Supplementary Information

Flow cytometry data analysis

During analysis, 47 subsets from different lymphocytes and myeloid cells were defined in a blinded fashion based on the panel that we designed, and nonspecific cells were gated out with use of dump channels (e.g., CD14 for monocyte exclusion). Dead cells were excluded during data analysis based on the binding of Aqua, a fixable viability dye (Invitrogen), that was also added to the tubes during the staining. Fold changes in the indicated lymphocyte subsets after IL-2 therapy were presented as the percentage of total viable lymphocytes, or the $CD4^+$ T cell subset. In some cases, fold changes in multiple subsets were heat-mapped by using an Excel conditional formatting tool that assigned specific colors (black to bright red for increases and black to bright blue for decreases) for the various ranges of fold change. In some data, absolute count or percentage in different lymphocytes subsets at baseline and 2 days after HD IL-2 therapy were calculated.

PBMC activation and intracellular cytokine staining

PBMCs from some patients were analyzed for IFN-γ and IL-2 production in Treg subsets after PMA/ ionomycin stimulation by using intracellular cytokine staining (ICS). PBMCs were thawed and washed in culture medium (RPMI 1640 with Glutamax, 1 mM pyruvate, 5% human AB serum, 50 μM 2-mercaptoethanol, penicillin/streptomycin). 1.2×10^6 cells were seeded per well in a 96-well U-bottom plate and stimulated with 50 ng/ml PMA plus 2 μg/ml ionomycin in culture medium for 1 hour in the incubator. Diluted GolgiStop (BD Biosciences) was added, and the cells were incubated for another 5 hours. For

detecting the production of IL-10, PBMCs were stimulated with PMA/ionomycin for 24 hours. The cells were harvested, washed, and stained for the Treg markers with use of anti-CD4-APC-H7, CD25-FITC, ICOS-PE, and Foxp3-Pacific Blue together with anti-IFN-γ-PE-Cy7, and anti-IL-2-PerCP-Cy5.5. The stained cells were washed and re-suspended in 1% *p*-formaldehyde, 0.25% ethanol in PBS and acquired on a FACScanto II flow cytometer using FACSDiva software.

Proliferation assay

PBMCs were labeled with 1 μM Cell Proliferation Dye eFluor® 670 (eBioscience) in PBS for 10 mins at 37°C in the dark. Labeling was then stopped, and cells were washed as recommended by the manufacturer's instruction.

Supplemental Table S1. Summary of patient clinical parameters before HD IL-2 therapy and best response after therapy[A]

Pt. #	Sex[B]	Age[C]	Stage[D]	Prior therapy (within 4 months before HD IL-2)	Best response after 2nd cycle of IL-2[E]
003	M	30	IV (M1a)	None	PD
005	M	55	IV (M1b)	Chemotherapy	PD
006	M	59	IV (M1b)	None	PD
008	M	59	IV (M1c)	Chemotherapy	PD
010	M	42	IV (M1c)	Surgery	PD
011	F	54	IV (M1b)	None	PR
012	M	45	IV (M1c)	None	CR
013	M	47	IV (M1b)	None	PR
014	F	43	IV (M1c)	None	PD
015	M	57	IV (M1b)	None	MR
017	F	47	IIIC	High-dose IFN-α	PD
018	F	30	IV (M1b)	None	PD
021	M	49	IV (M1b)	Surgery	SD
023	F	30	IV (M1c)	Surgery	PR
024	M	33	IV (M1c)	None	PD
027	F	52	IV (M1c)	Surgery	PD
028	M	34	IIIC	None	PR
029	F	25	IV (M1c)	Surgery	PD
030	M	57	IV (M1a)	gp100 vaccine, surgery	PD
031	F	47	IV (M1c)	Chemotherapy	PD
032	M	51	IV (M1c)	Surgery	PD
034	M	63	IV (M1c)	Surgery	PD
035	F	63	IV (M1b)	None	PD
036	F	49	IIIC	Surgery	MR
040	M	49	IV	Surgery	PD
048	F	34	IIIC	Surgery	CR
054	M	36	IV	High-dose IFN-α	PD
067	M	49	IIIC	Chemotherapy, Radiation	PD
071	M	55	IV (M1a)	Surgery/Radiosurgery	PD
072	M	49	IIB T3b	Surgery	PD
074	M	50	IV (M1a)	Surgery	PD
076	M	53	IV (M1a)	Surgery	PD
077	F	28	IV (M1a)	Chemotherapy, IFN, surgery	SD
078	M	50	-	High-dose IFN-α, Surgery	PD
080	M	38	IV (M1c)	Surgery	PD
082	F	33	IV (M1a)	High-dose IFN-α, Surgery	PD
086	F	30	IV	High-dose IFN-α, Surgery	PD
092	M	40	IV	Surgery, Immunotherapy	PD
093	M	37	IV (M1c)	Surgery	PD
016M[F]	M	40	IV (M1c)	None	PR
031M[F]	M	48	IV (M1b)	None	PD
032M[F]	F	45	IV (M1a)	None	SD

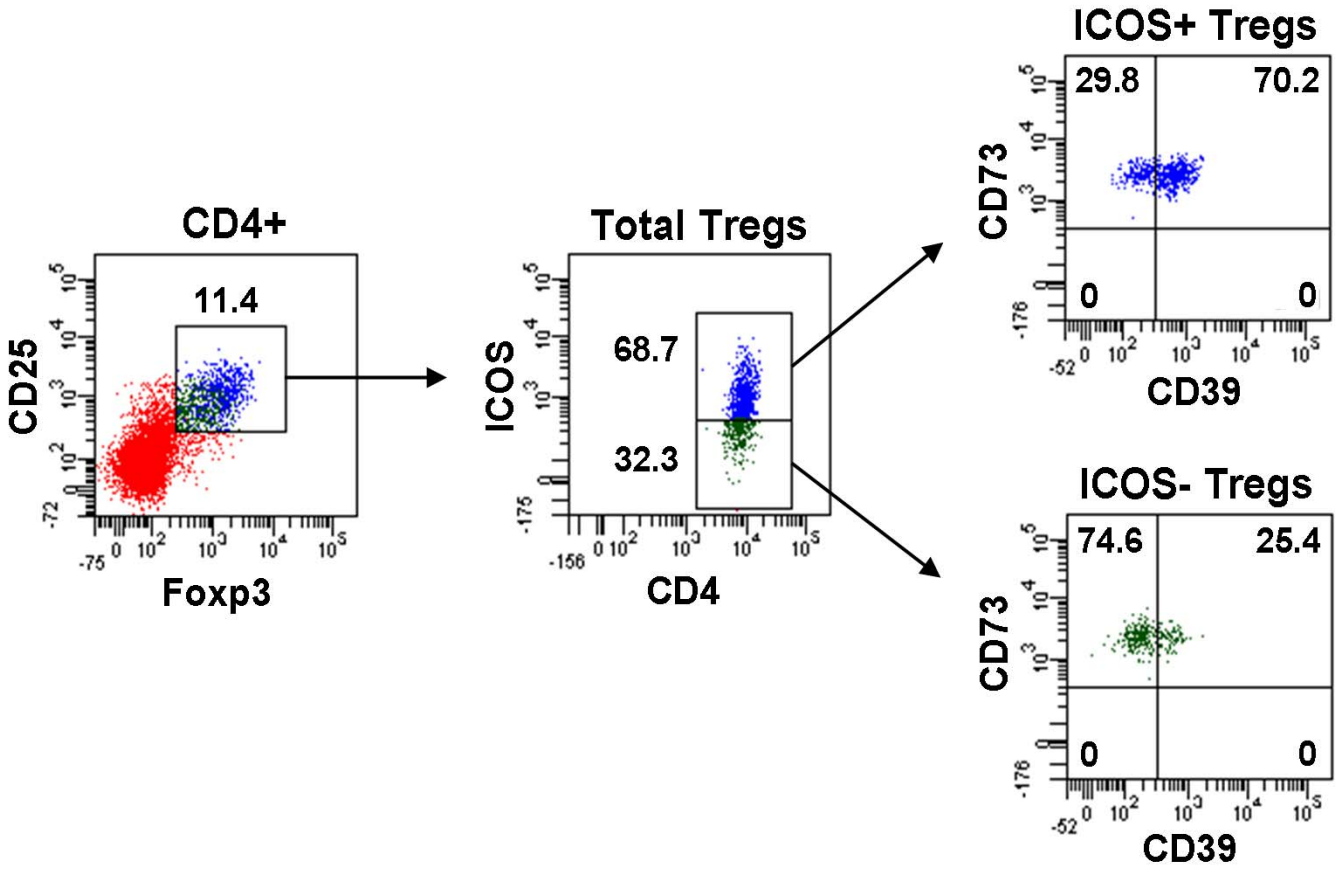
[A]All patients received at least two cycles of HD IL-2 therapy before evaluation of clinical response, except for three (006, 014, and 032) who were withdrawn from further therapy due to clear evidence of rapid disease progression after cycle 1 of therapy.
[B]14 of 25 patients studied (56%) were males, and 11 of 25 patients (44%) were females.
[C]Median age was 47 years (range: 25-63 years).
[D]Stage based on the American Joint Committee on Cancer (AJCC) TNM staging system (http://www.cancerstaging.org).
[E]The best response after the second cycle of IL-2 therapy was determined by using full body CT scans and scoring of responses using RECIST criteria by two radiologists. PD: progressive disease; SD: stabilization of disease; MR: mixed response; PR: partial response; CR: complete response. Patients with PD, SD, or MR were considered non-responders, whereas patients having a PR or CR were considered responders.
[F]Patients received HD IL-2 and MAGE-A3 + AS15.

Supplemental Table S2. Summary of patient age, sex, number of IL-2 infusions, and effects of HD IL-2 on major leukocyte subsets

Parameter	Tumor Response		P-value
	R	NR	
Age (years)	40	49	0.193
Gender:			
Male	4	23	0.686
Female	3	12	
Total (n)	7	35	
Number of IL-2 infusions:			
Cycle 1	7	7	0.889
Cycle 2	7	6	0.119
Lymphocyte count ($x10^6$/ml):			
Pre-IL-2	1.46	1.49	0.532
Post-IL-2	2.42	2.35	0.839
Monocyte count ($x10^6$/ml):			
Pre-IL-2	0.35	0.53	0.038
Post-IL-2	0.46	0.58	0.091
Neutrophil count ($x10^6$/ml):			
Pre-IL-2	2.57	3.04	0.612
Post-IL-2	3.20	3.19	0.774

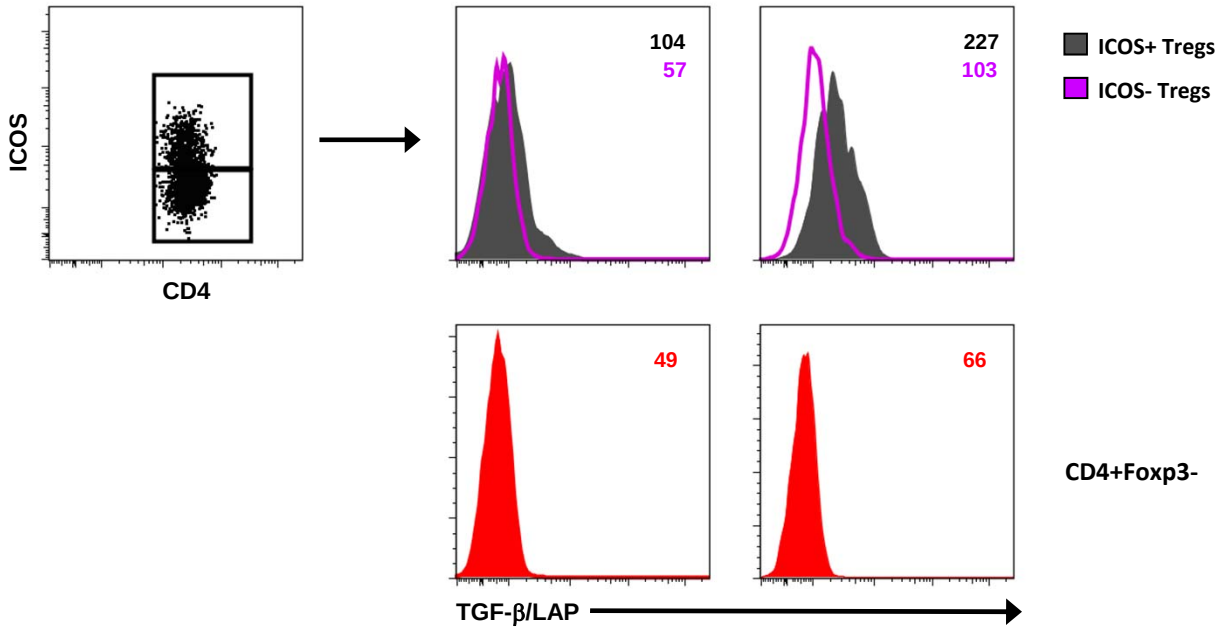
Abbreviations: NR: nonresponder; R: responder.

Figure S1



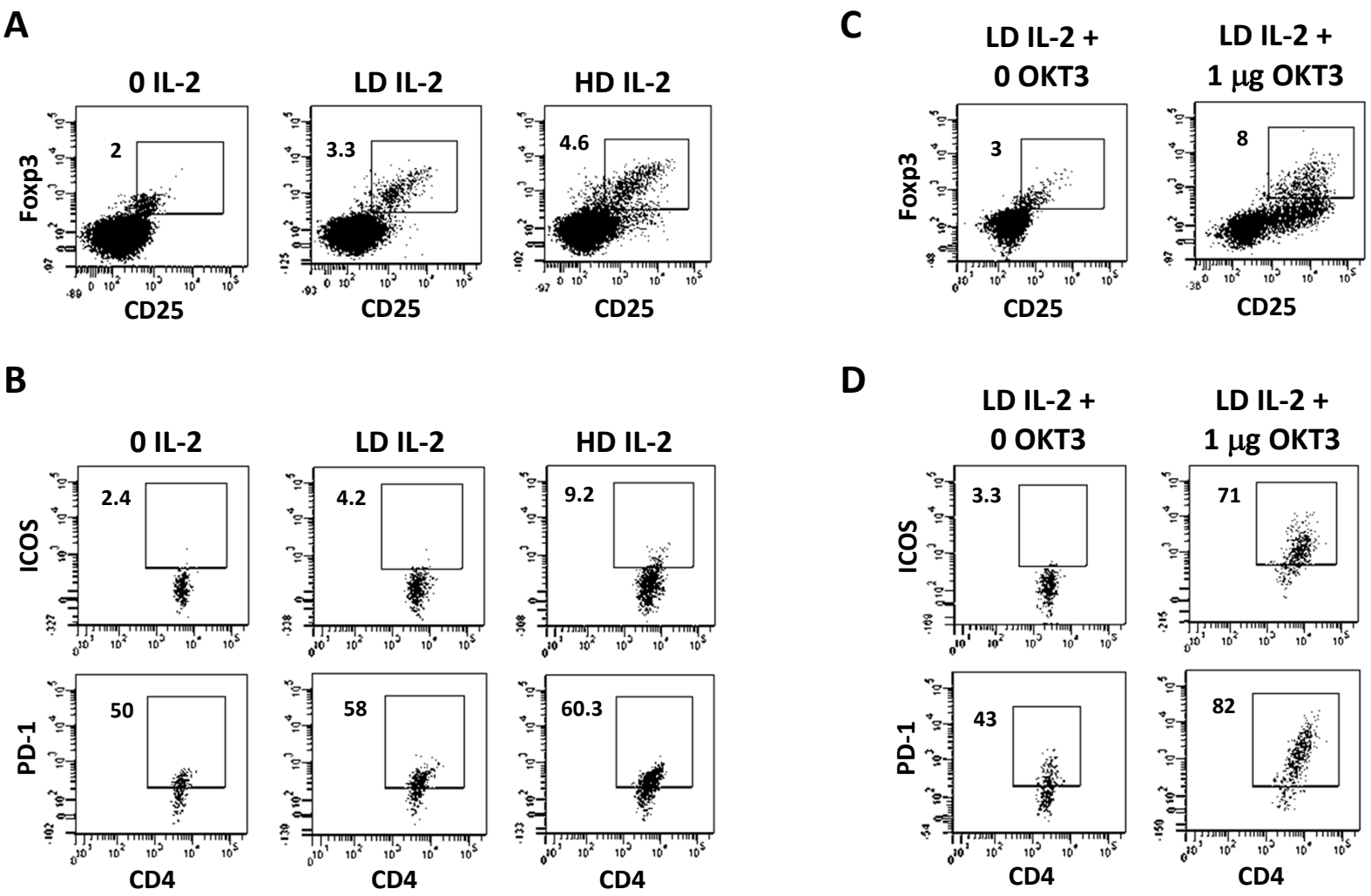
Supplemental Figure S1. $CD4^+CD25^+Foxp3^+ICOS^+$ T cells expanded in HD IL-2 patients co-express CD39 and CD73. PBMCs from a HD IL-2 patient were isolated 2 days after the last dose of IL-2 (cycle 1 of therapy) and freshly stained for CD4, CD25, Foxp3, ICOS, CD39 and CD73. Co-expression of high levels of CD73 together with high CD39 were found on $CD4^+CD25^+Foxp3^+ICOS^+$ T cells after HD IL-2 cycle 1. Frequencies are as indicated in each quadrant of the dot plots.

Figure S2



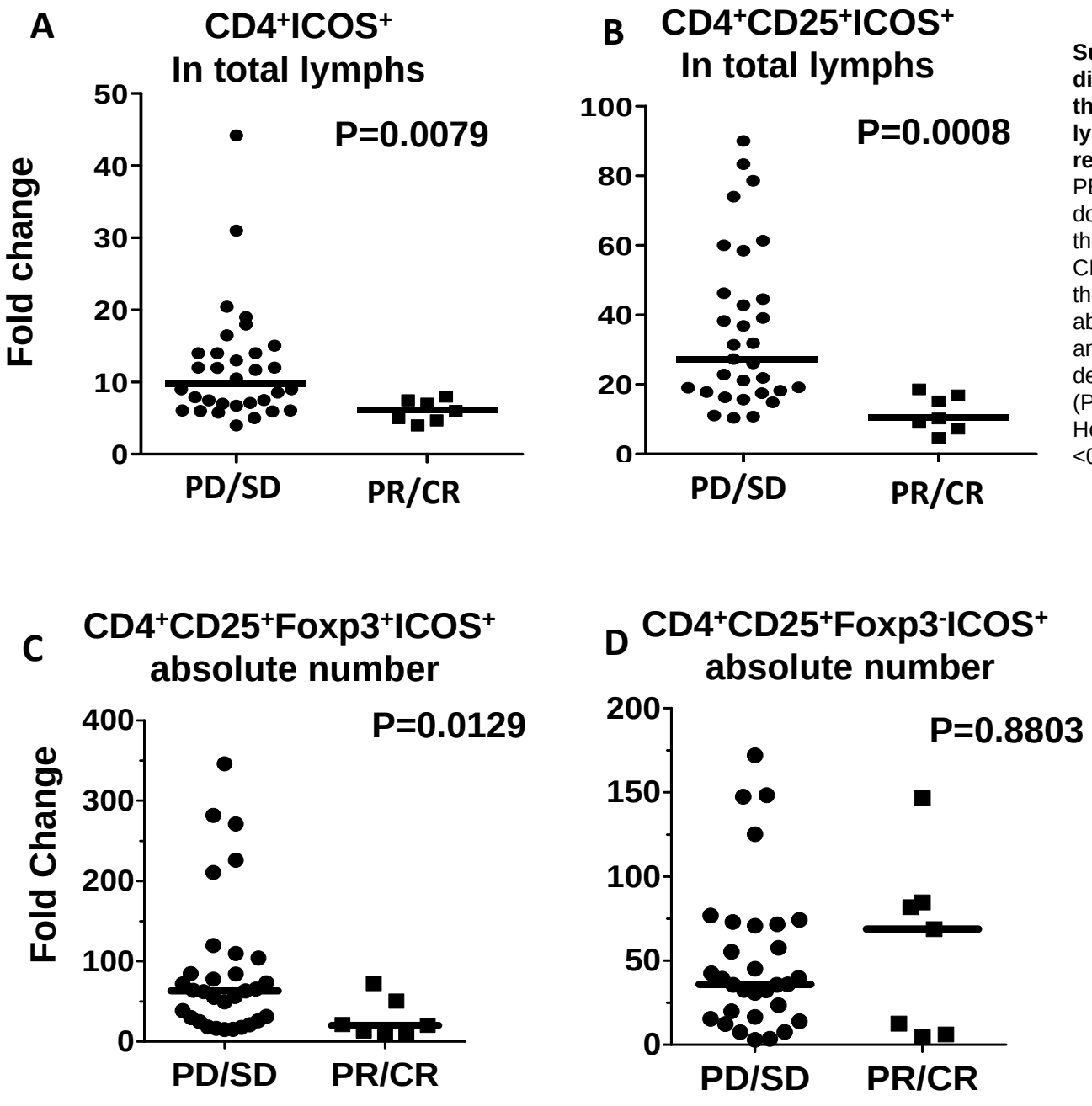
Supplemental Figure S2. The cell surface expression of TGF-β/LAP in ICOS^{+} and ICOS^{-} Treg cells as well as in CD4^{+}Foxp3^{-} T cells from two patients after cycle 1 of HD IL-2 therapy. The MFI are as indicated in histograms.

Figure S3



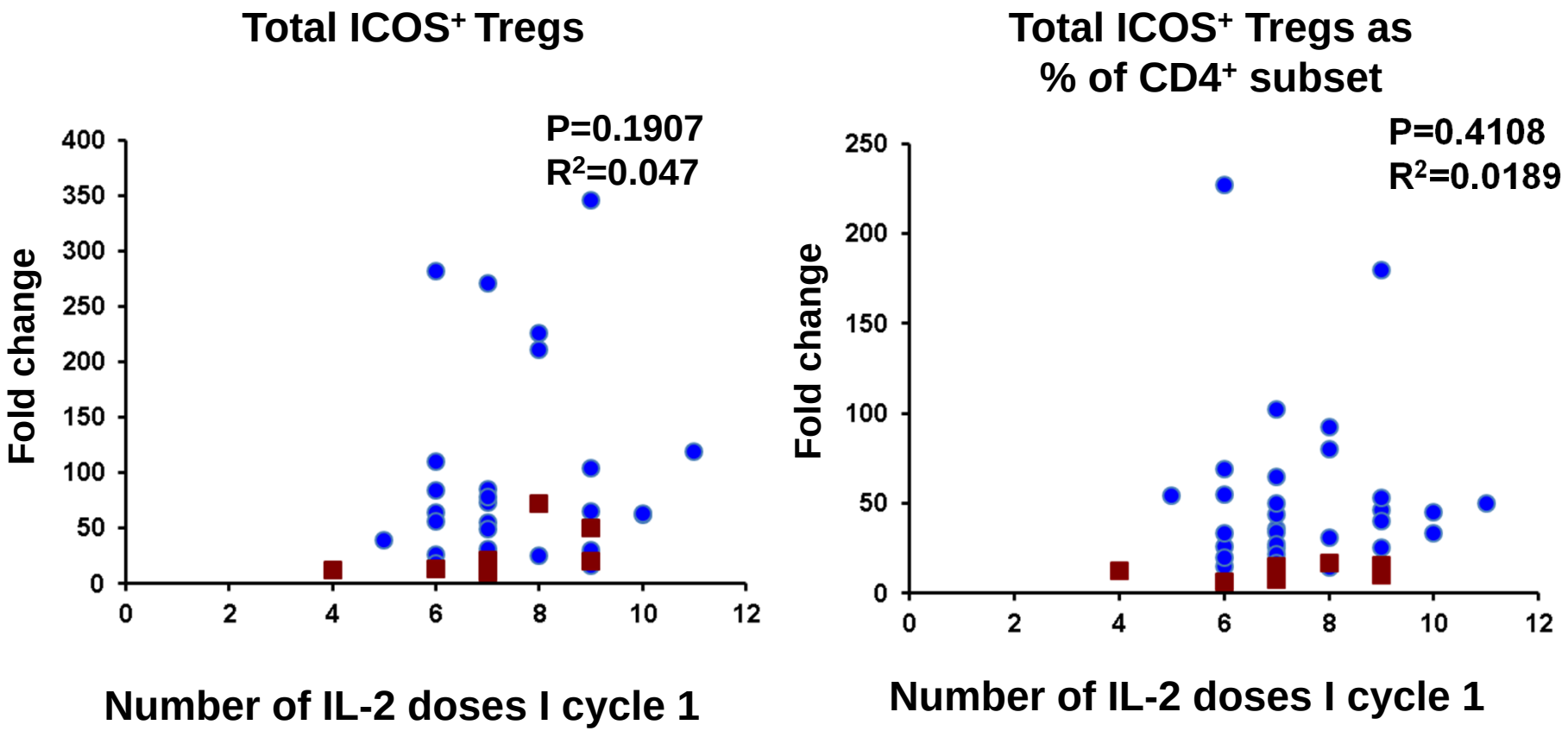
Supplemental Figure S3. IL-2 does not induce ICOS and PD-1, while TCR complex activation induces a large increase in both ICOS and PD-1 in CD4$^+$CD25$^+$Foxp3$^+$ Tregs. PBMCs from a Stage IV melanoma patient were cultured under the indicated conditions with low-dose (LD) IL-2 (300 IU/ml) or HD IL-2 (3,000 U/ml) for 72 hours with or without TCR stimulation using anti-CD3 (OKT3; 1 mg/ml coated in the wells). The cells were stained for CD4, CD25, Foxp3, ICOS, and PD-1 and analyzed by flow cytometry. (**A, C**) The expression of CD25 and Foxp3 by CD4$^+$ T cells that were stimulated with different IL-2 concentrations and with or without OKT3. (**B, D**). Dot plots depict the expression of ICOS and PD-1 by CD4$^+$CD25$^+$Foxp3$^+$ T cells under the stimulation with different IL-2 concentrations in the presence or absence of OKT3. Frequencies are as indicated in each quadrant of the dot plots.

Figure S4



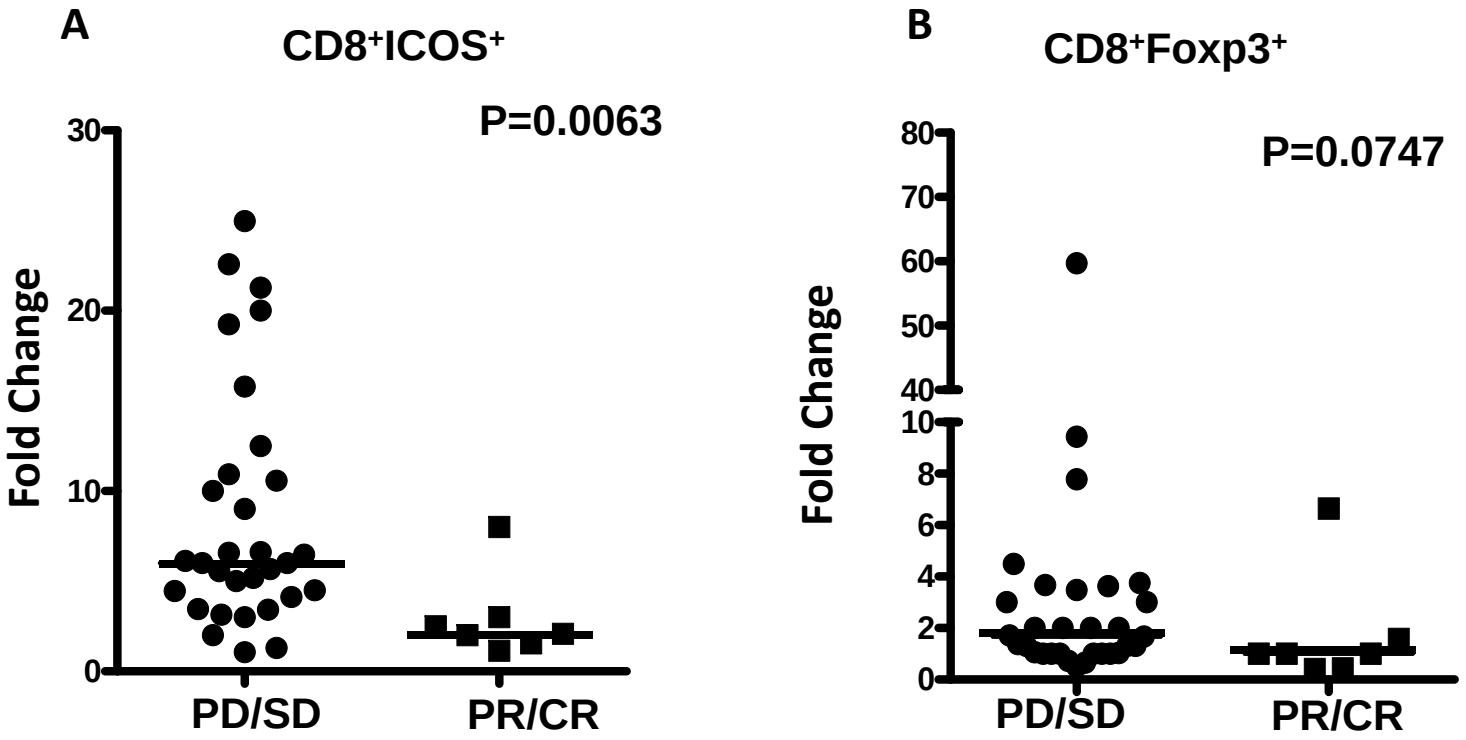
Supplemental Figure S4. Changes in different $CD4^+CD25^+$ subsets during IL-2 therapy as a percentage of total lymphocytes or as absolute number in responding and non-responding patients. PBMCs at baseline and two days after the last dose of HD IL-2 in cycle 1 were analyzed for the fold change of (**A**) $CD4^+ICOS^+$ and (**B**) $CD4^+CD25^+ICOS^+$ subsets as a percentage of the total viable lymphocyte gate. (C) The absolute number in $CD4^+CD25^+Foxp3^+ICOS^+$ and (D) $CD4^+CD25^+Foxp3^-ICOS^+$ were determined and plotted for non-responders (PD/SD, n=31) and responders (PR/CR, n=7). Horizontal bars represent median values. P <0.05 was considered significant.

Figure S5



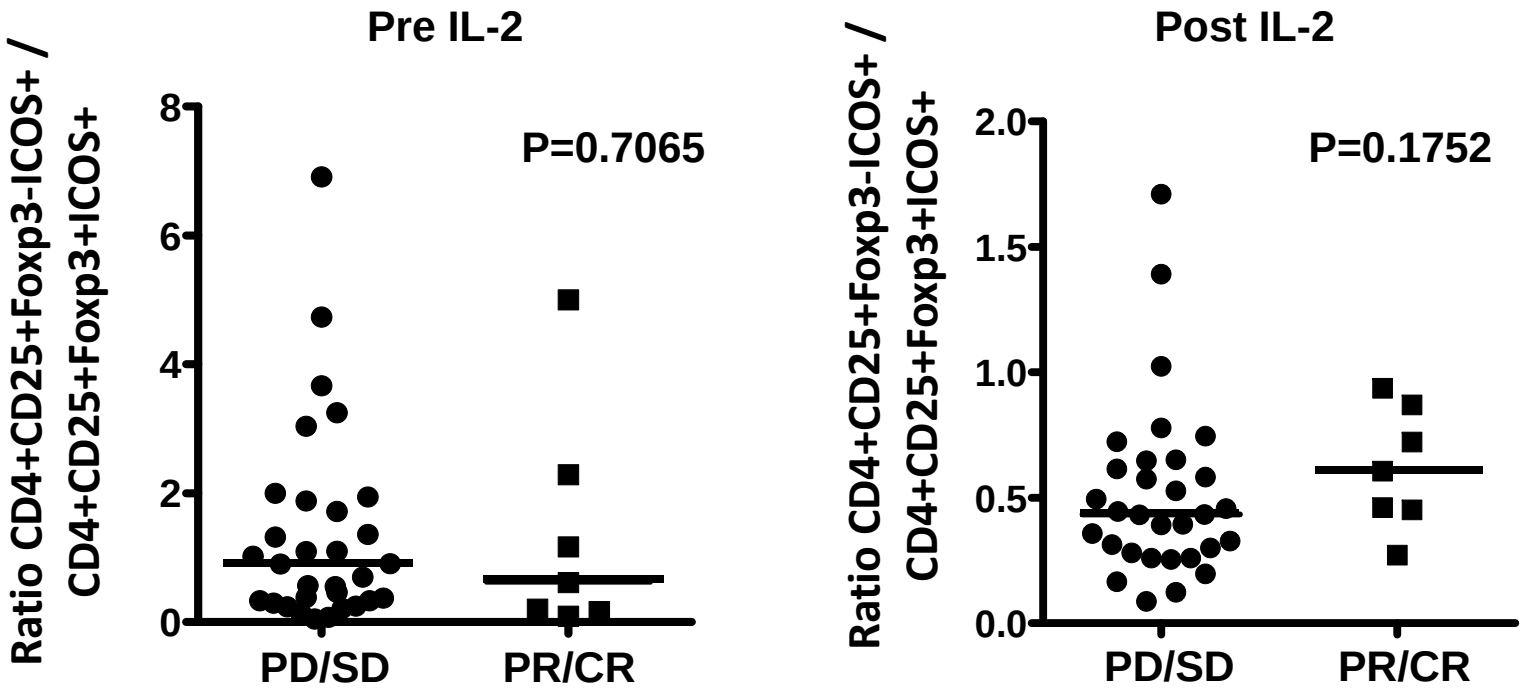
Supplemental Figure S5. The number of doses of IL-2 given in cycle 1 of HD IL-2 therapy does not correlate with changes in ICOS⁺ Tregs. The fold change in total ICOS⁺ Treg number (**A**) and the fold change in the percentage of ICOS⁺ Tregs in the CD4⁺ subset (**B**) for all patients (n=38) were plotted versus the number of doses or infusions of IL-2 received during cycle 2 of HD IL-2 therapy. Correlation analyses were performed using Spearman test.

Figure S6



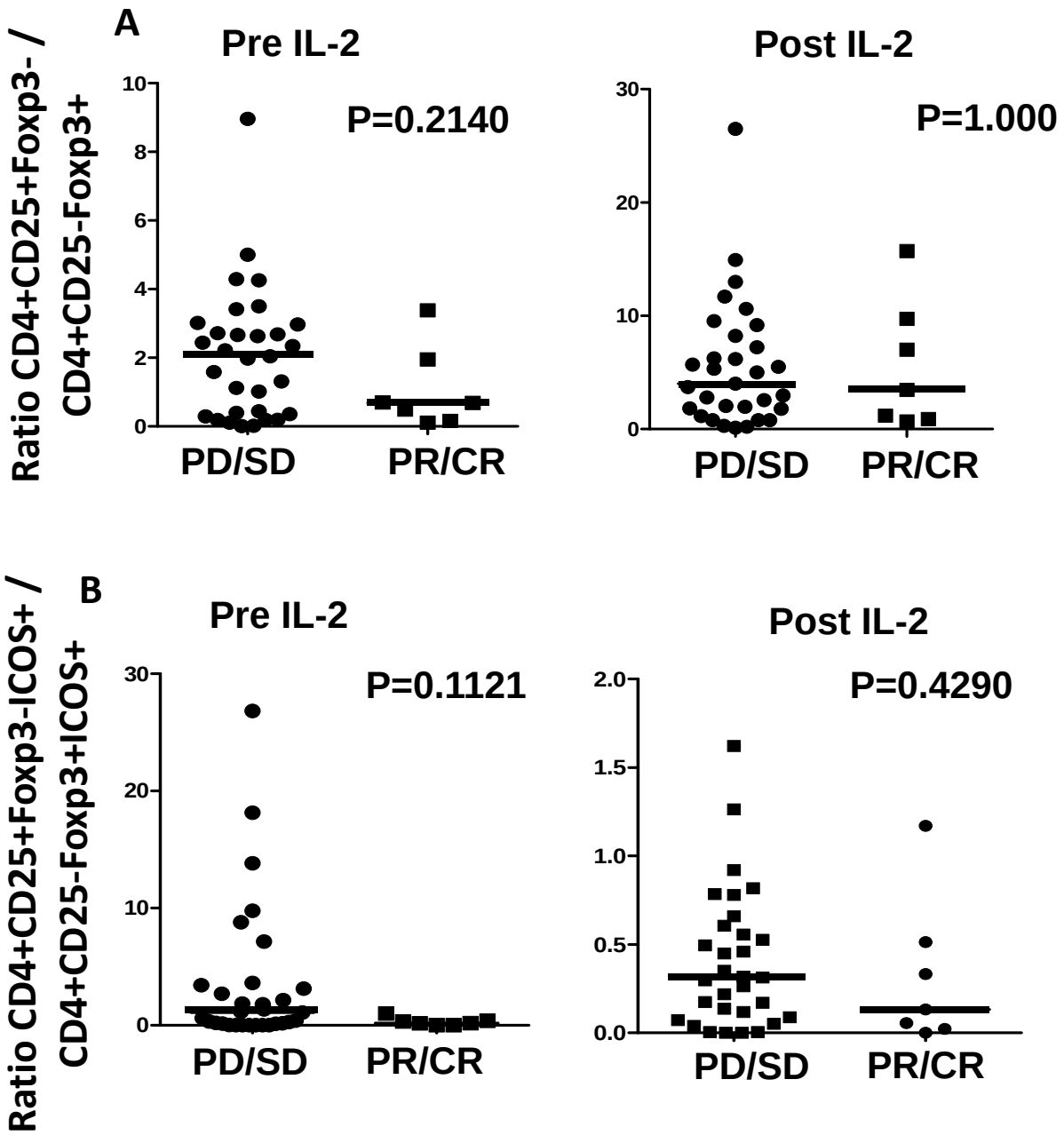
Supplemental Figure S6. Changes in $CD8^+ICOS^+$ and $CD8^+Foxp3^+$ cells during IL-2 therapy as a percentage of total lymphocytes in responding and non-responding patients. PBMCs at baseline and 2 days after the last dose of HD IL-2 in cycle 1 were analyzed as described in the Materials and Methods. The fold change in the gated (**A**) $CD8^+ICOS^+$ and (**B**) $CD8^+Foxp3^+$ subsets as a percentage of the total viable lymphocyte gate was determined and plotted for non-responders (PD/SD, n=31) and responders (PR/CR, n=7). Statistical analyses were performed with 2-tailed Mann-Whitney test. Horizontal bars represent median values. $P < 0.05$ was considered significant.

Figure S7



Supplemental Figure S7. Ratio of ICOS$^+$ non-Treg cells (CD4$^+$CD25$^+$Foxp3$^-$ICOS$^{+)}$ to ICOS$^+$ Treg (CD4$^+$CD25$^-$Foxp3$^+$ICOS$^+$) cells before and after HD IL-2 therapy in responding and non-responding patients. PBMCs at baseline and two days after the last dose of HD IL-2 in cycle 1 were analyzed as described in the Materials and Methods. The ratio of ICOS$^+$ non-Treg cells to ICOS$^+$ Tregs as a percentage of the total viable CD4$^+$ T cells gate was determined and plotted for non-responders (PD/SD, n=31) and responders (PR/CR, n=7). Horizontal bars represent median values. P <0.05 was considered significant.

Figure S8



Supplemental Figure S8. Ratios of $CD4^+CD25^+Foxp3^-$ to $CD4^+CD25^-Foxp3^+$ and $CD4^+CD25^+Foxp3^-ICOS^+$ to $CD4^+CD25^-Foxp3^+ICOS^+$ cells during IL-2 therapy in responding and non-responding patients. PBMC at baseline and two days after the last dose of HD IL-2 in cycle 1 were analyzed as described in the Materials and Methods. The ratio of gated (A) $CD4^+CD25^+Foxp3^-$ to $CD4^+CD25^-Foxp3^+$ and (B) ratio of $CD4^+CD25^+Foxp3^-ICOS^+$ to $CD4^+CD25^-Foxp3^+ICOS^+$ as a percentage of the total viable lymphocyte gate were determined and plotted for non-responders (PD/SD, n=31) and responders (PR/CR, n=7). Horizontal bars represent median values. P <0.05 was considered as significant.