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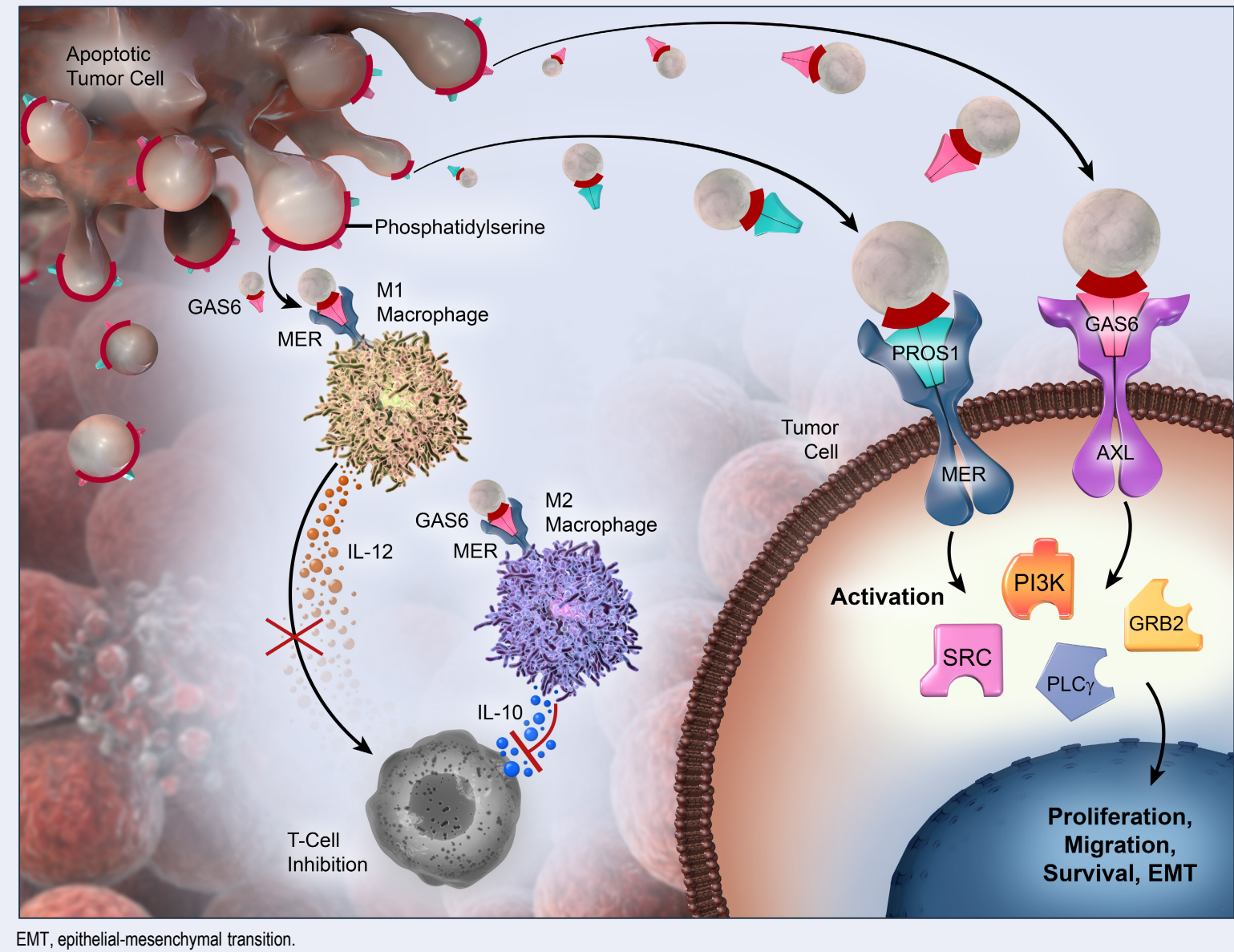
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Abstract

TYRO3, AXL, and MER constitute the TAM family of receptor tyrosine kinases (RTKs), which are amplified, translocated, or over-expressed in numerous types of human cancer. These RTKs play important roles in tumor growth, survival, cell adhesion, and migration as well as drug resistance. In addition, it has been shown that both AXL and MER are critical regulators of innate immunity, phagocytosis, and immune-suppressive activity. Therefore, targeting both AXL and MER kinases may not only impact the growth, survival, and malignant progression of neoplastic cells directly, but also has the potential to restore and enhance host immunity against cancers. INCB081776 is a potent inhibitor of AXL and MER that exhibits selective pharmacological activity and enhanced anti-tumor immune activity. In biochemical assays, INCB081776 effectively blocked autophosphorylation of AXL or MER including BaF3 cells transfected with constitutively active AXL or MER, AXL in H1299 tumor cells, or MER kinase in G361 tumor cells, with low nanomolar IC₅₀ values. In addition, INCB081776 inhibited activation of MER kinase in primary human macrophages with low nanomolar IC₅₀ potency. More importantly, in an *in vitro* functional assay, INCB081776 partially reversed M2 macrophage-mediated suppression of T-cell proliferation, and increased IFN γ in co-cultured macrophages and T cells. *In vivo*, INCB081776 administration to H1299 tumor-bearing mice dose-dependently inhibited the phosphorylation in tumors. Consistent with the proposed mechanism of action, INCB081776 potentially inhibited tumor growth in immunocompetent mice, but not in immunodeficient mice, demonstrating that a functional immune system is important for activity. Treatment was associated with dose-related increases in the percentage of tumor-infiltrating effector CD4⁺ and CD8⁺ T cells, as well as macrophages with the M1 phenotype. In addition, INCB081776 decreased the percentage of intratumoral M2 macrophages and monocytic myeloid-derived suppressor cell (M-MDSC) immune cell populations. In the 4T1 model, combining INCB081776 with anti-PD-L1 resulted in synergistic anti-tumor effects compared to either single agent. Collectively, these preclinical data support the hypothesis and potential therapeutic utility of INCB081776 as an immunotherapeutic agent capable of enhancing tumor immune surveillance mechanisms in cancer patients as a single agent and when combined with therapies mediating immune PD-L1 checkpoint blockade.

Role of AXL/MER in Tumor Promotion



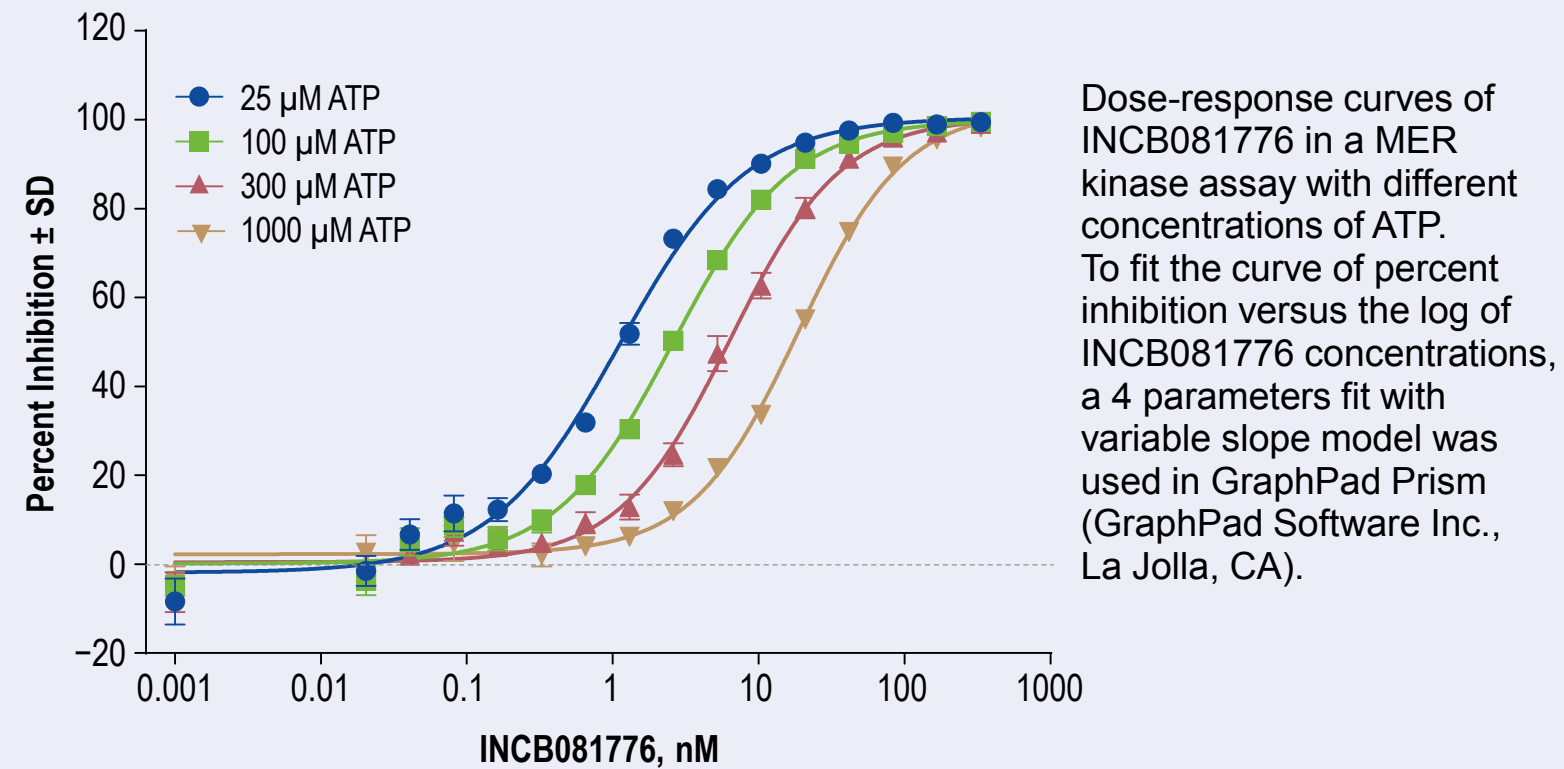
Biochemical and Cellular Potency of INCB081776 Against AXL, MER, and TYRO3

Recombinant Kinase	Biochemical IC ₅₀ (Mean \pm SD, nM)	BaF3 Cell Line	GI ₅₀ (Mean \pm SD, nM)
AXL	0.61 \pm 0.31	AXL	16 \pm 11
MER	3.17 \pm 1.97	MER	14 \pm 4.9
TYRO3	101 \pm 27	TYRO3	498 \pm 161

IC₅₀, half maximal inhibitory concentration; SD, standard deviation.

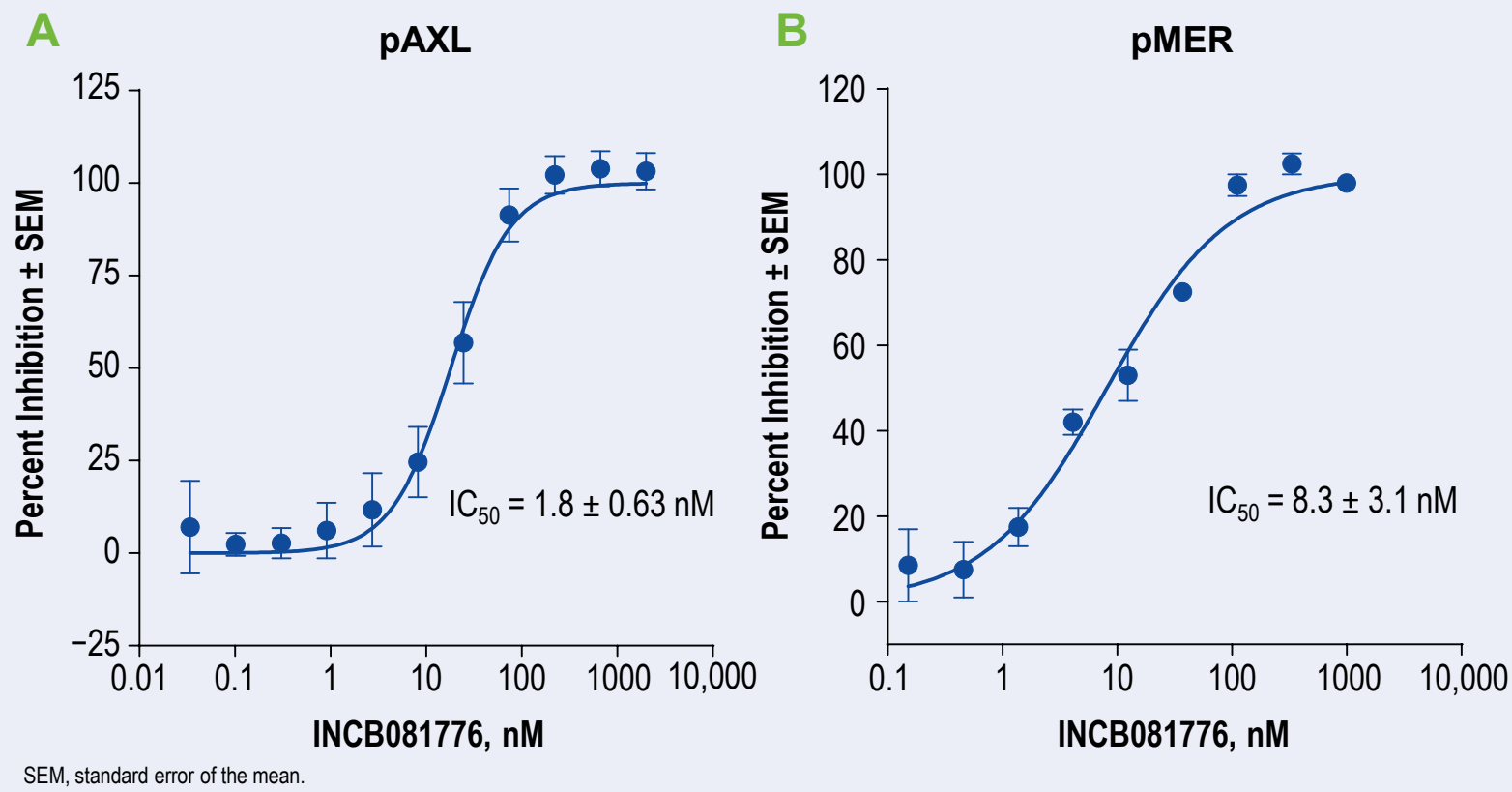
GI₅₀, concentration required for 50% growth inhibition.

INCB081776 Has an ATP-Competitive Mode of Inhibition



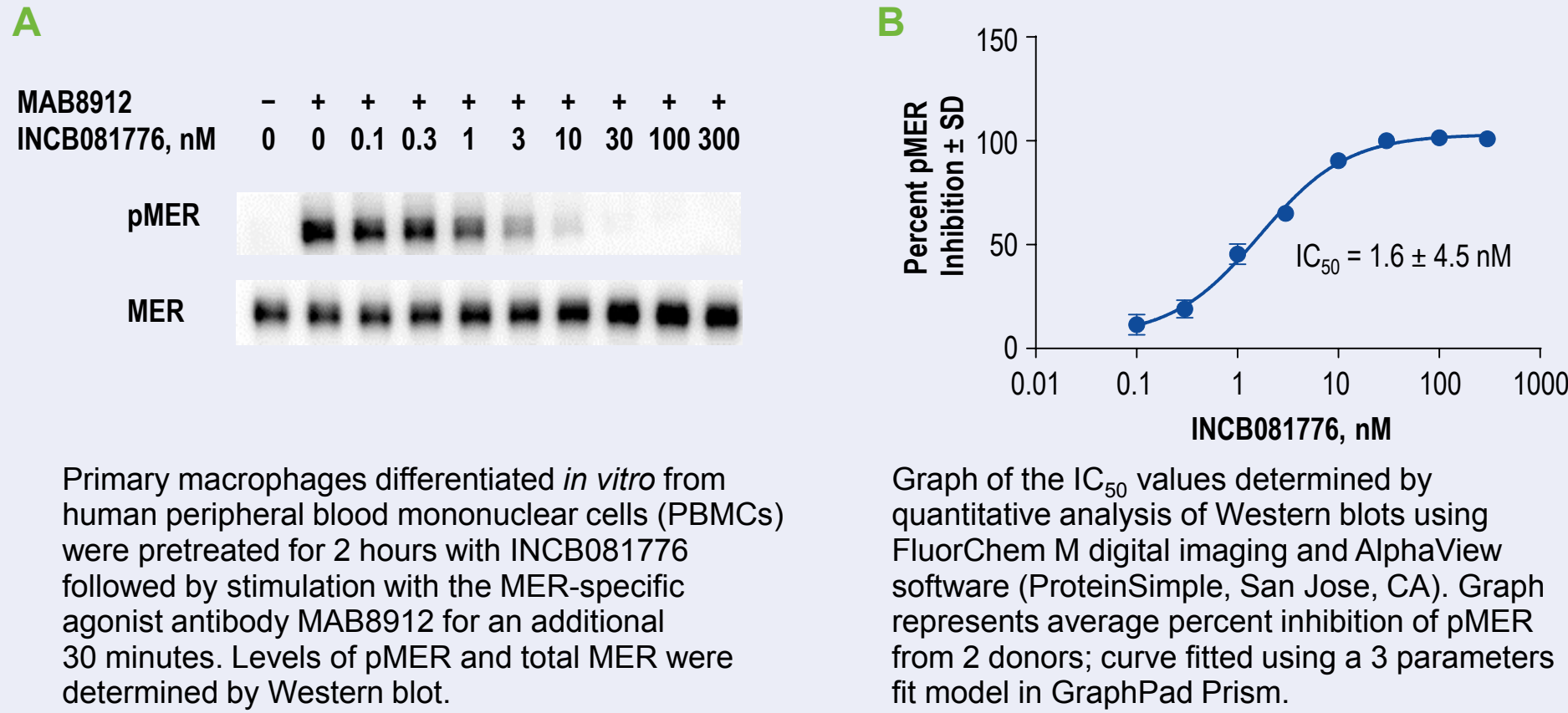
Dose-response curves of INCB081776 in a MER kinase assay with different concentrations of ATP. To fit the curve of percent inhibition versus the log of INCB081776 concentrations, a 4 parameters fit with variable slope model was used in GraphPad Prism (GraphPad Software Inc., La Jolla, CA).

Inhibition of pAXL and pMER in Tumor Cell Lines



- A. H1299 cells, which express high levels of phosphorylated AXL (pAXL), were pretreated with INCB081776 for 1 hour, then stimulated with recombinant human GAS6 for 15 minutes. Cell lysates were quantified for pAXL by ELISA.
- B. G361 cells, which express high levels of phosphorylated MER (pMER), were pretreated with INCB081776 for 1 hour followed by 30 minutes of incubation with a MER agonist antibody, MAB8912. Levels of pMER were quantified by ELISA. IC₅₀ values were determined by fitting the curve of percent inhibition versus the log of INCB081776 concentrations using sigmoidal dose response with variable slope in GraphPad Prism.

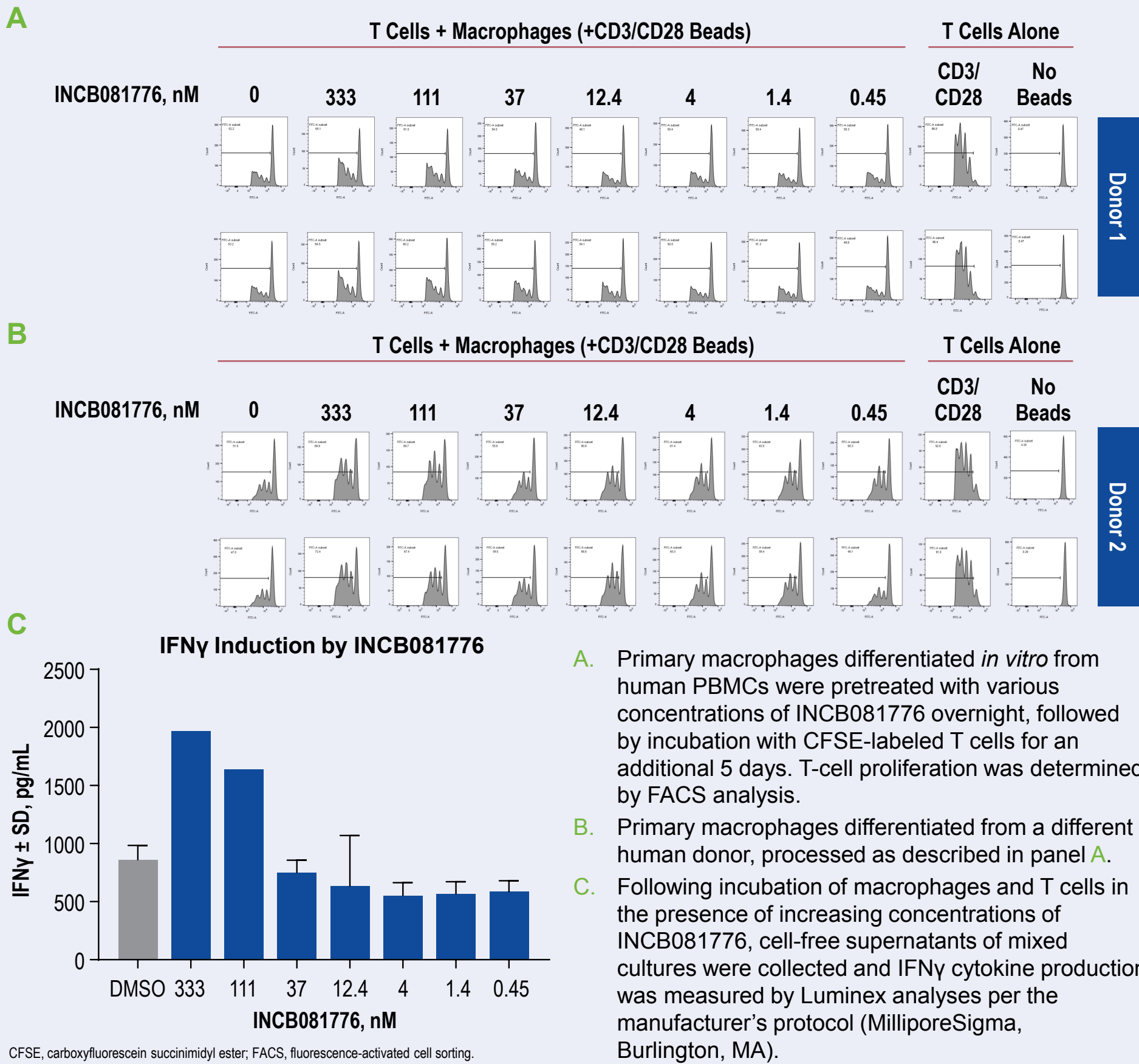
Inhibition of MER Kinase Activity by INCB081776 in Primary Human Macrophages



Primary macrophages differentiated *in vitro* from human peripheral blood mononuclear cells (PBMCs) were pretreated for 2 hours with INCB081776 followed by stimulation with the MER-specific agonist antibody MAB8912 for an additional 30 minutes. Levels of pMER and total MER were determined by Western blot.

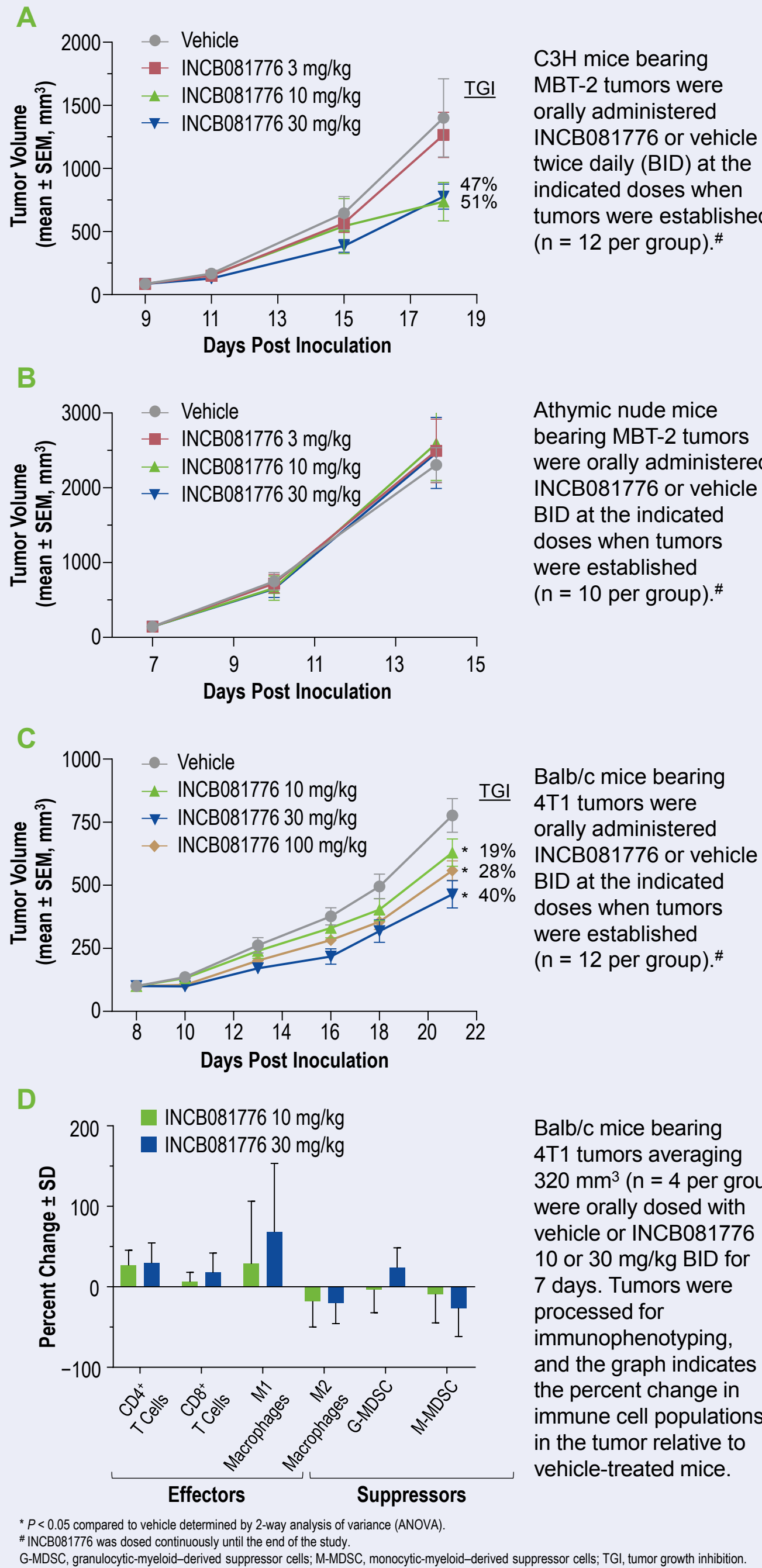
Graph of the IC₅₀ values determined by quantitative analysis of Western blots using FluorChem M digital imaging and AlphaView software (ProteinSimple, San Jose, CA). Graph represents average percent inhibition of pMER from 2 donors; curve fitted using a 3 parameters fit model in GraphPad Prism.

INCB081776 Treatment Partially Reverses Macrophage-Mediated Inhibition of T-Cell Proliferation



CFSE, carboxyfluorescein succinimidyl ester; FACS, fluorescence-activated cell sorting.

Single-Agent Antitumor Efficacy and Immunomodulatory Activity of INCB081776 in Syngeneic Mouse Models



C3H mice bearing MBT-2 tumors were orally administered INCB081776 or vehicle twice daily (BID) at the indicated doses when tumors were established (n = 12 per group).*

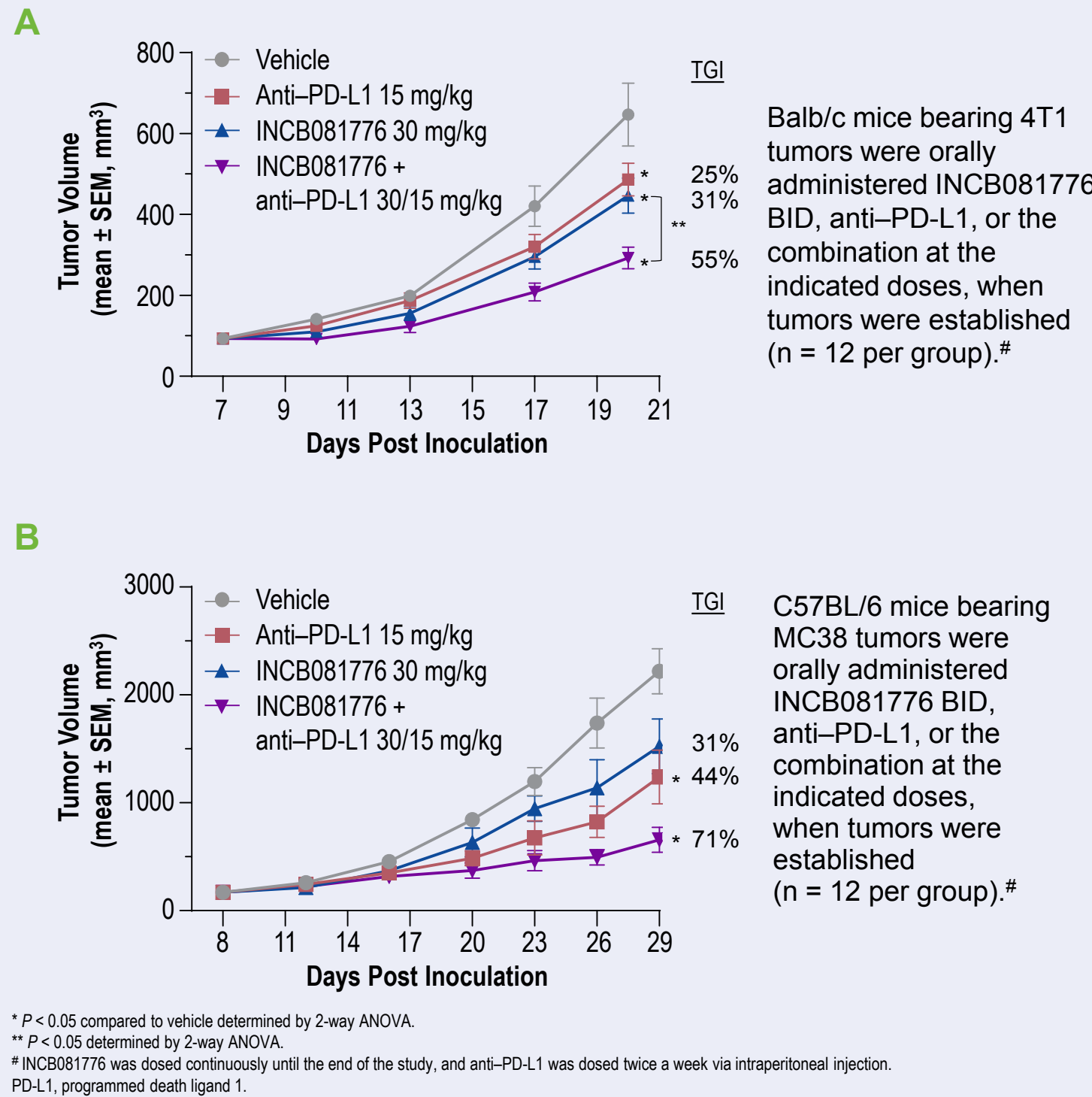
TGI, tumor growth inhibition.

Athymic nude mice bearing MBT-2 tumors were orally administered INCB081776 or vehicle BID at the indicated doses when tumors were established (n = 10 per group).*

Balb/c mice bearing 4T1 tumors were orally administered INCB081776 or vehicle BID at the indicated doses when tumors were established (n = 12 per group).*

Balb/c mice bearing 4T1 tumors averaging 320 mm³ (n = 4 per group) were orally dosed with vehicle or INCB081776 10 or 30 mg/kg BID for 7 days. Tumors were processed for immunophenotyping, and the graph indicates the percent change in immune cell populations in the tumor relative to vehicle-treated mice.

Increased Activity of INCB081776 in Combination With Anti-PD-L1 in 4T1 and MC38 Syngeneic Tumor Models



Balb/c mice bearing 4T1 tumors were orally administered INCB081776 BID, anti-PD-L1, or the combination at the indicated doses, when tumors were established (n = 12 per group).*

C57BL/6 mice bearing MC38 tumors were orally administered INCB081776 BID, anti-PD-L1, or the combination at the indicated doses, when tumors were established (n = 12 per group).*

* P < 0.05 compared to vehicle determined by 2-way ANOVA.
** P < 0.05 determined by 2-way ANOVA.
* INCB081776 was dosed continuously until the end of the study, and anti-PD-L1 was dosed twice a week via intraperitoneal injection. PD-L1, programmed death ligand 1.

Conclusions

- INCB081776 is a potent, highly selective, ATP-competitive inhibitor of AXL and MER, and is >30-fold selective against TYRO3
- INCB081776 partially reverses macrophage-mediated inhibition of T-cell proliferation
- INCB081776 inhibits tumor growth in multiple syngeneic models and increases the levels of intratumoral CD4⁺ T cells, CD8⁺ T cells, and M1 macrophages
- An intact immune system is required for efficacy with no INCB081776 activity observed in immunodeficient mice
- The combination of INCB081776 and anti-PD-L1 resulted in increased antitumor activity compared to monotherapy in the 4T1 and MC38 syngeneic models
- Selective AXL and MER dual inhibition represents an attractive approach for the treatment of cancer potentially as a single agent, as well as in rational combination with immunotherapy

Disclosures
All authors: Employment and stock ownership – Incyte Corporation.

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