Published OnlineFirst February 5, 2019; DOI: 10.1158/0008-5472.CAN-18-2022

Tumor Biology and Immunology

Cancer Research

Immuno-oncological Efficacy of RXDX-106, a Novel TAM (TYRO3, AXL, MER) Family Small-Molecule Kinase Inhibitor

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Abstract

Expression of the TAM (TYRO3, AXL, MER) family of receptor tyrosine kinases (RTK) has been associated with cancer progression, metastasis, and drug resistance. In immune cells, TAM RTKs can dampen inflammation in favor of homeostatic wound-healing responses, thus potentially contributing to the evasion of cancer cells from immune surveillance. Here we characterize the small-molecule RXDX-106 as a selective and potent pan-TAM RTK inhibitor with slow dissociation kinetics and significant antitumor activity in multiple syngeneic tumor models. Expression of AXL and MER on both immune and tumor cells increased during tumor progression. Tumor growth inhibition (TGI) following treatment with RXDX-106 was observed in wild-type mice and was abrogated in immunodeficient mice, suggesting that the antitumor activity of RXDX-106 is, in part, due to the presence of immune cells. RXDX-106–mediated TGI was associated with increased tumor-infiltrating leukocytes, M1-polarized intratumoral macrophages, and activation of natural killer cells. RXDX-106 proportionally increased intratumoral $CD8^+$ T cells and T-cell function as indicated by both IFNγ production and LCK phosphorylation (pY393). RXDX-106 exhibited its effects via direct actions on TAM RTKs expressed on intratumoral macrophages and dendritic cells, leading to indirect activation of other immune cells in the tumor. RXDX-106 also potentiated the effects of an immune checkpoint inhibitor, α-PD-1 Ab, resulting in enhanced antitumor efficacy and survival. Collectively, these results demonstrate the capacity of RXDX-106 to inhibit tumor growth and progression and suggest it may serve as an effective therapy against multiple tumor types.

Significance: The pan-TAM small-molecule kinase inhibitor RXDX-106 activates both innate and adaptive immunity to inhibit tumor growth and progression, indicating its clinical potential to treat a wide variety of cancers.

Introduction

Immune checkpoints can block cell expansion/activation, abrogate the immune response, and initiate return to homeostasis, but tumors have pathologically coopted these pathways to restrict effective antitumor responses (1). Targeted inhibition of these negative regulatory checkpoints has emerged as viable and promising immunotherapies for cancer (2). Antibodies that block CTLA-4, PD-1, or PD-L1 show clinical efficacy by boosting $CD4^+$ Th cells or by restoring activity of exhausted $CD8^+$ T cells, respectively (3, 4). Nevertheless, the response rate to checkpoint blockade remains ≤30% for many cancers, in part, due to the suppressive milieu in the tumor microenvironment (TME) stemming from suppressive innate immune cells, including myeloid-derived suppressor cells (MDSC; ref. 5), M2-like macrophages (6), dysfunctional dendritic cells (DC; ref. 7), and natural killer (NK) cells (8). Presumably, therapies that activate/polarize the innate immune system could synergize with checkpoint inhibitors to boost antitumor effects of the adaptive immune system (9).

One group of checkpoint molecules that regulate innate immunity are the TAM RTKs–TYRO3, AXL, and MER, which are broadly expressed on several immune cell lineages, including macrophages, DCs, and NK cells (10). With their ligands, GAS6 and PROS1, TAM RTKs help to resolve inflammatory signals and participate in wound healing. In $Mertk^{-/-}$ mice, inefficient clearance of apoptotic cells by monocyte-derived and epithelial cells, a process dependent on MER activation by GAS6 or PROS1 complexed with externalized phosphatidylserine (PtdSer) on apoptotic bodies (11, 12), leads to aberrant accumulation of apoptotic material, instigating inflammation. MER also suppresses the M1 macrophage/pro-inflammatory cytokine response

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-18-2022

Published OnlineFirst February 5, 2019; DOI: 10.1158/0008-5472.CAN-18-2022

while enhancing the M2 macrophage/anti-inflammatory production (13–15). On antigen-presenting cells (APC), Toll-like receptor (TLR) signaling upregulates AXL, which complexes with the type-I interferon receptor (IFNAR1), eliciting negative feedback on further TLR signaling (16). In addition, activated $CD8^+$ T cells upregulate PROS1 and externalize PtdSer; this complex, in turn, stimulates TAM receptors on APCs, reducing APC activity (17). Furthermore, TAM triple knockout mice (TAM TKO), although gestationally viable, develop chronic inflammation and systemic autoimmunity, multiple organ defects, and massive lymphoproliferative disease due to overactivation of the innate immune system and failure to clear apoptotic debris, leading to engagement of the adaptive immune response (18).

Abnormal overexpression of TAM RTKs and/or GAS6 has been documented in a wide range of malignancies (14, 19, 20). In contrast to its perceived inhibition of immune cell signaling, TAM receptor activation in tumor cells promotes survival and tumorigenicity (21, 22). In the TME, where resources such as oxygen and nutrients are scarce, TAM RTK signaling may be critical in providing survival cues (20, 23), while promoting chemoresistance, colony-forming potential, invasion/motility, and induction of epithelial-to-mesenchymal transition (EMT; refs. 14, 20, 21, 24). Thus, TAM RTKs may both dampen inflammation and activate/promote cancer cell progression, encouraging development of therapeutic agents that inhibit TAM receptors for cancer immunotherapy.

The frequent observation of $\geq$2 TAM RTKs coexpressed on immune cells (25) and the normal phenotype of single- or double-TAM knockout mice in contrast to the autoinflammatory and autoimmune phenotype of the TAM TKO (18), suggests a redundancy in TAM receptor activity in innate immune cells. Indeed, in response to selective TAM RTK agents (i.e., anti-AXL–specific inhibition), cancer cells upregulate other TAM RTK family members as a mechanism of resistance (26). In this report, we document the characterization of RXDX-106, a novel small-molecule inhibitor of all three TAM RTKs. RXDX-106 blocked TAM RTKs on both tumor and immune cells, both *in vitro* and *in vivo*. This activity reduced tumor progression, even in tumor cells that do not respond to TAM RTK inhibition, suggesting that RXDX-106 activated antitumor immunity. Indeed, RXDX-106 directly activated macrophages, leading to activation of NK and T cells, restoring tumor immunity. These results confirm the negative checkpoint activity of TAMs in tumor immunity and support the use of RXDX-106 to tip the immune rheostat in favor of an effective antitumor immune response.

Materials and Methods

Cell lines and reagents

Cell lines were purchased from Kerafast (MC38), Sigma-Aldrich (EMT-6), ATCC (CT26, NIH3T3), or DSMZ (Ba/F3), respectively, and maintained at $\leq$passage 10. Cell line authentication and pathogen testing was performed to confirm cell line identities and pathogen-free status (including *Mycoplasma* testing; IDEXX BioResearch). Micronized anhydrous free RXDX-106 (CEP-40783) was synthesized at Base Micro Technologies. Antibodies and qPCR primers utilized are listed in Supplementary Tables S1–S4.

In vivo studies

RXDX-106 *in vivo* oral dosing solution was made by suspending RXDX-106 in equal parts Oleic Acid (Thermo Fisher Scientific) and PEG400 (Sigma-Aldrich) at a concentration of 1 or 3 mg/mL, and animals were dosed orally every day. Female C57BL/6, BALB/c, athymic nude, BALB/c nude, SCID Beige, and CD45.1 C57BL/6 mice between 6 and 7 weeks of age were ordered from Charles River. C57BL/6 MMTV-PyMT (PyMT) female mice were bred in-house [University of California, San Diego (UCSD), San Diego, CA]. CD45.1.2 C57BL/6 mice were a kind gift from Dr. John Chang (University of California, San Diego, San Diego, CA). Bones from $Axl^{-/-}Mertk^{-/-}$ (AM) double knockout (AMdKO) mice were a kind gift from Dr. Greg Lemke (Salk Institute, La Jolla, CA). Animal experiments were conducted in accordance with guidelines established by the Explora Biolabs Institutional Animal Care Use Committee (IACUC; Ignyta ACUP# EB15-050).

Cells (1×10^6 cells/mouse, unless otherwise indicated) were implanted subcutaneously on the right flank of each mouse. Tumor-bearing mice were randomized and treated orally every day with vehicle or RXDX-106 at 30 mg/kg or at indicated doses. For studies with NIH3T3 cells expressing TYRO3, AXL, and MER, SCID/Beige animals were inoculated and treated on the indicated day with RXDX-106 orally every day when mean tumor volume reached approximately 140, 60, and 150 mm^3, respectively.

For TGI combination studies with anti-PD-1 antibody, MC38 and CT26 cells were implanted subcutaneously in the right flank of C57BL/6 and BALB/c mice, respectively. Mice were randomized and treated when tumors reached 130 mm^3. Animals were treated with 30 mg/kg RXDX-106 (orally every day) $\pm$ antibodies (intraperitoneally, two times/week; 10 mg/kg anti-PD-1 antibody or rat IgG2a antibodies). Survival was monitored for a maximum duration of 60 days or until tumor volume reached 2,000 mm^3. Isotype antibody had no effect on tumor growth. For immunophenotyping and RNA-seq combination study, animals were treated for 7 days with either vehicle or RXDX-106 (orally every day) $\pm$ antibodies (anti-PD-1 antibody or rat IgG2a antibodies (10 mg/kg) intraperitoneally 2$\times$/week. PyMT mice were treated orally every day with vehicle or RXDX-106 at 30 mg/kg at tumor onset.

Generation of bone marrow chimeric mice

Eight-week-old female CD45.1 mice were irradiated with 1,000 cGy and intravenously injected with 5×10^6 bone marrow (BM) cells from WT CD45.2 or AMdKO CD45.2, respectively. For some experiments, CD45.1 mice were intravenously injected with bone marrow cells from WT CD45.1.2 and AMdKO CD45.2 at a 1:1 ratio. Proper reconstitution was tested by FACS analysis of cheek blood after 6 weeks.

In vivo proliferation determined by BrdU incorporation

Mice were subcutaneously inoculated with 1×10^5 MC38/mouse and treated with 30 mg/kg RXDX-106 (or vehicle) for 1 week after tumors reached a size of 400 mm^3. Forty-eight and 24 hours before harvest, mice were intraperitoneally injected with 1 mg of BrdU/mouse (eBioscience). Tumor-draining lymph nodes (LN) and tumors were harvested and single-cell suspensions were stained for BrdU expression after DNase treatment according to the manufacturer's protocol. Samples were analyzed by FACS. Differences shown are not significant. Animals were housed in the UCSD Animal Care Facility, and all experiments were conducted in accordance with guidelines established by the UCSD IACUC (protocol #S06201).

Immunophenotypical analysis

For immunophenotyping, MC38 tumor-bearing mice were dosed orally every day with vehicle or RXDX-106 for 7 days or

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as indicated. Dissociated tumor cells were washed with PBS, stained with Fixable Viability Stain 780 (BD Biosciences) for 30 minutes at 4°C, and washed with FACS staining buffer. Cells were blocked with Mouse FcR Blocking Reagent (Miltenyi Biotec) prior to staining, and then cells were stained with antibodies for 30 minutes at 4°C. After washing with FACS buffer, cells were fixed with 1% paraformaldehyde. For intracellular staining of FoxP3 and IFNγ, Foxp3/Transcription Factor Staining Buffer Set (eBioscience) was used following the manufacturer's instructions. For detection of intracellular phospho-TAM, tumors were collected and dissociated with 1 mmol/L Na_3VO_4. Cells were first stained for surface markers, permeabilized with BD Cytofix/Cytoperm, and stained with phospho-TAM antibodies. Samples were acquired on BD FACS Celesta flow cytometer (BD Biosciences) and data analyzed with FlowJo software.

Statistical analysis

Statistical analysis was done either by ANOVA or two-tailed *t* test using GraphPad Prism (version 6.07; GraphPad Software, Inc.). *, *P* (*t* test) or FDR <0.05; **, *P* or FDR <0.01; ***, *P* or FDR <0.001; ****, *P* or FDR <0.0001.

Results

RXDX-106 is a pan-TAM RTK small-molecule inhibitor

The overexpression of multiple TAM receptors has been implicated in driving tumor growth and metastasis across multiple cancer types (14, 27). In addition, there is evidence of coexpression and functional redundancy of the receptors on immune cell types including macrophages, NK, and T cells (28–30). Hence, we developed RXDX-106, a small molecule, ATP-competitive inhibitor that targets all three TAM receptors. RXDX-106 bound to the kinase domains corresponding to TYRO3, AXL, and MER with an IC_{50} of 3.50, 0.69, and 1.89 nmol/L, respectively (Fig. 1A). In a broader activity-based kinase screen, we found that RXDX-106 is highly selective for the TAM RTKs with additional activity against the highly related c-MET/RON families (Supplementary Fig. S1A). Notably, RXDX-106 had slow dissociation kinetics indicative of a type II mode of inhibition (31) and remained bound to the TAM RTK targets at least 5-fold longer than other TAM RTK inhibitors tested (Supplementary Fig. S1B and S1C), suggesting more durable *in vivo* pharmacodynamics (32).

To corroborate the biochemical assays, target engagement of RXDX-106 was measured in NIH3T3 cells expressing human TYRO3, AXL, or MER. Receptor activation and signal transduction were dose-dependently inhibited by RXDX-106 with low nanomolar cellular IC_{50}s (Fig. 1B). Similarly, in a Ba/F3 cell line dependent on the ectopic expression of human AXL expression for cellular survival upon IL3 withdrawal, RXDX-106 inhibited cellular proliferation and viability with an IC_{50} of 0.31 nmol/L (Supplementary Fig. S1D). RXDX-106 also inhibited mouse TAM RTKs. In bone marrow–derived or peritoneal macrophages polarized with polyI:C or dexamethasone to express either AXL or MER, respectively, we observed dose-dependent inhibition of GAS6-dependent cellular signaling and inhibition of TAM RTK–dependent phagocytosis, a known readout for TAM RTK function (Supplementary Fig. S1E and S1F; refs. 30, 33).

We next investigated RXDX-106 efficacy *in vivo* using a classic transformation assay with NIH3T3 cells transduced with human TYRO3, AXL, or MER, allowing them to form tumors in mice (19, 34, 35). Mice implanted with NIH3T3_TYRO3, AXL, or MER cell lines treated with RXDX-106 showed rapid tumor regression, demonstrating *in vivo* efficacy of RXDX-106 (Fig. 1C). In a more physiologic *in vivo* setting, RXDX-106 inhibited the growth of primary mouse mammary tumors driven by oncogenic expression of the middle T antigen from polyoma virus (PyMT) in mammary tissues, although the differences were not significant due to control group variability (Supplementary Fig. S1G).

RXDX-106 efficacy is mediated by immunomodulatory function

TAM RTKs play key roles in both survival of tumor cells and in negative regulation of immune cell function. To determine whether RXDX-106 exerts its actions by targeting TAM RTKs directly on tumor cells or via immune cells, we compared the TGI of RXDX-106 in immunocompetent versus immunodeficient mice in three syngeneic mouse models. RXDX-106 significantly promoted TGI in the immunocompetent mice, whereas the effect in immunodeficient mice was not significant. Specifically, the TGI in the MC38 model was 61% ($P = 0.016$) in C57BL/6 versus 22% in athymic nude mice at day 18 (Fig. 2A). TGI in the Renca model was 60% ($P < 0.0001$) in BALB/c versus 19% in BALB/c nude mice at day 21 (Fig. 2B). The TGI in the EMT-6 model was 74% ($P = 0.023$) in BALB/c versus 17% in BALB/c nude mice at day 15 (Fig. 2C).

To investigate the effect of RXDX-106 on immune cells in the TME, MC38 tumor–bearing mice were treated with 1, 3, 10, 30, and 60 mg/kg RXDX-106 for 7 days (Fig. 2D, top), and $CD45^+$ TILs were measured by flow cytometry. A significant dose-dependent increase in TILs was observed with RXDX-106 (Fig. 2E), suggesting that RXDX-106 increased immune cell recruitment, survival, or proliferation in the TME. In addition, in a timecourse assay (Fig. 2D, bottom), this increase in immune cell infiltration was significant by day 5 (Fig. 2F) and at the transcript level by day 3 post-RXDX-106 as indicated by an increase in *Ptprc* (CD45) gene expression in the TME by RNA-seq that continued through day 9 (Fig. 2G). Notably, genes induced in the TME posttreatment with RXDX-106 were almost exclusively involved in immune function, as demonstrated by an unbiased global gene ontology analysis (GO) in which 16 of the top 20 gene pathways induced by RXDX-106 were immune-related (Fig. 2H; Supplementary Table S5). To further elucidate the significance of the immune system in mediating RXDX-106–induced TGI and to investigate the impact of other kinases inhibited by RXDX-106 (Supplementary Fig. S1A), we generated bone marrow chimeric mice by irradiation and reconstitution with bone marrow from either WT or $Axl^{-/-}Mertk^{-/-}$ double-knockout mice (hereafter WT BM or AMdKO BM) and transplanted them with MC38 cells (Fig. 2I). Reconstitution was >90% in all mice. We found no difference in tumor growth between WT and AMdKO BM mice at the basal state (Fig. 2J). In line with publications on AMdKO mice (18), we observed a hyperactivation of some immune cells in the spleen of AMdKO BM mice, represented by more $MHCII^{hi}$ macrophages and $CD44^+CD4^+$ and $CD8^+$ T cells (Fig. 2K). However, this hyperactivation did not transfer to the TME, in part, explaining why there was no TGI in MC38-challenged AMdKO BM mice. We also observed no difference in the number of MDSCs in the tumor (Supplementary Fig. S5E). Importantly, RXDX-106 was not able to inhibit tumor growth in the AMdKO BM mice (in contrast with WT BM mice; ref. Fig. 2J), suggesting that not only is its effect on TGI immune-mediated, but also that it solely acts on AXL and MER on immune cells. TYRO3, c-MET, RON, or other kinases that

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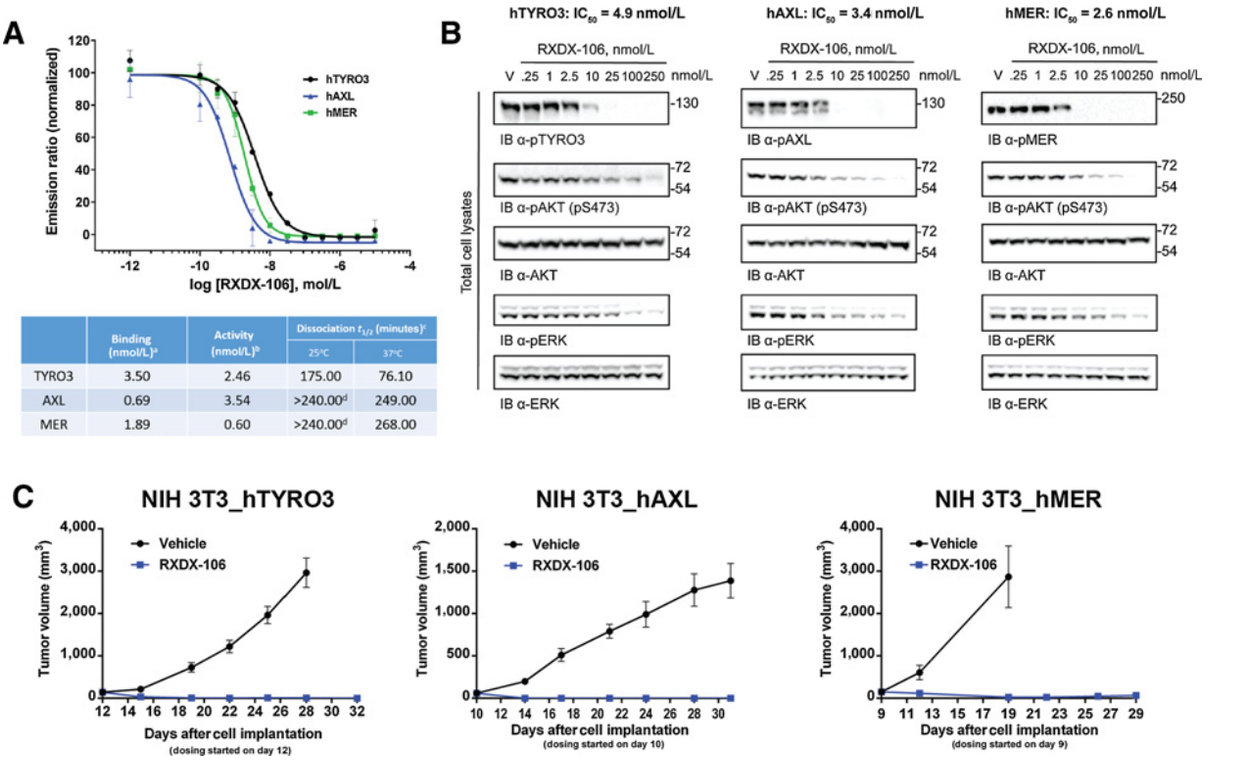

	Binding (nmol/L)[a]	Activity (nmol/L)[b]	Dissociation $t_{1/2}$ (minutes)[c]	
			25°C	37°C
TYRO3	3.50	2.46	175.00	76.10
AXL	0.69	3.54	>240.00[d]	249.00
MER	1.89	0.60	>240.00[d]	268.00

Figure 1.

RXDX-106 is a pan-TAM RTK inhibitor with durable target inhibition. **A,** Top, LanthaScreen equilibrium binding was performed and biochemical binding IC_{50} for TYRO3, AXL, and MER was determined. (bottom). Table shows calculated biochemical parameters for RXDX-106 as determined by LanthaScreen Kinase: [a]Binding and [b]Radiometric Kinase Activity Assay and [c]Dissociation Assay. [d]Determination limited to 4-hours due to signal decay. **B,** *In vitro* inhibition in NIH3T3 cells expressing human TAM RTKs. **C,** RXDX-106 mediates tumor regression in SCID/Beige mice harboring NIH3T3_TYRO3, AXL, or MER tumors, respectively. $n = 10$ mice/group.

RXDX-106 can inhibit (Supplementary Fig. S1A) do not seem to play a significant role in RXDX-106–mediated TGI.

RXDX-106 directly affects TAM RTK signaling *in vivo* on immune cells

Our data thus far suggest that RXDX-106 requires immune cells *in vivo* to mediate antitumor responses. Because TAMs are expressed on both tumor and immune cells, we sought to investigate whether RXDX-106 could directly kill tumor cells, leading indirectly to immune cell activation. We therefore examined the effect of RXDX-106 on tumor cell viability *in vitro*. Fig. 3A shows that RXDX-106 was more than 100-fold less potent ($IC_{50} = 2.67$ μmol/L) than staurosporine in killing MC38 cells, with an IC_{50} that is 1,000× higher than the IC_{50} to block *in vitro* TAM RTK kinase activity (Fig. 1B). Proliferation of tumor cells *in vivo*, as measured by Ki67, was also not affected by RXDX-106 (Fig. 3B; Supplementary Fig. S2A), suggesting that RXDX-106's antitumor effect was mediated by an immune response instead of direct antiproliferative activity on the cancer cell.

We next examined the expression of TAM RTKs on tumor versus immune cells *in vivo*. During tumor progression, an increase in cell surface MER was observed on both tumor cells and tumor-associated macrophages (TAMφ; $CD11b^{hi}Ly6G^{lo}Ly6C^{lo}F4/80^{hi}$) by flow cytometry, with a more significant increase observed on TAMφ (Fig. 3C; Supplementary Fig. S2B). This increase in expression was specific for TAMφ, because it was not seen on mMDSCs and gMDSCs (36). A further increase in overall MER levels in the TME was observed in tumor samples of RXDX-106–treated animals (Fig. 3D), most likely due to drug-mediated increases in TILs as RNA-seq analysis of $F4/80^+$, $CD49b^+$, and $CD90.2^+$-depleted tumor samples did not have increases in *Mertk* gene expression with RXDX-106 and only the $CD45^+$ population had detectable MER expression by Western blot analysis (Supplementary Fig. S2C and S2D). Similarly, a dose-dependent increase in *Mertk* gene expression was observed by RNA-seq analysis of whole tumors, in agreement with RXDX-106–mediated increases in MER-expressing TILs (Supplementary Fig. S2E). Notably, despite these increases in TAM RTK expression, a striking decrease in AXL and MER but not TYRO3 phosphorylation was observed, demonstrating the potent inhibitory effect of RXDX-106 in the TME (Fig. 3E and F). Inhibition of TAM RTKs by RXDX-106 occurred on both the MC38 tumor cells and TAMφ, as demonstrated by a decrease in phosphorylated TAM RTKs (pY779) in both $MHCII^{hi}$ and $MHCII^{lo}$ macrophage populations and on tumor cells (Fig. 3G; Supplementary Fig. S2F).

RXDX-106 increases innate immune responses to mediate antitumor effects

To further elucidate the cell types activated by RXDX-106 in the TME, immunophenotyping was performed on MC38 TILs from animals treated for 7 days with vehicle or RXDX-106 once mean tumor size reached 400 mm^3 (Supplementary Fig. S3A; Fig. 4A).

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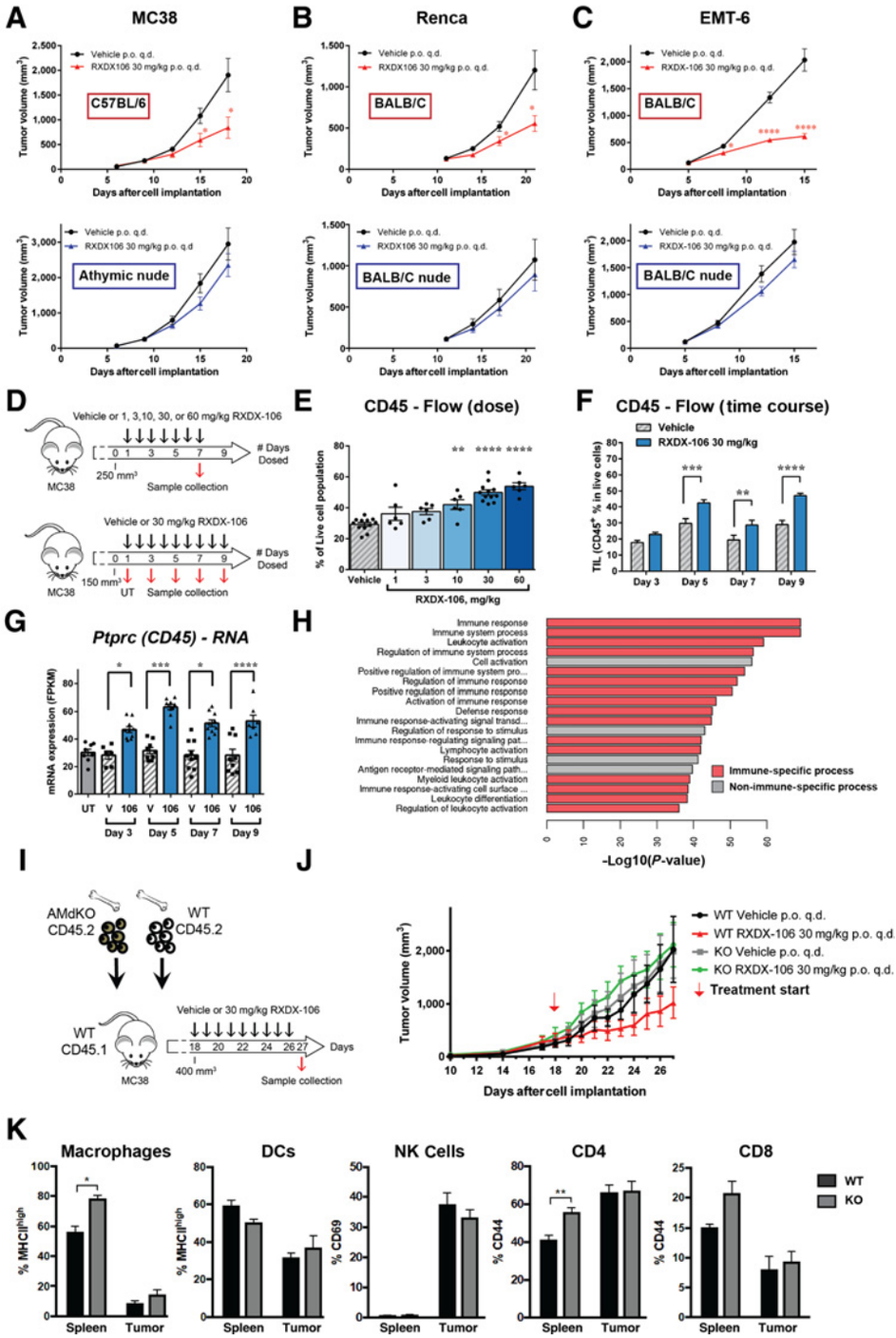

Figure 2.

RXDX-106 induces immune-mediated antitumor activity. MC38 (**A**), Renca (**B**), EMT6 tumor models in immunocompetent (top) or immunodeficient (bottom) mice (**C**). Treatment started post-randomization when tumor size reached approximately 200 mm^3 (MC38) or 120 mm^3 (Renca and EMT6). **D,** Schematic illustrating *in vivo* dosing/sample collection for dose escalation (top) or timecourse (bottom), respectively. Black arrows, dosing; red arrows, sample collection 2 hours post-dosing; UT, sample collection prior to treatment. **E,** $CD45^+$ population in MC38-tumor-bearing animals treated with 1, 3, 10, 30, or 60 mg/kg of RXDX-106 for 7 days. **F** and **G,** Whole tumor global gene and protein modulations by flow cytometry (**F**) and RNA-seq (**G**) in MC38 tumor-bearing animals treated for 3, 5, 7, or 9 days with 30 mg/kg RXDX-106. **H,** GO enrichment analysis with $-\log_{10} P$ values of top 20 GO terms enriched for differentially expressed genes (defined as $\log_2$ FC >1, FDR <0.01, and FPKM >1 in $\geq$2 mice) at day 5. Red bars, immune-related pathways; $n = 10$. **I** and **J**, Lethally irradiated CD45.1 mice transplanted with BM from CD45.2 WT or AMdKO were challenged with MC38 and tumor growth monitored post-treatment. **K,** Percentages of $MHCII^{hi}$ macrophages and DCs;$CD69^+$ of NK cells; $CD44^+$ of $CD4^+$ and $CD8^+$ T cells in the spleens and tumors; $n = 5$. Data are mean $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

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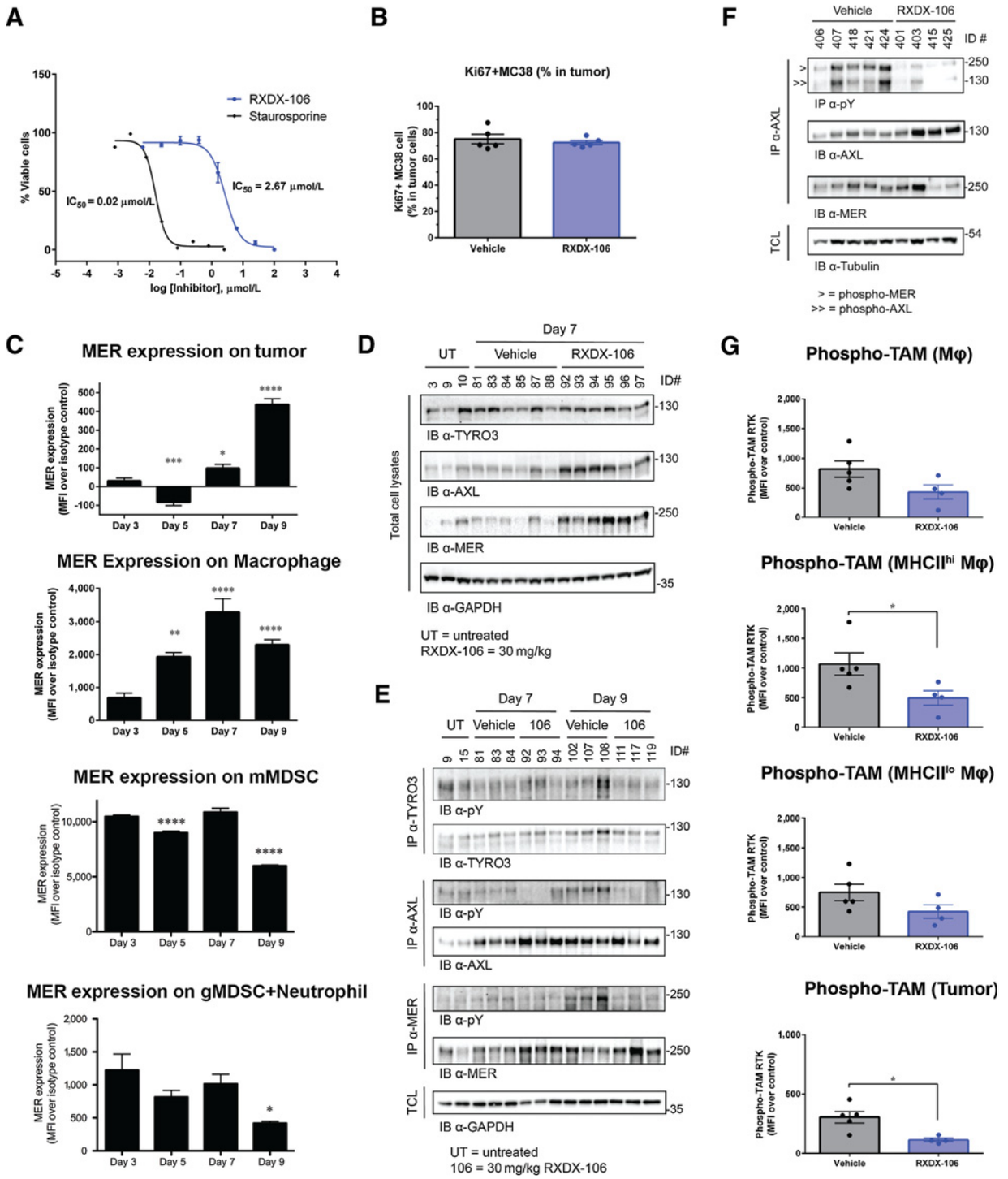

Figure 3.

RXDX-106 directly affects TAM RTKs on immune cells *in vivo*. **A,** *In vitro* cell viability assay in MC38 cells treated with RXDX-106 or staurosporine for 72 hours. **B–G,** Tumors from MC38 tumor-bearing mice were collected and analyzed for RXDX-106 effects on tumor cell proliferation and TAM RTK activation as illustrated in Fig. 2D (bottom). **B,** *In vivo* effect of RXDX-106 on tumor cell proliferation ($CD45^-Ki67^+$) by flow cytometry. **C,** TAM RTK expression on MC38 tumors ($CD45^-$), TAMφ-$CD11b^{hi}Ly6G^{lo}Ly6C^{lo}F4/80^{hi}$, mMDSCs-$CD11b^{hi}Ly6G^{lo}Ly6C^{hi}$, and gMDSCs/Neutrophils-$CD11b^{hi}Ly6G^{hi}Ly6C^{lo}$ during tumor progression. Statistical analysis relative to day 3. **D** and **E,** *Ex vivo* analysis of TAM RTK protein expression (**D**) and TAM RTK activation (**E**) in MC38 TME. **F** and **G,** Phosphorylation status of TAM RTKs in MC38 tumors 7-day posttreatment assessed in parallel by Western blot analysis (**F**) and flow cytometry (**G**). Data are mean ± SEM; $n = 8$–10. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

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Consistent with previous experiments (Fig. 2), RXDX-106 significantly mediated TGI (Fig. 4B) and increased TILs (Vehicle: 21.32% vs. RXDX-106: 38.41% in total live cells, $P < 0.0001$; Fig. 4C), with a striking increase in the percentage of macrophages (vehicle: 7.43% vs. RXDX-106:16.78% in total live, $P < 0.0001$; Fig. 4D). In addition, there was a dramatic increase in MHCII^{hi} (M1-like) macrophages (vehicle: 18.45% vs. RXDX-106: 38.88% in macrophages, $P < 0.0001$; Fig. 4E) and a significant decrease in MHCII^{lo}(M2-like) macrophages (vehicle: 16.15% vs. RXDX-106: 5.78% in macrophages, $P < 0.0001$; Fig. 4F), resulting in overall polarization of macrophages to an M1-like/antitumorigenic phenotype (Fig. 4G).

In addition to an increase in MHCII^{hi} macrophages, there was also a significant increase in NK cells in the TME (Fig. 4H). *Ex vivo* reactivation of NK cells in tumors with IL12/IL18/anti-NK1.1 Ab revealed significant activation of NK cells by RXDX-106 as demonstrated by increased IFNγ$^+$ NK cells from 67.8% (vehicle) to 76.0% (RXDX-106; $P = 0.047$; Fig. 4I). These phenotypic changes were also reflected at the transcriptional level, as transcripts associated with macrophages (F4/80$^+$) and NK cells (CD49b$^+$) were enriched in dissociated tumors. Moreover, in macrophages, RXDX-106 decreased M2-related genes, *Arg1*, *Fizz1*, and *Ym-1* and increased MHCII-related genes, *Ciita and H2-Ab*, and *Siglec1* (Fig. 4J). In NK cells, the activation genes *Tnfa*, *Cd69*, *Klrg1*, *Il2ra*, *Ifng*, and *Zbtb32* were increased by RXDX-106 (Fig. 4K). In addition, VEGF transcript and protein were significantly reduced in a dose-dependent manner throughout the study (day 3–9 posttreatment; Supplementary Fig. S3B–S3D), further indicating innate immune modulation. No significant changes in MDSCs or neutrophils were observed in the TME by RXDX-106 (Supplementary Fig. S3E). Finally, activation of innate cells correlated with activation of adaptive immunity, because in the same experiments, IFNγ production by CD8$^+$ and CD4$^+$ T cells was increased post-RXDX-106 treatment (Fig. 4L–N). In addition, increases in TNFα, FasL, IFNγ, and GranzymeB in tumor cell lysates and increases in PD-1 on CD8$^+$ T cells were observed with RXDX-106 (Supplementary Fig. S4A–S4E). These cytokine modulations and changes in overall immune cell numbers were largely undetectable in peripheral blood, indicating that the effects, mediated by RXDX-106, were limited to the TME (Supplementary Fig. S4F).

RXDX-106 enhances adaptive immune response via cell-extrinsic mechanisms

In addition to their role in modulating the innate immune response, TAM RTKs can also modulate activity of adaptive immune cells like T cells (37, 38). Therefore, we examined the effect of RXDX-106 on the adaptive immune response. Mice were inoculated with MC38 cells such that the growth rate of the tumor was slower relative to Fig. 4 (Fig. 5A and B). We found that RXDX-106 significantly induced the accumulation of CD8$^+$ but not total CD4$^+$ cells or Tregs in the tumor (Fig. 5C–E). The increase in CD8$^+$ cells ($P = 0.0036$) was likely due to enhanced proliferation because we found increased Ki-67$^+$CD8$^+$ T cells after RXDX-106 treatment ($P = 0.0065$; Fig. 5F). PD-1 expression and LCK phosphorylation (pY393) were both enhanced compared with vehicle treatment (Fig. 5G and H), suggesting that there might be increased signal transduction via the TCR.

To further determine the cell type directly affected by RXDX-106 and to better assess cell-intrinsic roles for TAM RTKs, we generated mixed bone marrow chimeric mice reconstituted with a 1:1 mixture of bone marrow from WT:*Axl*$^{-/-}$*Mertk*$^{-/-}$ (AMdKO) mice and treated them with RXDX-106 for 1 week after MC38 tumor establishment (Fig. 5I and J; Supplementary Fig. S5A). We reasoned that comparing KO with WT cells would inform us whether the inhibitor activated any immune cell type directly, in which case, the impact of RXDX-106 would be to activate WT cells and have no impact on KO cells. We first examined the reconstitution of WT and AMdKO cells in the tumor in the absence of RXDX-106. Fig. 5K shows that AMdKO macrophages and DCs reconstituted the tumor (but not the LN) better than WT macrophages and DCs, indicating a clear cell-intrinsic activity for TAM RTKs on myeloid cells in the absence of RXDX-106. Notably, this did not occur with T or NK cells, suggesting that TAM RTKs did not have basal activity in these cell types. Notably, RXDX-106 increased recruitment of T and NK cells into the tumor from both WT and AMdKO backgrounds and increased proliferation of both WT and KO CD8$^+$ T cells, indicating that those cell types could be activated indirectly by RXDX-106 (Fig. 5L). RXDX-106 likely cannot directly affect T cells because it did not have a significant impact in *in vitro* T-cell proliferation cultures (Supplementary Fig. S5B and S5C). Interestingly, in the mixed bone marrow chimera setting, MHCII was increased in both AMdKO and WT DCs and macrophages, suggesting that RXDX-106 also had cell-extrinsic effects on the myeloid lineage (in addition to cell-intrinsic role of TAM RTKs at the basal level in these cells). In contrast, this increase in MHCII was not observed in macrophages and DCs from mice reconstituted with complete bone marrow from AMdKO (Fig. 2I; Supplementary Fig. S5D), showing that RXDX-106 directly affected at least these cell types. These also suggest that RXDX-106 affects antigen presentation in the TME, which might be one reason for increased T-cell proliferation and TGI. Altogether, we conclude that RXDX-106 acts directly on WT macrophages and DCs in the chimeric setting, resulting in additional indirect effects on KO immune cells, including myeloid and lymphoid lineages.

RXDX-106 in combination with adaptive immune checkpoint inhibitors

We observed that RXDX-106 led to TGI by activation of both innate and adaptive cells, but the response remains partial (typically <60%). Notably, we found that PD-1 expression on CD8$^+$ cells was markedly increased (Fig. 5G; Supplementary Fig. S4E). Therefore, we evaluated the antitumor activity of RXDX-106 in combination with an anti-PD-1Ab in MC38 (Fig. 6A and B) and CT26 (Fig. 6C and D) syngeneic mouse models. In the MC38 model, animals treated with RXDX-106 alone or anti-PD-1Ab displayed similar TGI (TGI = 56% and 66% at day 17, respectively; Fig. 6A), but when given in combination, significantly enhanced TGI was observed (TGI = 86%). Median survival was also enhanced from 17 days (vehicle), to 24, 24, and 28 days for vehicle + anti-PD-1Ab, RXDX-106 alone, and combination, respectively (Fig. 6B, $P = 0.003$, 0.003, and <0.0001, respectively). Similarly, in the CT26 model, RXDX-106 alone and anti-PD-1Ab displayed similar TGI (TGI = 44% and 43% at day 25, respectively; Fig. 6C), although combination of RXDX-106 and anti-PD-1Ab showed significantly enhanced TGI (76%). In addition, survival was significantly enhanced. Median survival of vehicle, vehicle + anti-PD-1Ab, RXDX-106 alone, and combination were 25, 28, 28, and 36.5 days, respectively (Fig. 6D).

To dissect how the combination treatment increased overall survival, RNA-seq analysis was performed in the MC38 model on whole tumors 7 days posttreatment with vehicle or RXDX-106 ±

Published OnlineFirst February 5, 2019; DOI: 10.1158/0008-5472.CAN-18-2022

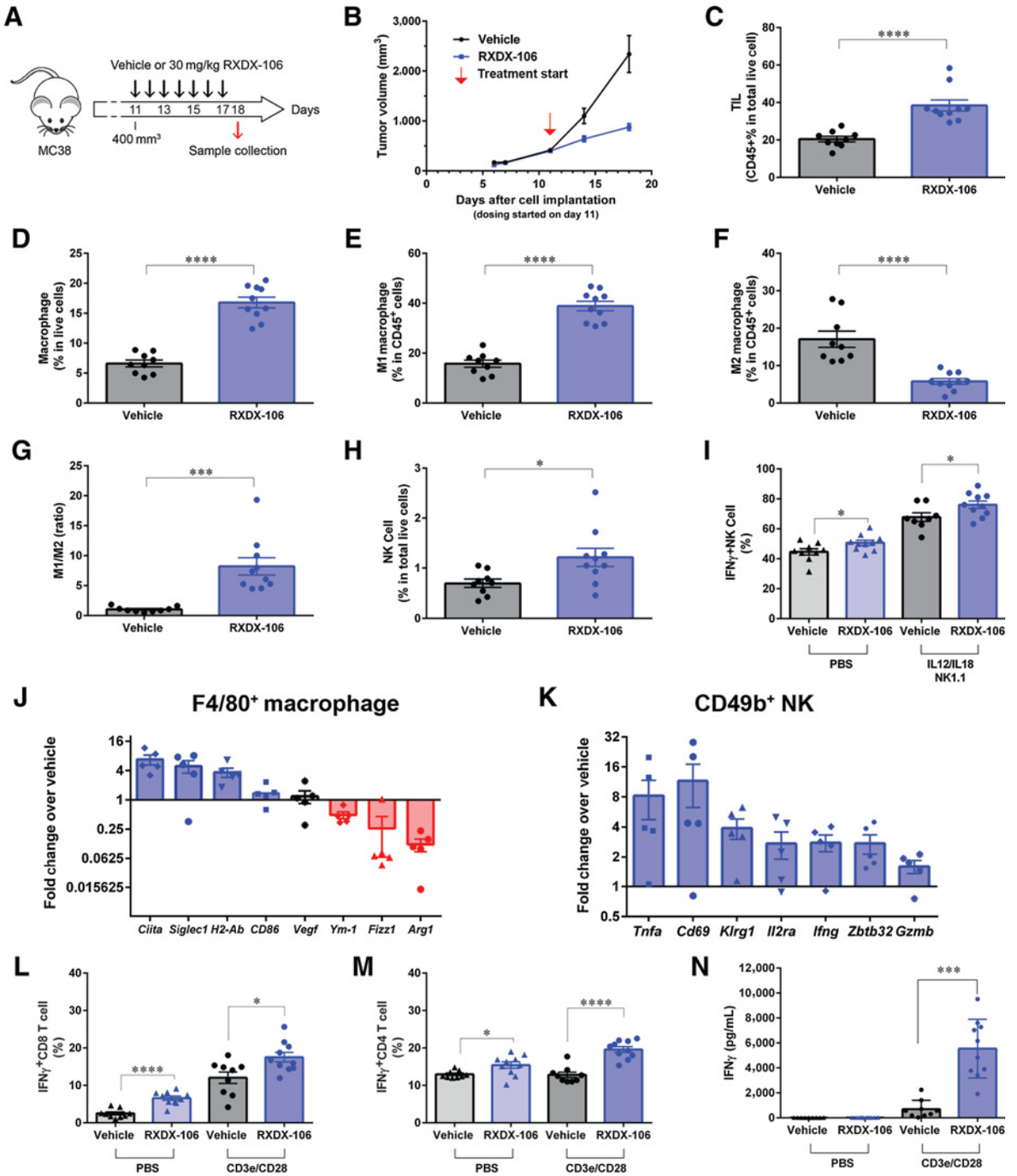

Figure 4.

RXDX-106 modulates the innate immune response and induces antitumoral immune response. **A** and **B,** Treatment began when average MC38 tumor size reached 400 mm^3 (day 11), and immunophenotyping was performed on day 18 (24 hours posttreatment). **C–K,** Tumor-infiltrating leukocytes (TILs-CD45$^+$; **C**), TAMφ-CD11b^{hi}Ly6G^{lo}Ly6C^{lo}F4/80^{hi} (**D**), MHCII^{hi} macrophages (M1-like; **E**), MHCII^{lo} macrophages (M2-like; **F**), M1/M2 ratio (**G**), tumor-infiltrating NK-NK1.1$^+$ CD335$^+$ (**H**), intracellular IFNγ expression by NK cells±IL12/IL18/anti-NK1.1Ab re-stimulation (**I**), F4/80$^+$ macrophages (**J**), and CD49b$^+$ NK cells (**K**) were enriched from RXDX-106–treated, MC38-derived tumors. RT-qPCR analysis was performed 7 days posttreatment. Fold changes (blue, up; red, down) were normalized to a control gene (18S rRNA) and are expressed relative to vehicle; $n = 5$ mice/group. **L** and **M**, Intracellular IFNγ expression in CD8$^+$ (**L**) or CD4$^+$ (**M**) T cells derived from MC38-dissociated tumors ± anti-CD3ε/CD28Ab. **N,** ELISA of IFNγ secretion in dissociated tumors ± anti-CD3ε/CD28Ab restimulation for 3 days; $n = 8$-10. Data are mean ± SEM; $n = 8$–10. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$.

Published OnlineFirst February 5, 2019; DOI: 10.1158/0008-5472.CAN-18-2022

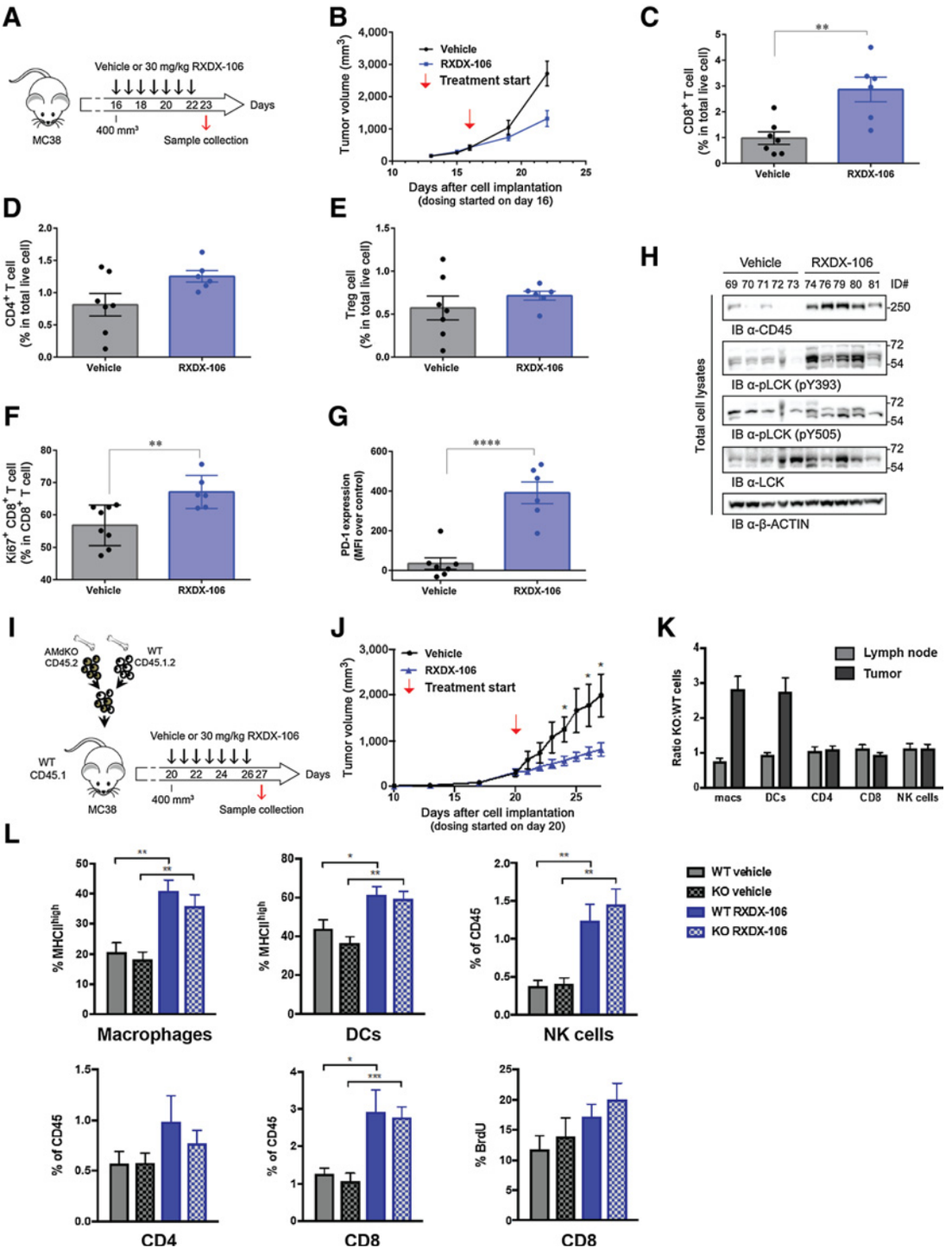

Figure 5.

RXDX-106 indirectly enhances adaptive immune responses by affecting innate immune cells. MC38 cells were inoculated at 1×10^5 cells/mouse so that tumor growth occurred over a longer time period. **A** and **B,** Treatment began when average tumor size reached 400 mm^3/day 16, and immunophenotyping was performed on day 23 (24 hours post-treatment). **C–G,** Tumor-infiltrating $CD8^+$ (**C**), $CD4^+$ (**D**), Treg- ($CD25^+FoxP3^+$) (**E**), $Ki67^+CD8^+$ cells (**F**), PD-1 (**G**) expression on $CD8^+$ T cells; $n = 10$. **H,** Western blot analysis of whole tumors containing tumor and immune cells. **I** and **J,** Irradiated CD45.1 WT mice were reconstituted with a 1:1 mixture of CD45.1.2 WT cells:CD45.2 AMdKO cells, challenged with MC38, and treated. Mice were intraperitoneally injected with 1 mg/mouse BrdU 48 and 24 hours before harvest. **K,** Ratios of AMdKO:WT in LNs and tumors. **L,** Percentages of $MHCII^{hi}$ macrophages and DCs, NK, $CD4^+$, and $CD8^+$ T cells of $CD45^+$ and BrdU incorporation of $CD8^+$ T cells in the tumors; $n = 7$. Data are mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Published OnlineFirst February 5, 2019; DOI: 10.1158/0008-5472.CAN-18-2022

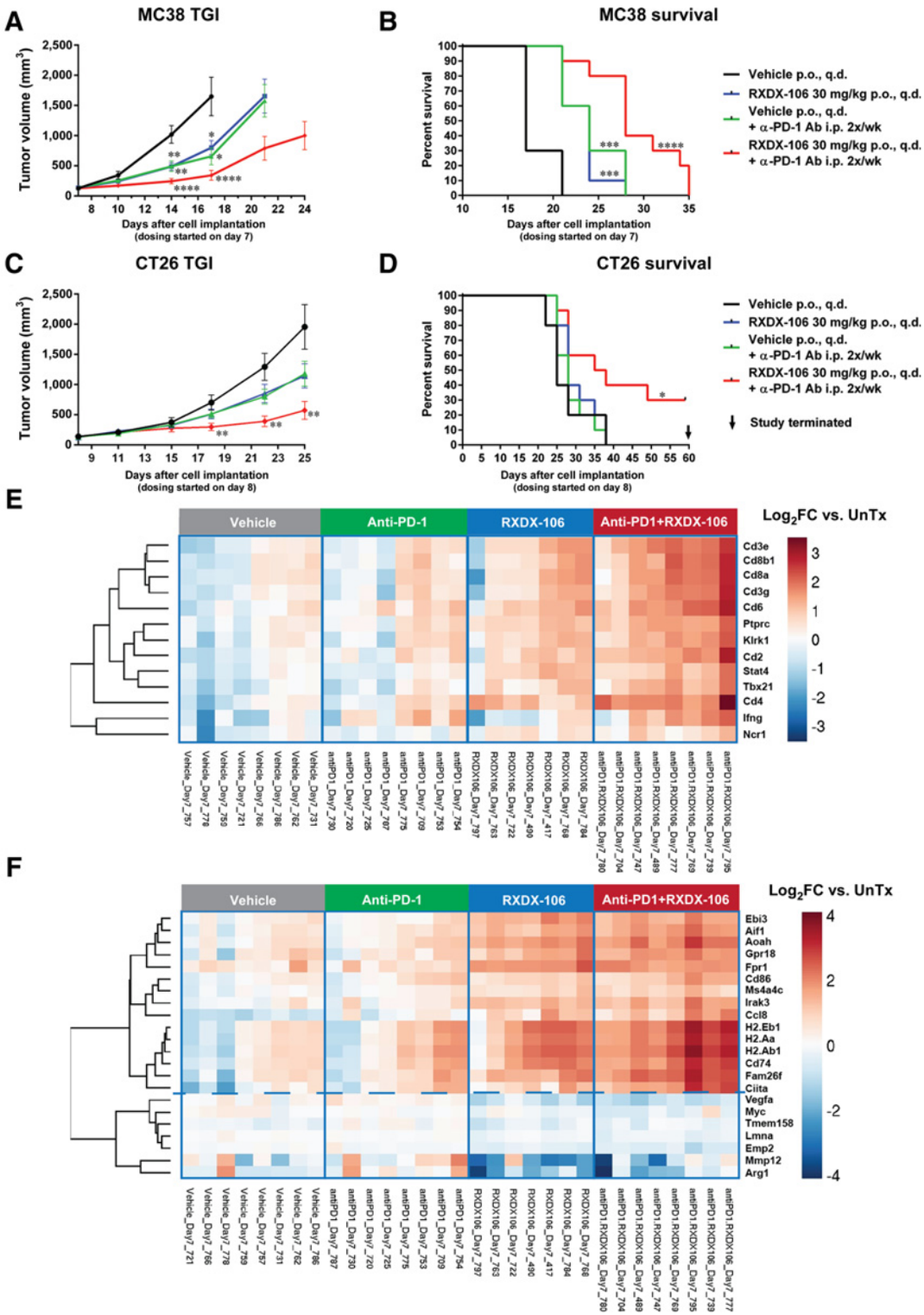

Figure 6.

RXDX-106 potentiates adaptive checkpoint inhibitors in multiple syngeneic models. *In vivo* efficacy of RXDX-106 in combination with anti-PD-1 antibody in MC38 (**A** and **B**) and CT26 (**C** and **D**) models (TGI: **A** and **C**; Survival: **B** and **D**; TGI–ANOVA; Survival–Mantel Cox test). **E** and **F**, Heat maps representing gene level expression of general immune cell markers (**E**) and genes implicated in M1/MHCII^{hi} or M2 macrophage phenotypes (**F**) modulated relative to untreated controls, at a significance level of FDR <0.01 and $\log_2$ FC >1. $n = 8$–10 mice/group. Data are representative of two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Published OnlineFirst February 5, 2019; DOI: 10.1158/0008-5472.CAN-18-2022

anti-PD-1 antibody. Depicted are heatmaps representing gene-level expression of individual genes that transcribe general immune cell markers (Fig. 6E), and genes previously implicated in M1/MHCII high or M2 macrophage phenotypes (Fig. 6F; refs. 39, 40). Unbiased analysis of all animals showed combination of RXDX-106 and anti-PD-1Ab increased T- and NK-cell–related genes including genes indicating activation, such as *Tbx21*, *Stat4*, *Ifng*, *Klrk1* compared with single-agent treatments. Among genes expressed in macrophages, MHCII-related genes, *Cd86*, and *Ccl8* were increased and M2 macrophage–related genes, such as *Arg1* and *Vegfa*, were decreased in combination group. The increases in immune activation and macrophage polarization observed previously in either the RXDX-106 alone and anti-PD-1Ab–treated groups was further enhanced in combination (Supplementary Table S6), supporting the hypothesis that RXDX-106 would act cooperatively with an adaptive checkpoint inhibitor.

Discussion

The TAM RTKs are emerging targets for cancer therapy as traditional oncodrivers and in immuno-oncology as key negative regulators of the innate immune response. Upregulation of TAM RTKs on cancer cells has been well documented, particularly as a mechanism of resistance to both chemotoxic agents and other TKIs and in the maintenance of EMT (14, 41, 42). Furthermore, recent evidence suggests compensatory upregulation of TAM RTK family members (i.e., MER) in response to selective TAM RTK inhibition such as with AXL-specific inhibitors (BGB324; ref. 26) and suggests that selective TAM RTK inhibition relative to pan-TAM RTK inhibition may have limited clinical benefit. Notably, the expression of TAM RTKs and c-MET in syngeneic tumor cell lines is often quite low (Supplementary Fig. S6A–S6D). In the context of the immune system, TAM RTKs are widely expressed on macrophages, DCs, MDSCs, NK, and T cells (16, 28, 43), and can inhibit tumor immunity. Here we demonstrate that expression of AXL and MER on immune and tumor cells increased during tumor progression, supporting development of RXDX-106 as a pan-TAM RTK inhibitor for cancer immunotherapy.

In an activity-based kinase screen, we found that RXDX-106 was highly specific for TAM RTKs with additional activity against c-MET/RON kinase families. Although off-target activity against LCK was observed in the *in vitro* biochemical assay, we did not observe any evidence of *in vivo* inhibition. In fact, tumor-bearing mice treated with RXDX-106 had increased LCK phosphorylation/activation (pY393), leading to increased T-cell activation *in vivo*, trending toward increased proliferation. When compared with other TAM inhibitors currently tested in preclinical or clinical settings, RXDX-106 had slower dissociation kinetics relative to BGB324 (AXL-specific), cabozantinib (c-MET/AXL), or merestinib (MST1R, FLT3, AXL, MERTK, TEK, ROS1, NTRK1/2/3, and DDR1/2, and MKNK1/2), which may translate to more durable target engagement and *in vivo* efficacy.

TAM RTK single, double, and triple knockout mice have been generated and are completely viable with no obvious developmental defects (18). However, the receptors play key homeostatic roles in the clearance of $>10^9$ apoptotic cells daily and particularly, $Mertk^{-/-}$ and TAM TKO mice progressively develop a plethora of phenotypes due to the inability of specialized phagocytes like Sertoli and RPE cells in the testes and eye, respectively, to clear PtdSer-expressing apoptotic cells/debris (11, 44, 45). RXDX-106 inhibited AXL and MER-dependent phagocytosis *in vitro* and *in vivo*, but we did not observe accumulation of apoptotic neutrophils in peripheral blood or indications of global autoimmunity (Supplementary Fig. S4F). In addition, tumor growth in WT mice transplanted with AMdKO BM cells was not decreased compared with mice transplanted with WT BM cells and the hyperactivation of immune cells was not seen in the TME, indicating that the intrinsic hyperactivated immune system of those mice was insufficient to inhibit tumor growth.

In addition to their role in phagocytosis, TAM RTKs function to negatively regulate the magnitude and duration of the innate immune response. In response to pathogens, AXL is upregulated and activated downstream of the TLR where it coopts IFNAR1 to induce SOCS1/3 signaling (16). The importance of the TAM RTKs in regulating the immune response is exemplified by the broad-spectrum autoimmunity observed in the TAM TKO mice, which have increased autoantibodies, enlarged lymph nodes, and splenomegaly (18). In addition, TAM RTKs on NK cells may promote tumor growth and metastasis via negatively regulating NK-mediated antitumor response, a process that requires both the kinase activity and Cbl-mediated internalization (28). In this report, we demonstrate that RXDX-106 significantly increased infiltration of immune cells into tumors (TIL) and augmented both innate and adaptive immune responses. Using bone marrow chimeric mice reconstituted with a mixture of WT and AMdKO cells, we found that RXDX-106 directly activated macrophages and DCs (derived from WT BM) but also indirectly activated macrophages, DCs, NK cells, $CD4^+$ and $CD8^+$ T cells (derived from AMdKO BM). This indirect activation likely resulted from increased antigen presentation and polarization of the innate response toward an M1-like state. We also showed that the global activation of immunity by RXDX-106 required AXL and MER because we did not observe these effects in RXDX-106–treated AMdKO BM macrophages and DCs in nonchimeric bone marrow–reconstituted animals (that lacked WT cells that could respond to RXDX-106).

Recent reports suggest that c-MET can also play a role in mitigating the immune response due to its expression on neutrophils in the context of adoptive T-cell transfer immunotherapy (46). Although RXDX-106 is 7-fold more potent against the TAM RTK family, we cannot rule out the possibility that RXDX-106 may exert a minimal immunomodulatory effect through c-MET. However, it is unlikely because we observed no TGI after RXDX-106 treatment in mice transplanted with AMdKO BM that still express c-MET. In addition, we did not observe changes in neutrophil number in either the TME or peripheral blood (Supplementary Fig. S4F), and only minimal expression of c-MET on tumor cells was observed in cell lines utilized. Because RXDX-106 did not decrease tumor growth in mice that had AMdKO immune cells, we conclude that its antitumor activity is solely mediated by AXL and MER on immune cells, not by TYRO3 or another kinase. It is unclear whether AXL, MER, or both need to be inhibited for our effects. Other studies have found that inhibiting MER alone using UNC2025 led to tumor regression but did not fully examine effects on immunity (47–49), in part, due to use of xenograft models. BGB324, a specific AXL inhibitor in clinical trials, has also demonstrated efficacy in the treatment of various cancers and also shows immune-activating activity (50, 51).

In the TME, RXDX-106 suppressed both transcript and protein levels of VEGF. In the TME, macrophages, MDSCs, and tumor cells are considered to be the major sources of VEGF. We believe that

Published OnlineFirst February 5, 2019; DOI: 10.1158/0008-5472.CAN-18-2022

RXDX-106 reduced VEGF production by tumor cells, at least as the inciting event because: (i) reduction of VEGF protein in tumors was observed at a lower dose (1 mg/kg) and at an earlier time point (day 3) compared with the induction of M1 polarization of macrophages by RXDX-106 (>30 mg/kg and at day 5–9); (ii) there was no noticeable change in the MDSCs in tumors of RXDX-106 treated mice (Supplementary Fig. S3E); and (iii) Transcripts for *Vegf* in macrophages were not changed. Nevertheless, M2-like TAMφ are known to produce more VEGF than M1-like macrophages (52). Moreover, the interaction between tumor cells and macrophages has been reported to increase VEGF production (53, 54), which in turn also recruits macrophage progenitors that can then differentiate into M2-like macrophages that produce more VEGF (55), creating a positive feedback loop (53, 54). Further studies are required to delineate how TAM RTKs control expression of VEGF in cancer and immune cells.

We also found that RXDX-106 treatment increased T-cell activation (increased *ex vivo* IFNγ production) and increased %$CD8^+$ cells. These effects were T-cell–extrinsic because RXDX-106 was able to act on AMdKO T cells in bone marrow chimeras, possibly by acting on WT innate immune cells that in turn activate $CD8^+$ T cells. To our knowledge, these studies are the first to demonstrate the role of TAM RTKs in regulating T-cell activity. Whether TAM RTK expression on T cells has additional direct functions still needs to be determined. Interestingly, a previous study from Carrera-Silva and colleagues showed that activated T cells upregulate PROS1 and concomitantly externalize PtdSer. This complex binds to TAM RTKs on APCs and suppresses their activity (17). It is unknown whether this complex is also seen in tumor-infiltrating T cells or whether T cells can present TAM RTK ligands to other T cells.

Using two syngeneic models, RXDX-106 and anti-PD-1 had similar effects but had more significant effects in combination. These results suggest merit in investigating combinations with other immune checkpoint inhibitors and identification of precise mechanisms of action governing observed potentiation.

In summary, RXDX-106 can restore and enhance immune function by modulating the local immune-suppressive TME. The unique mechanisms of activating both innate and adaptive immunity, and regulating cross-talk between immune and tumor cells, support clinical evaluation of RXDX-106 as an immunomodulatory agent for the treatment of a variety of cancers.

Disclosure of Potential Conflicts of Interest

J.D. Bui reports receiving commercial research grant from Ignyta and is a consultant/advisory board member of Ignyta. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Yokoyama, E.D. Lew, R. Seelige, C. Walsh, P.C. Fagan, J.Y. Lee, R. Nevarez, J. Oh, K.D. Tucker, M. Chen, H. Vaaler, K.M. Smith, J.D. Bui

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Yokoyama, E.D. Lew, R. Seelige, E.A. Tindall, C. Walsh, P.C. Fagan, J.Y. Lee, R. Nevarez, J. Oh, K.D. Tucker, A. Diliberto, G. Li, J.D. Bui

Writing, review, and/or revision of the manuscript: Y. Yokoyama, E.D. Lew, R. Seelige, E.A. Tindall, P.C. Fagan, J.Y. Lee, J. Oh, A. Diliberto, H. Vaaler, K.M. Smith, A. Albert, G. Li, J.D. Bui

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Yokoyama, E.D. Lew, E.A. Tindall, P.C. Fagan, J. Oh, K.D. Tucker

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Acknowledgments

We thank members of Ignyta and the RXDX-106 team for their support. We also acknowledge Drs. Gloria Hernandez-Torres, Greg Lemke, and Kaisa Happonen for technical assistance and manuscript comments.

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Received July 2, 2018; revised November 16, 2018; accepted January 30, 2019; published first February 5, 2019.

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Published OnlineFirst February 5, 2019; DOI: 10.1158/0008-5472.CAN-18-2022

Cancer Research
The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

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Cancer Res 2019;79:1996-2008. Published OnlineFirst February 5, 2019.