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Suppression of Regulatory T cells (T_{regs}) *in vivo* by Small Molecule Targeting of the Mucosa-Associated Lymphoid Tissue Lymphoma Translocation Protein 1 (MALT1)

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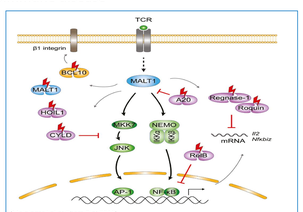
BACKGROUND

- Mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) is a protease and scaffold protein that mediates NF-kappaB signaling downstream of the T cell receptors (TCR)
- MALT1 is a molecular target in hematologic malignancies with constitutively active MALT1-signaling and Th_{17}-driven autoimmune disease indications. Pharmacologic inhibition of MALT1 has shown efficacy in preclinical models of autoimmune disease[1]
- Genetic evidence from MALT1-deficient mice suggest that MALT1 promotes the development of immune suppressive natural regulatory T cells (nT_{regs})[2]
- Mice with protease-dead MALT1 (MALT1-PD mice) develop autoimmunity and are less amenable to syngeneic tumor transplantation[3-7]
- We hypothesize that pharmacologic inhibition of MALT1 will enable selective suppression of tumor-associated T_{regs} and stimulate anti-tumor immunity[8]

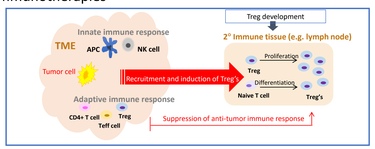


MALT1 function as a protease and scaffold downstream of the TCR and activates NF-kappaB and JNK-pathways in T_{regs}

SCIENTIFIC RATIONALE

Suppression of regulatory T-cells (T_{regs}) to enhance anti-tumor immune response

- Immune system plays a key role in tumor development
  - T_{reg} cells inhibit several types of immune cells and thereby suppress the anti-tumor immune response
  - Tumor cells recruit and induce development of T_{reg} cells
- Selective suppression of T_{reg} cells, without negatively affecting T_{eff} and other immune cells, is predicted to enhance the anti-tumor immune response in cancer patients and possibly potentiate other immunotherapies



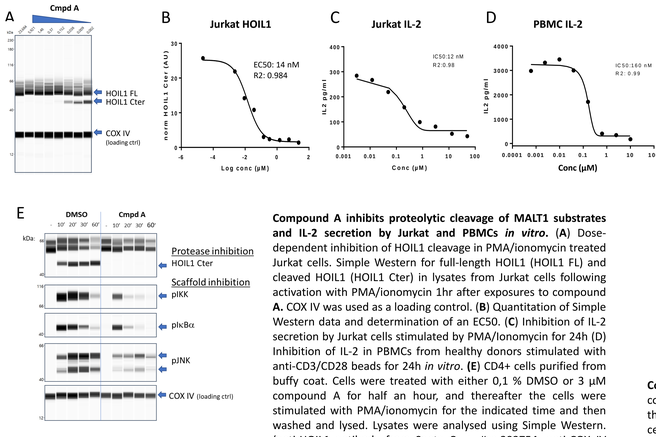
T_{regs} in the tumor microenvironment (TME)

METHODS

MALT1 enzyme assay. In-house produced MALT1 was assayed in using Ac-Leu-Arg-Ser-Arg-AMC as substrate.
Western blot. Whole-cell lysates were run with Protein Simple (Peggy Sue, size separation mode) using Size Master kit. A mix of anti-HOIL1 (#sc-393754, Santa Cruz), anti-pIKK (#2078), anti-pIkBα (#4814), pJNK (#4668) and anti-COX IV (#4850, all from Cell Signaling) was used.
IL-2 expression. IL-2 protein in medium of PMA/IO activated Jurkat cells was measured after 40 hours using Meso Scale human IL-6 tissue culture kit (MSD)
T_{reg} differentiation assay. To assess the impact of MALT1 inhibition on T_{reg} differentiation, CD4+ naïve T cells (CD4+CD45RA+CD127+CD25-) were flow sorted from healthy donor PBMCs and stimulated in IL-2 and TGF-beta containing medium with anti-CD3/CD28 for 5 days, rested for 5 days without stimulation, and analyzed for FoxP3 and CD25 expression.
IL-2 *in vivo*: The capacity to inhibit TCR-signalling was assessed by quantification of serum IL-2 following a single IV bolus of the anti-CD3 agonist antibody 145-2C11.
Effect on Tregs *in vivo*: The pharmacodynamic effects on FoxP3+ Tregs in the tumor, tumor-draining lymph nodes (TDLN) and distal lymph nodes (LN) were evaluated in the MB49 syngeneic mouse bladder cancer model.

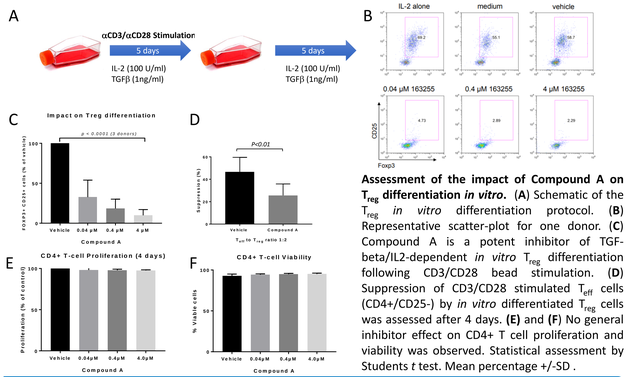
CHARACTERIZATION AND PK PROFILE

		Compound A[9]
PhysChem properties	Mw	448
	Solubility (Kin. @pH 7.4)	1
In vitro properties	MALT1 Human/Mouse Ki (nM)	11/5
	Jurkat HOIL1/IL-2 IC_{50} (nM)	14/12
	CC50 A549/CEM/MOLT-4/RAJI (µM)	>100/>50/20/>50
	Selectivity Thrombin/Trypsin/Cat S	>40-fold
	HLM/MLM (µl/min/mg)	7/10
	Caco-2 Papp (10^{-6} cm/s)	20
	fu plasma Human/Mouse (%)	1.1/2.6
In vivo properties	F Mouse (%)	93
	CL Mouse (mL/min/kg)	2
	Vss Mouse (L/kg)	0.9



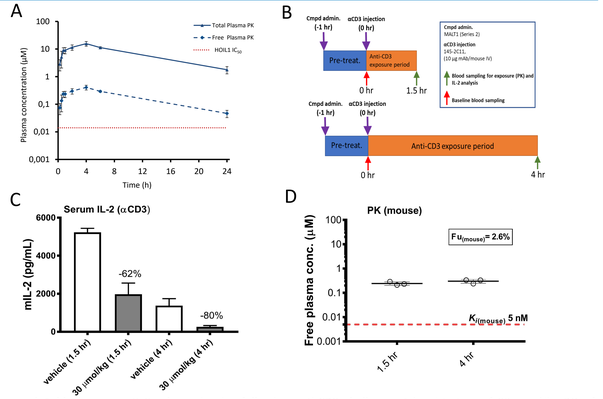
Compound A inhibits proteolytic cleavage of MALT1 substrates and IL-2 secretion by Jurkat and PBMCs *in vitro*. (**A**) Dose-dependent inhibition of HOIL1 cleavage in PMA/ionomycin treated Jurkat cells. Simple Western for full-length HOIL1 (HOIL1 FL) and cleaved HOIL1 (HOIL1 Cter) in lysates from Jurkat cells following activation with PMA/ionomycin 1hr after exposures to compound **A.** COX IV was used as a loading control. (**B**) Quantitation of Simple Western data and determination of an EC50. (**C**) Inhibition of IL-2 secretion by Jurkat cells stimulated by PMA/Ionomycin for 24h (D) Inhibition of IL-2 in PBMCs from healthy donors stimulated with anti-CD3/CD28 beads for 24h *in vitro*. (**E**) CD4+ cells purified from buffy coat. Cells were treated with either 0,1 % DMSO or 3 µM compound A for half an hour, and thereafter the cells were stimulated with PMA/ionomycin for the indicated time and then washed and lysed. Lysates were analysed using Simple Western. (anti-HOIL1 antibody from Santa Cruz, #sc-393754, anti-COX IV antibody from Cell Signaling Technologies #4850).

IN VITRO SUPPRESSION OF T_{REG} DEVELOPMENT



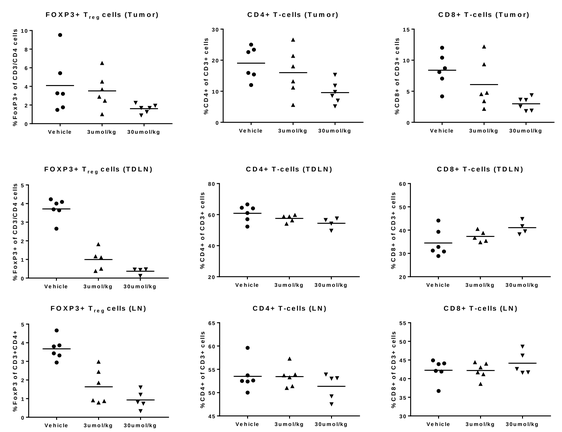
Assessment of the impact of Compound A on T_{reg} differentiation *in vitro*. (**A**) Schematic of the T_{reg} *in vitro* differentiation protocol. (**B**) Representative scatter-plot for one donor. (**C**) Compound A is a potent inhibitor of TGF-beta/IL2-dependent *in vitro* T_{reg} differentiation following CD3/CD28 bead stimulation. (**D**) Suppression of CD3/CD28 stimulated T_{eff} cells (CD4+/CD25-) by *in vitro* differentiated T_{reg} cells was assessed after 4 days. (**E**) and (**F**) No general inhibitor effect on CD4+ T cell proliferation and viability was observed. Statistical assessment by Students *t* test. Mean percentage +/-SD .

IN VIVO SUPPRESSION OF IL-2



Compound A blocks serum IL-2 release *in vivo* following anti-CD3 challenge. Administration of 30 µmol/kg PO of compound A to mice was well-tolerated and gave an average 24 hr free plasma exposure twenty-one times greater than the mouse K_i (data not shown). (**A**) PK profile of compound A showing total and free plasma concentration relative to cellular IC_{50} for HOIL1 cleavage. Similar exposure was observed after 10 day repeat dosing (not shown). (**B**) Schematic of *in vivo* experiment. (**C**) Mouse serum IL-2 levels 1.5 and 4 hrs after anti-CD3 treatment in the presence and absence (vehicle) of compound A. Average of N=3 is shown +/-SEM. (**D**) Free plasma concentration of 'A' in mouse plasma at 1.5 and 4 hrs following anti-CD3 in relation to K_i. Median of an N=3/time point is shown. Fu indicates fraction unbound (%) drug.

IN VIVO SUPPRESSION OF T_{REG}



Compound A reduces T_{reg} numbers in Tumor, Tumor-draining lymph nodes (TDLN), and distal Lymph Nodes (LN) in tumor-bearing mice. MB49 bladder cancer ($3x10^5$ cells) was inoculated on the right flank of C57/BL6 mice on day 1. Compound A (30 µmol/kg) was administered p.o. on day 8, 9, 10, 11. On day 12 the percentage of FOXP3+, CD4+ and CD8+ T-cells was analyzed in Tumor, TDLN, and LN.

CONCLUSIONS

- MALT1 inhibition by Compound A causes selective inhibition of human CD25+/FoxP3+ T_{reg} differentiation *in vitro* without inhibition of activation-induced proliferation of other T cell populations or apparent cytotoxicity
- *In vivo* inhibition of MALT1 causes a selective reduction of T_{regs} in Tumour-draining lymph node
- This novel small molecule approach to T_{reg}-targeting may improve the response to immune therapy for multiple cancer indications without additive/synergistic toxicities
- Investigations of *in vivo* anti-tumour effects of MALT1 inhibition are ongoing
- A chemistry program is in progress with the aim to select a final molecule for clinical development

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