

### CD40 AGONISM IS REQUIRED TO PROMOTE THE FUNCTION OF CROSS-PRESENTING DENDRITIC CELLS AND INCREASE TRIPLE NEGATIVE BREAST CANCER RESPONSE TO RADIATION AND CTLA4 INHIBITION

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**Background** Radiation therapy (RT) in combination with CTLA4 inhibition (CTLA4i) can induce anti-tumor immune responses in mice bearing immunotherapy-resistant tumors and in some cancer patients.<sup>1,2</sup> In mice, RT and CTLA4i cooperate to increase the clonality of tumor-infiltrated T cells, and promote the functional differentiation of Th1 CD4<sup>+</sup> T cells and effector memory, early activation and precursor exhausted CD8<sup>+</sup> T cells. However, the majority of mice bearing 4T1 and AT3 mouse triple negative breast cancer (TNBC) did not show deep tumor responses ( $\geq 90\%$  regression) to RT+CTLA4i. Such responses were significantly increased when a CD40-agonist antibody (CD40a) was added to RT+CTLA4i.<sup>1</sup> Here we analyzed tumor and draining lymph nodes (dLN) of 4T1-bearing mice to determine the mechanisms underlying CD40a benefit.

**Methods** Mice were randomized at day 12 post-tumor injection to treatment with: isotype control Ab; RT (8Gyx3); CTLA4i (9H10 mAb)+CD40a (FGK45 mAb); RT+CD40a; RT+CTLA4i; RT+CTLA4i+CD40a. Bulk RNAseq was performed on tumors from mice treated with control Ab, RT+CTLA4i, RT+CD40a or RT+CTLA4i+CD40a. The ImmunoSEQ platform was used to assess the TCR repertoire in paired tdLN and tumors from mice treated with control Ab, RT+CTLA4i or RT+CTLA4i+CD40a. The immune infiltrate was evaluated in tumors, tdLN and lungs by flow cytometry. Depletion of CD8 T cells was performed by 2.43 anti-CD8 antibody treatment started before RT and maintained through the experiment.

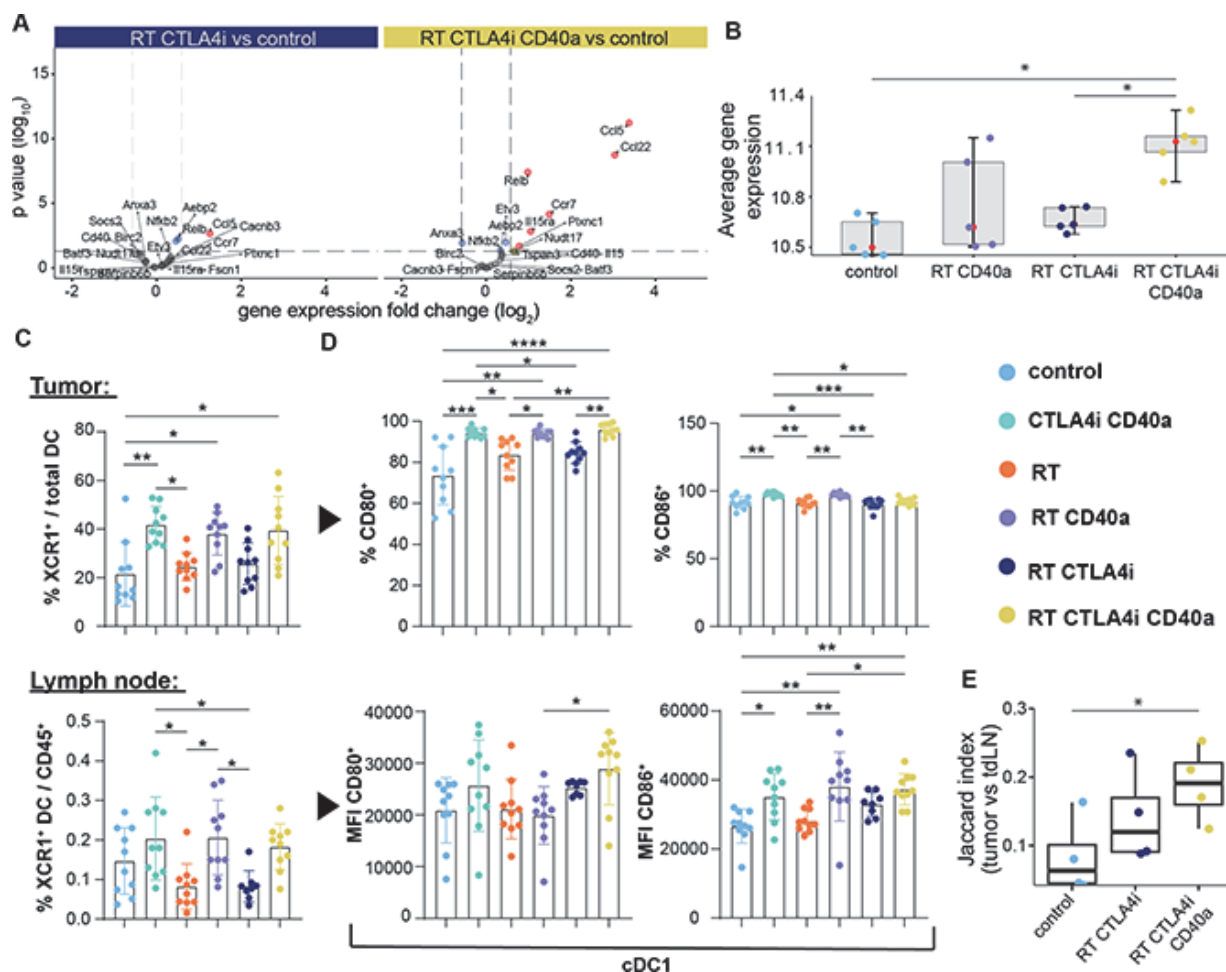
**Results** Depletion of CD8 T cells abrogated the anti-tumor effect of the triple combination treatment confirming that it relied on an adaptive immune response rather than on CD40a-mediated activation of tumoricidal macrophages. Consistently, neither RT nor CD40a induced changes in expression of activation markers by tumor macrophages. RNA-seq analysis revealed a significant increase in a gene signature of CD40a-activated cross-presenting conventional dendritic cells type 1 (cDC1)<sup>3</sup> only in tumors of mice treated with RT+CTLA4i+CD40a (figure 1A,B). Flow cytometry analysis showed a CD40a-driven increase in XCR1<sup>+</sup> cDC1 expressing costimulatory molecules CD80 and CD86 in tumors and dLN (figure 1C,D). Addition of CD40a to RT+CTLA4i also increased significantly AH1-specific TCR repertoire sharing between the tumor and dLN of each mouse as compared to controls (figure 1E).

**Conclusions** Thus, CD40a is required to increase cDC1, promote their activation and migration to the dLN, and ultimately improve CD8 T cell cross-priming. Overall, these results suggest that improving responses to RT+CTLA4i requires an intervention to sustain cDC1 function, at least for tumors with a dominant myeloid immunosuppressive microenvironment such as triple negative breast cancer.

#### REFERENCES

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**Ethics Approval** All mouse experiments were approved by the Institutional Animal Care and Use Committee at Weill Cornell Medicine.



**Abstract 860 Figure 1** BALB/c mice implanted with 4T1 tumors were treated with: isotype control Ab; RT (8Gyx3); CTLA4i+CD40a; RT+CD40a; RT+CTLA4i; RT+CTLA4i+CD40a. Tumor and tumor-draining lymph node (tdLN) were collected at day 17 after the first dose of CD40a for flow cytometry analysis (C) and at day 22 after treatment completion for analysis of gene expression (tumor, n=5/group) and TCR repertoire (tumor and tdLN, n=4/group) (A,B,D). (A,B) Volcano plots comparing the tumor expression of a gene signature of anti-CD40 activated cDC1 (Relb, Etv3, Batf3, Aebp2, Nfkb2, Cc122, Cc15, Il15, Ccr7, Il15ra, Plxnc1, Pmp, Cd40, Birc2, Fscn1, Anxa3, Cacnb3, Nudt17, Socs2, Tspan3, Serpinb6b) in untreated versus RT+CTLA4i (left) and RT+CTLA4i+anti-CD40-treated (right) 4T1 tumors (A). The average expression (generated via DESeq2) of the genes was determined as the arithmetic mean (red dot) of the log scale gene expression data in all treatment groups (B). For flow cytometry analysis of tumors (top panels) and tdLN (bottom panels), dendritic cells (DCs) were defined as CD11b<sup>+</sup> F4/80<sup>-</sup> CD11c<sup>+</sup> cells among CD45<sup>+</sup> cells and DC1 were further defined by expression of XCR1 (C). Percentage of DC1 positive for CD80 and CD86 in tumor and expression of CD80 and CD86 by DC1 in tdLN were measured (D). The Jaccard overlap index of the anti-tumor AH1-specific TCR repertoire between paired tumor and tdLN for each mouse was calculated to assess overlap between the two compartments (E). Flow cytometry data are shown as mean +/- SD, each dot represent one animal, n=10 per group. Populations were compared by Kruskal-Wallis and Dunn's post test for statistical significance. A t-test was used to evaluate statistical significance in both B and E. \*, \*\*, \*\*\* and \*\*\*\* indicate p-values <0.05, 0.01, 0.001 and 0.0001 respectively.

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