**Triggering co-stimulation directly in melanoma tumor fragments drives CD8\(^+\) tumor-infiltrating lymphocyte expansion with improved effector-memory properties**

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**Abbreviations:** ACT, adoptive T-cell therapy; DC, dendritic cell; TIL, tumor-infiltrating lymphocytes; TME, tumor microenvironment.

TIL from solid tumors can express activation/co-stimulatory molecules like 4–1BB/CD137, a sign of recent antigenic stimulation in the tumor microenvironment (TME). This activated state can be exploited *ex vivo* to enhance the expansion of tumor-reactive CD8\(^+\) TIL for adoptive cell therapy through direct addition of immunomodulators to tumor fragments in culture.

**Review**

Adoptive T-cell transfer (ACT) using tumor-infiltrating lymphocytes (TIL) has emerged to be a powerful treatment for metastatic melanoma. Current TIL expansion methods often involve an initial culture of small (6–9 mm\(^3\)) tumor fragments cut from excised tumors to induce the outgrowth of T cells using IL-2 or other T-cell growth factors.\(^1,2\) Although this initial tumor fragment culture step has been used to generate TIL for ACT for years, very little is known about the TME within early tumor fragments and whether it can be manipulated to enhance the outgrowth of tumor-reactive T cells. This new concept, together with recent data showing that T cells within solid tumors express markers of recent antigenic stimulation, such as co-stimulatory molecules of the TNF-R family, suggests that addition of immunomodulators to tumor fragment cultures may modify the types of T cells growing out of these tissue fragments. This can have significant impact on developing new approaches at generating improved TIL products from melanoma for adoptive transfer and also help tackle the new problem of developing TIL therapy for other solid tumor indications, where TIL expansion is more difficult.

CD8\(^+\) tumor-reactive TIL have emerged to be important in ACT.\(^3\) 4–1BB is a costimulatory molecule that is expressed on recently activated CD8\(^+\) T cells especially\(^4\) and is a marker of naturally occurring tumor reactive TIL.\(^5\) In a recent study,\(^6\) we hypothesized that T cells within the microenvironment of tumor fragments can express 4–1BB *ex vivo* for a sufficient amount of time in early cultures and can respond to an addition of a 4–1BB agonist (e.g., an agonistic antibody (Ab)) added directly to tumor fragments. Our hypothesis was that this *ex vivo* manipulation of T-cell costimulatory molecule could further induce the activation of the TIL, enhance the output and rate of CD8\(^+\) T-cell outgrowth, and augment the TIL effector-memory phenotype for ACT.

Our results indicated that adding an anti-4–1BB Ab during the initial isolation and expansion of the TIL resulted in increased CD8\(^+\) TIL expansion and tumor reactivity. 4-1BB co-stimulation also induced the expression of NFκB and anti-apoptotic genes within the CD8\(^+\) TIL subset coming out of the tumor fragments. We also investigated the role of 4–1BB ligation on other leukocyte subsets within the tumor fragment microenvironment. A number of other cell types, including dendritic cells (DC), macrophages, natural killer (NK) cells, and other stromal cells co-exist within these tumor fragments in early culture, and that many of these cells actually stay alive along with the activated T cells for at least 7 d in these fragments. Unexpectedly, we found that DC within the tumor fragments expressed 4–1BB and could be directly costimulated using 4–1BB agonists *in situ*. This was evidenced by the increased translocation of NFκB into the nucleus of DC isolated from the fragments (as found also in the activated CD8\(^+\) TIL) and upregulation of...
DC maturation markers (e.g., CD80, CD86, HLA class II). We also found that adding a blocking anti-HLA class I Ab decreased the output of CD8+ TIL from tumor fragments, suggesting that continued or “run-on” antigen presentation takes place in these ex vivo cultures.6 These results suggest that the tumor fragments used to isolate and expand TIL can be dynamic TMEs that can be altered to improve TIL expansion and the phenotype of TIL for ACT. We have also begun to examine what other downstream modulators triggered by 4-1BB agonist addition play a role in modulating TIL outgrowth, and whether these factors may be detrimental if induced at too high levels in the tumor fragment microenvironment.

Tumor fragment microenvironments may also contain many suppressive factors and cells, such as tumor cells, myeloid derived suppressor cells, M2 macrophages, T regulatory cells, TGF-β, and PDL-1 that may play inhibitory roles in TIL activation, expansion, and migration out of the fragment. Preliminary data (Chacon, et al. unpublished) found that the fragment cultures receiving anti-4–1BB Ab exhibited an increase in pro-inflammatory cytokines, such as IL-6 and IFNγ. Since IL-6 regulates dendritic cell differentiation and activates STAT3,7 we investigated the role of adding exogenous IL-6 cytokine to the fragment cultures with or without anti-4–1BB Ab. Interestingly, the addition of IL-6 resulted in a further increase in IFNγ production, NFκB and HLA class-II expression in the DCs, but had a detrimental effect on the TIL, largely abrogating the yield of CD8+ TIL from the tumor fragments. This may be due to the enhanced outgrowth of CD4+ T cells and/or negative effects on CD8+ T cells paradoxically, due to too much pro-inflammatory cytokines in the milieu. For example, high IFNγ levels can inhibit CD8+ T-cell division and induce apoptosis.8 The source of the increased IFNγ could be from the T cells themselves or from other resident leukocytes, such as macrophages and immature suppressive myeloid cells (MDSC) that can secrete high levels of IL-6.9 Thus, too much pro-inflammatory cytokine can be detrimental suggesting that caution is needed when using these immunomodulators which can induce “hyper-inflammation” in the TME and negative effects on T cells. Further studies will need to investigate whether the addition of anti-4–1BB Ab in some cases augments the negative barriers the cells and factors may have on the TIL, such as the IL-6 effect described here.

In addition to 4–1BB, other TNF-R family members, such as OX40, HVEM, LIGHT, and CD30 may also play a key role in modulating the tumor fragment microenvironment. For example, modulating OX40 signaling alone or together with 4–1BB signaling may inhibit T regulatory cells that suppress T cells within tumor fragments, and alter the expansion and phenotype of CD4+ T cells toward a more cytolytic than helper phenotype. A recent study demonstrated combining anti-CTLA-4 and anti-4–1BB Abs mediate the rejection of B16 melanoma tumors.10 Therefore, a combination of anti-4–1BB with checkpoint blockade inhibitors may demonstrate a superior role in modulating the TME.

In conclusion, early tumor fragment cultures from metastatic melanoma and predictably other solid tumors have emerged to be dynamic TMEs, containing a variety of cell types surviving and functioning for a significant amount of time in

**Figure 1.** Proposed model for 4–1BB activation during initial TIL expansion. We hypothesized that the environment within the fragment may be playing a role to expand the TIL when 4–1BB ligation is occurring since many cells can express 4–1BB. We observed that when the anti-4–1BB antibody was added to the cultures, the tumor-resident Dendritic Cells (DCs) had an increase in activation markers MHC-II, CD80 and CD86. 4–1BB ligation may be activating the DCs and either directly activating the TIL or indirectly through cytokines, resulting in the TIL to proliferate more and migrate out of the fragment at a faster rate than when 4–1BB ligation does not occur.
ex vivo culture. This offers a new opportunity to manipulate these microenvironments with immunomodulators besides just adding T-cell growth factors to improve the output and phenotype of TIL for adoptive transfer and improve clinical responses. One immediate pathway that can be exploited in this regard is the 4–BB co-stimulatory pathway, not only directly in emerging CD8+ T cells, but also in tumor-resident DC that may function to further activate CD8+ T cells emerging from tumor fragments (Fig. 1). These observations open up a new paradigm with which we can improve not only melanoma ACT, but also facilitate development of TIL therapy for other solid tumors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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