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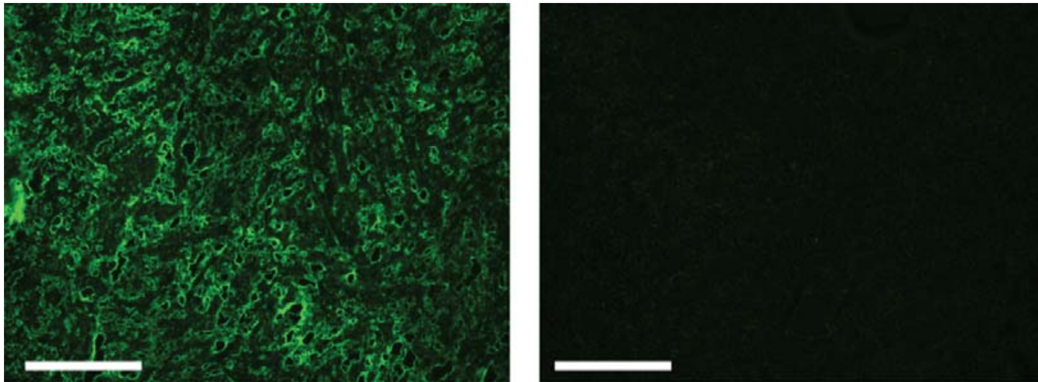
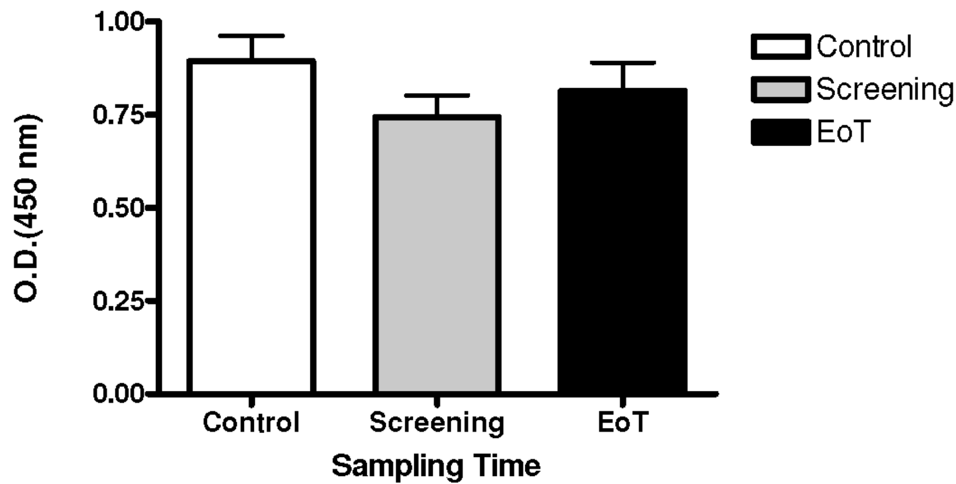
Supplementary Figure 1



Supplementary Figure 1

***Ex-vivo* detection of L19-IL2 in melanoma biopsies of study patients.** Biopsies were taken from melanoma study patients (n=2) 24 hours after injection of L19-IL2 (left panel, representative patient) and embedded in cryo-embedding compound. Sections were cut and fixed in 4% PFA. As negative control, tissue sections were taken from biopsies of melanoma patients (n=2) that were not previously treated with L19-IL2 (right panel). L19-IL2 was detected using rabbit anti-L19-IL2 serum, followed by Alexa Fluor 488 goat anti-rabbit antibody (Molecular Probes, Leiden, The Netherlands). Sections were mounted with fluorescent (DAKO, Glostrup, Denmark) and images were obtained using an Axioskop 2 mot plus (Carl Zeiss). Scale bars correspond to 100µm. The green fluorescence indicates presence of L19-IL2 within the melanoma tissue of a patient previously treated with L19-IL2, thus proving targeting of L19-IL2 to melanoma-infiltrated tissues.

Supplementary Figure 2



Supplementary Figure 2

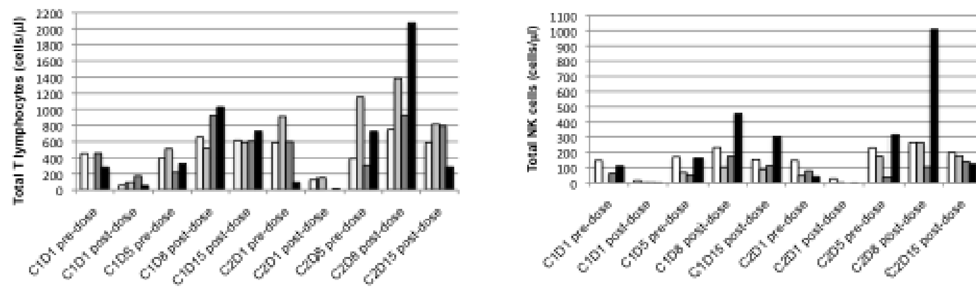
Analysis of the generation of human anti-fusion protein antibodies (HAFA) via a rabbit antiserum competition experiment. Two New Zealand rabbits were immunized at day 0 with 0.28 mg and at day 14 with 0.56 mg of L19-IL2 protein with incomplete Freund's adjuvant (subcutaneous injection, Zootecnica Il Gabbiano, Siena, Italy). At day 28 the two rabbits were blooded and sera were pooled, aliquoted and frozen at - 80°C. Nunc Maxisorp plates were coated with 10 µg/ml of L19-IL2 in PBS and incubated overnight at 4°C. The plates were washed four times with PBS and then blocked with 2% Non Fat Powder Milk in PBS (2 % MPBS) for 2 hours at 30°C. After washing the plate, 50 µL of the patient serum samples collected at screening day and at the end of treatment, were applied on plates at 1:100 dilution in 2% MPBS together with 50 µL of serum from rabbits immunized against IL19-IL2 diluted 1:10000 in 2% MPBS. Samples were incubated for 45 minutes at 30°C and then washed four times with 0.1% Tween 20 in PBS (PBS-T) and four times with PBS. Wells were incubated for 45 minutes at 30°C with a goat-anti-rabbit IgG HRP-conjugated antibody (Sigma), diluted 1:3000 in 2% MPBS. Development was performed after washing with PBS-T and PBS by using a POD substrate (Roche). The reaction was stopped with 1 M sulfuric acid (BDH Prolabo) and read at 450 nm.

Average values calculated on the samples at screening day (grey bar) and on the samples at end of treatment (EOT, black bar) from six patients are shown in the graph. The control (white bar) corresponds to the signal obtained with rabbit antiserum, in the absence of human serum.

The assay used for this experiment was validated by using a guinea pig serum immunized with L19-IL2. The guinea pig serum is able to produce a 17% inhibition of the rabbit serum when diluted 1:100 and a 49% inhibition when used at 1:10 dilution.

Supplementary Figure 3

A



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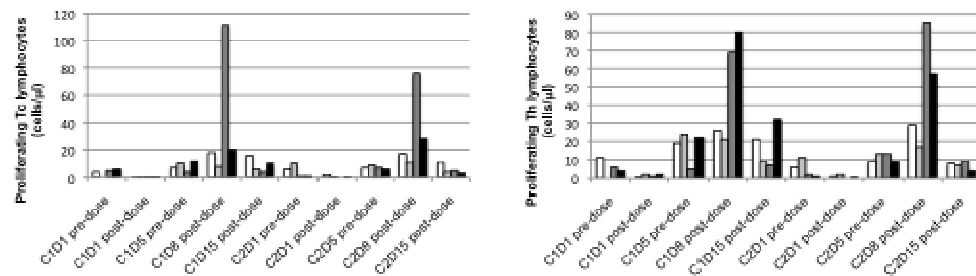
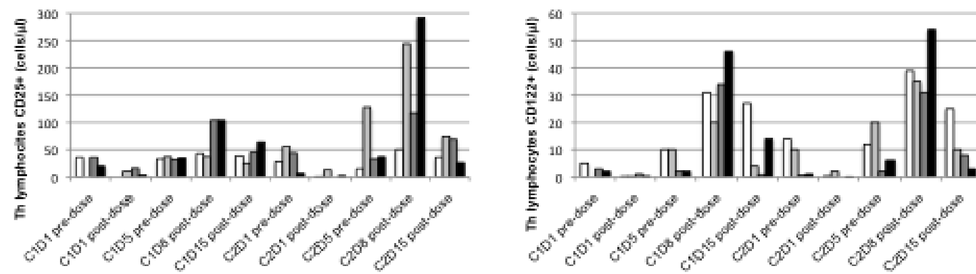


C



Supplementary Figure 3

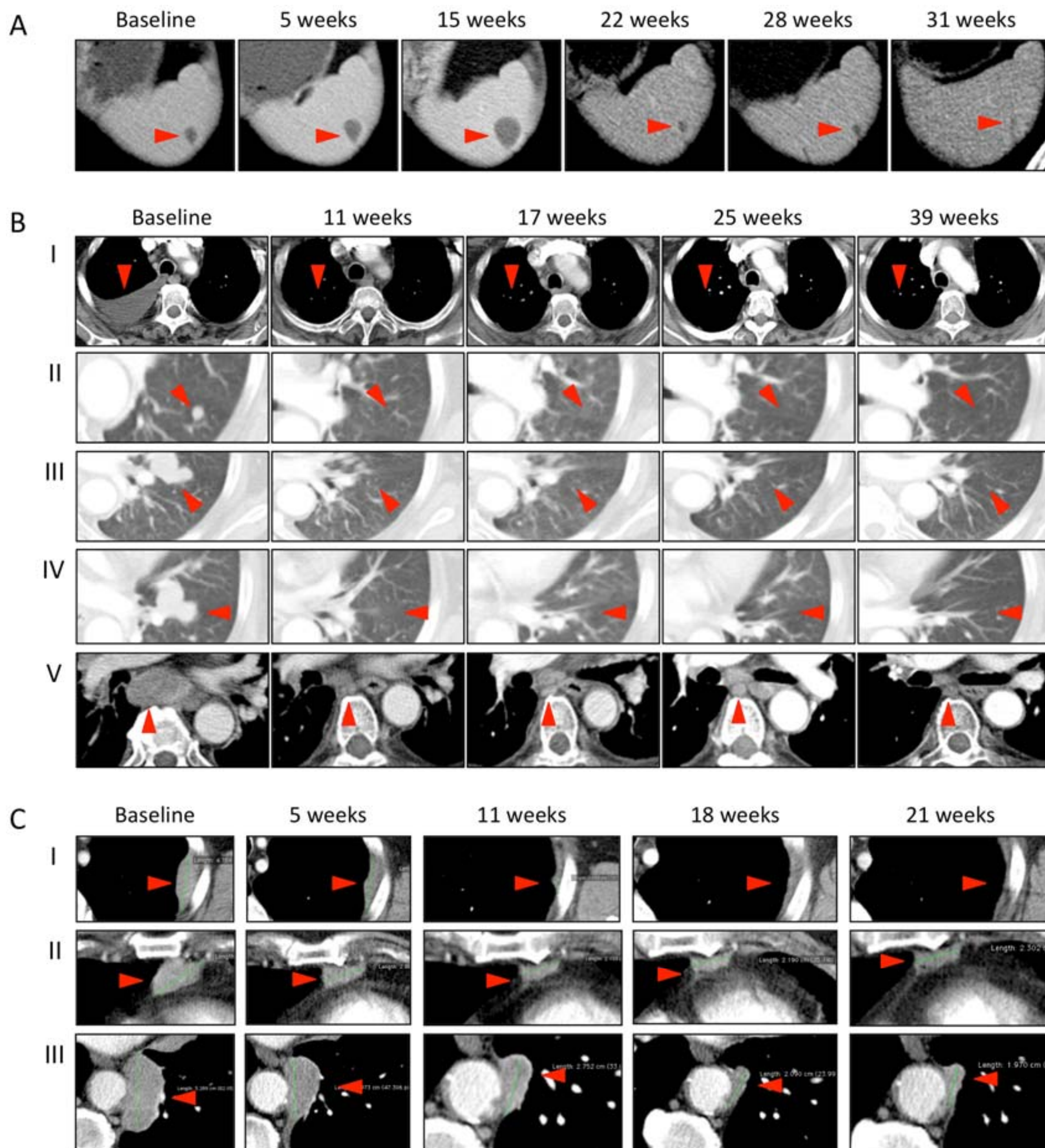
(A) Absolute number (cells/µl) of total T lymphocytes and total NK cells in four different patients (coded by different column shading), during the first two cycles of treatment; (B) Absolute number (cells/µl) of proliferating Tc lymphocytes (CD3+/CD8+/CD71+) and of proliferating Th lymphocytes (CD3+/CD4+/CD71+) in four different patients, during the first two cycles of treatment; (C) Absolute number (cells/µl) of CD25+ and CD122+ Th lymphocytes in four different patients, during the first two cycles of treatment.

The immunophenotyping analysis was performed by Accelera S.r.l., Italy. The study of the relative distribution of lymphocytes subsets in erythrocytes-lysed-

and-then-washed whole peripheral blood samples was performed by flow cytometry following a direct immunofluorescence staining method. The absolute number of each lymphocytes subset was obtained combining data from the flow cytometer (FACSCalibur Flow Cytometer, BD/Bioscience) and the hematology analyzer (ADVIA™ 120, Siemens). Four tubes per sample were prepared with different antibody mixtures in order to evaluate four sets of parameter: $mIgG_1$-FITC/$mIgG_1$-PE/$mIgG_1$-PerCP/$mIgG_2$-APC, CD4/CD8/CD3/CD71, CD19/CD56/CD3/CD71, and CD4/CD122/CD3/CD25. 100µl of TransFix (Cytomark) treated blood were dispensed in labeled tubes and incubated with the appropriate amount of antibody mixture for 20 minutes at RT in the dark. Red cells were lysed by incubation with ammonium chloride (Ammonium Chloride Solution, StemCell Technologies) for 15 minutes at RT in the dark, followed by a 5 minutes centrifugation at 400 g. After a washing step, 500µl of Stain Buffer (1x PBS, 2% FBS, 10mM sodium azide) were added to the stained sample and immediately acquired to the cytometer. A minimum of 5000 events in the lymphocytes region was collected.

The results show the typical transient pharmacodynamic effects of IL2 regarding stimulation of peripheral T lymphocyte subsets.

Supplementary Figure 4



Supplementary Figure 4 – CT documentation of responses. (A) CT images of the splenic lesion, indicated by the red arrows, of patient No. 6 (best response -31.25 and -33.33%, according to the clinical and central assessments, respectively) are shown. CT images were taken at baseline and at the following time-points indicated in figure. **(B)** CT images of a pleural effusion (I), three coin lesions (II, III, and IV), and a subcarenal lymph node (V) of patient No. 7 (best

response -100%) are indicated by red arrows. CT images were taken at baseline and at the following time-points indicated in figure. **(C)** CT images of a pleural thickening at the left thoracic wall (I), a retrosternal solid mass (II), and a paraortic solid mass (III) of patient No. 17 (best response -34.48% and -52.87%, according to the clinical and central assessments, respectively) are indicated by red arrows. CT images were taken at baseline and at the following time-points indicated in figure.

Supplementary Table 1

Patient N°	BOR (%)[a]	Duration of response (days)	Site of disease									Prior treatments		BRAF status	NRAS status	KIT status
			lung	soft tissue	lymph nodes	bone	intestine	liver	spleen	adrenal gland	kidney	chemo-therapy	radio-therapy			
7	-100	604[b]	✓	✓	✓							✓		wt	wt	wt
8	-67.54	7		✓	✓								✓	V600E	wt	wt
21	-56.37	325	✓	✓	✓									wt	Q61L	wt
20	-53.85	34			✓									n.a.	n.a.	n.a.
17	-52.87	82	✓	✓										n.a.	n.a.	n.a.
19	-45.45	86	✓		✓	✓								n.a.	n.a.	n.a.
26	-39.04	222	✓		✓									V600E	wt	wt
28	-38.8[c]		✓					✓						wt	Q61K	wt
13	-38.1	146		✓	✓									n.a.	n.a.	n.a.
6	-33.33	69		✓					✓			✓	✓	V600E	wt	wt
11	-24.44		✓		✓					✓		✓		n.a.	n.a.	n.a.
22	-20.27		✓		✓									wt	Q61R	wt
9	-14.26		✓	✓	✓							✓		n.a.	n.a.	n.a.
12	-6.51		✓					✓	✓			✓		n.a.	n.a.	n.a.
25	-5.71			✓	✓		✓			✓				V600E	wt	wt
31	-5.17						✓		✓					V600K	wt	wt
1	-1.58		✓	✓	✓					✓		✓	✓	n.a.	n.a.	n.a.
24	0		✓	✓	✓				✓	✓			✓	V600K	wt	L641L/F
18	3.33		✓	✓	✓	✓	✓							wt	wt	wt
16	5.7				✓			✓						n.a.	n.a.	n.a.
30	6.16				✓	✓			✓	✓				wt	wt	wt
29	6.68		✓		✓			✓						n.a.	n.a.	n.a.
15	7.39		✓	✓	✓					✓	✓	✓		n.a.	n.a.	n.a.
3	11.42		✓	✓	✓	✓						✓	✓	V600E	wt	wt
10	13.44		✓		✓	✓		✓				✓	✓	wt	wt	wt
23	15.18			✓	✓									n.a.	n.a.	n.a.
27	20.61		✓						✓					wt	Q61R	wt
4	29.75			✓	✓							✓	✓	V600E	wt	wt
14	58.54		✓	✓	✓									V600E	wt	wt
2	n.a.		✓	✓	✓							✓	✓	n.a.	n.a.	n.a.
5	n.a.		✓	✓	✓							✓	✓	n.a.	n.a.	n.a.
32	n.a.		✓											wt	Q61H	wt

A) Best overall response according to central rariological review
B) The date of last assessment is reported, however response is still on-going
C) The indicated percentage of target lesions shrinkage was accompanied by appearance of new lesions

Supplementary Table 1

Baseline characteristics and correlation to best overall response – The best overall responses (BOR) defined as the largest shrinkage in the sum of diameters of target lesions at any moment in time compared to baseline calculated by the central review, is indicated in the table. The table reports on the duration of the response, the sites of disease at baseline, prior treatments, and the BRAF, NRAS, and KIT mutational status of every patient.

Supplementary Material

Inclusion and exclusion criteria

Adult patients with histologically or cytologically confirmed unresectable metastatic (stage IV) non-uveal melanoma were included if following features applied: measurable disease defined as at least one lesion that could be accurately and serially measured per Response Evaluation Criteria in Solid Tumors (RECIST), cutaneous lesions measuring at least 1 cm were considered measurable; prior chemotherapy including dacarbazine for metastatic melanoma were allowed if treatment had completed > 6 months prior to study entry; fewer than three organs involved or cutaneous and/or subcutaneous metastases only; an Eastern Cooperative Oncology Group (ECOG) performance status of ≤ 2; a life expectancy of at least 12 weeks; absolute neutrophil count > 1500/µL; platelets 100000/µL; hemoglobin > 9.0 g/dL; total bilirubin < 2.0 mg/dL; alanine aminotransferase or aspartate aminotransferase ≤ 2.5 times the upper limit of normal (ULN), ≤ 5 x ULN for patients with hepatic involvement with tumor; LDH < 2 xULN; serum creatine < 1.5 x ULN. All acute toxic effects (excluding alopecia) of any prior therapy were to be resolved to National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events (CTCAE v3.0) Grade ≤ 1. Female patients of childbearing potential had to be negative for the serum pregnancy test at screening visit; moreover, they had to agree to use adequate contraceptive methods beginning at the screening visit and continuing until 3 months following last treatment with study drug.

Exclusion criteria: primary ocular melanoma; evidence of brain metastasis by computed tomography (CT) scan in the two months prior to study entry; previous or concurrent cancer that was distinct in primary site or histology from the cancer being evaluated in the study; history of HIV infection or chronic hepatitis B or C; presence of active infections (*e.g.*, requiring antimicrobial therapy) or other severe concurrent disease; recent history of acute or sub-acute coronary syndromes; heart insufficiency; uncontrolled hypertension; ischemic peripheral vascular disease; severe diabetic retinopathy; active autoimmune disease; history of organ

allograft or stem cell transplantation; recovery from major trauma; surgery and anti-tumour therapy within 4 weeks prior to study treatment; known history of allergy to IL2 and dacarbazine; breast-feeding female; previous *in vivo* exposure to monoclonal antibodies for biological therapy within 6 weeks prior to study treatment; previous dacarbazine treatment within 6 months prior to study entry; growth factors or immunomodulatory agents within 7 days of the administration of study treatment; corticosteroids or other immunosuppressant drugs that had to be taken on a long-term basis.

Screening visit procedures and assessments

At the screening visit, following data were collected and procedures performed: demographic data, medical and surgical history, including date and description of all prior anti-neoplastic procedures, documentation of allergies and any previous disease or surgery; inclusion and exclusion criteria as well as ECOG performance status were checked; tumor evaluation (according to RECIST 1.0), ECG, multiple-gated acquisition (MUGA) scan or echocardiography, assessment of baseline findings CTCAE version 3.0, assessment of concomitant medications and complete physical examination were performed; vital signs, height, weight were checked and body surface area was calculated; blood samples for standard safety laboratory examination, serum pregnancy test and HAFA detection as well as urine samples were taken; tumor biopsy on accessible cutaneous or sub-cutaneous lesions was performed.

Pharmacokinetic analysis of L19-IL2, dacarbazine and AICA in patients' serum

For pharmacokinetic analysis of L19-IL2, dacarbazine and 5-aminoimidazole-4-carboxamide (AICA), the main dacarbazine metabolite, blood samples were collected on day 1 of the first and second treatment cycle at each of the following time points: prior to L19-IL2 infusion, 30 and 55 minutes after start of L19-IL2 infusion, 30 and 55 minutes after start of dacarbazine infusion. Moreover, additional samples were collected after the end of the dacarbazine infusion at following time intervals: between 15 and 45 minutes, 45 and 75 minutes, 2.5 and 4.5 hours, 5.5 and 8.5 hours, and 18 and 22 hours. Serum samples were prepared at the study site and stored at –20°C until analysis. Determination of L19-IL2 in human serum was performed by DELFIA time-resolved Fluoroimmunassay (TR-FIA, Perkin Elmer). Briefly, Nunc Maxisorp ELISA Plates were coated with 20 µg/mL of the recombinant EDB domain of fibronectin (named 7B89) in PBS at 4°C, overnight. Plates were washed for 6 times with DELFIA Washing Buffer (Perkin Elmer) and blocked for 1 hour at room temperature (RT) with 300 µL of blocking solution (2% of ECL Advanced Blocking Agent, Amersham, in PBS supplemented with 1% Tween 20, Sigma). After washing, samples were diluted 1:4 in DELFIA Assay Buffer (Perkin Elmer) and were added to the plates (200 µL/well) and incubated for 2 hours at RT, with gentle shaking. Plates were washed and incubated 2 hours at RT with a 0.5 ng/µL solution of the Eu-labeled anti-human IL2 antibody (Perkin Elmer) in DELFIA Assay Buffer (200 µL/well). After washing, the plates were incubated with 200 µL/well of DELFIA Enhancement Solution (Perkin Elmer) for 5 minutes at 500 rpm in the dark and immediately read using the Victor 2V plate reader (Wallac Perkin Elmer).
Dacarbazine and AICA determination was performed by LC/MSMS. After protein precipitation, samples were analyzed by a HPLC coupled with a tandem mass spectrometer (Applied Biosystems MDS Sciex API 3200). Samples containing dacarbazine were light screened during the analysis.