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CD47 blockade enhances therapeutic activity of TCR mimic antibodies to ultra low density cancer epitopes

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We have discovered and developed two T cell receptor mimic antibodies (TCRm), ESKM, and Pr20M, specific for the Wilms tumor gene 1 product (WT1) derived peptide RMFPNAPYL, and the “Preferentially Expressed Antigen in Melanoma” (PRAME) derived peptide (ALYVDSLFFL), respectively, (1, 2), when presented by the human leukocyte antigen (HLA) class I haplotype HLA-A*02:01 on cancer cell membranes. These mAbs address highly cancer-specific, yet ultra-low density cancer targets and show therapeutic activity in murine models of human leukemia. However, the responses are incomplete and relapse is inevitable (2–4). The cytotoxicity of these TCRm is dependent on engagement of cytolytic effector cells and alone, these two antibodies do not possess the ability to kill

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Conflict of Interest

Melissa D. Mathias – Conflict of Interest: None

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tumor cells, activate complement, or block cell proliferation, unlike other some cancer specific TCRm antibodies. (3, 5)

CD47 is a trans-membrane protein broadly expressed on normal host tissue, and upregulated by tumors that inhibits innate immune responses including macrophage phagocytosis. CD47 transduces an anti-phagocytic signal via binding to its cognate ligand, SIRP α , expressed predominantly on myeloid derived cells including macrophages, dendritic cells, and neutrophils. A soluble truncated SIRP α protein variant, CV1, that potently antagonizes tumor cell CD47 binding to phagocyte SIRP α , improves macrophage-mediated antibody-dependent phagocytosis (ADCP) of cancer cells *in vitro*.

Anti-CD47 inhibitory mAbs are in clinical trials, but have significant toxicities of anemia and thrombocytopenia due to the ubiquitous expression of CD47 on red blood cells and platelets. (6, 7) CV1 has previously been shown to have modest activity alone *in vitro* and limited toxicity *in vivo*. The drug synergizes with mAbs that target high-density tumor antigens in murine models of multiple liquid and solid human tumors. (8) It is unknown if CD47 antagonism would also improve the efficacy of TCRm mAbs that target cell surface antigens present at levels as low as 1% of the antigen density of traditional mAbs, We asked if antagonizing CD47/SIRP α signaling would enhance TCRm antibody ADCP function and therapeutic activity against human leukemias.

We evaluated the ability of CV1, to enhance TCRm mAb-dependent macrophage phagocytosis of leukemia cells *in vitro* in HLA-A*02:01 positive human acute myeloid leukemia cell line, AML14, and human Ph' acute lymphoblastic leukemia cell line, BV173, which express both PRAME and WT1, as well as CD47. The HLA-A*02:01 negative cell line HL60 was used as a negative control. Blockade of leukemia cell CD47 with CV1 alone did not promote macrophage phagocytosis of AML14, BV173, or HL60 (Figure 1A). Pr20M alone did not promote ADCP of HL60 or BV173, but significantly increased phagocytosis of AML14 (Figure 1A). The combination of TCRm mAb and CV1 significantly increased macrophage phagocytosis of AML14 and BV173, but not the control HL60, indicating the effect was TCRm antigen-specific. As expected, the anti-CD47 blocking antibody, B6H12, induced a significant increase in ADCP of all three leukemia cell lines, and potentiated TCRm-mediated phagocytosis of AML14 and BV173. Phagocytosis EC₅₀ demonstrated the potency and specificity of the approach (Figure S1). ADCP with NSG mouse macrophages, showed improved phagocytosis with CV1 alone and significantly increased phagocytosis with CV1 and TCRm PR20 in combination (Figure 1B). Collectively, these results indicate that CD47 blockade is effective at improving ADCP *in vitro* of antibodies that target ultra-low density tumor antigens, such as TCRm mAbs.

IFN γ is a potent immunocytokine with pleiotropic effects, including induction of MHC Class I and II expression and increased antigen processing and presentation. (9) Anti-CD47 mAb therapy triggers a phagocyte type I and II interferon (IFN) response in the tumor microenvironment that presumably increases tumor cell surface peptide-MHC (pMHC) density. (10) As the epitope target of TCRm mAbs is presented by pMHC, we hypothesized that promoting IFN γ signaling may boost TCRm mAb effector functions by increasing target antigen density on the tumor cell surface. IFN γ treatment of AML14 and BV173

increased their HLA expression, resulting in increased binding of Pr20 (Figure S2) and ESK1 (not shown). IFN γ significantly increased expression of TCRm mAb epitopes of interest and increased macrophage-mediated ADCP of both AML14 and BV173 *in vitro* (Figure 1C). CV1 treatment alone did not increase HLA expression on either cell line.

We next asked if combination CV1 and TCRm therapy would improve potency *in vivo*. Dose titrations and dose scheduling was determined first (Figure S3). Although TCRm mAb or CV1 monotherapy significantly reduced leukemia burden in the AML14 model (Figure 2A), combination therapy had a markedly increased effect compared to either agent alone, with a 3 log reduction in leukemia burden relative to control untreated mice, a 10 fold reduction relative to the single agent groups (Figure 2A), and significantly improved survival for the combination therapy. (Figure 2B). These effects were generalizable, to the Ph+ ALL BV173, and to a second TCRm antibody (ESKM) (Figure 2C, S4). After therapy was stopped, leukemia relapsed not at the initial sites (bone marrow and spleen), but in lymphomatous nodules. At day 67, 3 weeks after the end of therapy, mice in combination therapy group with AML14 had lower tumor burden than at engraftment. (Figure S5)

In addition, we discovered that CV1 treatment caused elaboration of IFN γ *in vivo* (Figure S6). We hypothesized that some of the enhanced therapeutic activity was related to this release of IFN γ , which not only can directly activate phagocytosis further, but also cause a feed-forward mechanism unique to these TCRm antigenic systems in which the peptide epitope presentation on the target cells is up regulated by IFN γ .

Several factors may explain the dramatic therapeutic effects *in vivo* of combination CV1 and TCRm First, the *in vivo* microenvironment may positively alter macrophage effector function. Second, neutrophils express both SIRP α and Fc receptors and have been implicated in responses to anti-CD47 antibody treatment. Blocking SIRP α signaling may alter neutrophil transmigration, trafficking and therapeutic activity (11). Interestingly, relapses that occurred in BV173 engrafted mice were outside of sites with high phagocytic cell density. Third, the leukemias we evaluated preferentially engrafted in organs with high intrinsic numbers of phagocytic cells. Fourth, cross-species differences in Fc receptor biology, as well as alternative xenogeneic ligand-receptor interactions between human tumor cells and mouse immune effectors may alter antibody and immune cell function (12). NSG mice are B cell-, T cell-, and NK cell-deficient, and although they have intact IFN γ -dependent signaling, they have defective innate immunity and cytokine signaling pathways (13). While it is difficult to draw parallels between human and mouse systems, in the human, a greater variety of more potent effectors and an immunocompetent host that responds to pro-inflammatory signaling could allow even greater efficacy of this drug combination in the human patient. In addition, NSG mice have low circulating IgG levels that could compete with TCRm for Fc receptor interactions. (13)

We found no other up-regulated cytokines in the serum of CV1 treated mice other than IFN γ . IFN γ secretion caused by CV1 is likely contributing indirectly to the therapeutic effects seen through a new mechanism. IFN γ contributes directly to the innate and adaptive immune response (14). However, NSG mice have no T or NK cells. (15) Not surprisingly, we found that IFN γ -dependent signaling enhanced TCRm mAb dependent, macrophage-

mediated phagocytosis *in vitro*. This enhancement of phagocytosis was likely mediated through multiple mechanisms including direct IFN γ -dependent macrophage activation, as well as indirectly via TCRm specific mechanisms involving increases in tumor cell in HLA expression and antigen presentation. Thus, strategies that promote an IFN γ response, such as CD47 blockade (15), could uniquely potentiate the activity of TCRm, beyond what might be seen with traditional mAbs. This is consistent with the increase in serum concentrations of IFN γ we observed in mice treated with CV1. Notably, IFN γ alone did not cause cell cytotoxicity. Therefore, the combination of CV1 and TCRm in this specific milieu leads to remarkable tumor kill. Although we only quantified systemic cytokine levels, it is likely that the local concentration of IFN γ at sites where the targets and effectors are in contact is much higher, such as in the marrow, spleen and liver. It is also possible that other cytokines were also increased locally as a result of treatment, but not high enough to be detected in serum. Pro-inflammatory cytokine responses to CV1 monotherapy have not been reported. This novel feature of its activity may contribute to its usefulness as a cancer therapeutic. It will be important to determine if this effect is specific to leukemia, or also other cancers.

In conclusion, the greater than additive effect of these agents together *in vivo* is particularly unexpected given the extremely low epitope density of PRAME- and WT1-derived peptide epitopes. The synergy between CV1 and antitumor antibodies may be especially pronounced with TCRm compared to traditional mAbs since the targets of TCRm mAbs are presented by HLA and are thus regulated by cytokine signaling. Although we demonstrated the unusual therapeutic utility of antagonizing CD47 to potentiate the antitumor activity of TCRm, we anticipate this approach may be applicable to other mAbs that target a low cell surface density tumor antigen. This strategy could turn poorly efficacious antibodies into powerful antitumor therapeutics and significantly expand the possible cancer antigen targets of monoclonal antibodies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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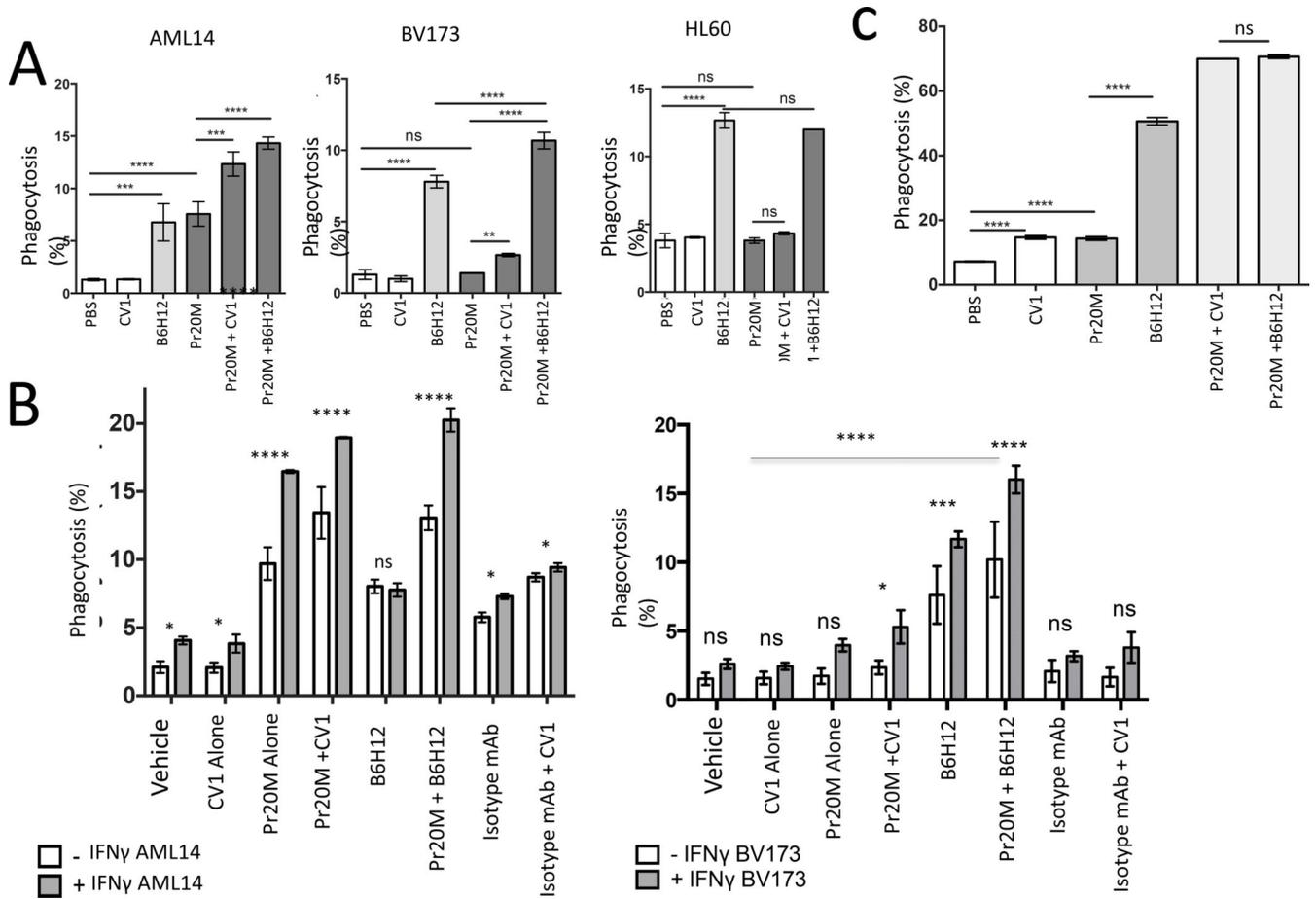


Figure 1. ADCP of leukemia cells in vitro

A) Human macrophage phagocytosis of AML14, BV173, and HL60 treated with various combinations of TCRm and CV1 quantified by flow cytometry. Experiments were completed in duplicate with various human donors. B) Left panel: AML14 cell line was pretreated with 100 ng/uL of IFN γ for 72 hours. Isolated human macrophages were incubated with pretreated AML14 cell line in the presence of 1) PBS, 2) CV1 alone, 3) Pr20M alone, 4) combination therapy with Pr20M and CV1, 5) positive control B6H12 (previously described), 6) B6H12 with Pr20M 7) irrelevant control mAb, and 8) irrelevant control mAb with CV1. All groups showed an increase in ADCP with IFN γ pretreatment. Increase was most significant in Pr20M alone, combination therapy, and B6H12 with Pr20M. Right panel: BV173 cell line was pretreated with 100 ng/uL of IFN γ for 72 hours. Isolated Human macrophages were incubated with pretreated BV173 cell line as above. All groups show an increase in ADCP with IFN γ pretreatment. Increase is significant in combination therapy, positive control, and positive control with Pr20. C) NSG-derived mouse macrophage ADCP of CSFE labeled AML14 cells quantified by flow cytometry. These experiments were performed in duplicate with consistent results.

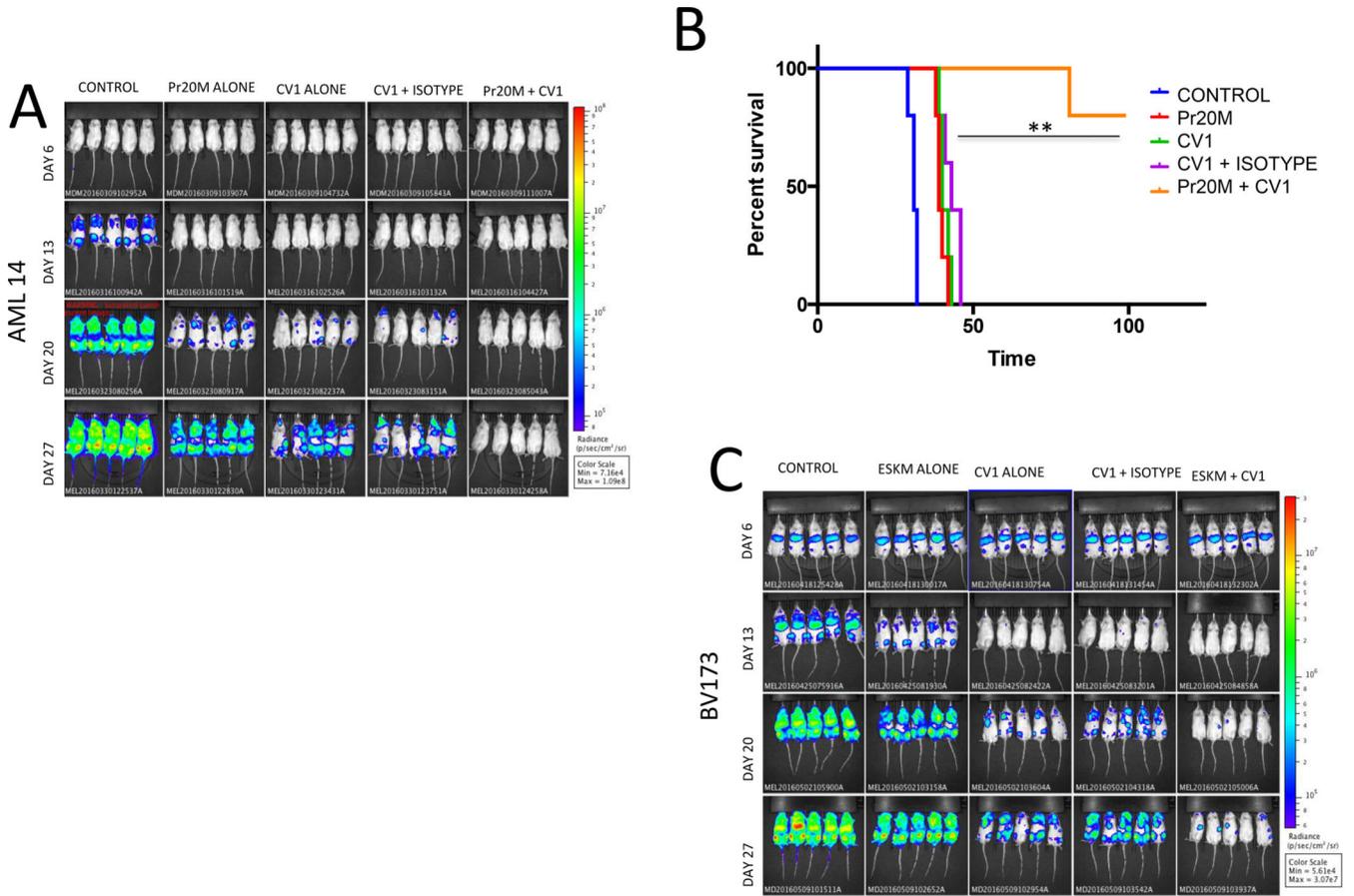


Figure 2. CV1 dose-response effects in vivo

A) Mice were engrafted via tail vein injection with 3 million cells/mouse of AML14 transfected with Luciferase gene. Mice were imaged via BLI on day 6. Mice were randomized to have equal group mean engraftment. Starting on day 6 after engraftment, mice were treated with either 200 μ g, 150 μ g, or 100 μ g of CV1 daily. Mice were imaged once a week for 3 weeks. B) Kaplan-Meier curve showing survival. Control and single treated groups had 100% death within 50 days. Experiment was truncated at 100 days at which time 4 of 5 mice in combination were alive. Log-rank test among all 5 groups indicated significant differences in overall survival among the groups ($p < 0.001$). C) Mice were engrafted via tail vein injection with 3 million cells/mouse of BV173 transfected with Luciferase gene. Mice were imaged via BLI on day 6. Mice were randomized into 5 groups of 5 mice each to have equal group mean engraftment. The 5 groups were: 1) control, 2) ESKM alone, 3) CV1 alone, 4) CV1 plus isotype TCRm antibody, and 5) CV1 + ESKM. Treatment started on day 6. ESKM was administered retro-orbitally biweekly at 50 μ g. CV1 was administered intraperitoneally daily at 100 μ g. Mice were imaged once a week for 3 weeks