Blocking DLL4, a notch ligand, effectively inhibit tumor growth by increasing non-vascular angiogenesis and decreasing the cancer stem cell (CSC) population. We are currently testing an anti-DLL4 antibody, democizumab, in Phase IIb trials in GCSCs, pancreatic, and breast cancer. DLL4 is also known to modulate immune responses. In the current study we examine the impact of anti-DLL4 on anti-tumor immune responses as a single agent and in combination with the key immune checkpoint inhibitor Inducible Costimulatory (ICOS). While the recent clinical success of PD1 and ICOS as single agents represents a new and promising cancer immunotherapeutic approach, high initial response rates are often associated with a lack of long-term durable effects in a significant number of patients. Therefore, we hypothesized that dual blockade of DLL4 and PD1 might further impact tumor growth by further enhancing anti-tumor immunity. Our data demonstrates that dual blockade of DLL4 and PD1 using antibodies not only reduces tumor growth, but also led to tumor rejection in 50% of CT26 tumor-bearing mice, similar to those treated with anti-PD1 alone (no tumor rejection was observed with single agent anti-DLL4 or PD1 treatment). Anti-DLL4 induced T cell-mediated IL2 production (Figure 1A). The anti-DLL4 treatment reduced IL17 production (Figure 1B).

These results show that dual targeting of DLL4 and PD1 may be an effective and durable cancer therapy by increasing IL2 and decreasing IL17. Anti-PD1 and anti-DLL4 combination produced more IL2, clearly indicating the role of DLL4 blockade in enhancing anti-tumor immunity. Therefore, anti-DLL4 significantly decreases IL-17a production and decreases frequency of splenic M-MDSC in CT26 mouse model. Balb/c mice were inoculated with 2x10⁴ CT26 cells, and at day 7 mice were treated with control and anti-DLL4 antibody twice weekly for 3 weeks. (A) IL17a ELISPOT (B-C) M-MDSC and G-MDSC FACS analyses. The combination of anti-DLL4 and anti-PD1 significantly reduces tumor growth and increases central memory (CM) and effector memory (EM) populations. Analyses were performed with BD Diva software. MDSC can be divided into two sub populations: Granulocytic MDSC (G-MDSC) and monocytic MDSC (M-MDSC). G-MDSC and monocytic MDSC (M-MDSC) G-MDSC have been characterized by CD11b+Ly6C+Ly6G− or CD11b+Gr1+monocytes, whereas M-MDSCs are characterized by CD11b+Ly6C−Ly6G− or CD11b−Gr1−. Effects of anti-DLL4 and anti-PD1 treatments were analyzed on day 7 and changes in dilution of DT4 dye used to calculate the proliferation by FACS. Treats and naive T cells were isolated using Ktek. Only combo increased IL2 while anti-DLL4 decreases total IL17a production (Figure 2A).

The combination of anti-DLL4 and anti–PD1 results in a long term antitumor memory of the cured mice. Anti-DLL4 and combo group significantly increases central memory (CM) and effector memory (EM) cells. Anti-DLL4 significantly decreases IL-17a production and decreases frequency of splenic M-MDSC. Anti-DLL4 induces IL2 production in splenic T cells. Anti-DLL4 and combo had increased IL2 after 1st re-challenge. Anti-DLL4 and combo group significantly increases central memory (CM) and effector memory (EM) cells.

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**RESULTS AND CONCLUSIONS**

**Anti-DLL4 significantly decreases IL-17a production and decreases frequency of splenic M-MDSC**

**Anti-PD1 and combo treatment reduces the suppressive function of Tregs**

**Co-targeting with anti-DLL4 and anti-PD1 significantly reduces tumor growth**

**Materials and Methods**

**In Vivo Studies:** The murine colon carcinoma (CT26-WT, ATCC CRL-2638) was obtained from American Type Culture Collection. Single cell suspensions were prepared and 1x10⁶ cells in 200 µl Matrigel were i.p. injected into the flanks of 7-8 week old Balb/c mice. One week following tumor inoculation, mice with palpable tumors were injected i.p. (individually with anti-PD1, anti-DLL4, or combination twice a week for 3 weeks). Isotype antibodies were used for isotype controls. Tumor growth was monitored by measuring two bleaching diameters of each tumor with an electronic caliper. Tumor volumes were calculated using the formula V=1/2 × l × w², with l the larger diameter and w the smaller diameter.

**ELISA:** Total spleenocytes were cultured in the presence and absence of tumor specific CDR T cell peptide i peptide in 10% media, followed by addition of proliferation media. Luminex Analysis: Cytochrome c in plasma were measured using Mouse Thy1 multikits (EMD Millipore) according to manufacturer’s instructions. The fluorescence intensity of the beads was measured using a Luminex-200 reader.

**Flow Cytometry:** Single cell suspensions of splenocytes or tumor digests were used with the indicated antibodies and their respective isotype controls. Cells were stained with anti-CD3 (1:100) and anti-CD8 (1:100) PE. Cells were washed with PBS (2% FCS PBS) and fixed with PBS 2% paraformaldehyde (pH: 7.4) for 30 minutes.

**Combination of anti-DLL4 and anti–PD1 resulted in a long term antitumor memory of the cured mice.** Anti-DLL4 and combo group significantly increases central memory (CM) and effector memory (EM) cells. Anti-DLL4 and combo group significantly increases central memory (CM) and effector memory (EM) cells.

**Summary**

Anti-DLL4 synergized with anti-PD1 activity and mediated the anti-tumor immune responses and lead to long-term immunological memory.

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