THE COMBINATION OF DLL4/VEGF-A BLOCKADE AND IMMUNOMODULATION CAN ELIMINATE MHC CLASS I NEGATIVE TUMORS IN MICE

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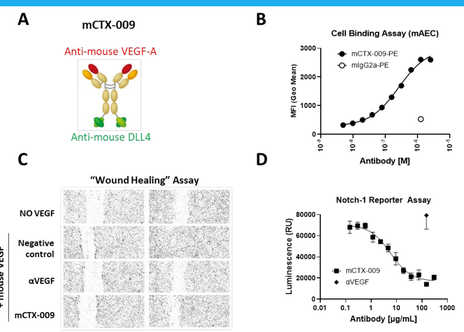
ABSTRACT

Class I negative tumors are resistant to immunotherapy. VEGF blockade has been demonstrated to alter the immunological composition of the tumor microenvironment. We set out to investigate the therapeutic potential of immunomodulation and neoangiogenesis disruption in mice with class I negative tumors. CTX-009 is a bispecific antibody targeting VEGF-A and DLL4. CTX-471 is a next generation CD137 agonist antibody that has been both epitope and affinity optimized for agonizing CD137. The combination of CTX-009 and CTX-471 significantly enhanced the anti-tumor activity of either antibody alone. Surprisingly, the combination of CTX-009 and CTX-471 maintained potent anti-tumor activity even in MHC-I deficient mouse tumors, which model loss of antigen presentation following progression on checkpoint blockade therapy in humans. NK cells and CD4 T cells emerged as key mediators of tumor growth control in this context. These findings suggest CTX-009's potential for improved clinical outcomes when combined with immunomodulatory agents, particularly in tumors resistant to immune checkpoint inhibitors. Both antibodies are currently being tested in human clinical trials.

INTRODUCTION

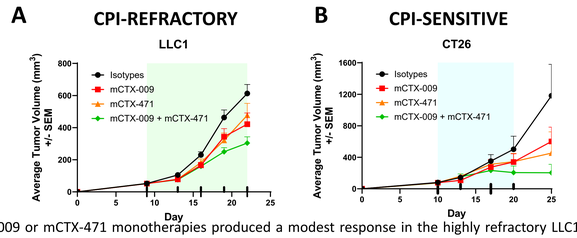
Blocking DLL4/Notch and VEGF/VEGF-receptor signaling is an important therapeutic strategy in oncology. CTX-009 is a recombinant bispecific antibody of the human IgG1 isotype which contains single chain variable fragments (scFvs) binding to DLL4 linked to the heavy chain of an antibody that binds and neutralizes the activity of human VEGF-A[1]. Phase 1 trials demonstrated promising activity in patients with a variety of solid tumors, both as a monotherapy and in combination with chemotherapy. A Phase 2 trial of CTX-009 in combination with paclitaxel, in patients with advanced biliary tract cancer treated in the second- and third-line settings, achieved a 37.5% overall response rate. Additional Phase 2 trials in colorectal and biliary tract cancers are ongoing. To explore the full potential of CTX-009, we set out to test in mouse syngeneic tumor models the combinations of a mouse surrogate version of CTX-009 (mCTX-009) and immunotherapy approaches, namely PD-1/PD-L1 pathway blockade or CD137 agonism.

1. A MOUSE CTX-009 SURROGATE BISPECIFIC ANTIBODY BINDS TO ENDOTHELIAL CELLS AND BLOCKS BOTH VEGF-A AND DLL4 ACTIVITY *IN VITRO*



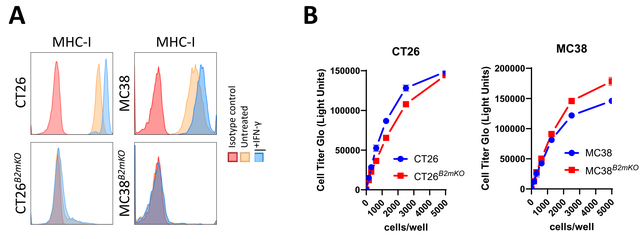
A) A bispecific antibody targeting murine VEGF and DLL4 was generated to model CTX-009 activity in isogenic tumor models (mCTX-009). The Kd of mCTX-009 to recombinant mouse VEGF-A and DLL4 were 2.22 pM and 13.1 nM respectively (comparable to CTX-009 binding to the human antigens [1]). The CH and CL regions of the IgG scaffold are of the murine IgG2a isotype, whereas the variable regions are human; **B)** mCTX-009 bound to mouse aortic endothelial cells (mAEC); **C)** mCTX-009 delayed the VEGF-dependent reconstitution of a cellular monolayer in the mAEC scratch assay; **D)** HEK293 cells were engineered to express luciferase upon Notch-1 and DLL4 binding. mCTX-009 blocked DLL4-induced luciferase expression in a dose-dependent manner (IC_{50}= 2.90e-8M).

2. SUPERIOR EFFICACY OF THE mCTX-009 AND mCTX-471 COMBINATION IN SELECTED CPI-SENSITIVE AND CPI-REFRACTORY MODELS



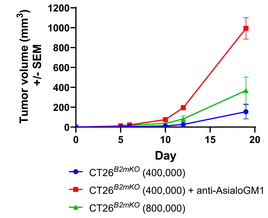
A) mCTX-009 or mCTX-471 monotherapies produced a modest response in the highly refractory LLC1 model, while their combination produced a markedly enhanced anti-tumor activity (green line). **B)** Similarly, combination of the two treatments produced curative responses (60% and trending lower at takedown) in the CPI responsive CT26 model. mCTX-009 was dosed every 3 days (upper thick tick marks, 6.8 mpk in LLC1, 5 mpk in the CT26 model), whereas mCTX-471 was dosed weekly (2 mpk in CT26, 5 mpk in LLC1, below axis thick tick marks). Shown is the average tumor volume of 9 (LLC1) or 7 (CT26) mice per group.

3. B2mKO NEGATIVE CELL LINES



A) Loss of HLA, commonly observed in patients with resistance to CPI, can be recapitulated in mouse isografts, albeit in an HLA-allele non-specific manner, by elimination of the B2m gene [3]. Deletion of B2m produced MHC-I null phenotypes in both CT26 and MC38 cell lines (bottom panels). Unlike the parental lines, IFN-γ fails to further induce the expression of MHC-I in $CT26^{B2mKO}$ and $MC38^{B2mKO}$ cells. **B)** Loss of B2M did not significantly affect their proliferation *in vitro*, however, tumorigenesis was severely affected.

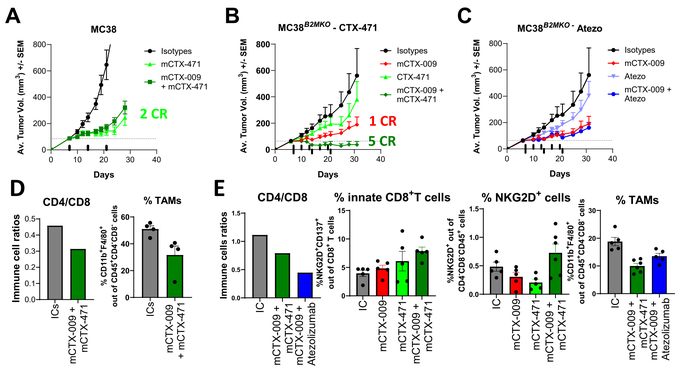
4. NK DEPLETION RESTORES B2mKO CELL ENGRAFTMENT



Loss of B2m did not significantly affect $CT26^{B2mKO}$ cell proliferation *in vitro*. However, engraftment in mice was affected. Mice rejected the standard cell inoculum used for engraftment of CT26 WT cells. Increasing the number of cells inoculated enabled $CT26^{B2mKO}$ engraftment to levels comparable to WT cells (data not shown). Treatment with NK-cell depleting antibody anti-Asialo GM1 restored engraftment to levels similar or better than seen with WT CT26. These data are consistent with literature suggesting a role for NK-cell mediated tumor cell killing in the absence of MHC-I [3].

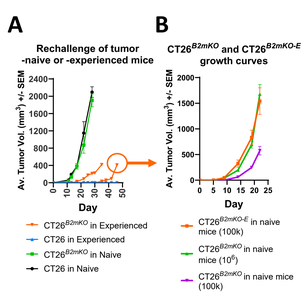
5. SUPERIOR EFFICACY OF THE mCTX-009 + mCTX-471 COMBINATION IN $MC38^{B2mKO}$ TUMORS

A) MC38 tumors were responsive to mCTX-009 + mCTX-471 treatment ($1x10^5$ cells/mouse, mCTX-471 @1.5 mpk, q7dx2). **B)** MC38 cells that were rendered MHC-I negative by B2m chain deletion responded to mCTX-009 (5 mpk, q3dx5) or mCTX-471 (1.5 mpk, q7dx3), with 1 (out of 8) mice completely responding in each treatment group. The efficacy was significantly increased by the combination of these antibodies which led to 62.5% cures (5 out of 8 mice). Of note, ten times more $MC38^{B2mKO}$ cells were inoculated to ensure tumor formation. **C)** In the same model, the combination of Atezolizumab (3 mpk, q3dx3) and mCTX-009 (5 mpk, q3dx5) controlled the tumor growth with no complete responders. **D)** TIL analysis by flow cytometry. In MC38 tumors, mCTX-009 + mCTX-471 increased the proportion of $CD8^+$ T cells and decreased the amount of tumor associated macrophages (TAMs). The proportions of $CD8^+$ T cells in control tumors were approximately twice the amount of $CD4^+$ T cells. Combination treatment increased the $CD4^+$T cells/$CD8^+$T cells ratio. **E)** In the control $MC38^{B2mKO}$ tumors, $CD4^+$ TILs were slightly more numerous than $CD8^+$ as previously shown by others [3]. Treatment with mCTX-009 and mCTX-471 or Atezolizumab increased the relative amount of $CD8^+$ T cells (left panel), with stronger effect of mCTX-009 + Atezolizumab combination. Since an increase in $CD8^+$ T cell infiltrate did not correlate with better efficacy (B, C), we hypothesized that the treatment altered the phenotype or the function of infiltrating T cells. Based on recent work by Lerner [4], we asked whether antibody combinations generated NKG2D-expressing innate killer cells. Both $NKG2D^+CD8^+T$ and $NKG2D^+CD4^-CD8^-$ cells were detected in $MC38^{B2mKO}$ tumors, which were positive for NKG2D ligands. mCTX-471 worked with mCTX-009 better than Atezolizumab in increasing the amount of these "innate killers". Compared to mCTX-471 or mCTX-009 single agents, however, differences did not reach statistical significance (middle two panels). Of note, resident TAMs proportion in $MC38^{B2mKO}$ was less than half of the total $CD45^+$ TILs in MC38 parental cell. Both combination treatments further reduced the fraction of infiltrating TAMs, suggesting that the CTX-009 + CTX-471 combination might help eradicating $MC38^{B2mKO}$ tumors by potentiating innate cell killing and alleviating immunosuppression by infiltrating TAMs.



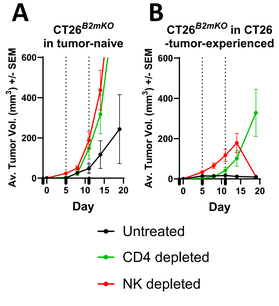
6. ESCAPE OF B2mKO CELLS AS A MODEL OF CPI PROGRESSION

A) Mice cured from CT26 tumors by either mCTX-471 or mCTX-471 + mCTX-009 (Experienced) or age matched tumor-naïve mice were challenged with either the CT26 or $CT26^{B2mKO}$ cells. Tumor experienced mice were capable of dramatically delaying or completely suppressing the growth of both cell types, whereas tumor-naïve mice did not. Note that $1X10^6$ $CT26^{B2mKO}$ cells were needed to produce growing tumors (N =5). **B)** $CT26^{B2mKO}$ tumors that eventually grew in experienced mice were re-inoculated in naive mice. A 10-fold lower inoculum was required for $CT26^{B2mKO}$ escaper ($CT26^{B2mKO\text{-}E}$) to produce tumors similar to $CT26^{B2mKO}$, suggesting the tumors acquired resistance.



7. REJECTION OF $CT26^{B2mKO}$ CELLS BY mCTX-009 + mCTX-471 CURED MICE IS CRITICALLY DEPENDENT ON BOTH $CD4^+$ T AND NK CELLS

A) Tumor-naïve mice were challenged with $CT26^{B2mKO}$ cells ($1x10^6$ per mouse, s.c.) in NK cells or $CD4^+$ T cell depleted animals (i.p. injections of depleting antibodies at day -1, 5 and 11 (100 µg anti-asialoGM1 and 200 µg anti-CD4T cells clone GK1.5 per mouse respectively, 5 mice/group, thick ticks). **B)** Same as in A, but in CT26-tumor-experienced mice. Since depletion of NK and CD4 cells rendered CT26-tumor-experienced mice more vulnerable to the MHC-I negative tumor cell challenge, it is possible that a mechanism involving NK cell memory might be mediating the rejection of these tumors. Hence, we hypothesize that the combination of mCTX-009 and mCTX-471 could re-establish anti-tumor immunity in patients whose tumors have either downregulated or lost MHC-I expression.



SUMMARY AND CONCLUSIONS

As a standalone treatment, mCTX-009 demonstrated significant antitumor activity in the CT26, MC38, 4T1, and LLC1 isograft models. mCTX-009 in combination with CTX-471 was more effective in several isograft models, including the IO resistant LLC1 model. Notably, this antitumor activity was even seen when tumors were rendered MHC-I negative due to B2M gene deletion, which recapitulates a CPI resistance mechanism observed in post CPI patients [2] [3]. In the $MC38^{B2mKO}$ and $CT26^{B2mKO}$ (not shown) models, the combination of CTX-009 and CTX-471 maintained potent anti-tumor activity. Preliminary investigations into the mechanism of action suggest this combination might re-establish a tumor rejection axis, potentially primed by the absence of MHC-I, which involves NK and CD4 T cells. **Our findings indicate that the combination of CTX-009 and CTX-471 could be considered not only as a first-line treatment option, but also as a viable alternative where previous immunotherapy was ineffective.**

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