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Supplementary Materials for

CD25 Blockade Depletes and Selectively Reprograms Regulatory T Cells in Concert with Immunotherapy in Cancer Patients

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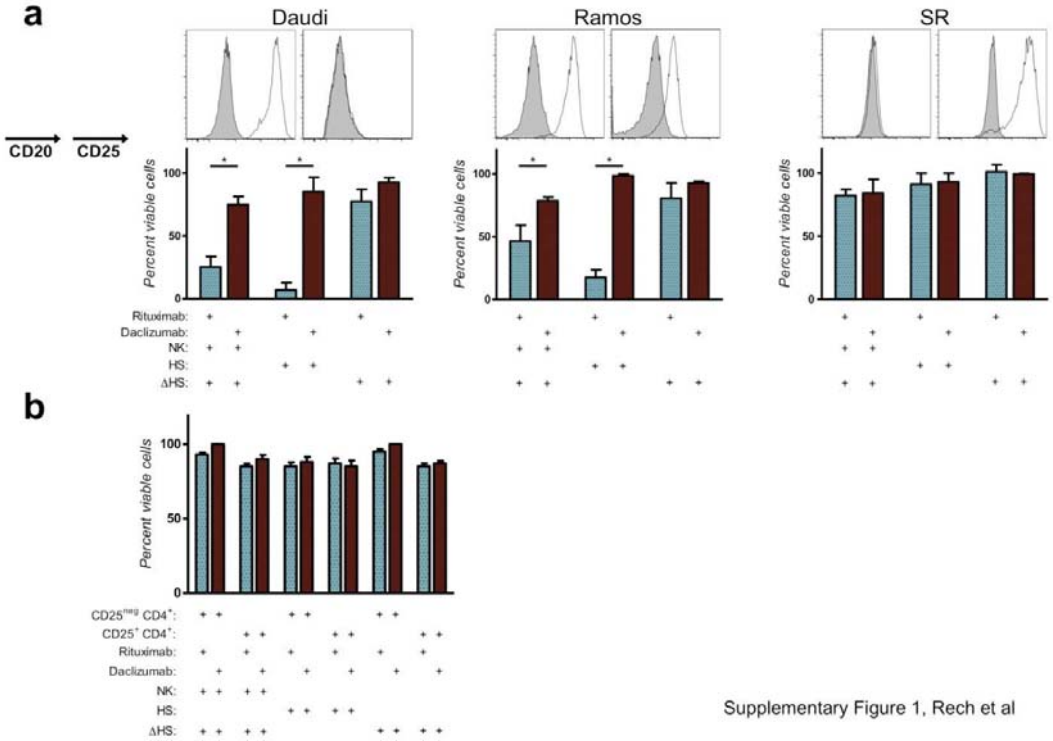
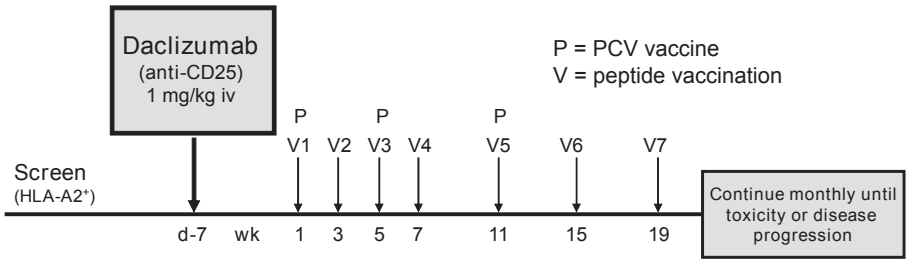
Published 16 May 2012, *Sci. Transl. Med.* **4**, 134ra62 (2012)
DOI: 10.1126/scitranslmed.3003330

The PDF file includes:



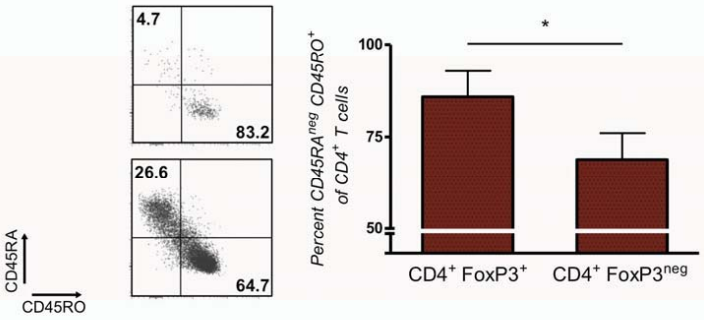
Supplementary Figure 1, Rech et al

Supplementary Figure 1 Daclizumab does not elicit cytotoxicity of CD25-expressing (a) lymphoid cell lines or (b) CD4 T cells. For each cell line tested, CD20 and CD25 expression are shown as unshaded histograms (compared to isotype control shaded histograms). Experimental in vitro conditions were designed to evaluate antibody-dependent cellular cytotoxicity (ADCC) (NK: + and □HS: +), complement-mediated cytotoxicity (CMC) (HS: +) and direct cytotoxicity (□HS: +) of daclizumab (red bars) compared to the CD20 mAb rituximab (blue bars). NK, natural-killer cell containing PBMC; □HS, heat-inactivated human AB serum; HS, non-heat-inactivated human AB serum. Three to 5 independent experiments for each condition were performed; data are shown as the mean for all experiments +/- SE. *, $p<0.05$ by Student's t test.



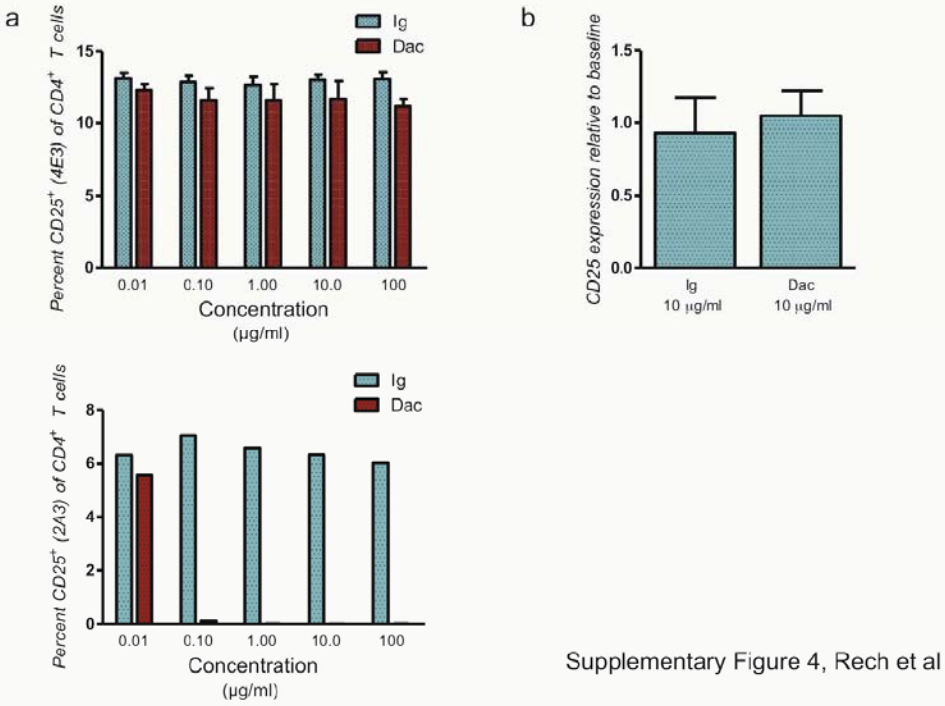
Supplemental Figure 2, Rech et al

Supplementary Figure 2 Clinical trial schema.



Supplementary Figure 3, Rech et al

Supplementary Figure 3 CD45RA and CD45RO phenotype of FoxP3^{+} CD4 T cells from patients with metastatic breast cancer. Left, an individual patient (upper panel, gated on CD4+ FoxP3+ cells; lower panel, gated on CD4+ FoxP3^{neg} cells); right, summary data for 4 patients (mean +/- SD; * $p<0.05$ by Student's t test).

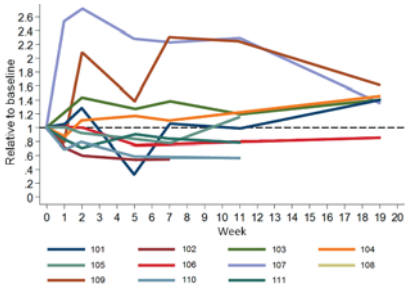


Supplementary Figure 4, Rech et al

Supplementary Figure 4 (a) Daclizumab does not block binding of the CD25 mAb clone 4E3 (upper panel), but daclizumab does block binding of CD25 mAb clone 2A3 (lower panel). CD4 T cells were pre-incubated with increasing concentrations of daclizumab (Dac) or IgG1 (Ig) *in vitro* at 4 C for 1 hr and then evaluated by flow cytometry. Representative data from one of 5 independent experiments are shown. $p<0.05$ (Student's t test) only for 2A3 analysis for Dac at the four highest concentrations compared to Dac 0.01 µg/ml. (b) Daclizumab does not trigger endocytosis of the CD25 antigen. CD4 T cells were incubated with daclizumab or IgG1 *in vitro* overnight at 37 C and then evaluated by flow cytometry using CD25 mAb 4E3. Representative data from one of 5 independent experiments are shown (mean +/- SD); $p>0.05$ (Student's t test).

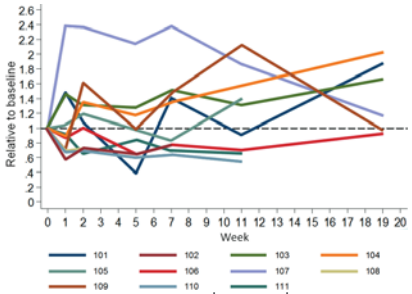
a

Fraction CD4$^+$ of total lymphocytes



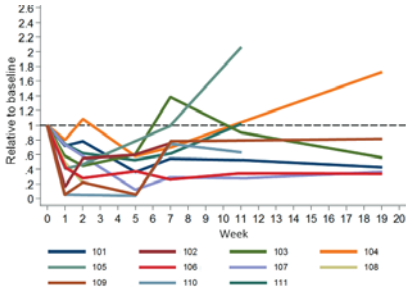
b

Absolute count CD4$^+$ of total lymphocytes



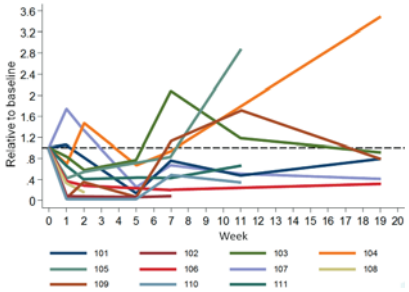
c

Fraction FoxP3$^+$ of CD4$^+$ lymphocytes



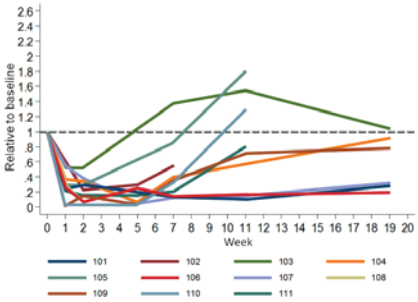
d

Absolute count FoxP3$^+$of CD4$^+$ lymphocytes



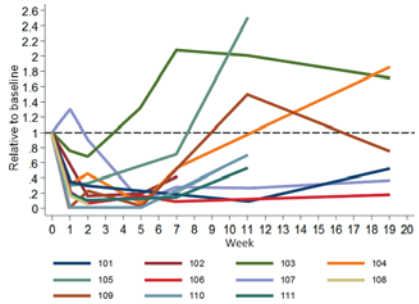
e

Fraction FoxP3$^+$ CD25$^+$of CD4$^+$ lymphocytes



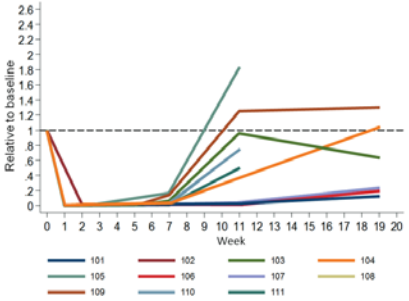
f

Absolute count FoxP3$^+$ CD25$^+$ of CD4$^+$ lymphocytes



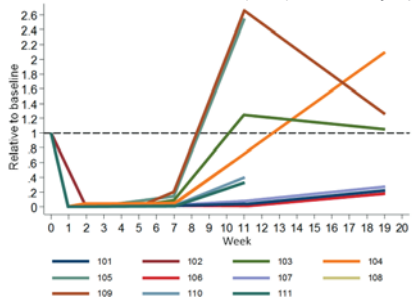
g

Fraction FoxP3$^+$ CD25$^+$ (2A3) of CD4$^+$ lymphocytes



h

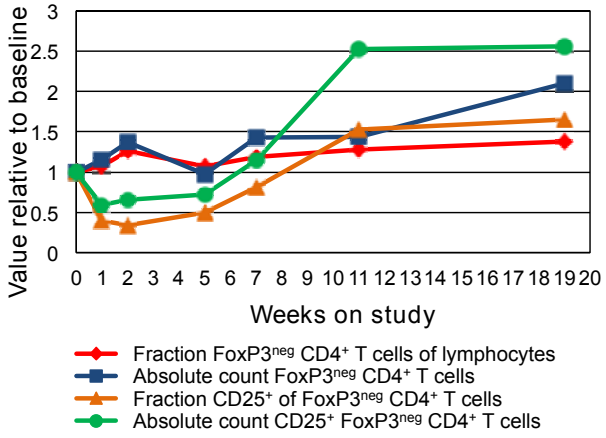
Absolute count FoxP3$^+$ CD25$^+$ (2A3) of CD4$^+$ lymphocytes



Supplementary Figure 5, Rech et al

Supplementary Figure 5 Change in T cell populations shown separately for each patient on study for (**a,b**) total CD4 T cells, (**c,d**) FoxP3+ CD4 T cells, (**e,f**) CD25+ FoxP3+ CD4 T cells as identified by the non-blocked CD25 mAb 4E3, and (**f,h**) CD25+ FoxP3+ CD4 T cells as identified by the blocked CD25 mAb 2A3. Values are shown as fractions (**a, c, e, g**) or absolute counts (**b, d, f, h**). Each curve is annotated and color-coded by the three digit unique patient identifier number with the key shown below each panel.

a



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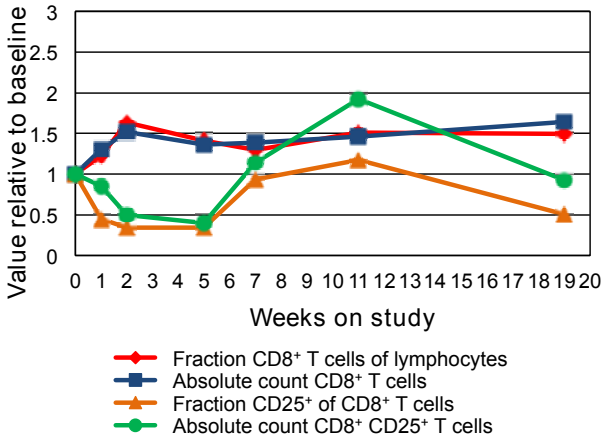
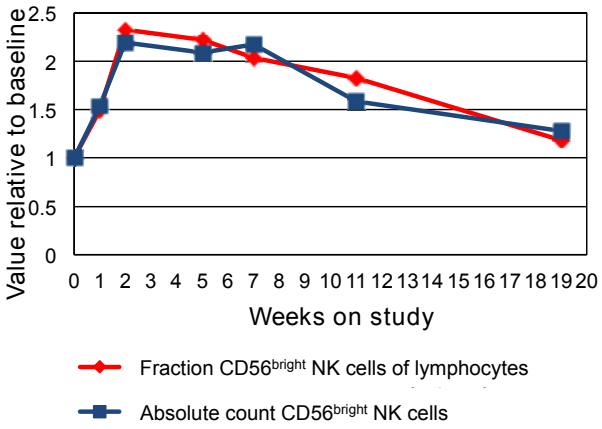


Supplementary Figure 6 Rech et al

Supplementary Figure 6 Impact of daclizumab administration on (**a**) FoxP3^{neg} CD4 T cells and (**b**) CD8 T cells. Peripheral blood samples obtained from patients before and at various times after a single infusion of CD25 mAb daclizumab were analyzed by flow cytometry, shown for each time point as the mean for all patients normalized to individual baseline values. Daclizumab was given on week 0. In (a), the fraction (red diamonds) and absolute counts (blue squares) of total FoxP3^{neg} CD4 T cells as well as the fraction (brown triangles) and absolute count (green circles) of CD25+ FoxP3^{neg} CD4 T cells as identified by the non-blocked monitoring CD25 mAb 4E3. For the fraction of CD25+ FoxP3^{neg} CD4 T cells, $p \leq 0.01$ at weeks 1, 2, 5 and 7; for the absolute count, $p < 0.01$ at weeks 1 and 2, and $p = 0.04$ at week 5. In (b), the fraction (red diamonds) and absolute counts (blue squares) of total CD8 T cells as well as the fraction (brown triangles) and absolute count (green circles) of CD25+ (4E3) CD8 T cells are shown. For the fraction of CD25+ CD8 T cells, $p \leq 0.01$ at weeks 1, 2, 5, 7 and 19, and $p = 0.03$ at week 11; for the absolute count, $p < 0.05$ at weeks 1, 2 and 19.

a



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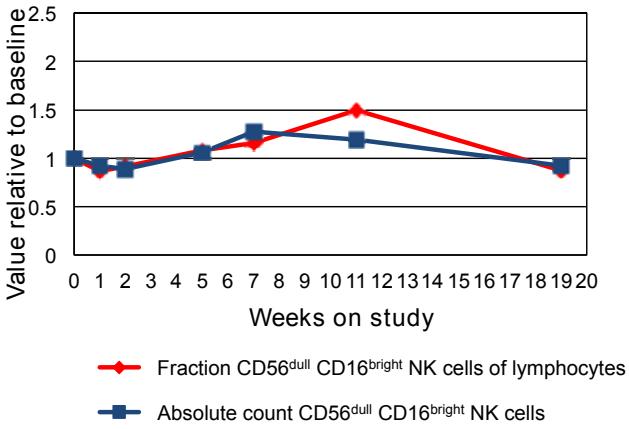

Supplementary Figure 7, Rech et al

Supplementary Figure 7 Impact of daclizumab on natural killer (NK) cell populations. Peripheral blood samples obtained from patients before and at various times after a single infusion of CD25 mAb daclizumab were analyzed by flow cytometry for (**a**) $CD56^{bright}$ NK cells or (**b**) $CD56^{dull}$ $CD16^{bright}$ NK cells, shown for each time point as the mean for all patients normalized to individual baseline values. Daclizumab was given on week 0. In each panel, the fraction (red diamonds) and absolute count (blue squares) are shown for the particular NK subset. For $CD56^{bright}$ NK cells, $p<0.05$ at weeks 2, 5 and 7 (fraction and absolute count).

Supplementary Table 1. Patient characteristics

Patient	Age	Prior treatment*	Hormone Receptor positive?	Her2/neu positive?	# vaccines received	Time on study (months)	Best clinical response†
101	43	H, T	Y	Y	5	3	PD
102	48	C, T, R	N	Y	4	2	PD
103	48	C, T	N	Y	16	14	SD
104	50	C	N	N	8	7	SD
105	61	C, H	Y	Y	5	4.5	PD
106	54	H, R, T	Y	N	6	7	SD
107	43	H	Y	N	16	13	SD
108	35	C	N	Y	2	1	NE
109	55	H	Y	N	7	5	SD
110	52	C, H, R	Y	N	4	2	PD
111	59	H	Y	N	6	5	SD

* Abbreviations: C, chemotherapy; H, hormonal therapy; R, radiation; T, trastuzumab

† Abbreviations: SD, stable disease; PD, progressive disease; NE, not evaluable

Supplementary Table 2. Treatment-emergent patient adverse events and abnormal laboratory values

Toxicity	Grade 1 (# events)	Grade 2 (# events)	Grade 3 (# events)	Grade 4 (# events)
CONSTITUTIONAL				
Chills	7			
Fatigue	21	2		
Headache	3	2		
DERMATOLOGY/SKIN				
RLE[1] injection site induration/erythema	24	45		
RLE injection site pruritus	29	1		
LLE[2] injection site induration/erythema	24	45		
LLE injection site pruritus	31	1		
Deltoid[3] injection site induration/erythema	3	2		
Rash around mouth/cheeks after vaccinations	4			
INFECTION				
Upper respiratory infection	2	1		
MUSCULOSKELETAL				
Edema (pretibial)	1			
NEUROLOGY				
Parasthesias in hands	1			
PAIN				
Generalized pain	7	1		
Pain to tumor site(s)	5	14		
RLE injection site pain	18	2		
LLE injection site pain	16	2		
Prevnar site (deltoid)	2	1		
Abdominal (gallstones)	1			
Jaw/head	1			
Muscular (right side)	1			
PULMONARY				
Sinusitis	1			
Wheezing	1			
SEXUAL/REPRODUCTIVE				
Vaginal discharge, idiopathic		1		
LABORATORY (grade 2-4 only)				
Elevated AST		1		
Leukopenia		1	1[4]	
Lymphopenia			1[5]	
Granulocytopenia				1[4]

[1] RLE, right lower extremity (site of hTERT peptides)
[2] LLE, left lower extremity (site of CMV and Sur1M2 peptides)
[3] Site of PCV injection
[4] Occurred in patient 111 with history of cyclic leukopenia; developed these lab abnormalities at the time of the second vaccine which resolved at the next visit, without clinical significance.
[5] Occurred in patient 102 with history of extensive prior therapy and grade 2 lymphopenia at baseline that became grade 3 one week after daclizumab then resolved at the next visit, without clinical significance.

Supplementary Table 3. T cell subset analysis after daclizumab (corresponds to Fig. 3B).

Changes relative to baseline for fraction of cells, all patients		Week 1	Week 2	Week 5	Week 7	Week 11	Week 19
$CD4^+$ T cells	n	11	11	9	10	8	6
	mean ± SE	1.04 ± 0.16	1.22 ± 0.19	1.02 ± 0.20	1.16 ± 0.20	1.25 ± 0.23	1.35 ± 0.11
	P value	0.76	0.44	0.19	0.99	0.68	0.7
$Foxp3^+$ $CD4^+$ T Cells	n	11	11	9	10	8	6
	mean ± SE	0.47 ± 0.08	0.48 ± 0.09	0.36 ± 0.08	0.70 ± 0.10	0.82 ± 0.20	0.70 ± 0.22
	P value	<0.001	<0.001	<0.001	0.12	0.31	0.13
$CD25^+$ 4E3 $Foxp3^+$ T cells	n	10	11	8	10	8	6
	mean ± SE	0.28 ± 0.06	0.23 ± 0.05	0.24 ± 0.12	0.44 ± 0.13	0.82 ± 0.24	0.59 ± 0.15
	P value	<0.001	<0.001	<0.001	0.002	0.11	0.13
$CD25^+$ 2A3 $Foxp3^+$ T cells	n	10	11	8	10	8	6
	mean ± SE	0.002 ± 0.001	0.008 ± 0.003	0.009 ± 0.004	0.04 ± 0.02	0.67 ± 0.23	0.58 ± 0.20
	P value	<0.001	<0.001	<0.001	<0.001	0.1	0.41