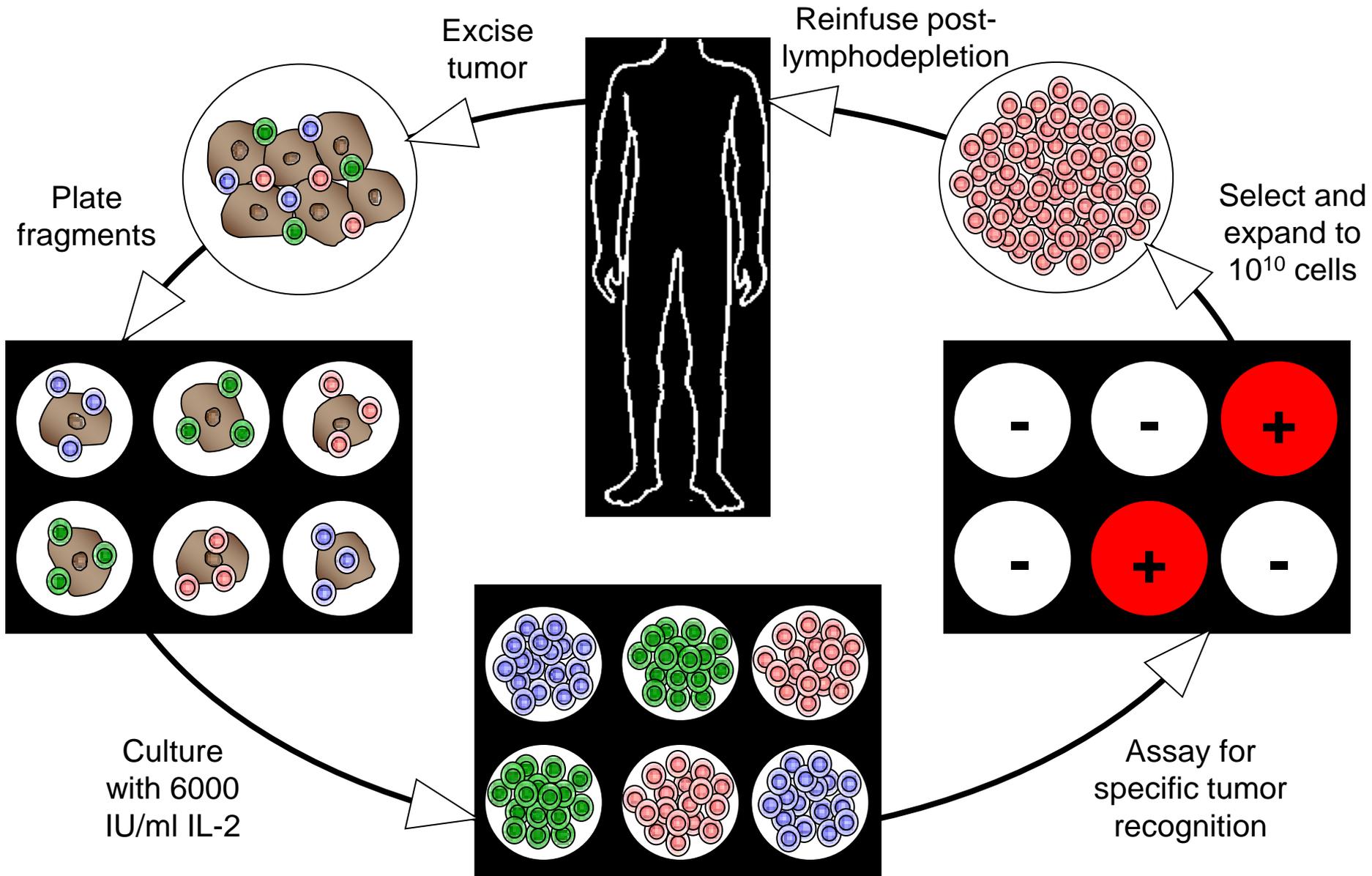


Adoptive transfer of tumor infiltrating lymphocytes (TIL)



Preparative Regimens for Cell Transfer

	Days													
	-7	-6	-5	-4	-3	-2	-1	0	1	2	3			
Non-myeloablative	Cy	Cy	Flu	Flu	Flu	Flu	Flu						Cells IL-2	
Ablative (200cGy)		Cy Flu	Cy Flu	Flu	Flu	Flu							TBI Cells IL-2	IL-2 IL-2 CD34+
Ablative (1200cGy)	Cy Flu	Cy Flu	Flu	Flu	Flu	Flu TBI	TBI	TBI					Cells IL-2	IL-2 IL-2 IL-2 IL-2 CD34+

Cell Transfer Therapy

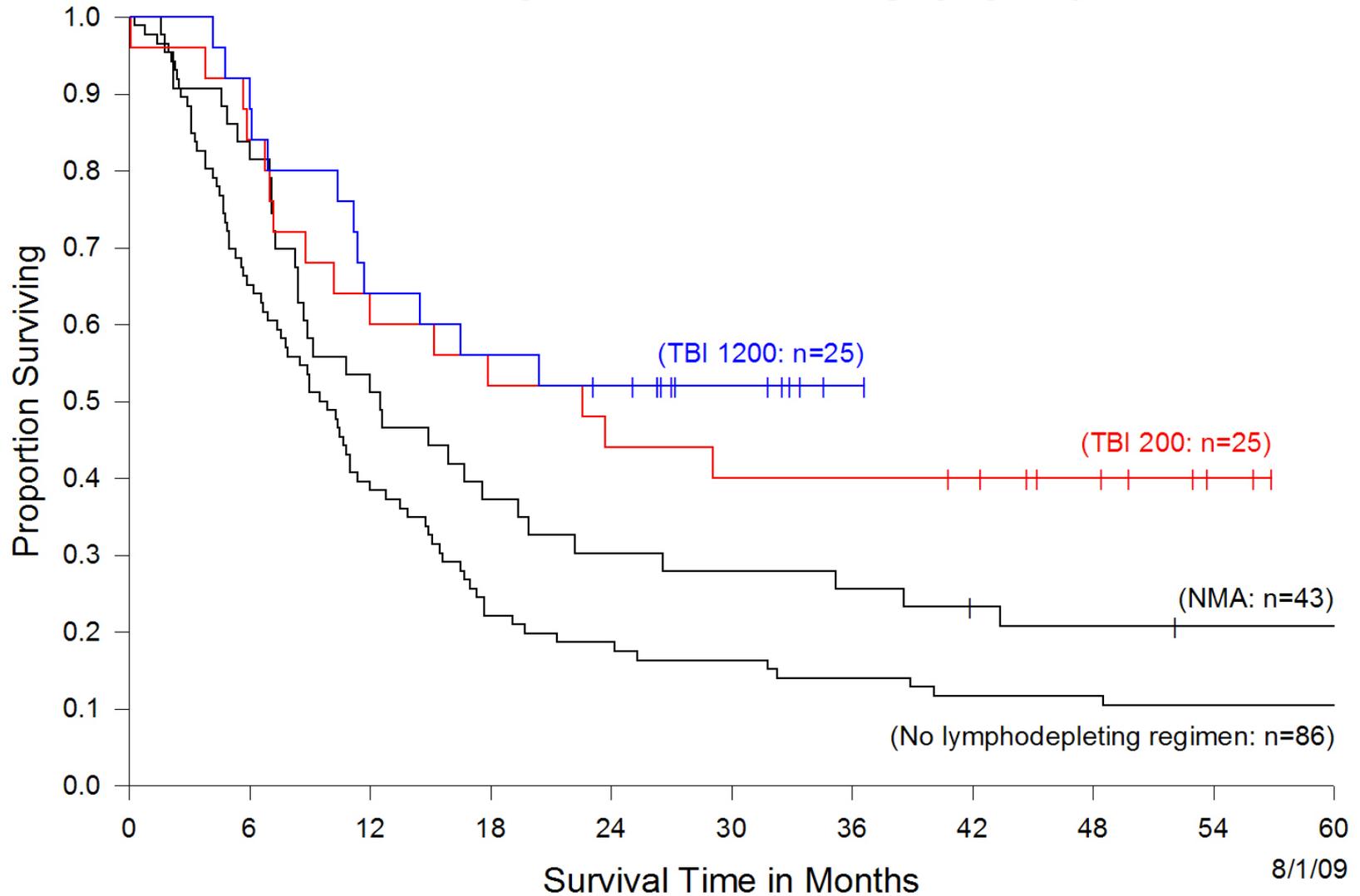
(5/1/10)

Treatment	Total	PR	CR	OR (%)
No TBI	43	16 (84, 36, 29, 28, 14, 13, 11, 8 8, 7, 4, 3, 3, 2, 2, 2)	5 (82+, 78+, 76+, 75+, 61+)	21 (49%)
200 TBI	25	10 (57+, 51+, 14, 9 6, 6, 5, 4 3, 3)	3 (65+, 61+, 54+)	13 (52%)
1200TBI	25	10 (42+, 35+, 21, 13, 7, 6, 6, 5 3, 2)	8 (45+, 41+, 41+, 36+, 35+, 35+, 34+, 19)	18(72%)

(52 responding patients: 42 had prior IL-2, 21 had prior IL-2 + chemotherapy)

(16 complete responses: 15 ongoing at 34 to 82 months)

Survival of Patients with Metastatic Melanoma Treated with Autologous Tumor Infiltrating Lymphocytes and IL-2



Conclusion

Cell transfer immunotherapy can mediate the regression of metastatic cancer in humans.

Autologous peripheral lymphocytes genetically modified to express anti-tumor T cell receptors can mediate cancer regression in vivo.

A major issue confronting the gene therapy of cancer using autologous lymphocyte transfer is the choice of targets.

Most targets have involved tumor antigens. In this protocol we propose to target the tumor vasculature.

Rationale for the anti-VEGFR2 Gene Therapy Protocol

Vascular endothelial growth factor (VEGF) stimulates tumor angiogenesis by binding to its receptor, VEGFR2.

Antibodies to VEGF (bevacizumab) interfere with tumor angiogenesis and improves survival in several metastatic cancers (FDA approved).

Redundancy in angiogenic pathways limits the effectiveness of VEGF.

VEGFR2 is the main receptor on for VEGF and is upregulated on tumor vasculature.

Destruction of cells bearing VEGFR2 may destroy tumor vasculature and result in an effective treatment for multiple cancer types.

VEGFR-2 antibodies

DC101: Rat IgG against mouse VEGFR-2 (Flk-1)

In mouse tumor models, DC101 treatment has been demonstrated to

- decrease the intratumoral interstitial fluid pressure
 - decrease microvessel density
 - normalize the tumor vasculature
 - enhance penetration of chemotherapeutic drugs into tumors
 - decrease the proliferation of tumor cells
 - inhibit tumor growth
-

KDR-1121 (IMC-1121B): A fully human anti-human VEGFR-2 (KDR) antibody
- currently being evaluated in Phase II/III clinical trials (n=9).

Both of these antibodies were developed by *Imclone Systems Inc.*

**Prewett *et al.* Cancer Res 1999; Jain RK. Nat Med (2001); Lin MI Cancer Cell (2004);
Tong *et al.* Cancer Res (2004); Jain RK. Science (2005)**

CAR Targeting VEGFR2 for the Gene Therapy of Cancer

Goal

Destroy existing tumor vasculature

Induce tumor necrosis

Advantages

Selective for tumor vasculature

Potentially active in all solid tumors

Easy access of cells to vascular endothelium

Endothelial cells genetically stable

Reduced risk of acquired resistance

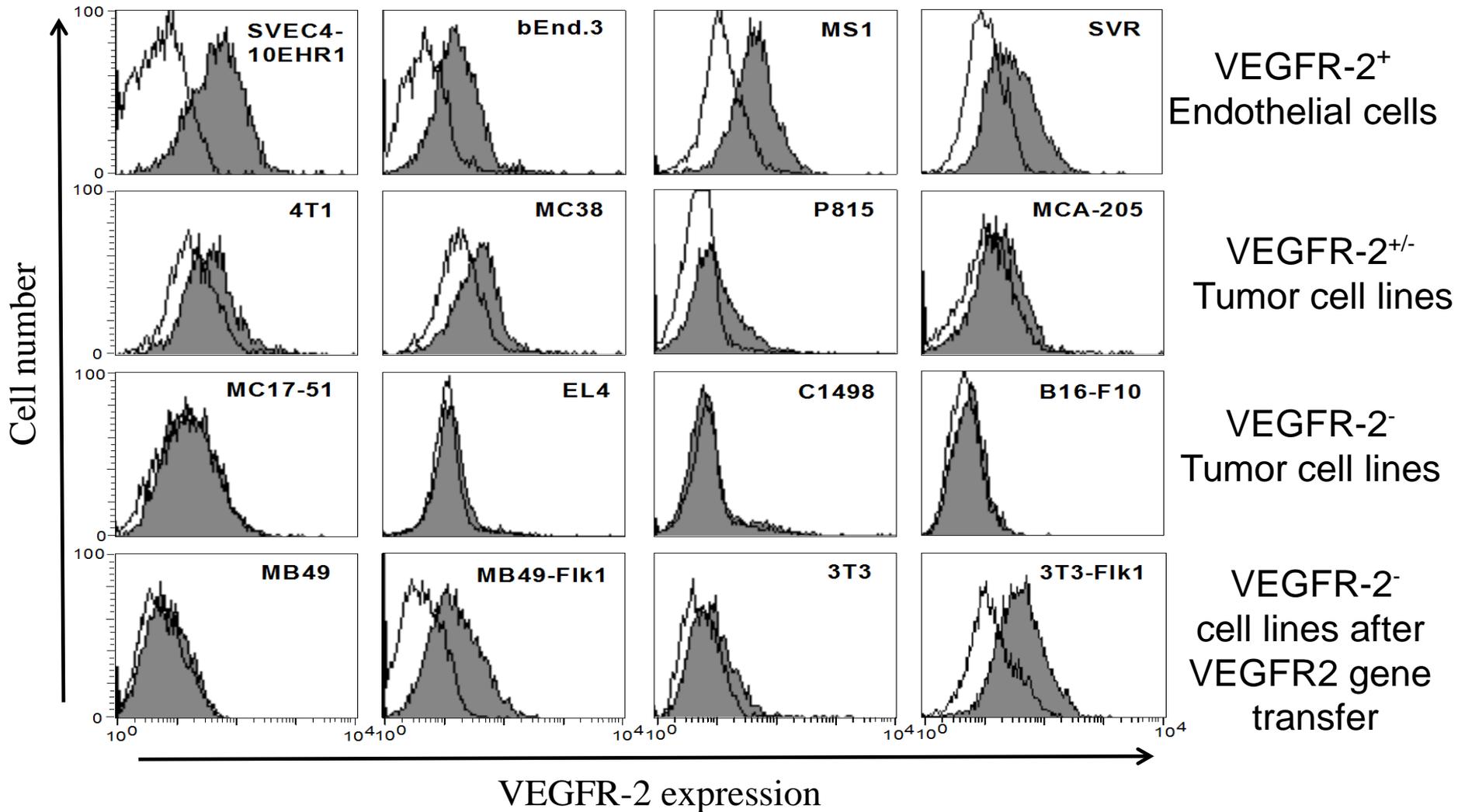
Blood vessel destruction lends to tumor death

Potentially complementary to other regimens

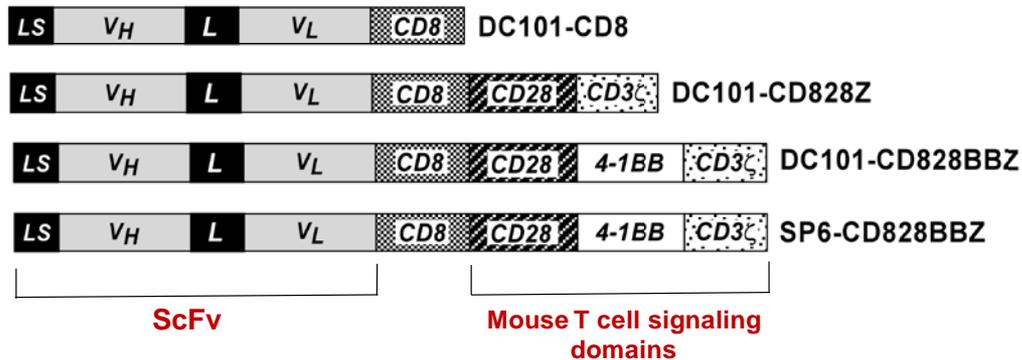
Disadvantages

Low level of VEGFR2 on some normal vessels

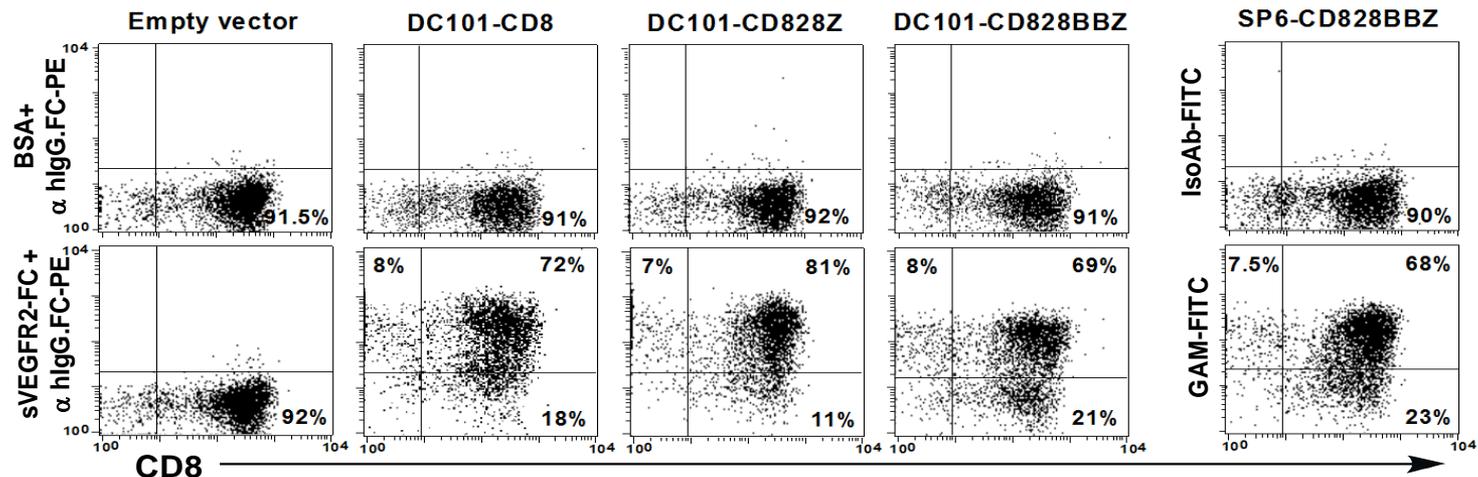
VEGFR-2 expression on mouse cells



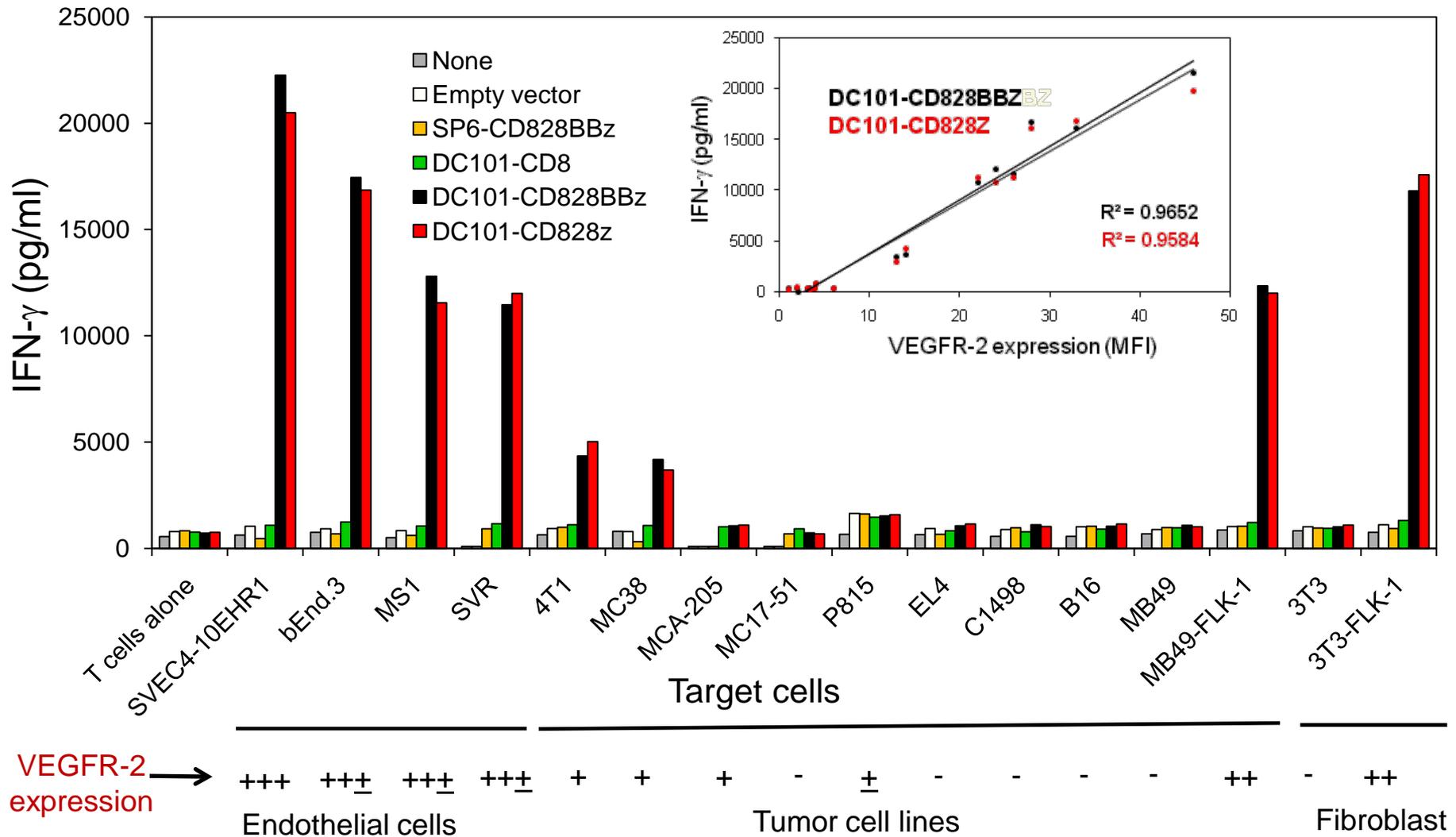
Retroviral vector-mediated expression of DC101-CAR in mouse T cells



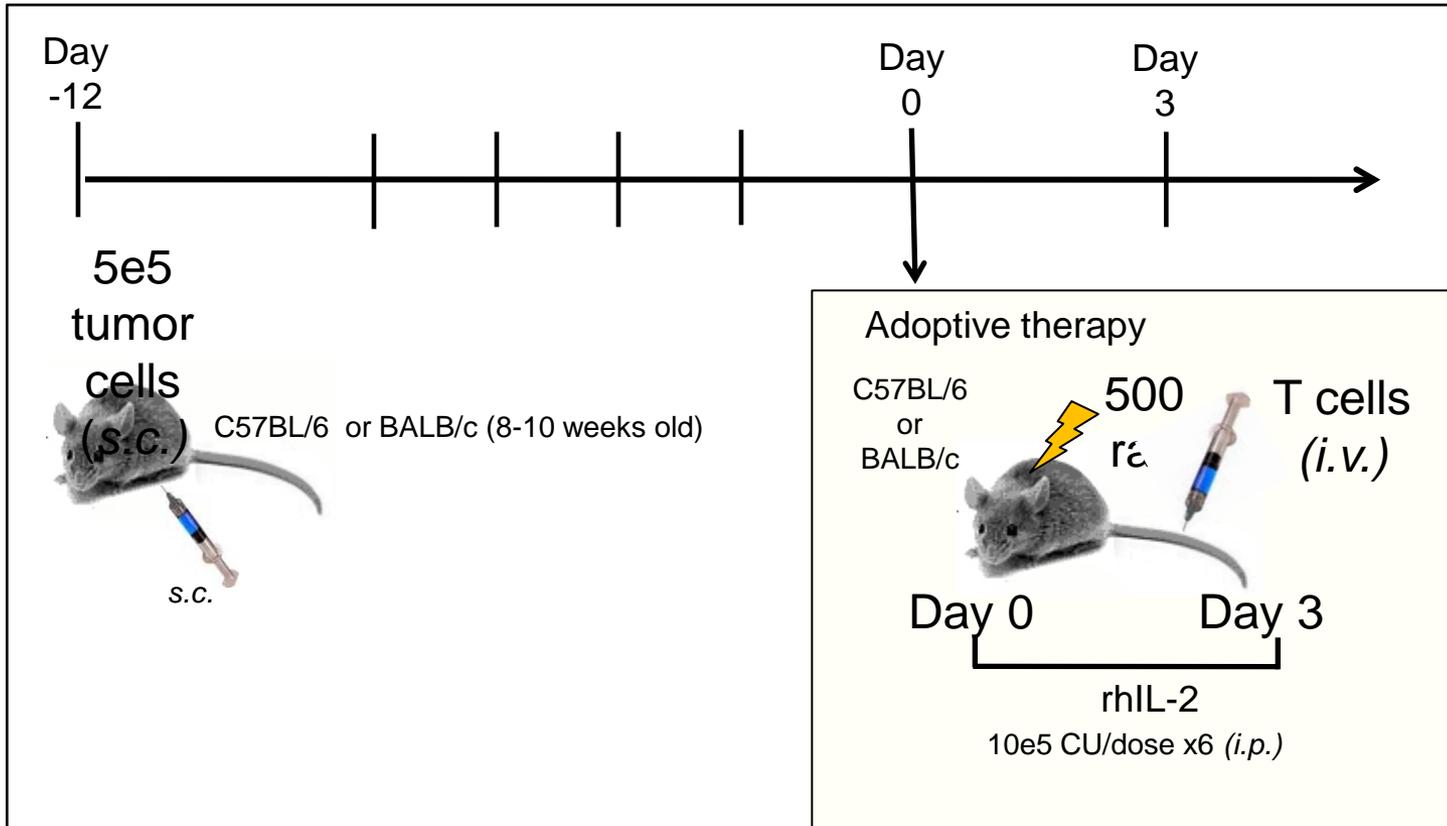
	DC101-CD8	DC101-CD828Z	DC101-CD828BBZ	SP6-CD828BBZ
% Tdxn	80.9 ± 2.0	84.5 ± 2.7	78.5 ± 4.9	86.2 ± 3.6
MFI	111.9 ± 8.4	93 ± 7.3	76.3 ± 5.2	84.8 ± 5.4



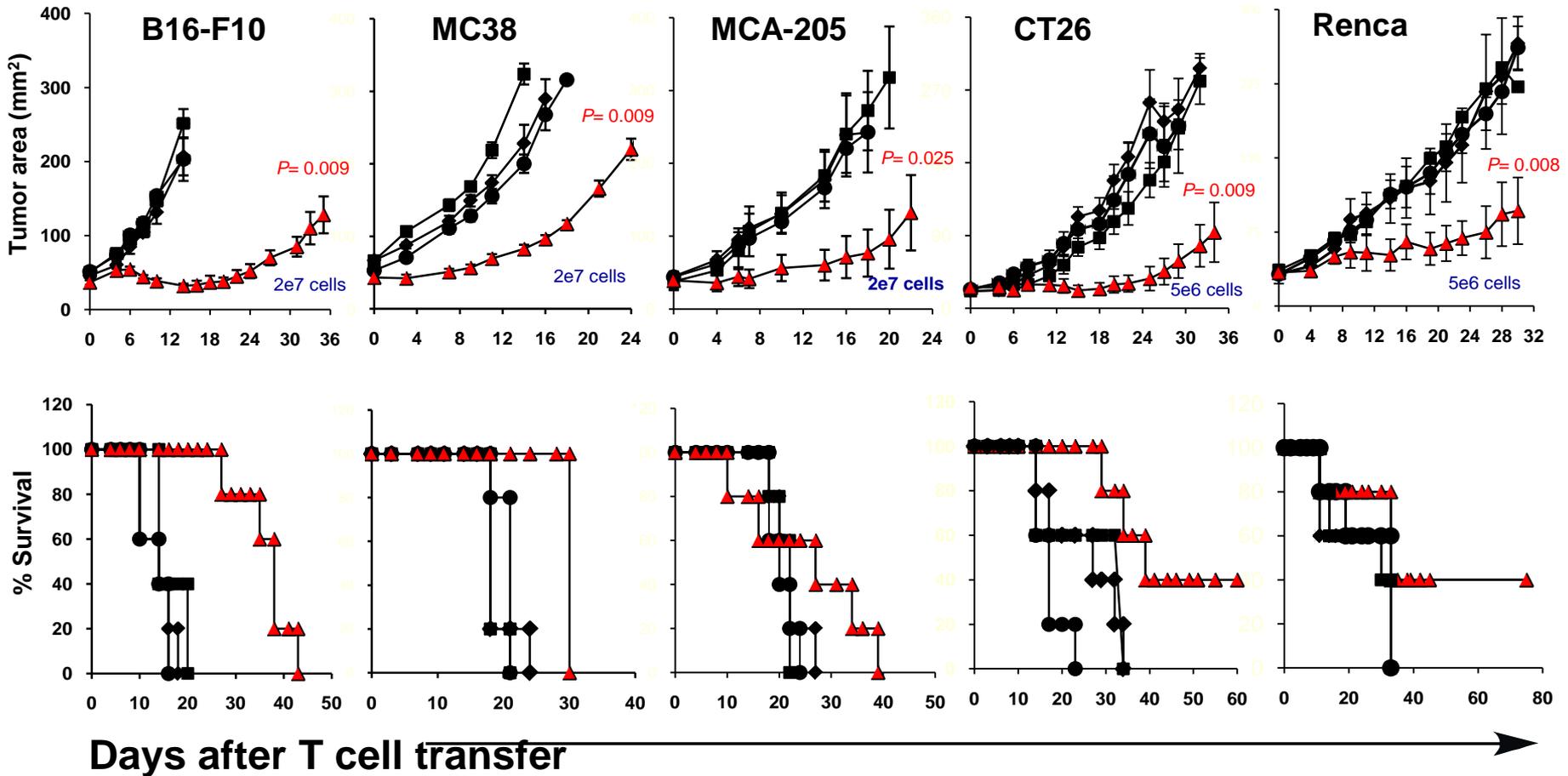
DC101 CAR transduced mouse T cells specifically recognized VEGFR-2⁺ mouse cells



Adoptive T cell transfer (ACT) protocol



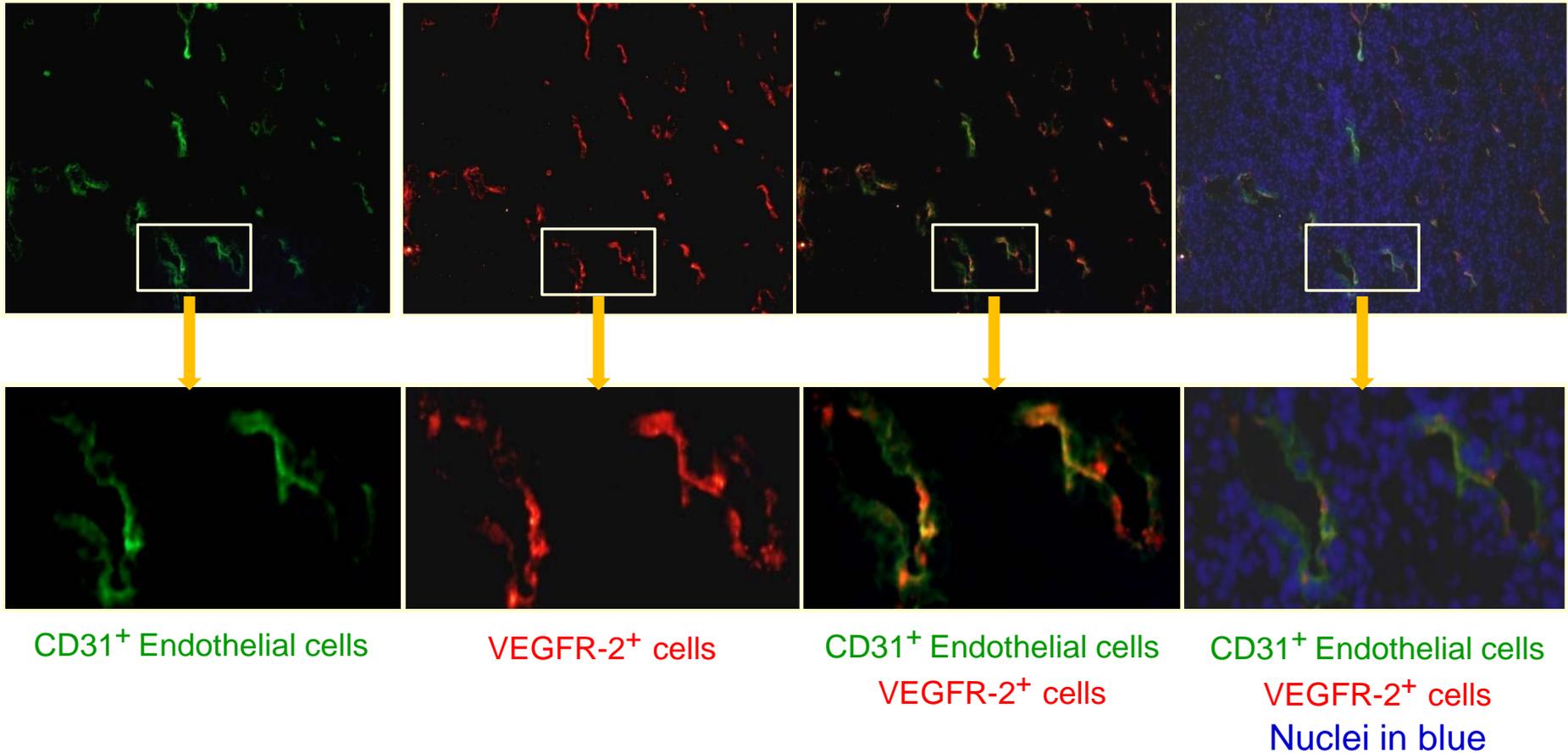
Adoptively transferred VEGFR-2 CAR engineered syngeneic T cells induced regression of multiple established solid tumors in two strains of mice



● No treatment ■ Empty vector Td T cells+ IL-2
 ◆ IL-2 alone ▲ DC101 CAR Td T cells+ IL-2

VEGFR-2 expression is restricted to endothelial cells in tumor vessels of subcutaneous B16 melanoma in C57BL/6 mice

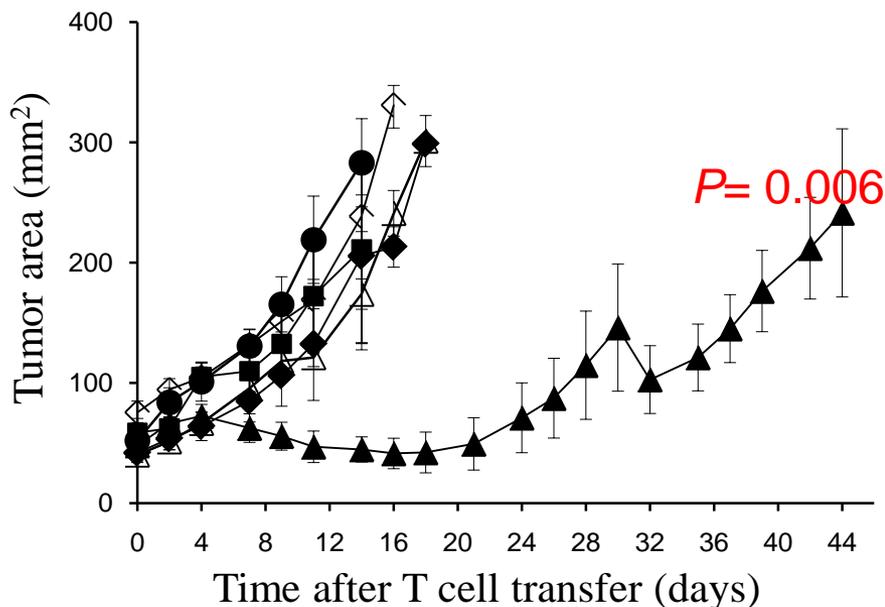
Immunofluorescence analysis of B16 tumor



In vivo antitumor effect of VEGFR-2 CAR modified T cells is cell-mediated and required CAR-mediated intracellular signaling

B16 tumor

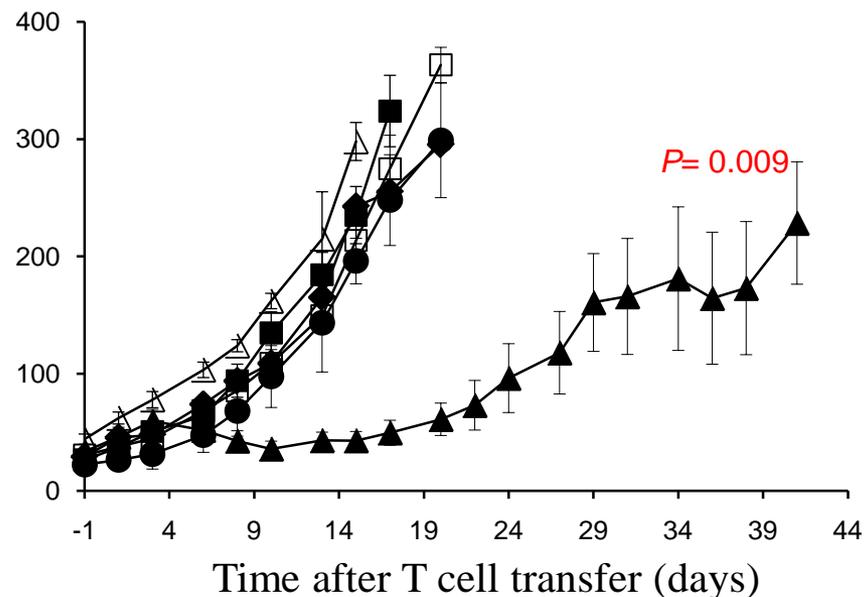
DC101 CAR T cells vs DC101 Ab



- No treatment
- ◆ IL-2 alone
- ◇ Rat IgG + IL + IL-2
- △ DC101 Ab + IL-2
- Empty vector + IL-2
- ▲ DC101 CAR + IL-2

DC101 CAR vs empty vector: $p=0.009$
 DC101 CAR vs DC101 ab: $p=0.006$

DC101 CAR +/- T cell signaling

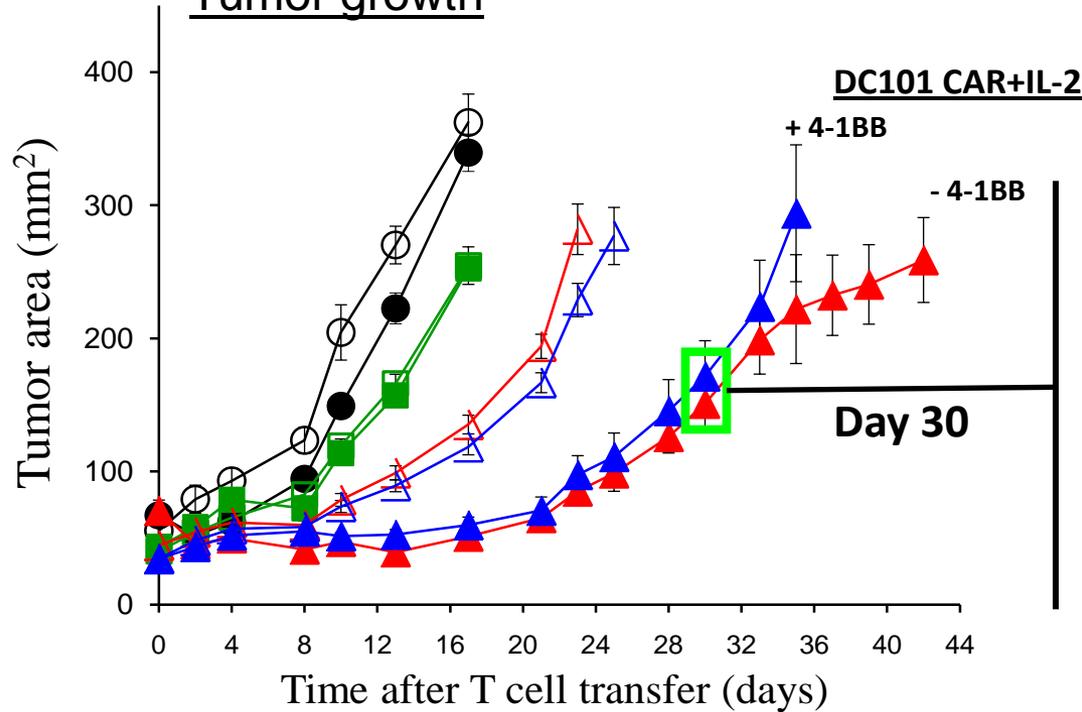


- No Treatment
- ◆ IL-2 + Rat IgG
- Empty Vector + IL-2
- SP6-CAR + IL-2
- △ DC101-CD8 + IL-2
- ▲ DC101-CAR + IL-2

DC101 CAR vs DCD101-CD8: $P=0.009$
 DC101 CAR vs Sp6 CAR: $P=0.009$
 DC101 CAR vs empty vector: $P=0.009$

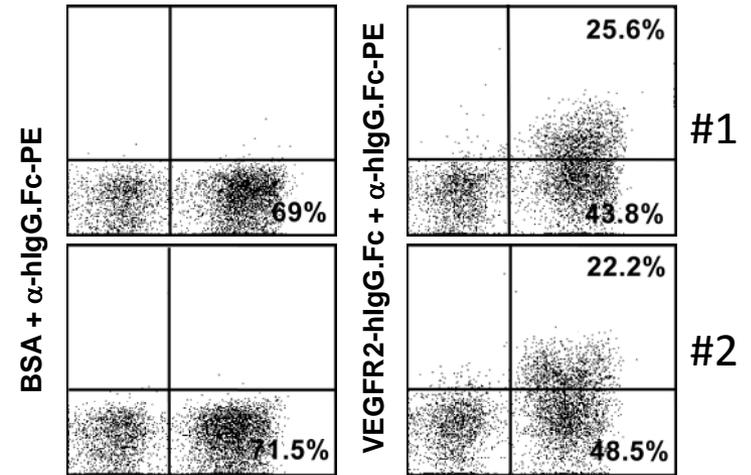
4-1BB signaling sequences enhanced the persistence of DC101-CAR modified T cells in the tumor

Tumor growth

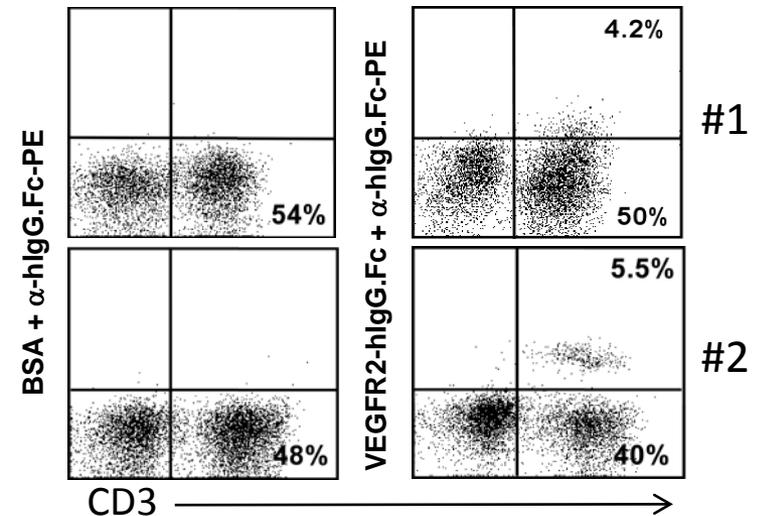


- IL-2 alone
- No Treatment
- Empty vector Td T cells +IL-2
- Empty vector Td T cells
- ▲ DC101(-41BB) Td T cells+ IL-2
- ▷ DC101(-41BB) Td T cells
- ▲ DC101(+41BB) Td T cells+ IL-2
- ▷ DC101(+41BB) Td T cells

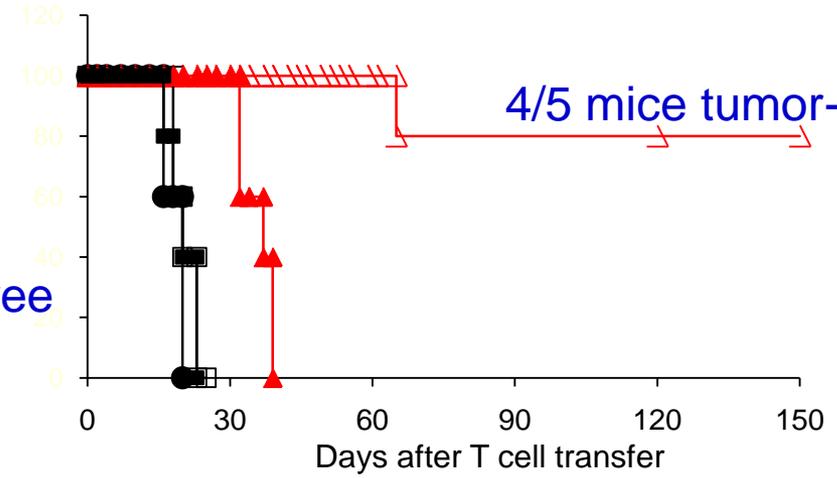
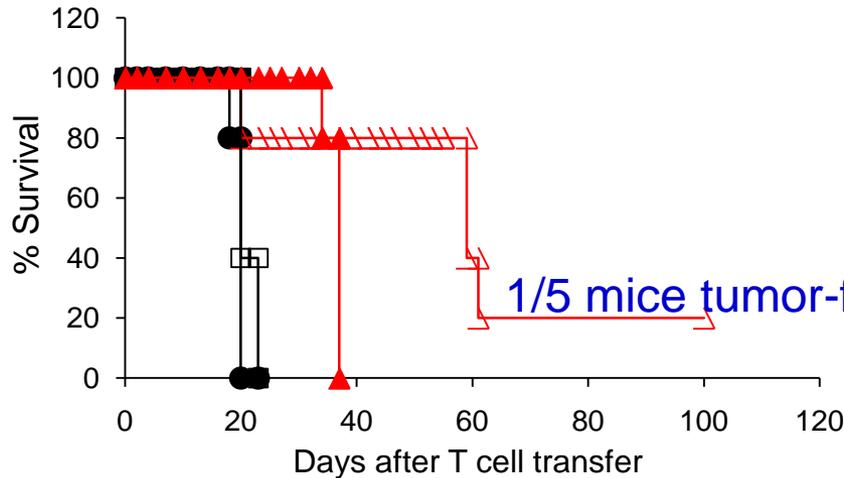
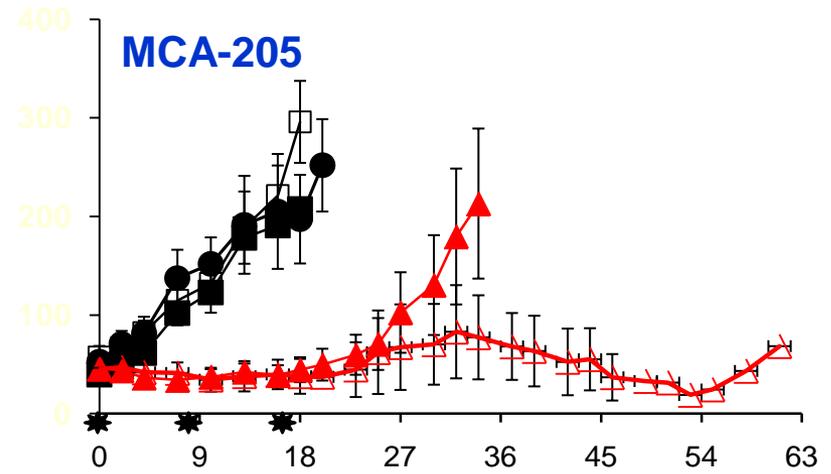
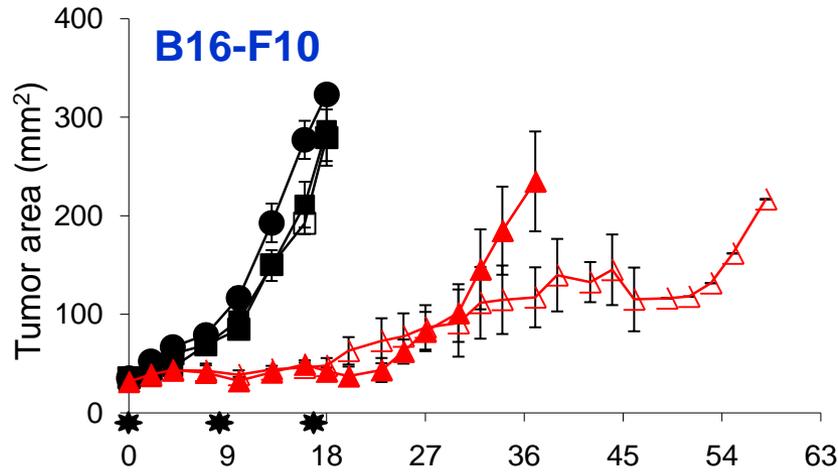
DC101-CD828BBZ



DC101-CD828Z



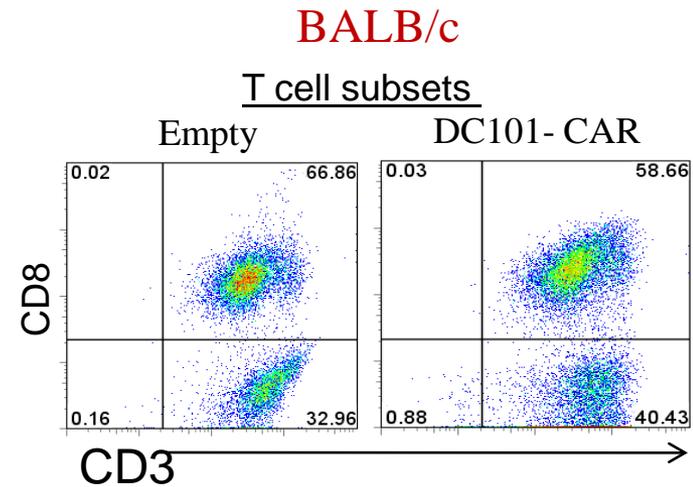
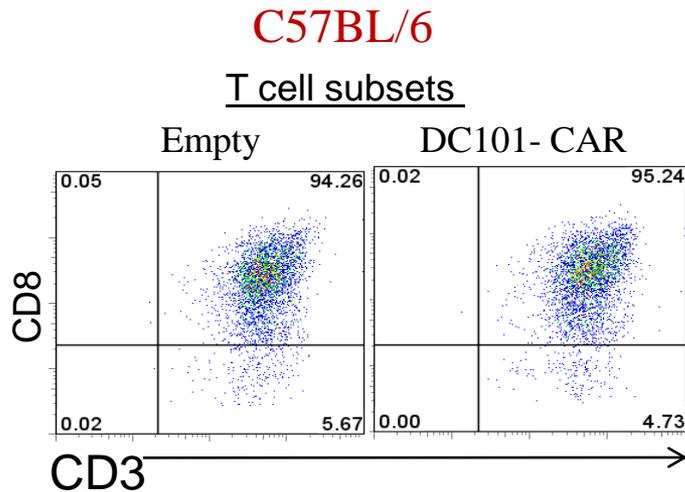
Adoptive transfer of 3 sequential doses 5e6 DC101 CAR transduced T cells into tumor bearing mice increased the tumor regression and tumor-free survival



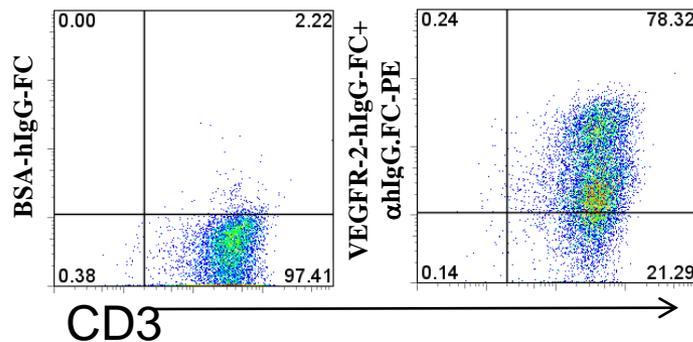
Groups labeled in open symbols received 2 additional doses of 5e6 T cells plus IL-2 on day 7 and 16.

- No treatment
- 2e7 empty vector td T cells + IL-2
- ▲ 2e7 DC101 CAR td T cells + IL-2
- 5e6 empty vector td T cells+IL-2 (x 3 doses at 7-10 days interval)
- ◻ 5e6 DC101 CAR td T cells+IL-2 (x 3 doses at 7-10 days interval)

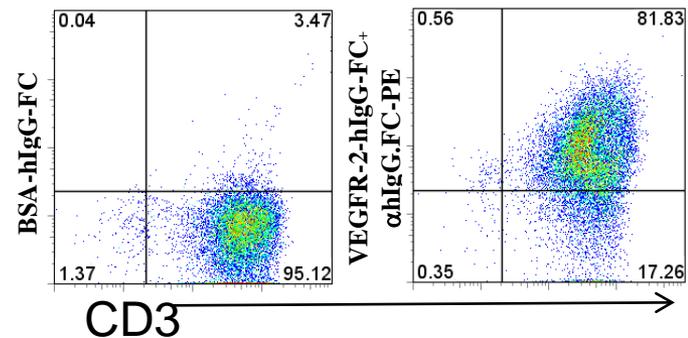
Differences in the phenotype of CD3⁺ T cells of C57BL/6 and BALB/c mice after ConA/IL-7 stimulation and 5 days *in vitro* culture



CAR expression in DC101 CAR transduced T cells



CAR expression in DC101 CAR transduced T cells



CD3⁺ T cells were purified from mouse spleen, stimulated with ConA/IL-7 for 24 hours, transduced, and cultured for 5 days before adoptive transfer.

Summary of mortality and tumor-free survival of tumor bearing C57BL/6 mice treated with DC101 CAR transduced T cells

Single dose of 2e7 DC101 CAR transduced T cells

No. of cells transferred	No. of mice treated	Mortality	Tumor-free survival
Unseparated CD3 ⁺ T cells (>90% CD8 ⁺)	135	0	0
Purified CD3 ⁺ CD8 ⁺ T cells	5	0	0
Purified CD3 ⁺ CD4 ⁺ T cells	5	2	0
1e7 CD4 ⁺ plus 1e7 CD8 ⁺ purified T cells	5	1	0

Multiple doses of DC101 CAR transduced unseparated CD3⁺ T cells (>90% CD8⁺)

No. of cells transferred	No. of mice treated	Mortality	Tumor-free survival
2 doses of 2e7 cells at 15 days interval	10	0	3 mice, >180 days
3 doses of 5e6 cells at weekly interval	20	0	7 mice, >60 days

Mice received 5Gy TBI prior to receiving the first dose of cells

Summary of treatment effects and toxicity DC101 CAR transduced T cells in BALB/c mice bearing various established tumors

Unseparated CD3⁺ (CD4⁺ plus CD8⁺) T cells

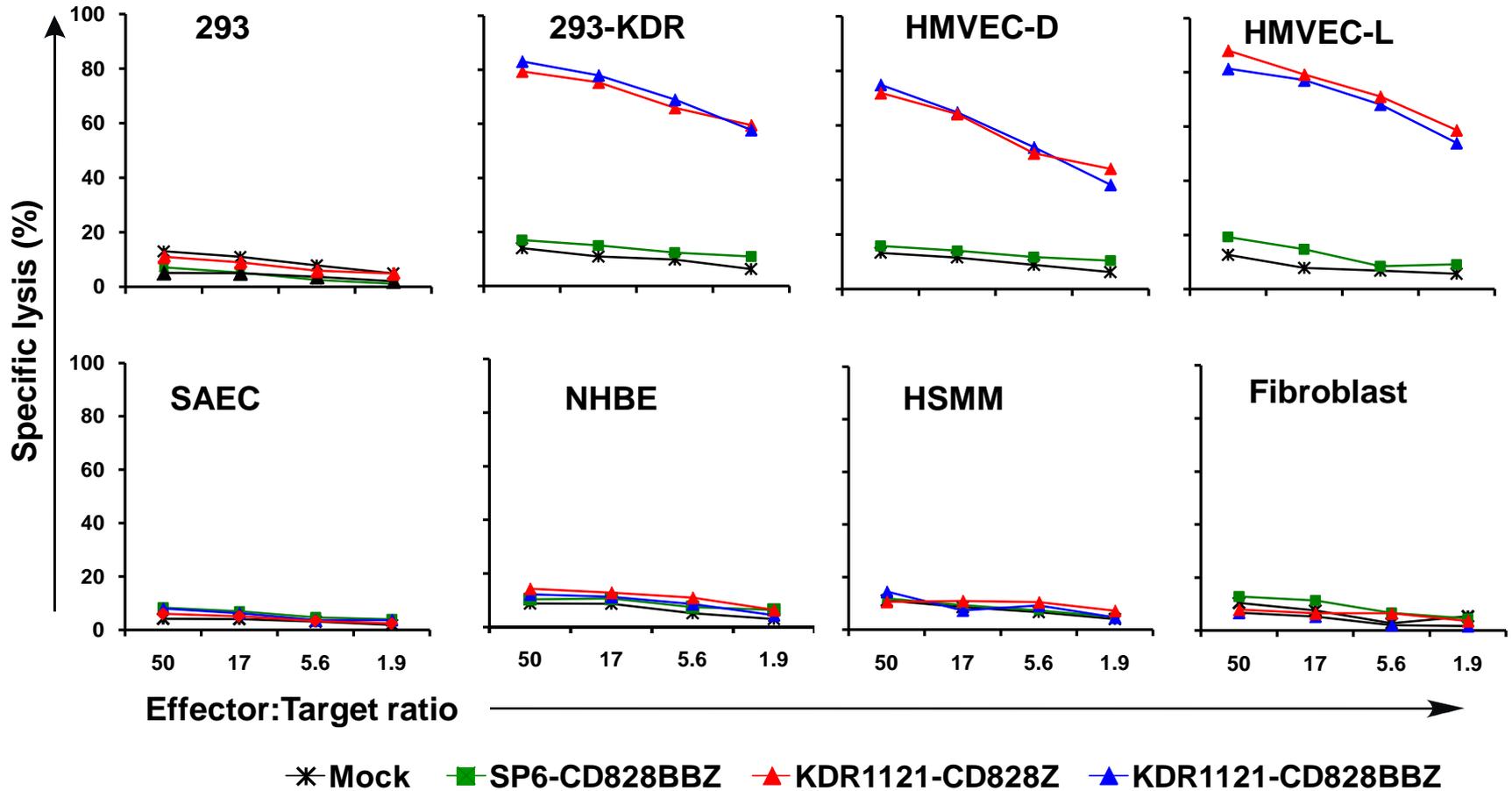
No. of cells transferred	Number of mice treated	Mortality	Tumor-free survival
2e7 DC101 CAR	33	21	8; >75 days
5e6 DC101 CAR	25	4	5; >65 days
Total	58	25	13

Purified CD3⁺CD8⁺ T cells

No. of cells transferred	Number of mice treated	Mortality	Tumor-free survival
2e7 DC101 CAR	15	0	5 mice; >75 days
5e6 DC101 CAR	10	0	2 mice; > 65 days
Total	25	0	7

All mice received 5Gy TBI and 6 doses of IL-2 at 1e5 CU/dose

KDR CAR transduced human T cells specifically killed the primary endothelial cells and 293 cells expressing VEGFR-2



293: Human embryonic kidney epithelial cell line
HMVEC-D: Human dermal micro-vessel endothelial cells
HMVEC-L: Human lung micro-vessel endothelial cells

SAEC: Human small airway epithelial cells
NHBE: Normal human bronchial epithelial cells
HSMM: Human smooth muscle myoblasts

SUMMARY

- **Adoptive therapy of DC101 CAR transduced mouse T cells resulted in significant regression of multiple types of established syngeneic solid tumors in mice and extended the survival rate of mice.**
- **Intracellular signaling sequences are necessary for the functionality of DC101 CAR.**
- **IL-2 coadministration enhanced the *in vivo* antitumor effect of DC101 CAR modified T cells, whereas 4-1BB signaling influenced the persistence of the T cells at the tumor site without additional therapeutic benefit.**
- ***In vivo* antitumor activity of DC101 CAR modified T cells directly correlated with the number of infused T cells.**
- **There were no treatment related adverse effects documented in tumor treatment experiments in C57BL/6.**
- **However, treatment related toxicities were observed in tumor bearing BALB/c mice treated with higher numbers (2e7) of DC101 CAR transduced T cells in conjunction with rhIL-2 that could be eliminated by purifying CD8 cells without loss of therapeutic efficacy.**

PROTOCOL TITLE

**Phase I/II Study of Metastatic Cancer Using Lymphodepleting
Conditioning Followed by Infusion of Anti-VEGFR2
Gene Engineered CD8 Lymphocytes**

Principal Investigator: Steven A. Rosenberg, M.D., Ph.D.

Anti-VEGFR2 Gene Therapy: Objectives

Primary objectives:

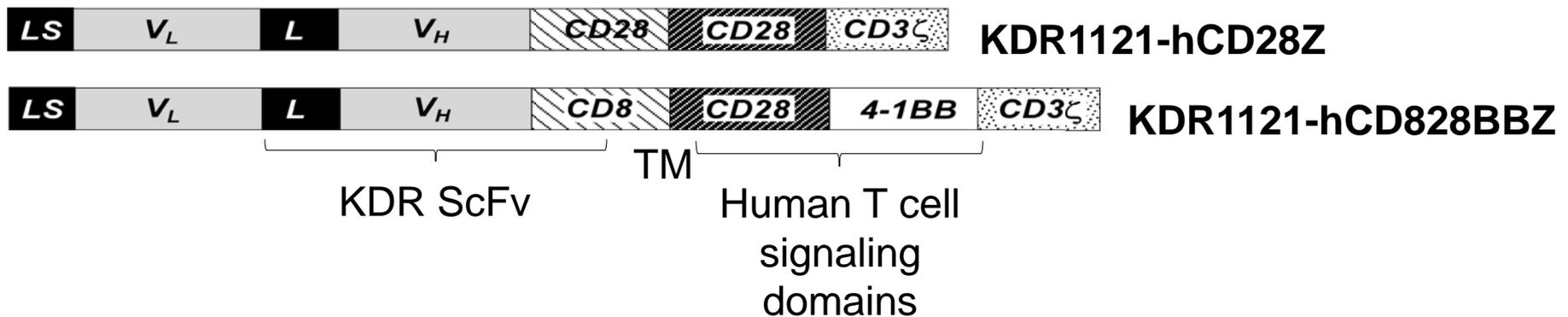
- To evaluate the safety of the administration of anti-VEGFR2-CAR engineered CD8+ peripheral blood lymphocytes in patients receiving a non-myeloablative conditioning regimen and aldesleukin
- Determine if the administration of anti-VEGFR2-CAR engineered CD8+ peripheral blood lymphocytes and aldesleukin to patients following a non-myeloablative but lymphoid depleting preparative regimen will result in clinical tumor regression in patients with metastatic cancer

Second objective:

- Determine the in vivo survival of CAR gene-engineered cells

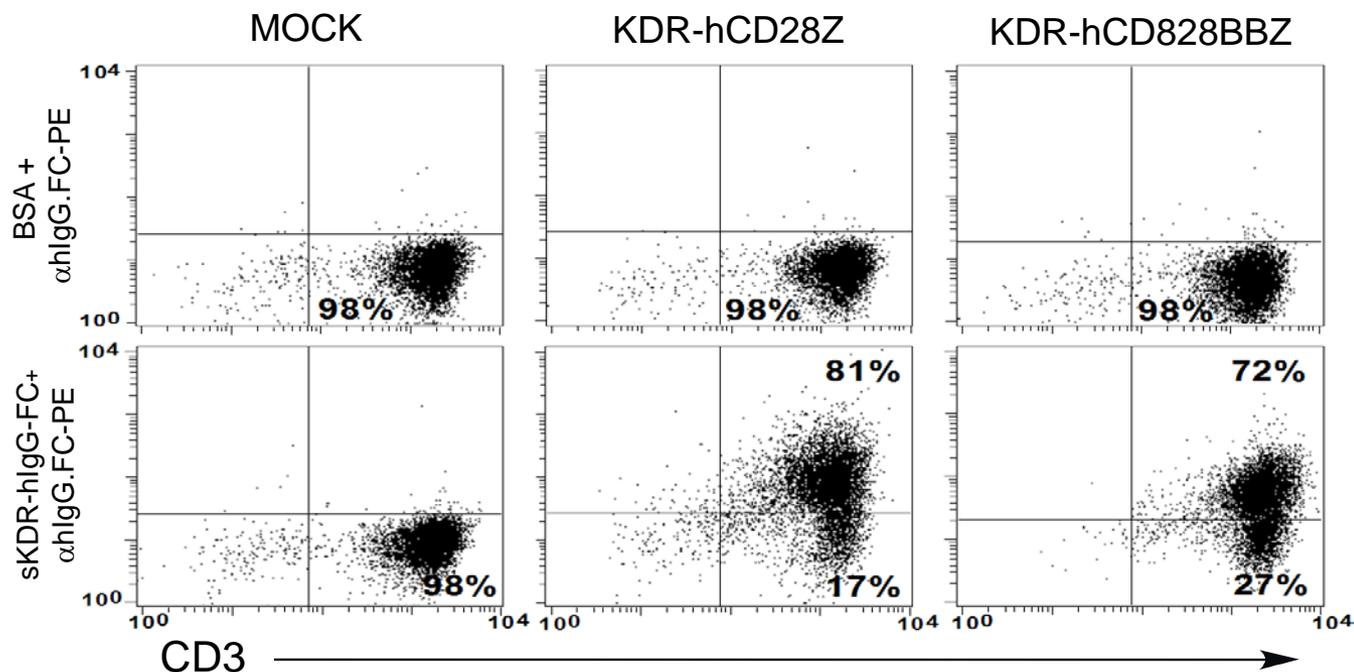
Construction and evaluation of retroviral vectors encoding CAR against human VEGFR-2 (KDR)

[KDR-1121 \(IMC-1121B\)](#): A fully human anti-human VEGFR-2 (KDR) antibody - currently being evaluated in Phase II/III clinical trials (n=9).



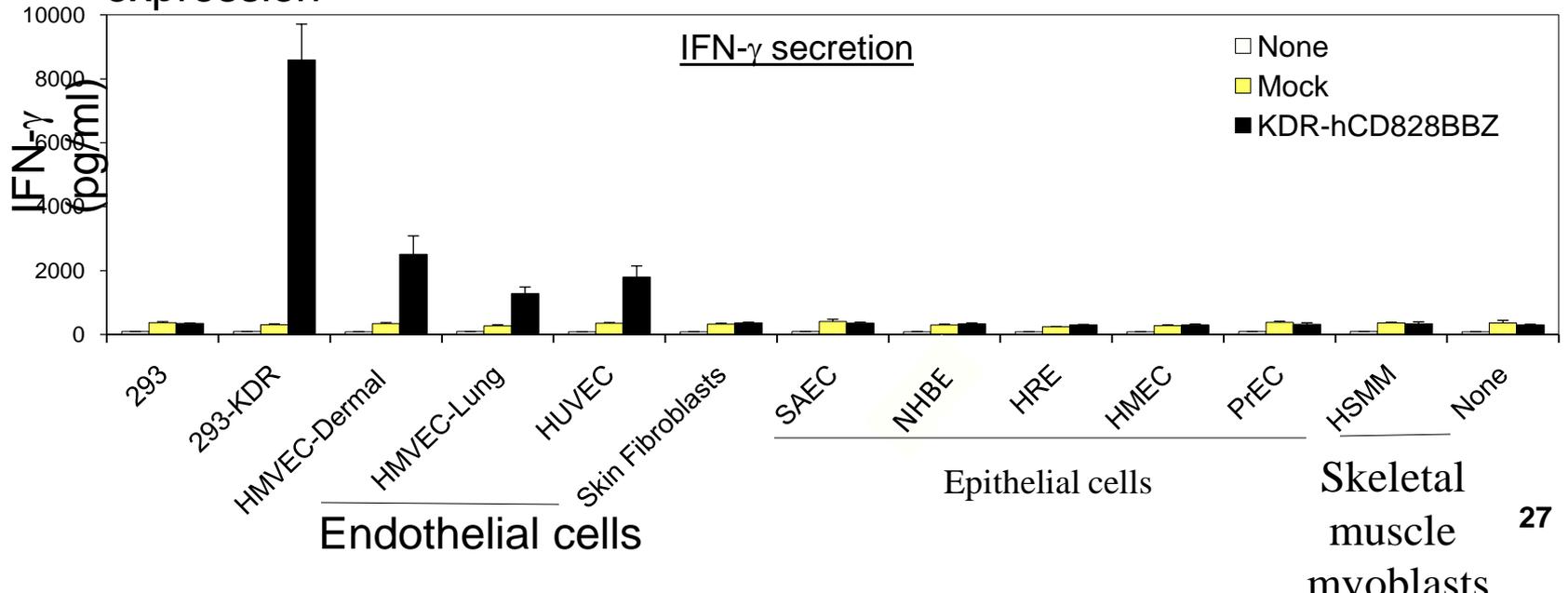
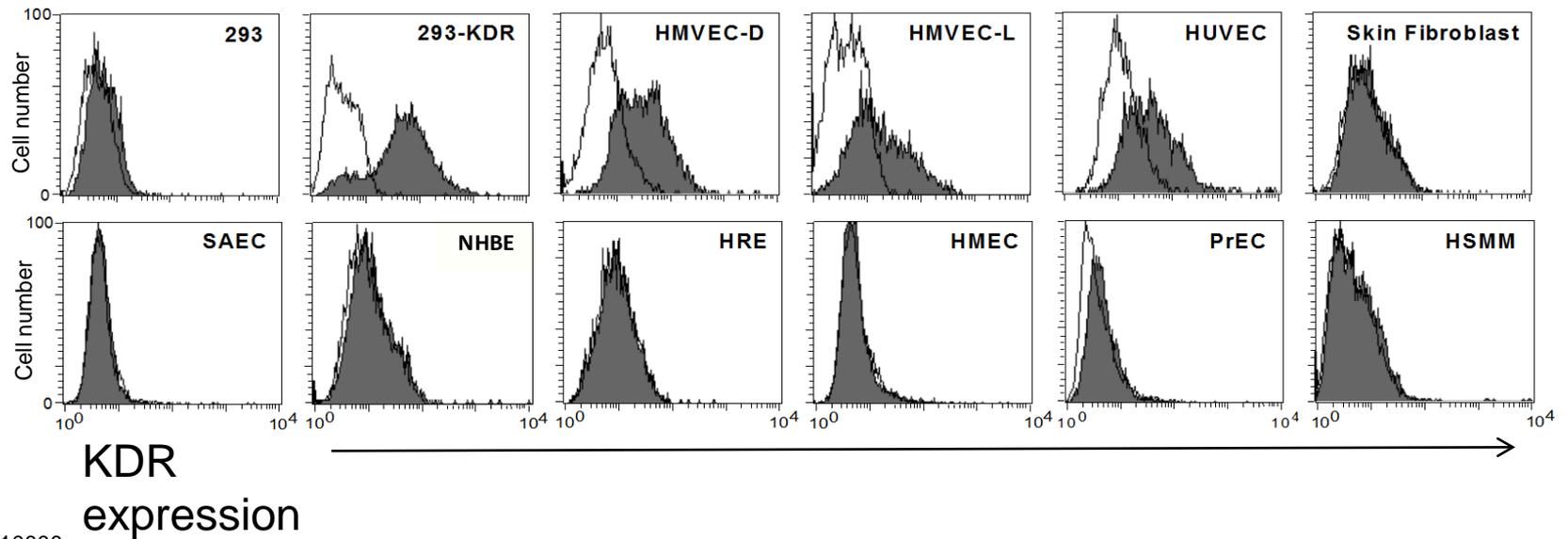
The codon optimized KDR ScFv comprising the V_L and V_H sequences fused by a 218 linker was designed and synthesized using a web-based DNA codon optimization algorithm (Gao, A. et al. (2004) using the primers generated by the Ugene software.

Expression of KDR CAR in transduced primary human peripheral blood lymphocytes



	KDR1121-hCD28Z	KDR1121-hCD828BBZ
% Tdxn	85 ± 1.7	79 ± 1.5
MFI	160 ± 16	123 ± 21

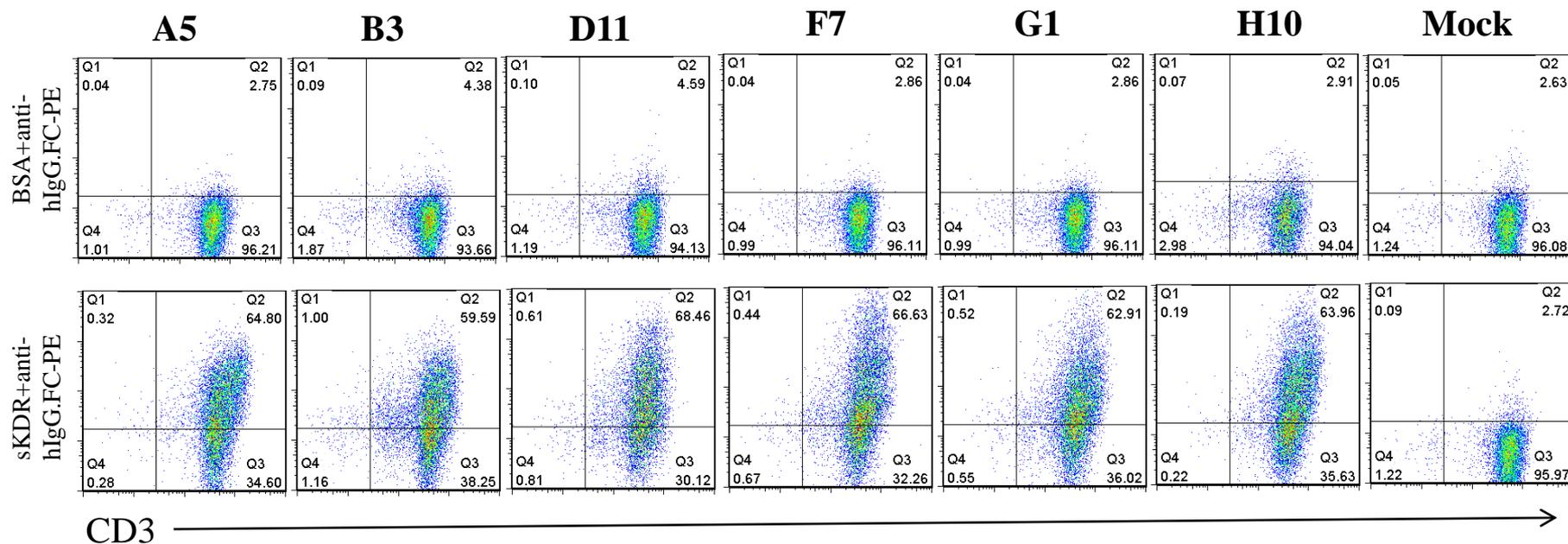
KDR CAR transduced human PBLs specifically recognized KDR expressing primary human endothelial cells



In vitro expansion of primary human PBLs transduced with KDR CAR retroviral supernatants from stable PG13 packaging clones

REP1day14

Donor-2: Roahen, James O



	A5	B3	D11	F7	G1	H10	Mock
% Tdxn	64.8	59.6	68.5	66.6	63.0	64.0	2.7
MFI	63.0	51.2	79.8	89.9	54.4	61.7	6.6

Anti-VEGFR2 Gene Therapy: Eligibility

Metastatic cancer with measurable disease

Patients must have progressed after at least one systemic standard care for metastatic disease if known to be effective

≥ 18 years old

ECOG 0 or 1

Life expectancy > 3 months

ANC > 1000 mm³

Platelets $> 100,000$ mm³

Hemoglobin > 8 g/dl

Creatinine ≤ 1.6 mg/dl

No brain metastases

No active infections or major illnesses of the cardiovascular, respiratory, renal systems

Anti-VEGFR2 Gene Therapy: Treatment Regimen

Enrich CD8+ cells from leukopheresis (Miltenyi apparatus)

Stimulate with OKT-3, transduce with anti-VEGFR2 CAR and expand

Infusion:

d-7 to d-1: Cy/flu preparative regimen

d0: single infusion (3 patients/cohort)

Cohort 1: 10^6 cells

2: 10^7

3: 10^8

4: 10^9

5: 10^{10}

6: 10^{10} to 5×10^{10}

Mandatory safety assessments:

2 week delay after the first patient in any cohort

2 week delay after last patient in cohort prior to enrollment in next cohort

Anti-VEGFR2 Gene Therapy: Treatment Regimen (cont.)

Dose limiting toxicity (DLT):

All grade 3 and 4 toxicities except:

- (1) myelosuppression due to preparative regimen**
- (2) occurring within 24 hours of cell infusion reversible in < 8 hours by acetaminophen and/or diphenhydramine**

If 2 DLTs develop drop to next lower dose and treat at least 6 patients

MTD is highest dose at which ≤ 1 patient develops DLT

Phase II protocol at MTD: 21 evaluable patients if ≥ 2 responses, 41 total patients)

Cohort 1: Metastatic melanoma and renal cancer

Cohort 2: Other cancer types

Anti-VEGFR2 Gene Therapy: Safety Considerations

No toxicity in C57BL/6 or BALB/c mice infused with 2×10^7 CD8 enriched CAR transduced cells

(2×10^7 cells in 25 gm mouse = 8×10^8 /kg)

Starting dose: 10^6 cells (in 70 kg human; 1.4×10^4 /kg)

**Thus: 57,000 fold lower cell dose in human on per kg basis
20 fold fewer cells in human than safely tolerated by mice**

Anti-VEGFR2 Gene Therapy: Certificate of Analysis

Test	Method	Limits	Result	Initials/ Date
Cell Viability	trypan blue exclusion	> 70%		
Total viable cell number	visual microscopic count	Between 10^6 and 5×10^{10} cells		
Identity	FACS	> 90% CD3 + CD8+ after transduction		
Tumor reactivity	□IFN release vs. VEGFR2 cell line	> 200 pg/ml and > 2 times background		
CAR expression	FACS analysis of the transduced cells	PBL, > 30%		
Microbiological studies	gram stain	no micro-organisms seen		
	aerobic culture	no growth		
	fungal culture	no growth		
	anaerobic culture	no growth		
	mycoplasma test	negative		
Endotoxin	limulus assay	< 5 E.U./kg		
RCR	S+L Assay RCR-PCR	negative		

