Supplementary Methods for:

Local Delivery of Interleukin-12 using T cells Targeting Vascular Endothelial Growth Factor Receptor-2 Eradicates Multiple Vascularized Tumors in Mice

Dhanalakshmi Chinnasamy, Zhiya Yu, Sid P. Kerkar, Ling Zhang, Richard A. Morgan, Nicholas P. Restifo, and Steven A. Rosenberg

Surgery Branch, National Cancer Institute, Clinical Research Center, Bethesda, MD 20892

Requests for reprints: Steven A. Rosenberg, Surgery Branch, National Cancer Institute, Clinical Research Center, Building 10-CRC, Room 3-3940, 10 Center Drive, MSC 1201, Bethesda, MD 20892. Phone: 301. 496.4164; Fax: 301402-1738; E-mail: *SAR@mail.nih.gov*

*To whom correspondence should be addressed. E-mail: *SAR@mail.nih.gov*

Materials and Methods

Cell culture. The murine tumor lines used in this study were B16-F10 (melanoma), MCA-205 (sarcoma), MC38 (colon adenocarcinoma), MB49 (bladder carcinoma), MC17-51 (sarcoma), CT26 (colon carcinoma). B16-F10, MCA-205, and MC38 were obtained from the cell culture depository of Surgery Branch, National Cancer Institute (NCI), Bethesda, MD. CT26 cell line was obtained from Dr. Weiss (NCI-Frederick, Frederick, MD). The retroviral packaging cell line 293GP was obtained from ATCC. All the mouse tumor lines described above were maintained in

R10 (RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Invitrogen Corp.). The 293GP cells were maintained in D10 (DMEM from Invitrogen Corp.) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin). Mouse T cells were cultured in RPMI 1640 containing 10% heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.05mM 2-mercaptoethanol, 0.1 mM MEM Non-essential Amino Acids, 1 mM pyruvate, 2 nM L-glutamine (all from Invitrogen Corp), and 30 IU/ml of recombinant human interleukin (rhIL)-2 (Chiron). All the cells were cultured at 37^{o}C under 5% CO_2 and 95% humidity.

Retrovirus production and transduction of mouse T cells. The retroviral vector constructs used in this study are schematically illustrated in Figure 1A and are described in the corresponding figure legend. Details of the molecular sequences and methods used to generate these retroviral constructs have been described previously (1, 2, 3). The anti-VEGFR2 CAR (DC101 CAR) expressing retroviral vector supernatant was produced from a stable phoenix Eco producer clone as described previously (1). The empty retroviral vector without transgene and the murine IL-12 expressing retroviral vector supernatants were generated in 293-GP packaging cell line stably expressing retroviral GAG and POL proteins as previously described (1). Briefly, the 293GP cells were seeded onto poly-d-lysine coated 100mm plates (BD Biosciences) and transfected with 5 µg of pCL-Eco plasmid expressing the ecotropic envelope protein (Imgenex) and 10 µg of the MSGV1-Empty (Empty), MSGV1-IL12 (Flexi-IL-12) or the pSERS11-NFAT-IL12.PA2 (NFAT-IL12) plasmid DNA using lipofectamine 2000 reagent (Invitrogen Corp)

following the manufacturer's instructions. Viral supernatants were harvested 36-48 hours post transfection and used to transduce mouse T cells.

Transduction of mouse T lymphocytes. To obtain mouse T lymphocytes, spleens from 8-10 week-old mice were crushed through a 40μ cell strainer (BD biosciences) and subjected to red blood cell lysis. $CD3^+$ T cells were purified using Dynal mouse T cell negative isolation kit (Invitrogen Corp). Cells were stimulated for 24-48 hours with 2 μg/ml Concanavalin A (Sigma), and 1 ng/ml recombinant mouse IL-7 (R&D Systems) in RPMI 1640 media containing 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.05mM 2-mercaptoethanol, 0.1 mM MEM Non-essential Amino Acids, 1 mM pyruvate, 2 nM L-glutamine (all from Invitrogen Corp), and 30 IU/ml of recombinant human interleukin (rhIL)-2 (Chiron) prior to transduction. Mouse T cell transductions and culture were performed essentially as described previously (1). Cultured cells were adoptively transferred 3-5 days post transduction (> 95% $CD8^+$ T cells).

Detection of anti-VEGFR-2 CAR and IL-12 expression in transduced T cells. Ectopic expression of anti-VEGFR2 CAR on retrovirally transduced mouse T cells was detected by indirect immunofluorescence with soluble mouse VEGFR2-hIgG.Fc fusion protein (R&D Systems), followed by staining with a phycoerythrin (PE)-labeled goat anti-human IgG.Fc (α-hIgG.Fc) antibody (eBioscience). Cells were co-stained with FITC-labeled anti-mouse CD8 antibody (BD Pharmingen) or isotype control IgG. Aliquots of cells stained with bovine serum albumin (BSA; Sigma) and PE-labeled α-hIgG.Fc antibody were served as control to determine the specificity of CAR staining and the mean fluorescence intensity (MFI) of its expression on

transduced T cells by fluorescence activated cell sorting (FACS). Expression of IL-12 in transduced T cells was determined by intracellular staining using the cytofix/cytoperm kit (BD Pharmingen) followed by flow cytometry. NFAT response promoter driven IL-12 expression in transduced T cells was determined with or without 4-hour stimulation with 20 ng/ml phorbol 12-myristate 13-acetate (PMA) and 2.2 μmol/l ionomycin (both from Sigma). Cells were stained with allophycocyanin (APC)-labeled rat anti-mouse IL-12 antibody (BD Pharmingen) to determine IL-12 expression. The staining specificity was assayed using isotype-matched control antibodies. For analysis, the relative log fluorescence of stained cells was determined using a FACS Calibur ^{TM} flow cytometer (BD Biosciences). Immunofluorescence data were analyzed using FlowJo software (Tree Star).

Cytokine release assays. Transduced mouse T cells were tested for specific reactivity against target cells expressing VEGFR-2 using cytokine release assays using a commercially available enzyme-linked immunosorbent assay (ELISA) kits (mouse IL-12p70 and IFN-γ, Endogen; TNF-α, R&D Systems). On day 2 post-transduction $1x10^5$ transduced mouse T cells were co-cultured with $1x10^5$ target cells in 200 μl culture volume in individual wells of a 96 well micro titer plate for 18-24 hours as indicated in Figure 2B. Where indicated, the transduced T cells were treated with PMA (20 ng/ml) and ionomycin (1 μg/ml) for 4 hours to induce activation of T cells and NFAT-responsive promoter.driven IL-12 expression. Cytokine secretion was measured in culture supernatants diluted to the linear range of the assay. The assays were performed in triplicate wells and values are represented as mean ± standard error of the mean (SEM).

Tumor models and adoptive transfer. Mice at 6-7 weeks of age (n ≥ 5 for all groups) were injected subcutaneously (*s.c.*) with 5e5 syngeneic tumor cells and 10 to 12 days later treated with indicated numbers of syngeneic $CD3^+$ mouse T cells transduced with various retroviral vectors by intravenous (*i.v.*) injection. Starting volumes of tumors ranged from 40 to 80 mm^2. Unless indicated otherwise, lymphopenia was induced by nonmyeloablative (5 Gy) total body irradiation (TBI) of mice on the day of adoptive transfer. All experiments were performed in a double blinded, randomized fashion, and independently at least twice with similar results. Each treatment group included a minimum 5 mice. Serial, blinded tumor measurements were obtained and the products of perpendicular diameters were plotted ± SEM.

Evaluation of in vivo persistence of adoptively transferred T cells. Spleens and tumors from 3 mice in treatment and control groups were harvested at the indicated time points and single cell suspensions were made by crushing the tissues through a 40-μm cell strainer. Dead cells were removed by centrifugation with Lympholyte M. Live cells were further enriched by removing the dead cells from the tumor cell suspensions by density gradient centrifugation with Lympholyte-M (Cedarlane Laboratories). Splenocytes were obtained after red cell lysis. Phenotype of lymphocytes in the cell preparations was determined by direct staining of cells with APC-conjugated rat anti-mouse CD3 and PE-labeled mouse anti-mouse Ly5.1 (both from BD Pharmingen). Phenotype of the $CD11b^+Gr1^+$ myeloid cell subsets were determined by using fluorescein isothiocyanate (FITC) labeled CD11b and APC labeled anti-Gr-1 antibodies (both from BD Pharmingen). Aliquots of cells were stained with relevant isotype control antibodies to determine the specificity of staining. Percentage of $Ly5.1^+$ lymphocytes and $CD11b^+Gr1^+$ myeloid subsets in the total viable fraction of the tumor cell preparations and the splenocytes

were determined by FACS. Absolute cell numbers $Ly5.1^+$ or $CD11b^+Gr1^+$ cells were determined by multiplying the percentage of the respective cell subsets by the total number of viable cells.

References

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