of immunocytokines or other biologics in intraläsional applications to surgery or systemic treatments.

It seems reasonable to allow the treatment of all injectable lesions, including new metastases developing after the start of therapy over a defined interval of time, and to assess the duration of benefit for the patient in terms of both the absence of local progression and “time to stage IV.” A long period with local control of disease and absence of distant metastasis implies that patients will not have to undergo toxic treatments for metastatic disease, which may accelerate the emergence of mutations and resistant cancer cells (40–45).

Recently, in a preclinical study, Schwager and colleagues (46) have shown that L19–IL2 is able to induce complete tumor remissions in 100% of treated mice when administered in a single intratumoral injection in combination with L19–TNF. Interestingly, the two agents did not lead to cures when administered as single agents. The effect could be obtained only in immunocompetent mice (but not in nude mice), indicating the requirement of T cells for the complete eradication of tumors. Both of these therapeutic agents have been extensively studied individually in clinical trials (20, 21, 47, 48), and the antigen recognized by the L19 antibody has an identical sequence in mice and humans. These findings provided a rationale for a combination trial in melanoma, which is currently ongoing.

In conclusion, intratumoral L19–IL2 treatment elicited objective responses in a high percentage of lesions. The targeted form of IL2 yielded results similar to those of the untargeted cytokine, but at lower cumulative doses and with a more favorable schedule and treatment duration. In intratumorally treated patients, we observed a sustained decrease of circulating immunosuppressive MDSCs and promising data regarding the time to distant progression as well as OS.

Disclosure of Potential Conflicts of Interest
R. Weide is a consultant/advisory board member for Philogen. T.K. Eigentler is a consultant/advisory board member for Bristol-Myers Squibb. D. Neri has an ownership interest (including patents) in Philogen S.P.A. and is a consultant/advisory board member for the same. R. Gutzmer has received other commercial research support and project support from Roche, Johnson & Johnson, Novartis, and Pfizer. He has received honoraria from the speakers bureaus of Bristol-Myers Squibb, Roche, GlaxoSmithKline, Pfizer, Janssen, Merck Sero, MSD/Merck, and Amgen, and he is a consultant/advisory board member for Bristol-Myers Squibb, GlaxoSmithKline, Novartis, Roche, and MSD. C. Garbe has received commercial research support from Bristol-Myers Squibb, GlaxoSmithKline, Roche, and SOBI and is a consultant/advisory board member for Bristol-Myers Squibb, GlaxoSmithKline, MSD, Philogen S.P.A., Roche, and SOBI. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: B. Weide, D. Neri, C. Garbe
Acquisition of data (provided individuals, acquired and managed patients, provided facilities, etc.): B. Weide, T.K. Eigentler, A. Pfuggfelder, H. Zelba, A. Martens, I. Giovannoni, R. Gutzmer, J.C. Becker, C. Garbe


Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Martens, G. Pawelec, L. Giovannoni, G. Elia


References


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