1 # Supplemental Material

2

3 ## Methods

4 *Generation of TIL for infusion*

5 TIL for infusion were cultured from a small resected tumor sample or biopsy, as previously
6 described (1, 2). In brief, TIL were cultured in T cell medium (Iscoves Modified Dulbecco's
7 Medium (IMDM) with penicillin (100 IU/ml), streptomycin (100 µg/ml) and L-glutamine (4mM)
8 (all from Life Technologies, Breda, the Netherlands), and 7.5% heat inactivated pooled human
9 serum (Sanquin, Bloodbank, Amsterdam, the Netherlands) supplemented with interleukin-2 (IL-
10 2, 1000 IU/ml, Aldesleukin, Clinigen Healthcare BV, Netherlands) for a total period of 14-21
11 days. Next, the TIL were expanded according to the described Rapid Expansion Protocol (REP)
12 (1) for another 14 days before harvesting and cryopreservation, until further use. TIL were
13 released for infusion when they met the release criteria with respect to phenotype (>80% T or
14 NK cells), viability (>70% viable cells) and if negative in microbial contamination tests.

15

16 *Immunophenotyping of PBMC*

17 Cryopreserved PBMC collected before and during treatment were thawed in IMDM plus 10%
18 FCS and 30 µg/ml DNase (Roche, 10104159001) and washed twice with FACS buffer, consisting
19 of PBS with 0,5% BSA (Sigma). PBMC samples were immunophenotyped using a 40-marker panel
20 and multispectral flow cytometry. Fluorophore selection and panel design were done according
21 to the following rules: 1) minimize fluorochrome pairs with very high similarity indices, 2) select
22 combinations with lowest possible complexity index, 3) assignment fluorophores based on
23 primary, secondary and tertiary antigen classification as described (3). All antibodies were
24 titrated, and the optimal titer of the antibody was based on performance comparison between

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25 the single stained sample and the multi-color sample. Unmixing accuracy was tested for each
26 individual antibody using beads and single stained cells. Details on antibodies, titers and
27 unmixing are listed in Supplemental Table 1. First, samples were stained with LIVE-DEAD zombie
28 UV fixable amine-reactive dye at room temperature for 20 minutes, after which the cells were
29 washed, and incubated with 50 µl PBS/0.5%BSA containing 2.5 µl human Trustain FcX blocking
30 solution (Biolegend) for 10 minutes on ice to block Fc receptors. Next, the cells were stained for
31 30 minutes at room temperature in the dark with the cell surface antibodies in two consecutive
32 rounds with sufficient washing in between. Intracytoplasmic/nuclear staining was performed
33 using the True-Nuclear Transcription factor buffer set (Biolegend) according to manufacturers'
34 instruction. Finally, the cells were washed twice, stored in FACS buffer (PBS/0.5% BSA).
35 Acquisition of the samples was done within 24 hours on a 5-laser Aurora Cytek™ spectral
36 analyzer (Cytek Biosciences, CA, USA) with SpectroFlo acquisition software (version 3). Data
37 analysis was done by high-dimensional single cell data analysis using optSNE dimensionality
38 reduction followed by FlowSOM consensus metaclustering using the cloud-based OMIQ data
39 analysis software. The different cell populations were visualized and quantified.
40
41 *Lymphocyte function/proliferation*
42 The proliferative potential of peripheral blood mononuclear cells (PBMC) was evaluated using a
43 proliferation assay, as previously described (4). Briefly, cryopreserved PBMC were thawed in
44 IMDM plus 10% FCS and 30 µg/ml DNase and stimulated in 6-fold with the previously described
45 memory response mix (MRM, 2x concentrated, 50000 c/w), influenza matrix 1 protein-derived
46 overlapping peptides (FLU-M1, 5 µg/mL per peptide, 50000 c/w) or CD3/CD28-activation beads
47 (ratio 1:4 beads to cells, Dynabead, 10000c/w) for 6 days. After harvesting 50 µL supernatant
48 per well for cytokine analysis, [3H]-Thymidine (Perkin Elmer) was added to the wells for 16

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49 hours before the cells were harvested. [3H]-Thymidine uptake was determined by Wallac
50 Microbeta Trilux (Perkin Elmer) and used to calculate proliferation. Cells cultured in the
51 presence of medium only were included as negative controls. A positive response was defined
52 as a stimulation index (SI) of at least 3.
53
54 *APC function*
55 The antigen-presenting capacity of PBMC was determined in a mixed lymphocyte reaction
56 (MLR). Patients' PBMC were thawed in IMDM plus 10% FCS and 30 μg/ml DNase, irradiated
57 (3000 rad) to prevent proliferation, washed, and resuspended in IMDM plus 10% human AB
58 serum. Next, they were plated in a 1:1 ratio with third party PBMC and total volume of 200
59 μL/well in 96 well plates. Irradiated PBMC alone, as well as third party PBMC alone, were used
60 as negative controls. At day 6, 100 μL supernatant per well was harvested for cytokine analysis,
61 and [3H]-Thymidine (50 μL/well of 10 μCi/mL) was added for an additional 16 hours. Cells were
62 harvested and [3H]-Thymidine uptake was determined by Wallac MicroBeta TriLux and used to
63 calculate proliferation. A positive response was defined as an SI of at least 3 (4).
64
65 *Immunophenotyping of tumor infiltrating lymphocytes (TIL)*
66 To assess the phenotypic characteristics of TIL batches used for infusion, cryopreserved
67 reference vials of TIL were thawed in IMDM plus 10% FCS and 30 μg/ml DNase, washed twice
68 and resuspended in FACS-buffer. Next, the samples were divided into multiple samples and
69 stained with separate antibody panels for activation/inhibitory, memory, homing and regulatory
70 T cell markers, respectively as we previously described (2) according to our standard protocols
71 (5). In brief, TIL batches were first stained with LIVE-DEAD Fixable yellow amine-reactive dye
72 (Yellow ArC-Qdot585, ThermoFisher Scientific, L34959) at room temperature for 20 minutes,

73 after which the cells were washed, and incubated with PBS/0.5%BSA/10%FCS for 10 minutes on
74 ice to block Fc receptors. Next, the cells were stained for 30 minutes on ice and in the dark with
75 fluorochrome-conjugated antibodies. Intracytoplasmic/nuclear staining was performed using
76 the transcription factor buffer set (BD Pharmingen) according to manufacturers' instruction.
77 Details on antibodies used are listed in supplemental Table 2. After staining the cells were
78 washed twice and fixed in 1% paraformaldehyde before data acquisition on a BD LSR Fortessa
79 (BD Biosciences). Data analysis was done by high-dimensional single cell data analysis using
80 optSNE dimensionality reduction followed by FlowSOM consensus metaclustering using the
81 cloud-based OMIQ data analysis software. The different cell populations were visualized and
82 quantified.
83
84 *Autologous tumor cells and ovarian cell lines*
85 Small tumor fragments were cryopreserved for later use as autologous target cells in assays
86 conducted to detect tumor cell-reactivity of TIL batches. Ovarian carcinoma cell lines were
87 previously established in our laboratory (COV318, COV362.4, COV413B, COV434, COV504,
88 COV641 II, Ref COV lines), obtained from ATCC (A2780, CAOV3, OVCAR3 and SKOV3, ATCC,
89 Manassas,VA, USA) or kindly provided by Inge Marie Svane (EOC.TIL 04, EOC.TIL 11 and GY-
90 1508.06, CCIT-DK, Herlev Hospital, Copenhagen, Denmark (6)). Authentication of the cell lines
91 was performed by HLA-genotyping at the department of Immunohematology and Blood bank of
92 the LUMC and all cell lines were regularly tested to be mycoplasma negative. The cell lines
93 obtained from I.M. Svane were cultured in RPMI/glutamax supplemented with 10 % heat
94 inactivated Fetal Calf Serum (FCS, Life Technologies), penicillin (100 IU/ml), streptomycin (100
95 µg/ml), 0,5% fungizone, insulin (10ug/ml) and sodium pyruvate (1mM). All other cell lines were
96 cultured in 'tumor cell medium' (i.e. Dulbecco's minimal essential medium (Life Technologies,

97 Breda, the Netherlands) with 8% heat inactivated FCS, penicillin (100 IU/ml), streptomycin (100
98 µg/ml) and L-glutamine (4 mM). All culture media and supplements were obtained from Life
99 Technologies. Tumor cells were treated with medium alone or IFN-gamma (IFNg, 100 IU/ml for
100 24-48 h, Preprotech) to upregulate HLA-expression, before being used as target cells.
101
102 *Tumor-reactivity of TIL.* The antigen-specificity of the infusion product was tested against a
103 broad panel of EOC cell lines that were (partially) matched for at least one HLA-class I allele with
104 the corresponding patient. If available, autologous tumor cells were also tested. Briefly, $1.5x10^4$
105 T cells (effector cells) were co-cultured with $3x10^4$ target (tumor) cells in a total volume of 150 µl
106 B cell medium (i.e. T cell medium with 8% FCS instead of human serum) in triplicate wells of a
107 round-bottom 96-well plate. Medium alone and Staphylococcal Enterotoxin B (SEB, 0,5 µg/ml)
108 were used as negative and positive controls, respectively. In case that autologous tumor
109 material was used as target, equal parts of small tumor fragments were plated in the test and
110 control wells, and co-cultured with TIL or medium alone, respectively. After overnight
111 incubation at 37 °C the supernatant was harvested and, as a read-out of tumor-reactivity, the
112 IFNg secretion was determined by ELISA (Elisa Flex, Mabtech, Nacka Strand, Sweden) according
113 to manufacturer's recommendations. Specific cytokine production was defined by a cytokine
114 concentration above the cut-off value (IFNg 50 pg/ml) and >2x the concentration of the medium
115 control.
116
117 *Cytokine profile of TIL.* To characterize the cytokine profile potentially released upon activation
118 of the infused TIL, TIL were stimulated with CD3/CD28 activation beads (Dynabeads,
119 Thermofisher ratio 1:4 beads to T cells) or medium alone as negative control. After incubation

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120 for 24 h supernatant was harvested and used to analyze the IFNg production by ELISA (as
121 above).
122
123 *Statistical analysis.* Survival and PFS were estimated according to the Kaplan-Meier's method
124 using GraphPad Prism version 9.3.1. 1 for Windows (GraphPad Software, La Jolla California USA).
125 Paired and independent analyses were performed on the data generated by FACS analysis on
126 both the T cell products and PBMC. To compare data following a normal distribution either a
127 paired or unpaired t-test was used, when the assumption of normality was violated a Wilcoxon
128 signed rank test was performed for paired comparisons and a Mann-Whitney U test was
129 performed for unpaired comparisons. In case of multiple comparisons a Friedman for paired
130 comparisons or Kruskal-Wallis for unpaired analysis both with Dunn's correction for multiple
131 comparisons was used.

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133

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155 **Supplemental Tables**

Supplemental Table 1. Antibody panel spectral flowcytometry for immunophenotyping of PBMC samples.

laser	Detector	Fluorochrome	Antigen	Clone name	Company	Lot	Dilution	Unmixing
UltraViolet laser	UV2	BUV395	CD45RA	HI100	BD	276592	320	with beads
	UV4	Zombie UV	L/D	NA	biolegend	NA	2400	with cells
	UV7	BUV496	CD16	3G8	BD	1099494	80	with cells
	UV9	BUV563	CD39	TU66	BD	1162869	40	with beads
	UV10	BUV615	ICOS	DX29	BD	276620	80	with beads
	UV11	BUV661	CD1c	F10/21A3	BD	276617/1288139	40	with cells
	UV14	BUV737	CD86	2331 (FUN-1)	BD	258384	160	with beads
	UV16	BUV805	CD8	SK1/HIT8a	BD	195680/1200765	160	with cells
violet laser	V1	BV421	CD161	HP-3G10	biolegend	B334264	10	with beads
	V2	SB436	CD123	6H6	TFS	2196734/2271503	40	with beads
	V3	PacBlue	CD15	W6D3	biolegend	B273508	20	with beads
	V4	BV480	CD33	P67.6	BD	276608	640	with beads
	V6	BV510	CD11c	B-Ly6	BD	149997/1235761	40	with cells
	V7	PacOrange	CD3	UCHT1	exbio	2081485	20	with cells
	V8	BV570	CD45RO	UCHL1	biolegend	B326241	40	with beads
	V10	BV605	CD163	GHI/61	biolegend	B306652	10	with beads
	V11	BV650	PD1	EH12.2H7	biolegend	B322203	40	with beads
	V13	BV711	CD103	Ber-ACT8	biolegend	B305675	80	with beads
	V14	BV750	CD56	5.1H11	biolegend	B305755	80	with beads
	V15	BV785	CD28	CD28.2	biolegend	B332622	80	with cells
blue laser	B1	BB515	CD141	1A4	BD	212775	160	with beads
	B2	AF488	Foxp3	259D	biolegend	B315166	40	with beads
	B3	Spark Blue 550	CD14	63D6	biolegend	B314183	320	with cells
	B8	PerCP	CD45	Hi30	biolegend	B331249	160	with beads
	B9	PerCP/Cy5.5	CD11b	ICRF44	biolegend	B328101	80	with cells
	B10	PerCP/eF710	CD274/PD-L1	MIH5	TFS	2236348/224625	20	with beads
yellow green laser	YG1	PE	CLec9a	8F9	biolegend	B309940	80	with cells
	YG2	CF568	CD4	C4-206	antibodies online	18C0330	160	with cells
	YG3	PE/Dazzle 594	CD206	15-feb	biolegend	B329923	20	with beads
	YG4	PE/Fire640	CD25	M-A251	biolegend	B321902/B332511	40	with cells
	YG5	PE/Cy5	Tim3	F38-2E2	biolegend	B312192	40	with beads
	YG6	PE/Fire700	CD127	AO19D5	biolegend	B321904	40	with beads
	YG9	PE/Cy7	KLRG1	SA231A2	biolegend	B317449	40	with cells
	YG10	PE/Fire810	HLA-DR	L243	biolegend	B341939	160	with cells
red laser	R1	APC	NKG2a	Z199	beckman Coulter	200054	20	with beads
	R2	Alexa647	CD68	Y1/82A	biolegend	B311503	40	with beads
	R3	Spark NIR 685	CD19	HIB19	biolegend	B324543	160	with beads
	R4	APC/R700	Lag3	T47-530	BD	1114707	80	with cells
	R7	APC/Fire750	CCR7	G043H7	biolegend	B306864/B338244	20	with cells
	R8	APC/Fire810	CD27	QA17A18	biolegend	B315671	320	with beads

For each Ab the detector, conjugated fluorochrome, antigen, clone name, company, lot, dilution and unmixing details are given.

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Supplemental Table 2. Antibody panels used for flowcytometry analysis of ACT/TIL product

	Antibody	Antigen	Fluorochrome	Clone	Dilution	Supplier
Regulatory T cell panel	1	CD3	V500	UCHT1	30	BD Biosciences
	2	CD4	AlexaFluor700	RPA-T4	50	BD Biosciences
	3	CD8	BB700	HIT8a	400	BD Biosciences
	4	CD25	PE-Cy7	2A3	50	BD Biosciences
	5	CD127	BV650	A019D5	40	Biolegend
	6	CD45RA	APC-H7	HI100	150	BD Biosciences
	7	Foxp3*	PE-CF594	259D/C7	150	BD Biosciences
	8	CTLA4*	BV421	BNI3	40	BD Biosciences
	9	KI67*	FITC	20Raj1	50	eBiosciences
	10	Helios*	APC	22F6	100	BD Biosciences
	11	Tbet*	PE	eBio4B10	80	eBiosciences
Inhibitory marker panel	1	CD3	V450	UCHT1	40	BD Biosciences
	2	CD4	PE CF594	RPA-T4	50	BD Biosciences
	3	CD8	APC-Cy7	SK1	40	BD Biosciences
	4	CD56	AF700	B159	10	BD Biosciences
	5	CTLA-4	PE-Cy5	BNI3	10	BD Biosciences
	6	PD-1	PE-Cy7	EH12.2H7	10	Biolegend
	7	TIM3	BV605	F38-E2E	80	Biolegend
	8	NKG2a	PE	Z199	20	Beckmann Coulter
Memory marker panel	1	CD3	V450	UCHT1	40	BD Biosciences
	2	CD4	PE CF594	RPA-T4	50	BD Biosciences
	3	CD8	APC-Cy7	SK1	40	BD Biosciences
	4	CD25	BV605	2A3	20	BD Biosciences
	5	CD27	V500	M-T-271	200	BD Biosciences
	6	CD28	FITC	CD28.2	10	BD Biosciences
	7	CD45RA	PerCP-Cy5.5	HI100	30	Biolegend
	8	CD45RO	PE	UCHL1	20	BD Biosciences
	9	CD62L	AF700	DREG-56	80	Biolegend
	10	CD95	PE-Cy7	DX2	80	Biolegend
	11	CCR7	A647	3D12	40	BD Biosciences
Homing marker panel	1	CD3	V450	UCHT1	40	BD Biosciences
	2	CD4	PE CF594	RPA-T4	50	BD Biosciences
	3	CD8	APC-Cy7	SK1	40	BD Biosciences
	4	CD25	FITC	2A3	25	BD Biosciences
	5	CD45RA	PerCP-Cy5.5	HI100	30	Biolegend
	6	CD45RO	PE-Cy7	UCHL1	40	BD Biosciences
	7	CXCR3	AF700	1C5	20	BD Biosciences
	8	CCR4	PE	1G1	10	BD Biosciences
	9	CCR6 (CD196)	BV605	G034E3	10	Biolegend
	10	CCR10	APC	6588-5	30	Biolegend

*For each Ab the conjugated fluorochrome, clone name, dilution used, and supplier are given. *indicates Ab that were used for staining of intracellular markers.*

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