Supplementary appendix

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Supplement to: Heery CR, O'Sullivan-Coyne G, Madan RA, et al. Avelumab for metastatic or locally advanced previously treated solid tumours (JAVELIN Solid Tumor): a phase 1a, multicohort, dose-escalation trial.

This appendix is a supplement to: Heery CR, O'Sullivan-Coyne G, Madan RA, Cordes L, Rajan A, Rauckhorst M, et al., Avelumab for metastatic or locally advanced previously treated solid tumours: a phase 1a dose-escalation cohort of the multicohort JAVELIN Solid Tumor trial

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Table of contents

Supplementary Methods: Cytokine release by human peripheral blood mononuclear cells	Page 2
Table S1. In vitro cytokine release by pooled PBMCs in the presence of LPS	Page 3
Table S2. In vitro cytokine release by pooled PBMCs in the presence of PHA	Page 3
Figure S1. Serum concentrations of IFN-γ, IL-6, and TNF-α for patients in the avelumab 10 and 20 mg/kg dose groups	Page 4
Figure S2. Evidence of clinical activity with avelumab in phase 1 dose-escalation cohort	Page 10
Table S3. Evaluation of standard and PD-L1–expressing immune cell subsets at baseline, day 15, day 43, and day 127	Page 11

Supplementary Methods: Cytokine release by human peripheral blood mononuclear cells

Studies were conducted to assess the potential of avelumab to induce *in vitro* cytokine release from human peripheral blood mononuclear cells (PBMC) obtained from healthy donors who were exposed to avelumab in vitro in the presence of lipopolysaccharide (LPS) or phytohaemagglutinin (PHA).

In a first experiment, isolated PBMCs from eight male and eight female human donors were pooled and tested separately. Cells were treated with avelumab concentrations ranging from 0·01–14 µg/mL. LPS 10 µg/mL and alemtuzumab (Campath) 30 µg/mL were used as positive controls. The following Th1 and Th2 cytokines were measured using xMAP Luminex multiplex assay after 6 and 24 hours of exposure using Luminex 10-plex and single-plex assay kits: granulocyte macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, tumor necrosis factor α (TNF-α), monocyte chemotactic protein 1 (MCP-1), and IFN-inducible protein 10 (IP-10). As expected, positive control compounds induced release of pro-inflammatory cytokines by PBMCs. For example, LPS induced release of multiple cytokines (IFN-γ, IL-1β, IL-6, IL-8 and TNF-α) by PBMC from both male and female donors, with 100–200-fold increases over baseline for TNF-α and IL-6 (Table S1). Alemtuzumab induced a modest seven-fold increase in release of TNF-α compared with baseline. Exposure to avelumab for 6 hours resulted in weak responses, with only a three-fold increase in TNF-α release observed in male PBMC. Effects were greater after 24 hours compared with 6 hours of exposure, with TNF-α release six-fold and 11-fold higher than baseline in male and female PBMC, respectively. IL-6, IP-10 and MCP-1 showed a two-fold increase over baseline in female PBMC only.

In a second experiment, cytokine release profiles were assessed in PBMC from 16 human donors (eight male and eight female) that had been prestimulated with PHA. In these studies, avelumab was immobilised to 24 well polystyrene microtitre plates using a wet pre-coating technique (for method descriptions, refer to Findlay et al. 2010 and Stebbings et al. 2007), and avelumab concentrations of 2–2000 µg/mL were tested. LPS 10 µg/mL and alemtuzumab 300 µg/mL were used as positive controls. Table S2 shows release of IL-6 and TNF-α, important cytokines involved in acute phase reactions and other immune reactions. Avelumab caused a notable release in IL-6 and TNFα, with strong increases observed at the lowest avelumab concentration tested (i.e. 2 µg/mL). Different profiles were obtained in activated PBMCs from male vs female donors, in that alongside the IL-6/TNF-α response, GM-CSF and IL-10 were also induced; IL-10 was likely a direct response to the initial acute phase response. Weaker and sporadic increases in IL-8 and IL-1β were also observed in PBMCs from both sexes.

Based on these results, the risk of systemic cytokine release could not be excluded and further investigations to clinically evaluate this risk were conducted in patients enrolled in the phase 1a dose-escalation trial of avelumab. Cytokine analysis was performed to evaluate levels of RANTES (regulated on activation, normal T cell expressed and secreted) and various interleukins (including IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, and IL-17), TNF-α, and IFN-γ. Serum samples used for analysis were obtained on study days 1 (baseline), 3 (48 ± 6hr), 8, 15, 29, 43, 45, 50 and 85 in the 1, 3, 10, and 20 mg/kg treatment cohorts. Figure S1 shows levels of IFN-γ, IL-6 and TNF-α for the 10 and 20 mg/kg dose groups. TNF-α was transiently increased to 11·0 ± 10·5 pg/mL (mean ± standard deviation) in 10 mg/kg cohort samples on Day 8, and IFN-γ was transiently increased to 2·7 ± 1·9 pg/mL in the 10 mg/kg cohort samples on Day 3 and returned to baseline levels of 1·9 ± 1·6 pg/mL on Day 15. Concentrations of IFN-γ were low overall and did not exceed 6 pg/ml in individual subjects throughout the dosing interval. Overall, cytokine levels varied in serum with dose and time, but no clear pattern emerged.

References

Findlay L, Eastwood D, Stebbings R, et al. Improved in vitro methods to predict the in vivo toxicity in man of therapeutic monoclonal antibodies including TGN1412. J Immunol Methods 2010; **352**: 1–12. doi: 10.1016/j.jim.2009.10.013.

Stebbings R, Findlay L, Edwards C, et al. "Cytokine storm" in the phase I trial of monoclonal antibody TGN1412: better understanding the causes to improve preclinical testing of immunotherapeutics. J Immunol 2007; **179**: 3325–31. doi: 10.4049/jimmunol.179.5.3325.

Table S1. *In vitro* cytokine release by pooled PBMCs (500,000 cells/well) from male (M) and female (F) healthy donors following treatment with LPS 10 μg/mL, alemtuzumab (Campath) 30 μg/mL, or avelumab 0·01–14 μg/mL. For avelumab, the highest fold-increase observed is reported. Hyphens (-) indicate no change in cytokine level from baseline.

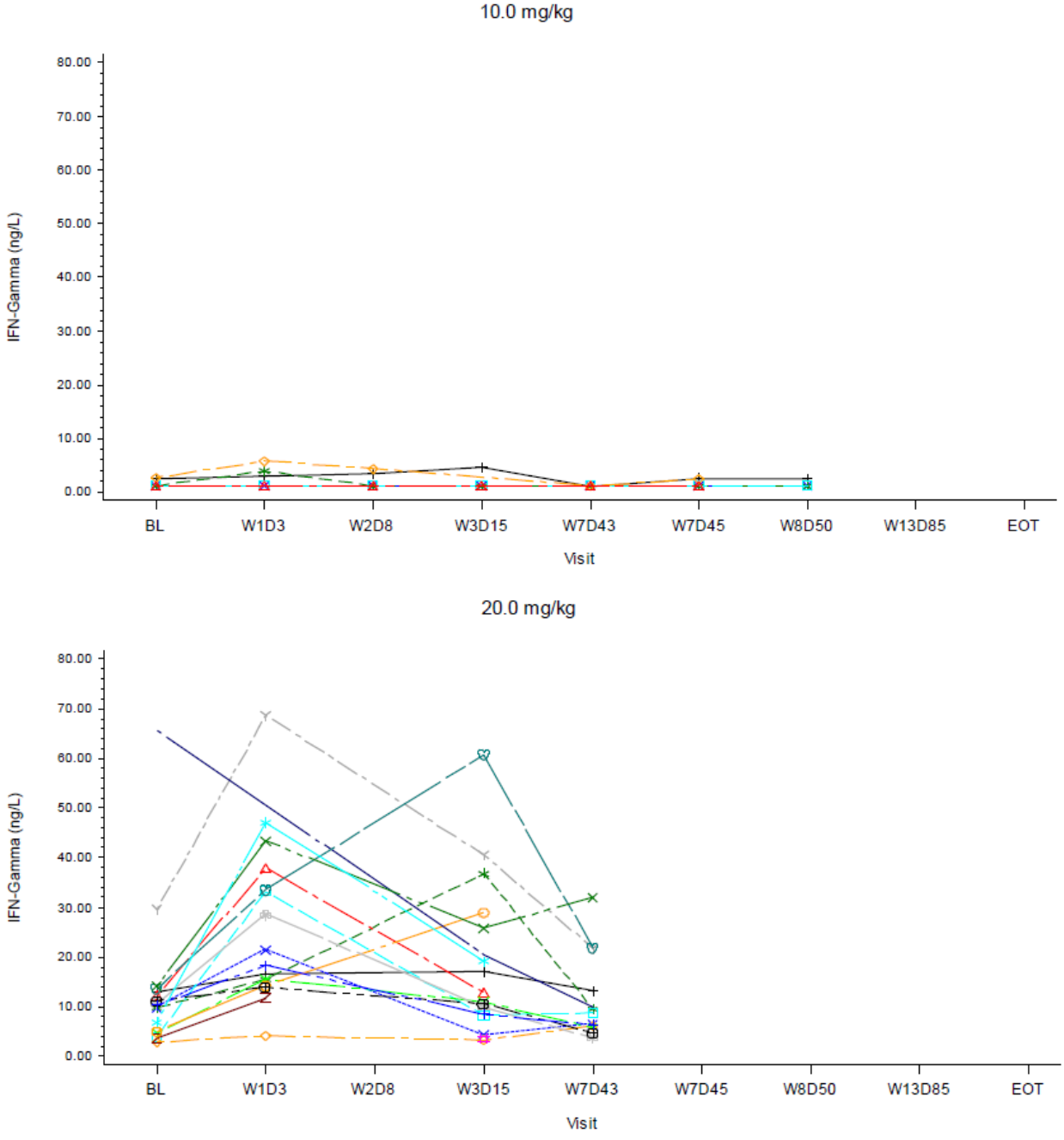
	Fold-increase over baseline															
	6 hours								24 hours							
Cytokine	Vehicle* alone		LPS (10 μg/mL)		Alemtuzumab (30 μg/mL)		Avelumab (0·01–14 μg/mL)		Vehicle* alone		LPS (10 μg/mL)		Alemtuzumab (30 μg/mL)		Avelumab (0·01–14 μg/mL)	
	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
GM-CSF	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IFN-γ	-	-	-	-	-	-	-	-	-	-	20	4	-	-	-	-
IL-10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IL-1β	-	-	7	6	-	-	-	-	-	-	8	2	-	-	-	-
IL-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IL-4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IL-5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
IL-6	-	2	70	58	-	-	-	-	-	-	200	140	-	-	-	2
IL-8	-	2	4	2	2	2	-	-	-	-	-	-	-	-	-	-
IP-10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
MCP-1	-	2	-	-	2	2	-	-	-	-	-	-	2	3	-	2
TNF-α	-	5	130	130	7	6	3	-	-	-	110	80	4	3	6	11

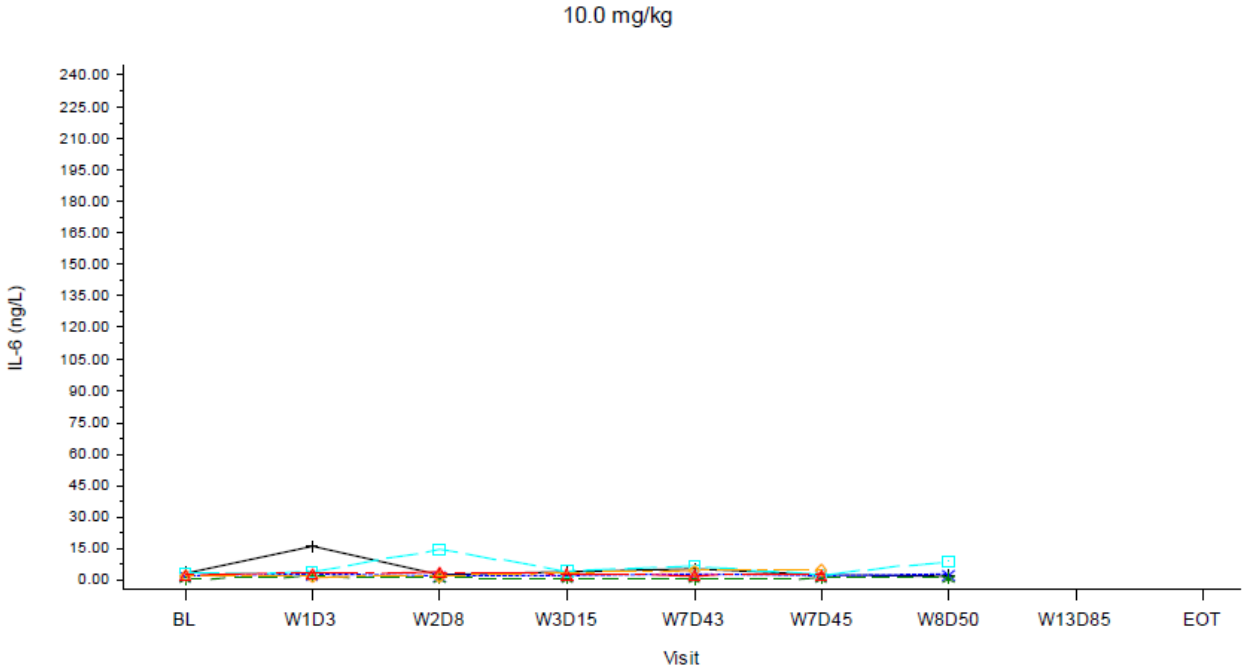
* Vehicle per mL of water was D-mannitol (51 mg), glacial acetic acid (0·6 mg), polysorbate 20 (0·5 mg), and sodium hydroxide (0·3 mg).

Table S2. *In vitro* cytokine release by pooled PBMCs (500,000 cells/well) from male (M) and female (F) healthy donors (mean and standard deviation) prestimulated with phytohaemagglutinin (PHA), and treated with LPS 10 μg/mL, alemtuzumab (Campath) 300 μg/mL, or avelumab 2–2000 μg/mL immobilised to microtitre plates. For avelumab, the highest fold-increase observed is reported.

24-hour prestimulation with PHA (2·5 μg/mL)		Fold-increase over baseline											
		6 hours						24 hours					
Cytokine		LPS (10 μg/mL)		Alemtuzumab (300 μg/mL)		Avelumab (2–2000 μg/mL)		LPS (10 μg/mL)		Alemtuzumab (300 μg/mL)		Avelumab (2–2000 μg/mL)	
		M	F	M	F	M	F	M	F	M	F	M	F
IL-6	Mean	79	83	4	37	6	7	84	305	5	144	18	9
	SD	55	80	2	32	3	3	49	251	3	172	9	4
TNF-α	Mean	66	10	10	4	11	36	31	3	29	1	30	62
	SD	82	7	3	3	7	13	52	1	13	1	20	14

Figure S1. Serum concentrations of IFN-γ, IL-6, and TNF-α for patients in the avelumab 10 and 20 mg/kg dose groups, shown as line plots of individual patients and box plots.

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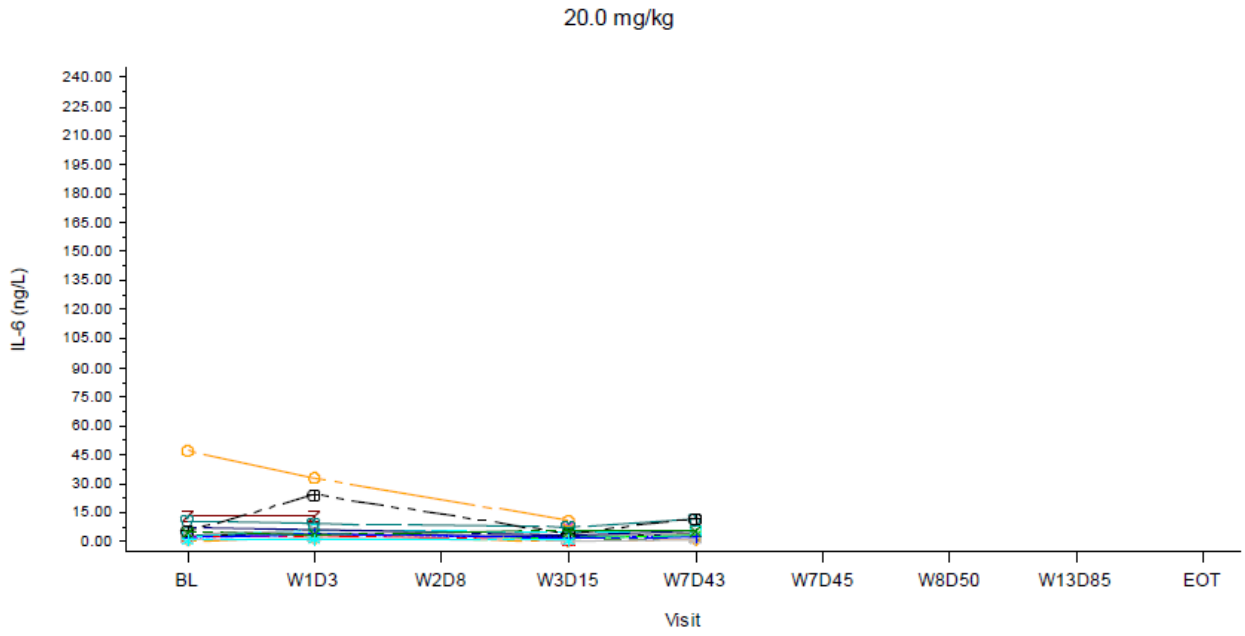
IL-6 (ng/L)
240.00
225.00
210.00
195.00
180.00
165.00
150.00
135.00
120.00
105.00
90.00
75.00
60.00
45.00
30.00
15.00
0.00
BL
W1D3
W2D8
W3D15
W7D43
W7D45
W8D50
W13D85
EOT
Visit


20.0 mg/kg
IL-6 (ng/L)
240.00
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W7D43
W7D45
W8D50
W13D85
EOT
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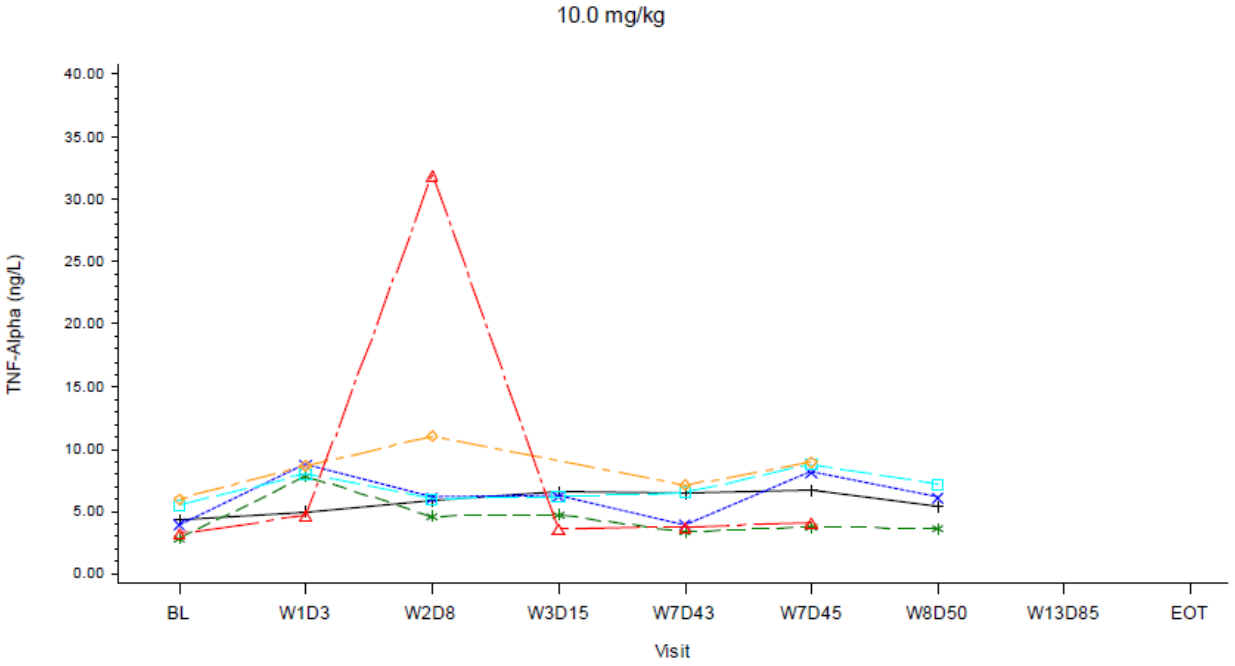
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TNF-Alpha (ng/L)
40.00
35.00
30.00
25.00
20.00
15.00
10.00
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BL
W1D3
W2D8
W3D15
W7D43
W7D45
W8D50
W13D85
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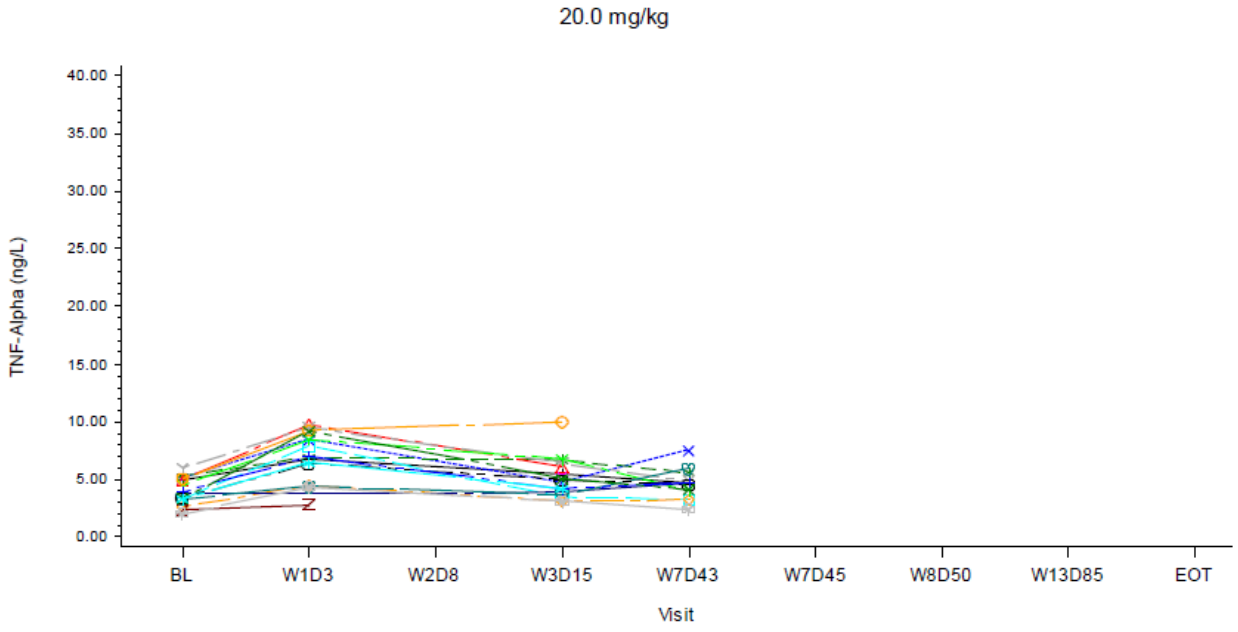
20.0 mg/kg
TNF-Alpha (ng/L)
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W3D15
W7D43
W7D45
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W13D85
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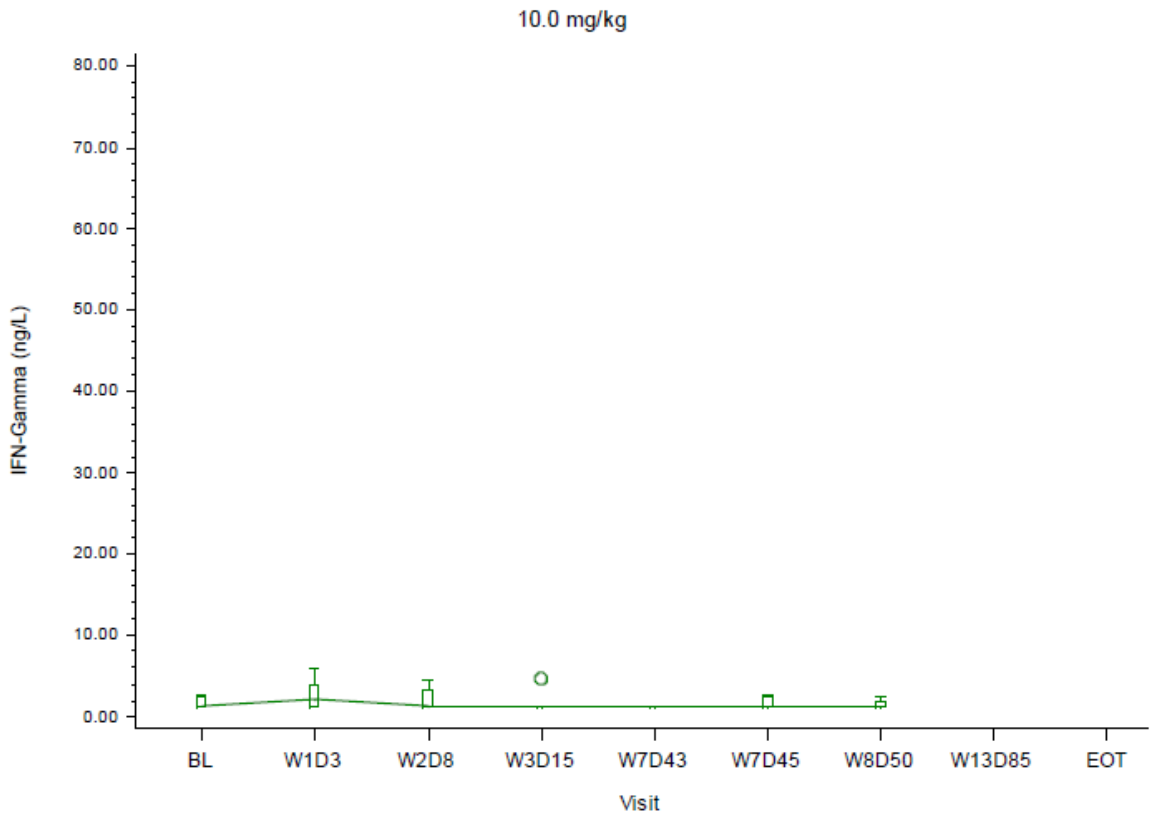
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IFN-Gamma (ng/L)
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W13D85
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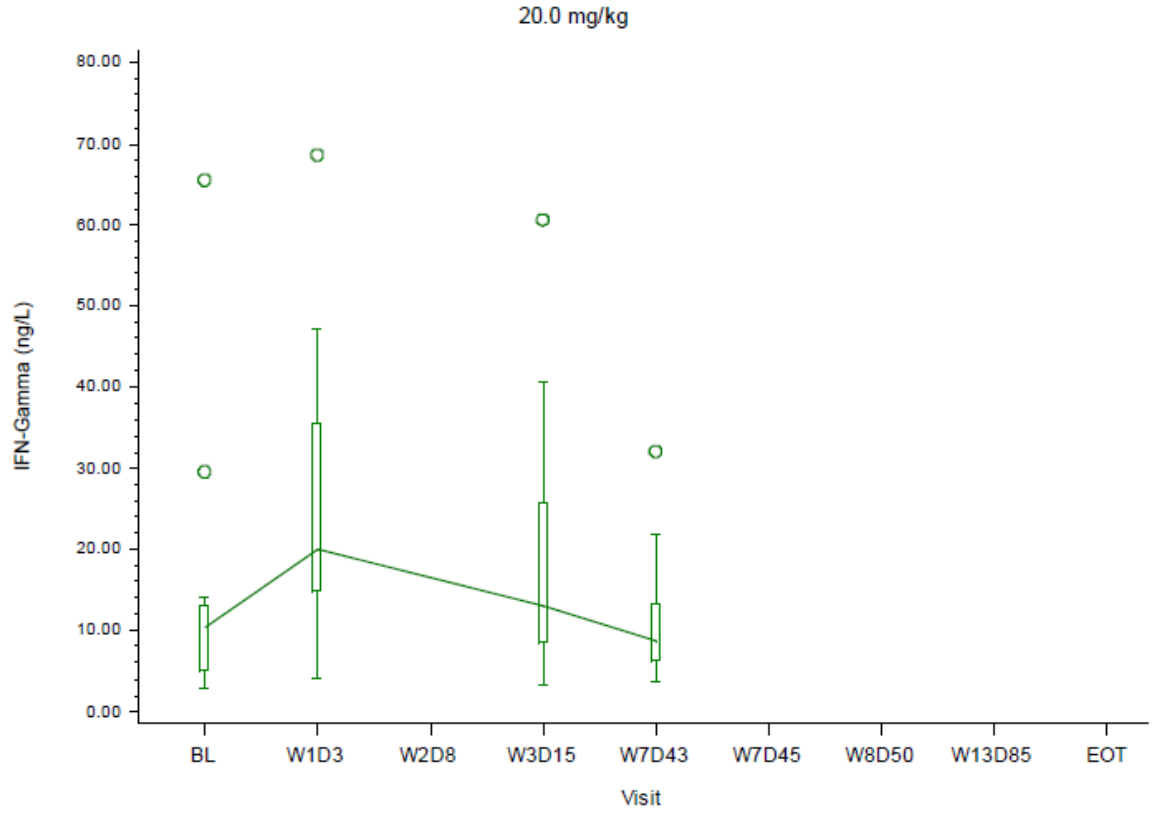
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IFN-Gamma (ng/L)
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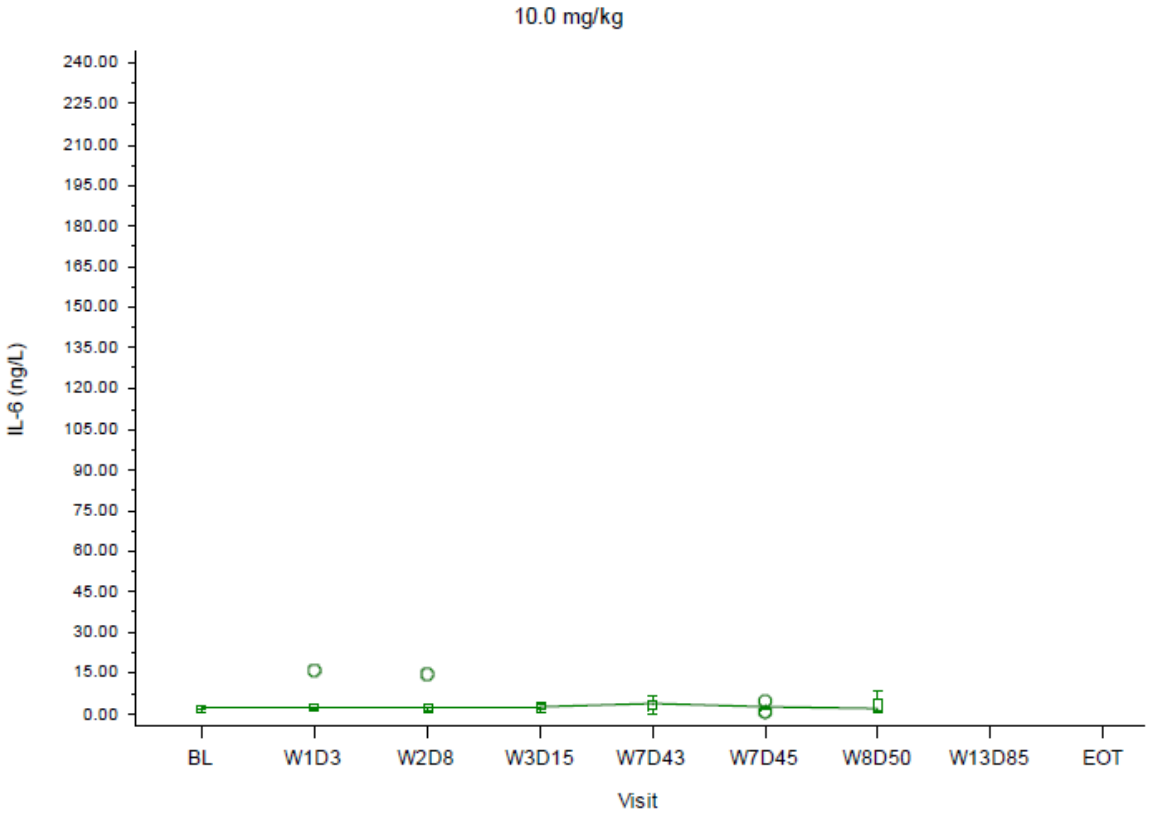
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IL-6 (ng/L)
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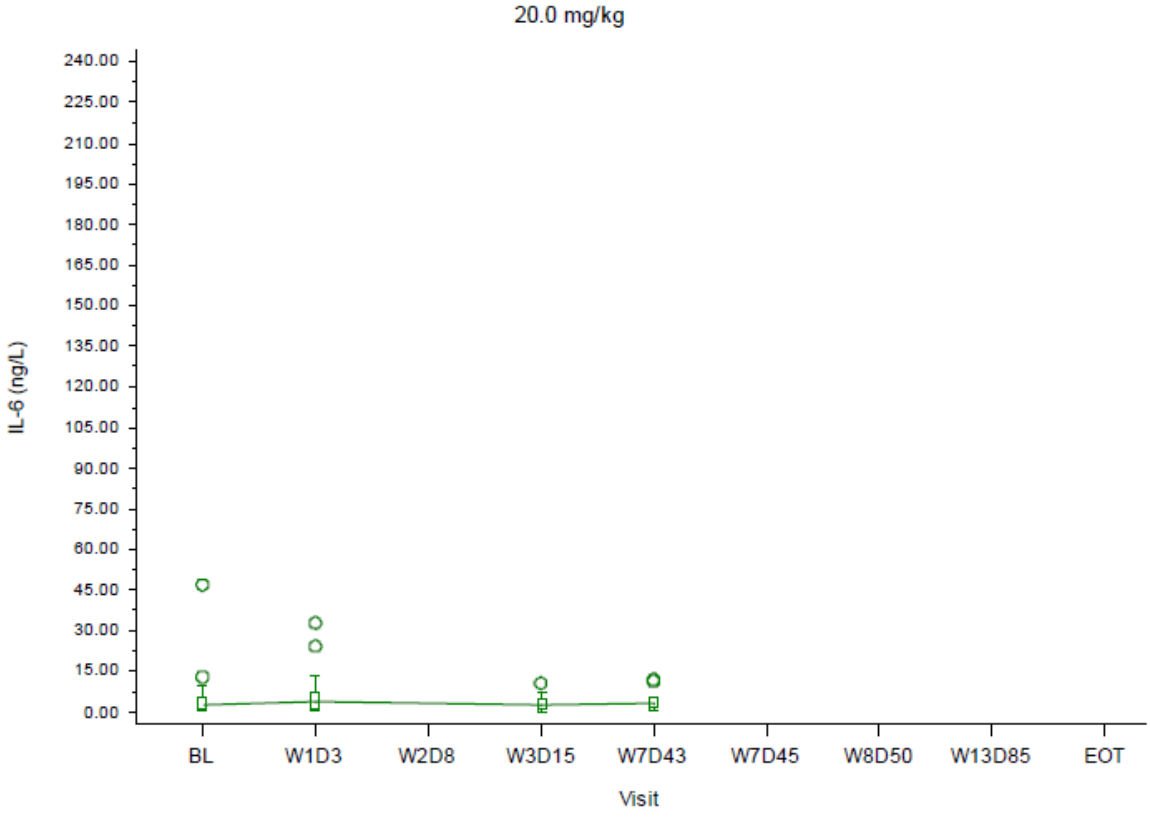
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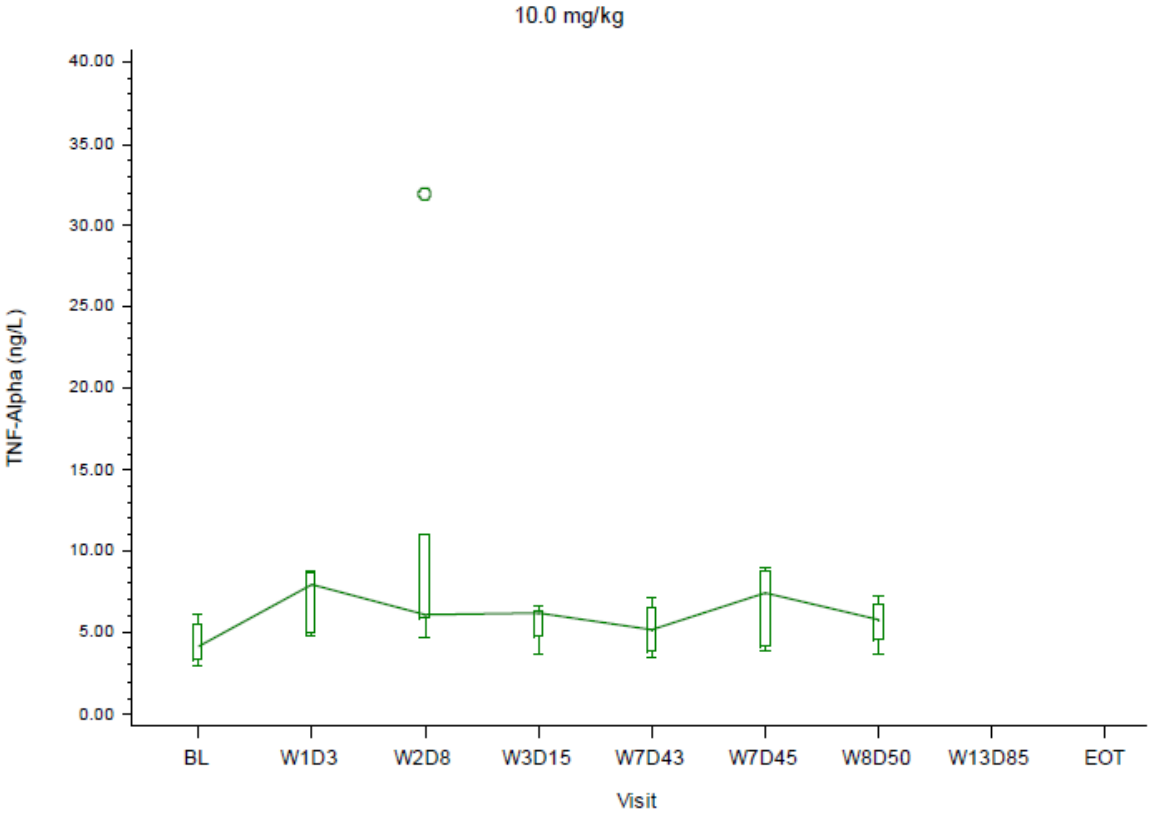
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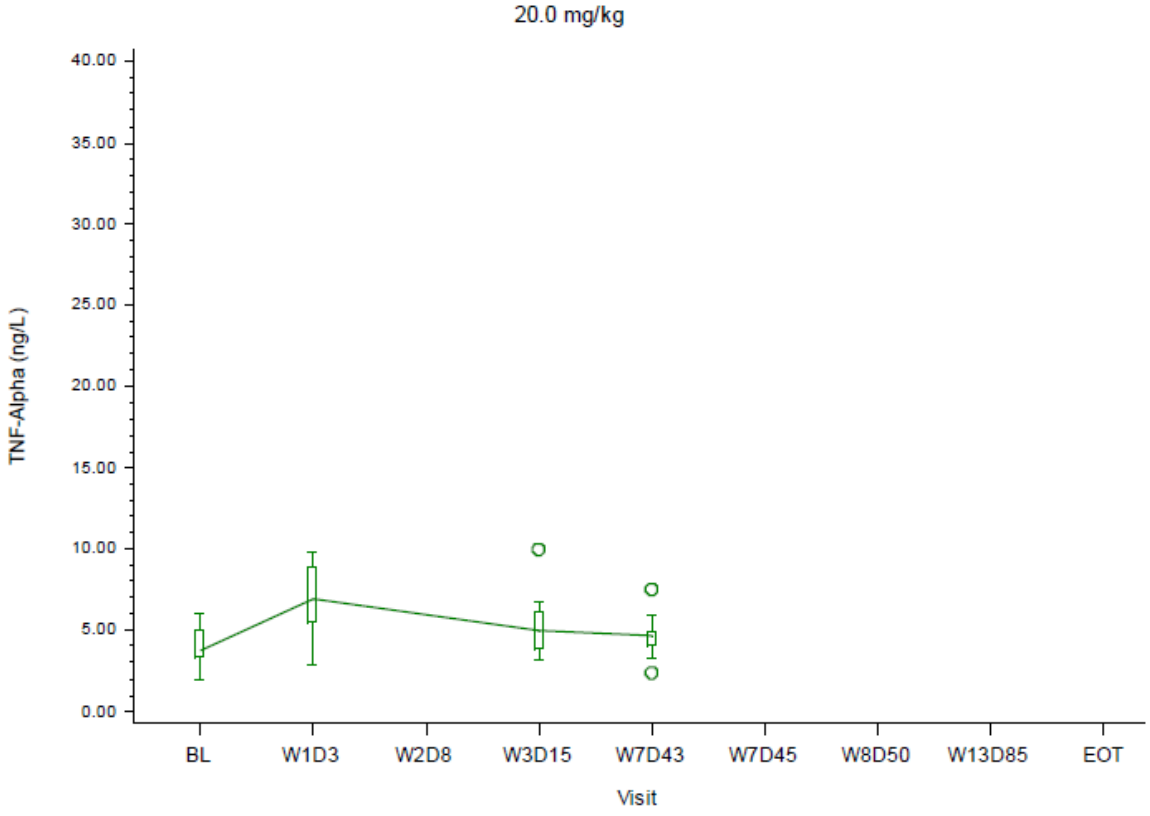
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BL
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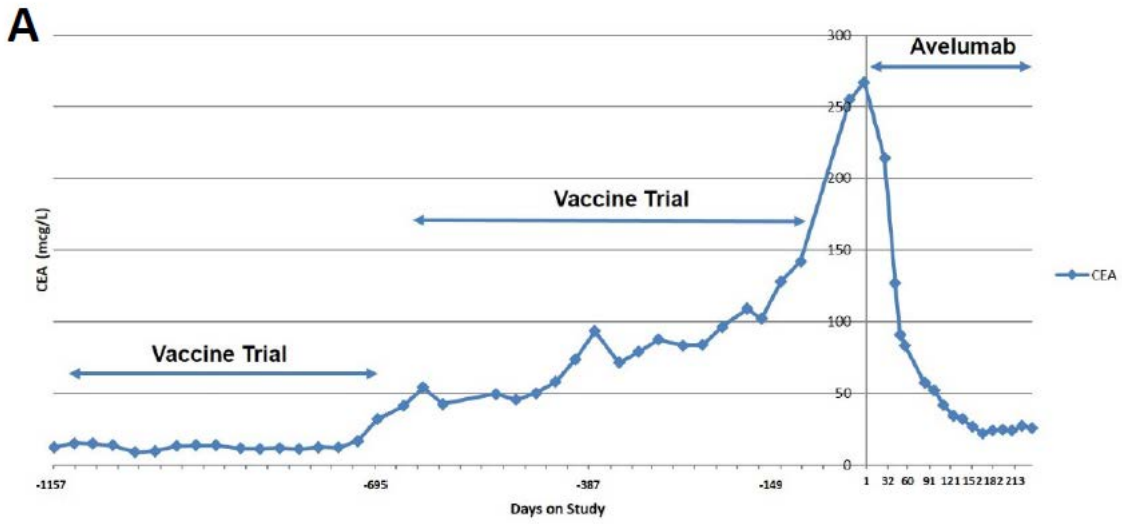
Figure S2. Evidence of clinical activity with avelumab in phase 1 dose-escalation cohort

(A) Serum carcinoembryonic (CEA) values in a patient with metastatic colorectal cancer and rising CEA at study enrolment decreased significantly over a 5-month period following initiation of avelumab therapy at dose level 3. Notably, this patient had been treated with two prior therapeutic cancer vaccines. His tumour was *KRAS* wild type and microsatellite stable. (B, C, D) Pretreatment computed tomography scans from a patient with progressive metastatic mesothelioma. Posttreatment images demonstrated a reduction in tumour size in two pulmonary parenchymal metastases (E, F), whereas the pleural mass did not shrink (G), indicating a mixed response in this patient.



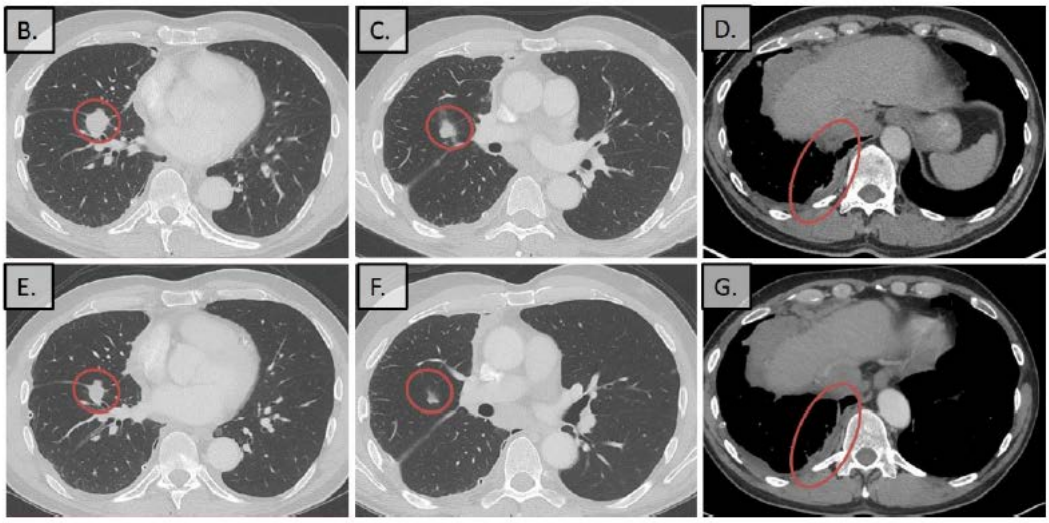


Table S3. Evaluation of standard and PD-L1–expressing immune cell subsets at baseline, day 15, day 43, and day 127

Values indicate median % of PBMCs and interquartile range (first and third quartiles).

	Pre	Day 15 (n=19)	Pre	Day 43 (n=14)	Pre	Day 127 (n=16)
Standard subsets						
CD4	26 (21-41)	28 (18-41)	24 (20-38)	25 (21-40)	33 (23-40)	28 (17-35)
CD8	16 (9-19)	13 (10-18)	16 (9-28)	13 (10-25)	15 (11-17)	14 (7-18)
Treg	1·6 (1·3-1·8)	1·6 (1·4-2·2)	1·7 (1·4-2·4)	1·7 (1·5-2·1)	1·4 (1·4-1·8)	1·2 (0·9-1·4)
NK	6 (3-9)	5 (4-8)	7 (5-10)	5 (4-9)	7 (4-10)	5 (4-7)
NKT	1·9 (0·9-4·6)	1·6 (1·0-5·1)	1·9 (0·9-5·0)	1·9 (0·8-4·2)	1·8 (0·9-3·1)	1·5 (0·7-3·0)
B cells	9 (2-12)	7 (3-11)	9 (3-12)	9 (3-11)	9 (5-14)	8 (6-13)
cDC	0·3 (0·3-0·4)	0·3 (0·2-0·4)	0·4 (0·3-0·5)	0·4 (0·4-0·5)	0·3 (0·2-0·4)	0·2 (0·2-0·4)
pDC	0·3 (0·2-0·3)	0·3 (0·2-0·3)	0·2 (0·2-0·3)	0·2 (0·2-0·3)	0·2 (0·1-0·3)	0·3 (0·2-0·5)
MDSC	4 (3-8)	6 (3-11)	4 (3-7)	6 (2-9)	4 (2-7)	5 (4-8)
PD-L1–expressing subsets						
PD-L1+ CD4	0·09 (0·06-0·09)	0·08 (0·06-0·09)	0·09 (0·06-0·11)	0·07 (0·05-0·09)	0·07 (0·05-0·09)	0·07 (0·05-0·10)
PD-L1+ CD8	0·04 (0·03-0·07)	0·04 (0·03-0·07)	0·05 (0·03-0·09)	0·04 (0·03-0·08)	0·04 (0·03-0·05)	0·04 (0·01-0·06)
PD-L1+ Treg	<0·01	<0·01	<0·01	<0·01	<0·01	<0·01
PD-L1+ NK	0·07 (0·05-0·09)	0·07 (0·04-0·11)	0·08 (0·05-0·14)	0·06 (0·05-0·10)	0·04 (0·03-0·08)	0·05 (0·04-0·07)
PD-L1+ NKT	0·04 (0·03-0·07)	0·04 (0·03-0·06)	0·04 (0·03-0·07)	0·04 (0·03-0·06)	0·02 (0·01-0·03)	0·02 (0·01-0·04)
PD-L1+ B cells	0·7 (0·2-1·2)	0·8 (0·3-1·3)	0·8 (0·3-1·3)	0·8 (0·3-1·3)	1·2 (0·4-1·9)	1·1 (0·4-1·5)
PD-L1+ cDC	0·01 (0·01-0·02)	0·01 (0·01-0·02)	0·01 (0·01-0·02)	0·01 (0·01-0·02)	0·02 (0·01-0·04)	0·01 (0·01-0·02)
PD-L1+ pDC	0·02 (0·01-0·05)	0·01 (0·01-0·03)	0·02 (0·01-0·04)	0·02 (0·01-0·06)	0·02 (0·01-0·07)	0·05 (0·02-0·25)
PD-L1+ MDSC	0·5 (0·4-1·1)	0·9 (0·5-1·6)	0·5 (0·3-0·8)	0·5 (0·4-1·0)	0·6 (0·3-1·8)	1·3 (0·5-2·8)

cDC=conventional dendritic cell; MDSC=myeloid-derived suppressor cell; NA=not available; NK=natural killer; NKT=natural killer T cell; pDC=plasmacytoid dendritic cell; PD-L1=programmed death-ligand 1; Treg=regulatory T cells.