Co-Targeting of Delta-like ligand 4 (DLL4) and vascular endothelial growth factor A (VEGF) with Programmed Death 1 (PD1) blockade inhibits tumor growth and facilitates anti-tumor immune responses.

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ABSTRACT

Blocking DLL4, a Notch ligand, effectively inhibits tumor growth by increasing non-functional angiogenesis and decreasing the cancer stem cell (CSC) population. Preclinical studies have demonstrated inhibition of tumor growth by anti-DLL4 treatment and have led us to enter demcizumab, an anti-DLL4 mAb, into ongoing clinical trials. Vascular endothelial growth factor A (VEGF A) also plays a central role in inducing tumor angiogenesis. VEGF signaling is also involved in recruiting immune suppressive myeloid cells. Therefore, targeting VEGF could induce favorable immune responses against cancer. We developed a bispecific monoclonal antibody that blocks both DLL4 and VEGF which is in phase 1 clinical trials. In the present study we compare the impact of anti-DLL4 in combination with anti-VEGF and anti-DLL4 in combination with anti-VEGF and anti-Programmed Cell Death Protein 1 (PD1) on anti-tumor immune responses. Our data demonstrate that the triple blockade of DLL4-VEGF-PD1 significantly inhibited tumor growth with more pronounced tumor regression. Anti-DLL4 treatment reduced IL17 production, an effect not observed with anti-PD1, blockade of DLL4-VEGF or DLL4-VEGF-PD1, suggesting that blocking DLL4 alone and together with VEGF or VEGF and PD1 might have different mechanisms for regulating immune responses. Anti-PD1 increased specific $CD8^+$ T cell-mediated IFN-gamma production while decreasing IL6. Interestingly, IL2 was increased at the tumor site by blockade of DLL4-VEGF-PD1 compared to controls. Since IL2 is required for secondary population expansion of $CD8^+$ memory T cells, increased IL2 in the triple combination group suggests potential for increased T cell activation, maintenance and memory T cell function, as compared to single agent anti-DLL4 and anti-PD1. Memory $CD8^+$ T cell frequencies were increased within the total $CD8^+$ T cell population by DLL4-VEGF-PD1 triple blockade. Therefore, these results show that co-targeting of DLL4 and VEGF with PD1 might be an effective and durable anti-cancer therapy in part by promoting anti-tumor immune responses and inhibiting pro-tumor immune responses.

METHODS

In Vivo Studies: The murine colon carcinoma (CT26-WT, ATCC CRL-2638) and renal carcinoma (Renca, ATCC CRL-2947) were obtained from American Type Culture Collection. Single cell suspensions of CT26 or Renca tumor cells were injected subcutaneously into the flanks of 7-8 week old Balb/c mice. Seven to 10 days following tumor inoculation, mice were randomized and dose *i.p.* Two bisecting diameters were measured with electronic calipers, and tumor volumes were calculated using the formula: $V=0.5ab^2$, with a as the larger diameter and b as the smaller diameter.

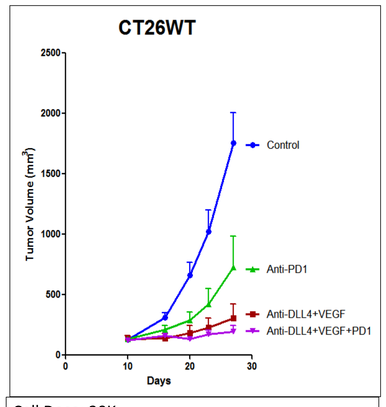
ELISPOT: Splenocytes were cultured in the presence and absence of tumor specific CD8 T cell peptide in RPMI+5% HI FBS for 48 hrs followed by the ELISPOT assay as described by manufacturer's instruction.

MDSC Suppression Assay: To determine the impact of MDSCs on T cell proliferation, MDSCs were isolated from pooled splenocytes from treated groups and naïve T cells were isolated from unused mice using Kits from Miltenyi Biotech according to the manufacture's instruction. Naive T were labeled with violet tracking dye (VTD), and co-cultured with different numbers of isolated splenic MDSCs in the presence of anti-CD3 and anti-CD28 beads for 96 h. Changes in dilution of VTD dye was used to calculate the proliferation by FACS.

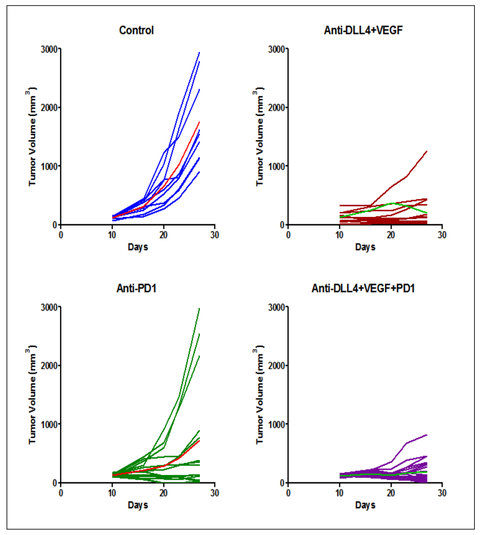
Flow Cytometry: Single cell suspension of splenocytes or tumor digests was used to stain with indicated antibodies and their isotype controls. Cells were treated with Anti-CD16/CD32 for Fc block. Cells were washed twice with FACS buffer (2% FCS in HBSS), fixed with 2% formaldehyde (v/v) in PBS analyzed using FACS Canto II. Analyses were performed with the Diva software. MDSC can be divided into two sub populations: Granulocytic MDSC (G-MDSC) and monocytic MDSCs (M-MDSC). G-MDSCs have been characterized by $CD11b+Ly6G+Ly6C^{low}$ or $CD11b+GR1^{high}$ phenotype, whereas M-MDSCs are characterized by $CD11b^+Ly6G^-Ly6C^{high}$ or $CD11b+GR1^{low}$. Effector CD8 T cell population are characterized by $CD44^{high}CD62L^{-/low}$ and central memory CD8 T cells are characterized by $CD44^{high}CD62L^{high}$ on CD8 T cells.

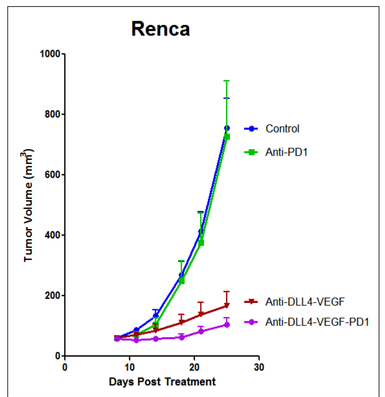
RESULTS

Anti-DLL4+VEGF Improves Anti-Tumor Activity of PD1 in both PD1 responsive (CT26WT) and non-responsive (RENCA) Murine Cancers

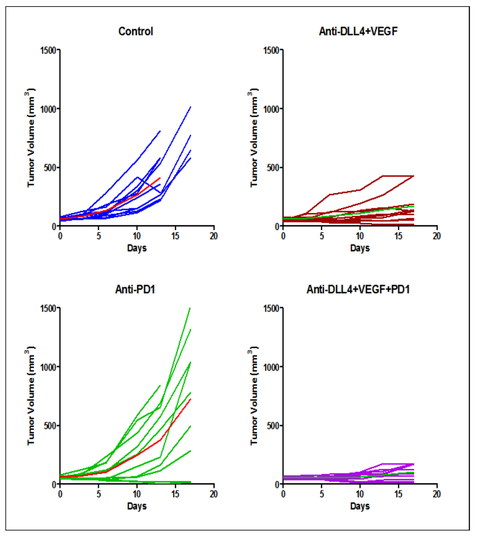


Cell Dose: 30K
Dose: Start at day10 (130mm3) for 3 weeks
anti-PD1 (250mg/dose, 2QW)
anti-DLL4 (5mg/kg, QW)
anti-VEGF (2.5mg/kg, QW)
isotype control antibodies (20mg/kg, 2QW)0

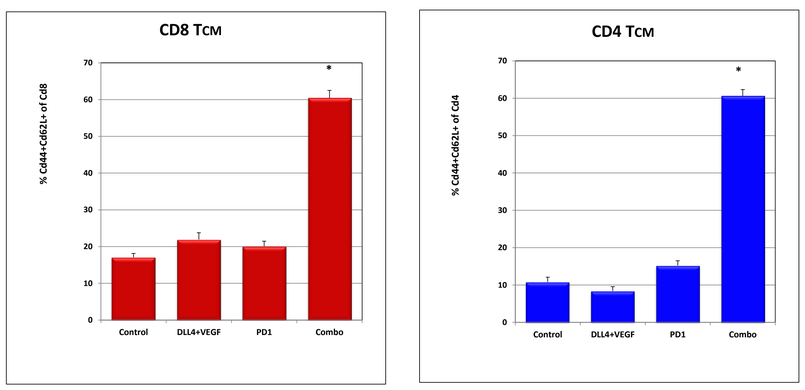




Cell Dose: 500K
Dose: Start at day10 (130mm3) for 3 weeks
anti-PD1 (250mg/dose, 2QW)
anti-DLL4 (10mg/kg, QW)
anti-VEGF (10mg/kg, QW)
isotype control antibodies (20mg/kg, 2QW)

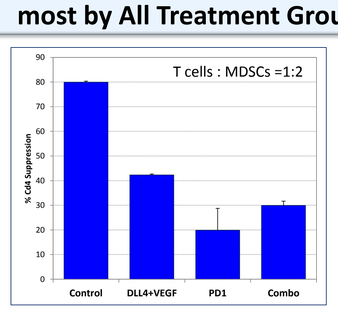


Co-blockade of DLL4, VEGF and PD1 Significantly Increased Central Memory (CM) T cells in the spleens.

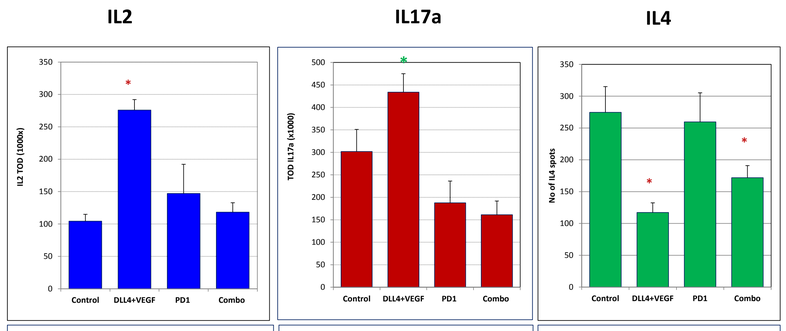


At day 30, spleens from control, Anti-DLL4+VEGF, Anti-PD1 and Anti-DLL4+VEGF+PD1 (Combo) groups were analyzed for CD8 and CD4 central memory populations by FACS. Central memory populations is identified by high CD44 and low CD62L expression levels in CD4 and CD8 T cells.

MDSC mediated T cell Inhibition were reduced most by All Treatment Groups



Only Anti-DLL4+VEGF Increased IL2 and IL17a and both Anti-DLL4+VEGF and Triple Combo Decreased IL4 Production

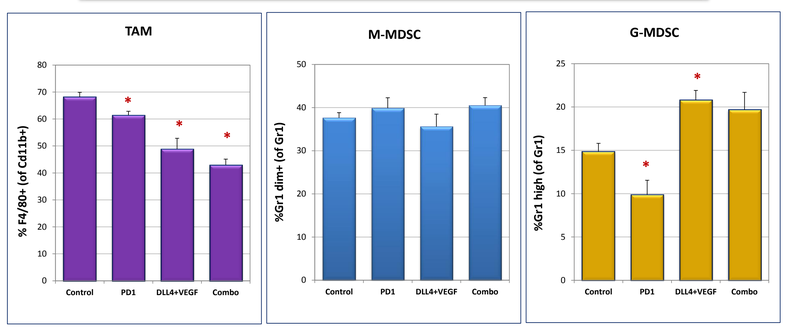


IL2 ELISPOT using splenocytes indicated that DLL4+VEGF block increased IL2 production to the level similar to that of DLL4+PD1 block (data not shown). P=0.00005

IL17a ELISPOT using splenocytes indicated that DLL4+VEGF block increased IL17a production (*=0.009).

IL4 ELISPOT using splenocytes indicated that DLL4+VEGF block as well as triple combo decreased IL4 production, indicating this effect is mediated by DLL4+VEGF blockade. P=0.01

Tumor Associated macrophages were reduced most by Anti-DLL4+VEGF+PD1



Tumor associated macrophages were reduced most by Anti-DLL4+VEGF+PD1 > Anti-DLL4+VEGF > Anti-PD1.

There was no difference in M-MDSC. In previous study DLL4+PD1 block decreased M-MDSC. By adding anti-VEGF, this effect was lost.

G-MDSCs was reduced by anti-PD1 as shown previously, and DLL4-VEGF block slightly increased G-MDSCs.

CONCLUSION

Anti-(DLL4+VEGF+PD1) resulted in

- tumor regression in mouse colon and renal cancer models
- generated both Cd4 and Cd8 T central memory cells.
- IL2 and IL17a production was increased and IL4 was decreased
- TAM was reduced but MDSC did not change

Therefore, combining bi-specific anti-DLL4/VEGF antibody with anti-PD1 might provide an effective and durable anti-cancer therapy not only by targeting tumor angiogenesis and cancer stem cells, also in part by promoting anti-tumor immune responses and inhibiting pro-tumor immune responses via mechanisms different from those of anti-DLL4+PD1 combination.