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Combined OX40 ligation plus CTLA-4 blockade
More than the sum of its parts

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Abbreviations: CTLA-4, cytotoxic T-lymphocyte-associated protein 4; PD-1, programmed death-1; SPAS-1, stimulator of prostatic adenocarcinoma specific T cells-1; TRAMP, transgenic adenocarcinoma of the mouse prostate

It is becoming clear that combination strategies will be necessary to augment cancer immunotherapy. We report that combination anti-OX40/anti-CTLA-4 mAb immunotherapy improves survival by enhancing effector T cell expansion and function, even while inducing Th2 cytokine production. Furthermore, IL-4 blockade in addition to combination therapy significantly improved anti-tumor efficacy.

Co-stimulatory (OX40, 4–1BB) and co-inhibitory (CTLA-4, PD-1) receptors promote or restrict T cell activation, respectively, and play a vital role in regulating the course of an immune response. Numerous studies have demonstrated that modulation of these pathways can dramatically enhance T cell activation. One such method to achieve this is through the use of monoclonal antibodies (mAb) targeting T cell co-inhibitory receptors, known as checkpoint inhibitors. Pre-clinical studies demonstrated that CTLA-4 blockade with an anti-CTLA-4 mAb was effective at improving tumor regression and enhancing survival. While this was true for more immunogenic tumors, additional therapies were needed to induce regression of poorly immunogenic tumors. Recently, it was demonstrated that anti-CTLA-4 mAb (ipilimumab) improved survival in patients with metastatic melanoma.[1] Although only 10–20% of patients respond to anti-CTLA-4, combined anti-CTLA-4/anti-PD-1 therapy resulted in ~50% response rates demonstrating the clinical potential of combination immunotherapy.[2]

An alternative strategy to augment anti-tumor immunity is to promote T cell activation directly through co-stimulatory receptors. Our group and others have shown that ligation of the TNF receptor family member, OX40, with an agonist anti-OX40 mAb significantly enhanced T cell cytokine production, expansion, and anti-tumor immunity.[3] A recently completed phase I clinical trial demonstrated the immunologic effects of anti-OX40 in patients with cancer, highlighting the therapeutic potential of OX40 agonists.[4] Despite similarities in their ability to elicit anti-tumor immunity, there are notable differences in the immune response following treatment with anti-OX40 or anti-CTLA-4 mAb. OX40 ligation elicited more cytokine-producing and memory CD4 T cells than CTLA-4 blockade, while CTLA-4 blockade specifically depleted Treg cells in the tumor.[5,6] Whether anti-OX40 similarly depletes intratumoral Treg cells is unknown. Anti-OX40 and anti-CTLA-4 seem to have differing mechanisms for boosting CD8 T cell expansion and survival. CTLA-4 blockade indirectly enhanced CD8 T cell function through cell extrinsic effects, while agonist anti-OX40 mAb directly and indirectly boosted CD8 T cell function.[7,8] Therefore, we hypothesized that OX40 ligation plus CTLA-4 blockade would greatly enhance anti-tumor immunity by enhancing effector T cell survival and function, while inhibiting the function of Treg.

A recent study examining intratumoral injection of anti-OX40 and anti-CTLA-4 along with the TLR agonist CpG demonstrated enhanced overall survival and Treg cell depletion in a murine lymphoma model.[9] Our data take this in a different direction, demonstrating that systemic administration of anti-OX40/anti-CTLA-4 mAb, in the absence of TLR agonists, significantly improves survival and primary tumor regression in the poorly immunogenic TRAMP-C1 mouse model of prostate cancer and in MCA-205 sarcoma tumor-bearing mice.[10] Monotherapy with either agent alone was insufficient to enhance survival. Combination therapy augmented the frequency of proliferating polyclonal effector CD8 T cells. Furthermore, combination therapy induced $Tbet^{hi}Eomes^{hi}$ CD8 T cells, which is associated with effector CD8

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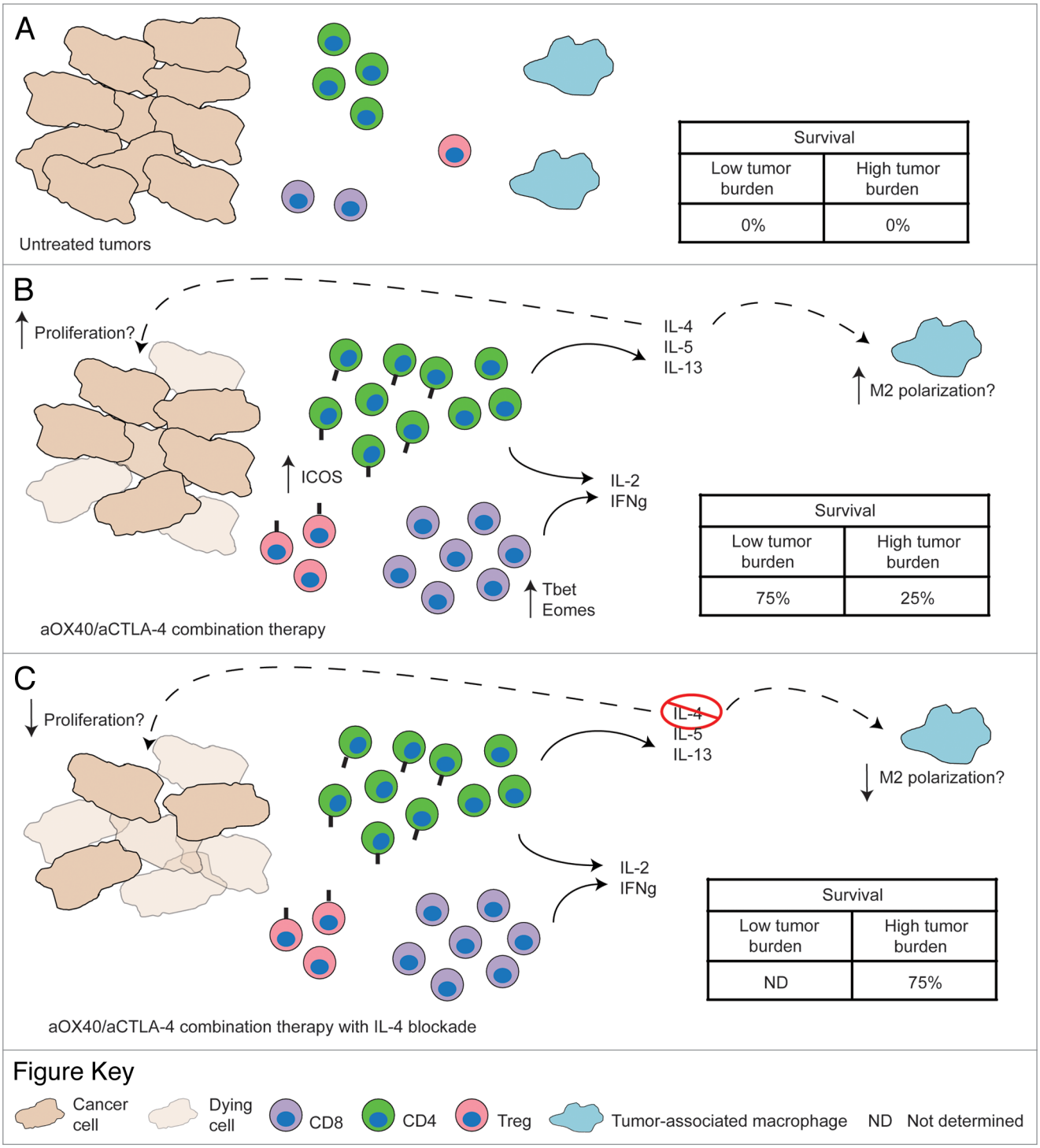

Figure 1. Schematic representation of the mechanisms by which combined anti-OX40/anti-CTLA-4 mAb immunotherapy enhances tumor regression and survival. (**A**) Untreated tumors exhibit minimal T cell infiltration, resulting in 100% mortality due to unchecked tumor growth. (**B**) Combined anti-OX40/anti-CTLA-4 mAb therapy induces tumor regression and enhances survival by augmenting effector CD4 and CD8 T cell survival and expansion. This is in spite of Th2 cytokine production, which may promote the proliferation of cancer cells and the induction of suppressive tumor-associated macrophages. (**C**) IL-4 blockade with combined anti-OX40/anti-CTLA-4 mAb significantly enhances tumor regression and survival in the presence of a large tumor burden.

T cell differentiation (**Fig. 1**). Moreover, we observed a significant expansion in tumor-associated antigen-specific (SPAS-1) CD8 T cells in the TRAMP-C1 model, which was accompanied by increased IFN-γ production. These data suggest that combining anti-OX40 and anti-CTLA-4 therapy with tumor-specific vaccination may further enhance the anti-tumor CD8 T cell response. With numerous vaccination strategies currently being evaluated in clinical trials, the potential synergy of this triple combination holds great promise.

Further mechanistic studies revealed that combination therapy enhanced the expansion of effector (FoxP3$^-$) CD4 T cells as compared with either monotherapy alone. Interestingly, while intratumoral injection of anti-OX40/anti-CTLA-4 mAb depleted Treg cells,[9] we found that systemic therapy led to a slight increase in Treg cells within the lymph nodes, while the tumor remained unchanged relative to controls. Likewise, we observed no change in the suppressive capacity of Treg cells isolated from mice receiving combination immunotherapy. One noticeable difference was an increase in ICOS expression on CD4 effector and Treg cells, which has been linked to long-term survival in patients and may be one explanation for why we observed enhanced survival despite no effect on Treg cells present within the tumor.

Perhaps the most striking finding of our study was regarding cytokine expression. While we observed an increase in IL-2 and IFN-γ production by CD8 T cells, we were surprised to find an increase in both Th1 (IFN-γ and IL-2) and Th2 (IL-4, IL-5, IL-13) cytokines by CD4 T cells following combination therapy (**Fig. 1**). We noted that these were distinct populations of CD4 T cells, as they were either IFN-γ$^+$Th2 cytokine$^-$, or IFN-γ$^-$Th2 cytokine$^+$. Moreover, the increase in IL-4 expression could not be attributed to Treg or NKT cells. Increased Gata-3 expression, which is associated with Th2 polarization in CD4 T cells, corresponded with increased IL-4 production and was primarily detected in the T-bet^{low} CD4 effector population.

To determine whether IL-4 expression following combination therapy was important for enhancing survival and tumor regression in the TRAMP-C1 model, we examined the effects of combination therapy with IL-4 blockade. IL-4 blockade in conjunction with either anti-OX40 or anti-CTLA-4 alone had no effect. However, IL-4 blockade delivered with combination anti-OX40/anti-CTLA-4 therapy led to a profound increase in long-term survival. The mechanism by which IL-4 restricts the efficacy of combination therapy is unknown, but may reflect the proliferative effects of IL-4 directly on the tumor or the induction of suppressive tumor-associated macrophages (**Fig. 1**). Together, these data indicate the efficacy of combined anti-OX40/anti-CTLA-4 therapy and suggest that the induction of potentially detrimental Th2 responses should be considered when translating these studies into patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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