SUPPLEMENTAL DATA

$S1P_1$ modulator-induced $G_{\alpha i}$ Signaling and β-Arrestin Recruitment Are Both Necessary to Induce Rapid and Efficient Reduction of Blood Lymphocyte Count *in vivo*

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MOLECULAR PHARMACOLOGY

SUPPLEMENTAL MATERIALS AND METHODS

GTPγS assay. Membrane preparations of CHO cells expressing recombinant hS1P1 receptors were used for (^{35}S)-GTPγS binding assay to measure intracellular $G_{\alpha i}$ protein activation following agonist binding. The assays were essentially performed as described previously (Bolli et al., 2010). EC_{50} values were calculated with the IC_{50} Witch software (Actelion Pharmaceuticals Ltd.) using the following settings: minimum defined by the no-effect level of the lowest compound concentration, maximum defined by the maximal response of the compound (smart max), no weighting and variable slope.

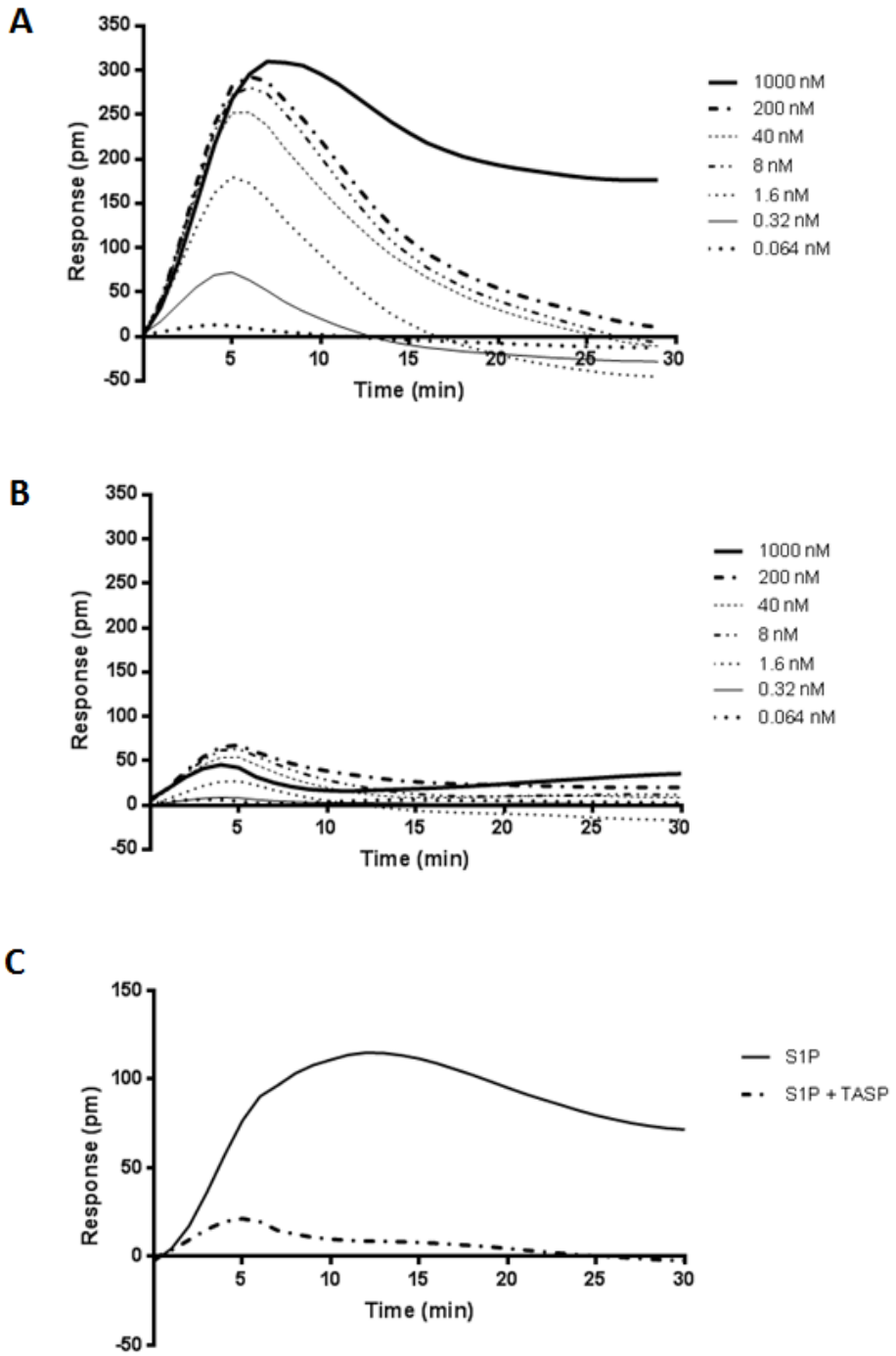
EPIC desensitization assay. 10'000 CHO cells stably expressing human $S1P_1$ receptor (CHO-h$S1P_1$) or rat $S1P_1$ receptor (CHO-r$S1P_1$) were seeded in uncoated (CHO-h$S1P_1$) or fibronectin-coated (CHO-r$S1P_1$) 384-well EPIC® sensor microplates (Corning) and grown for 24 h in Ham's F12 medium supplemented with 10 % charcoal-treated FBS, 300 µg/ml (CHO-h$S1P_1$) or 1000 µg/ml (CHO-r$S1P_1$) geneticin, 100 U penicillin and 1 µg/ml streptomycin. The medium was removed, the wells filled with 40 µl starvation medium (Ham's F12 with 0.05 % fafBSA and 2 % DMSO) and the plate loaded in the EPIC® reader (Corning) for 2 h equilibration. After the baseline dynamic mass redistribution (DMR) measurement, 10 µl of test compounds in starvation medium were added and a real-time DMR changes recorded for 30 minutes. Next, the plate was placed at 37°C in a humidified CO_2 incubator. After 16 h incubation, the plate was washed twice with assay buffer (HBSS with 20 mM Hepes, 0.05 % fafBSA and 2 % DMSO), the wells filled with 40 µl assay buffer and the plate loaded in the EPIC® reader (Corning) for 2 h equilibration. After the baseline dynamic mass redistribution (DMR) measurement, 50 nM of S1P in assay buffer were added and a real-time DMR changes recorded for 30 minutes. The data from EPIC® Analyser software (Corning) were exported to Microsoft Excel and used to determine compound potency with the IC_{50} Witch software (Actelion Pharmaceuticals Ltd.). For determination of compounds EC_{50} the following settings were used: minimum defined by the vehicle control (DMSO), maximum defined by the maximal response of the

compound (smart max), no weighting and variable slope. For determination of desensitization IC_{50} the following settings were used: minimum defined by the maximal response of the compound (smart min), maximum defined by the no-effect level of the compound (smart max), no weighting and variable slope.

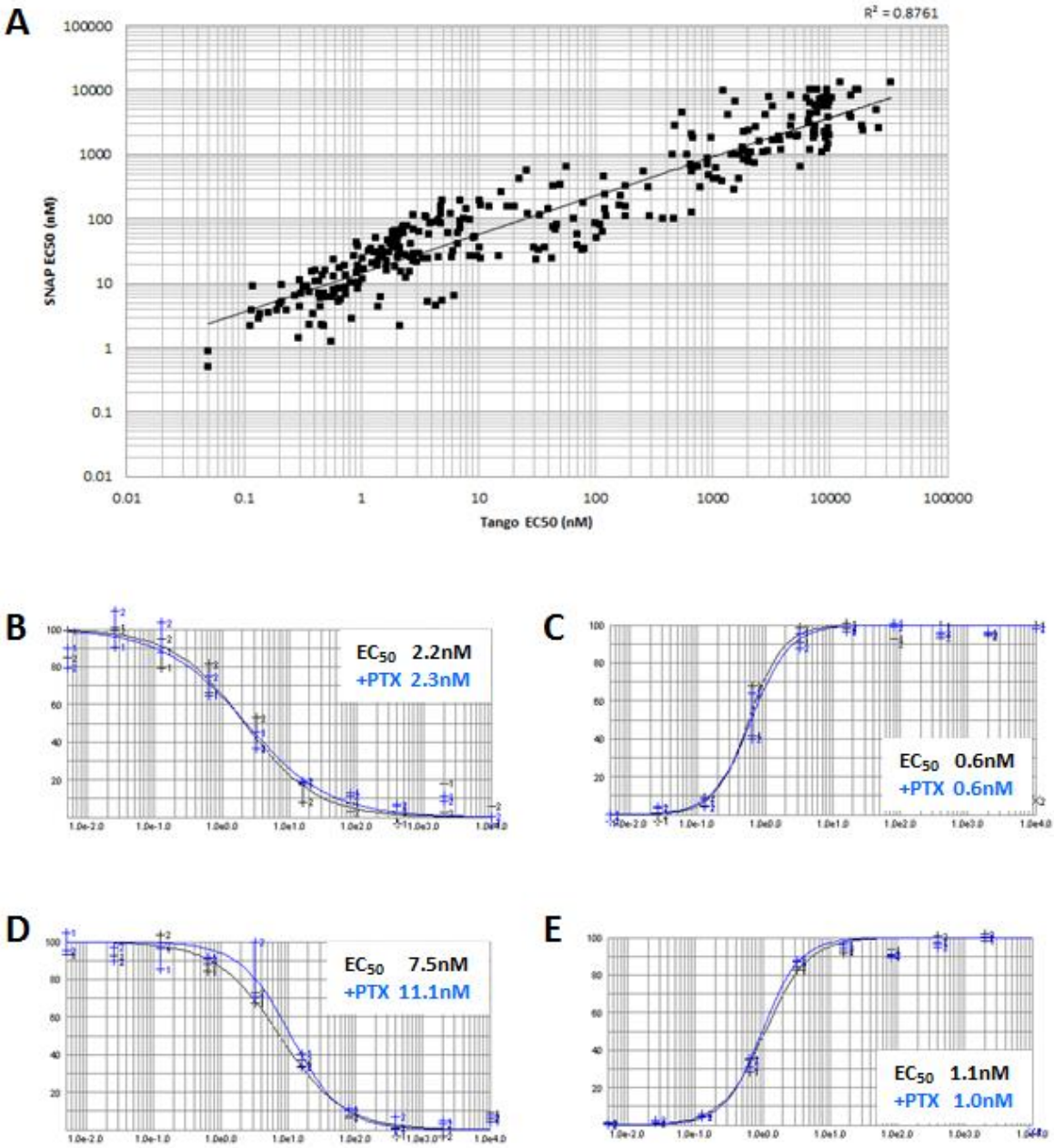
	EPIC			GTPγS		
	geomean	*geosdev*	*n*	geomean	*geosdev*	*n*
Ponesimod	1.1	*1.55*	*4*	7.1	*1.17*	*2*
D2	13.9	*1.79*	*6*	9.1	*1.28*	*2*
D3	29.1	*1.47*	*5*	41.7	*1.34*	*2*
D3i	63.3	*1.51*	*4*	54.9	*1.65*	*2*
D3-2	167.9	*1.40*	*2*	215.4	*1.04*	*2*
D4	62.7	*1.10*	*2*	108.4	*1.13*	*2*
D4i	61.1	*1.22*	*2*	87.2	*1.64*	*2*
D5c	159.8	*1.06*	*2*	186.4	*1.08*	*2*

EC_{50} (nM)

Supplemental Table 1. Comparison of compound potencies in different assays measuring $G_{\alpha i}$ activity of $S1P_1$ receptors.

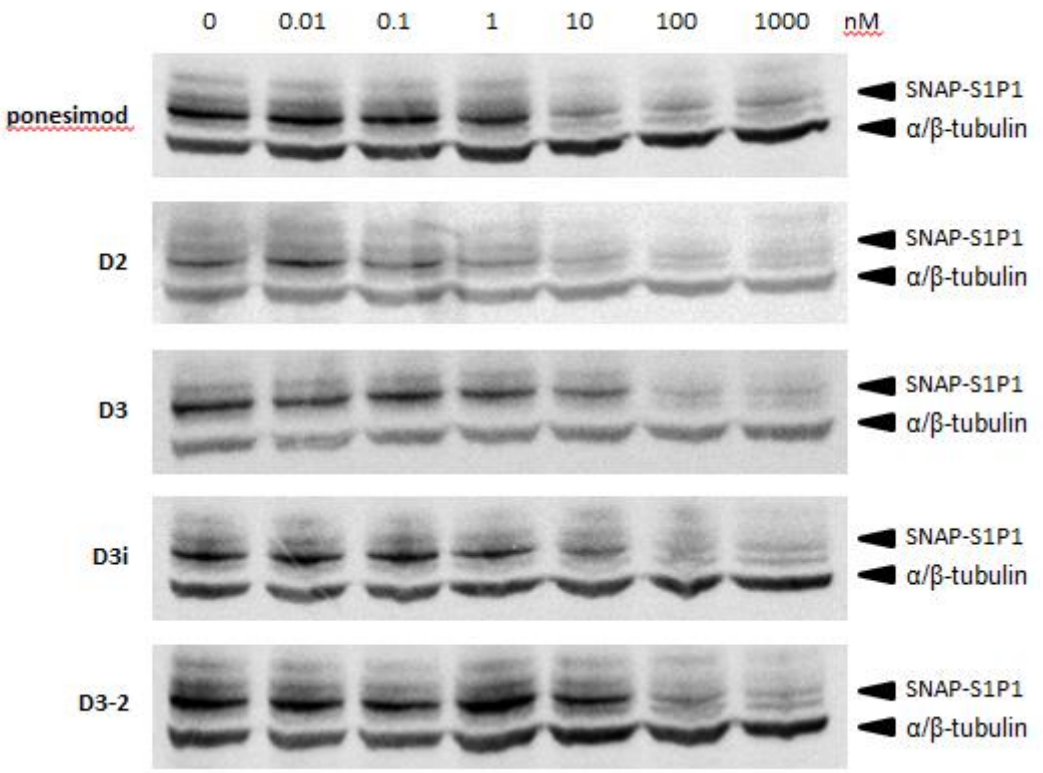


Supplemental Figure 1. Real-time EPIC traces in CHO-S1P$_1$ cells.
CHO cells stably expressing S1P$_1$ receptor were stimulated with a dose response of S1P in absence (A) or presence of PTX (300 ng/ml O/N) (B). (C) Response to 50 nM S1P in presence of 10'000 nM TASP0277308.

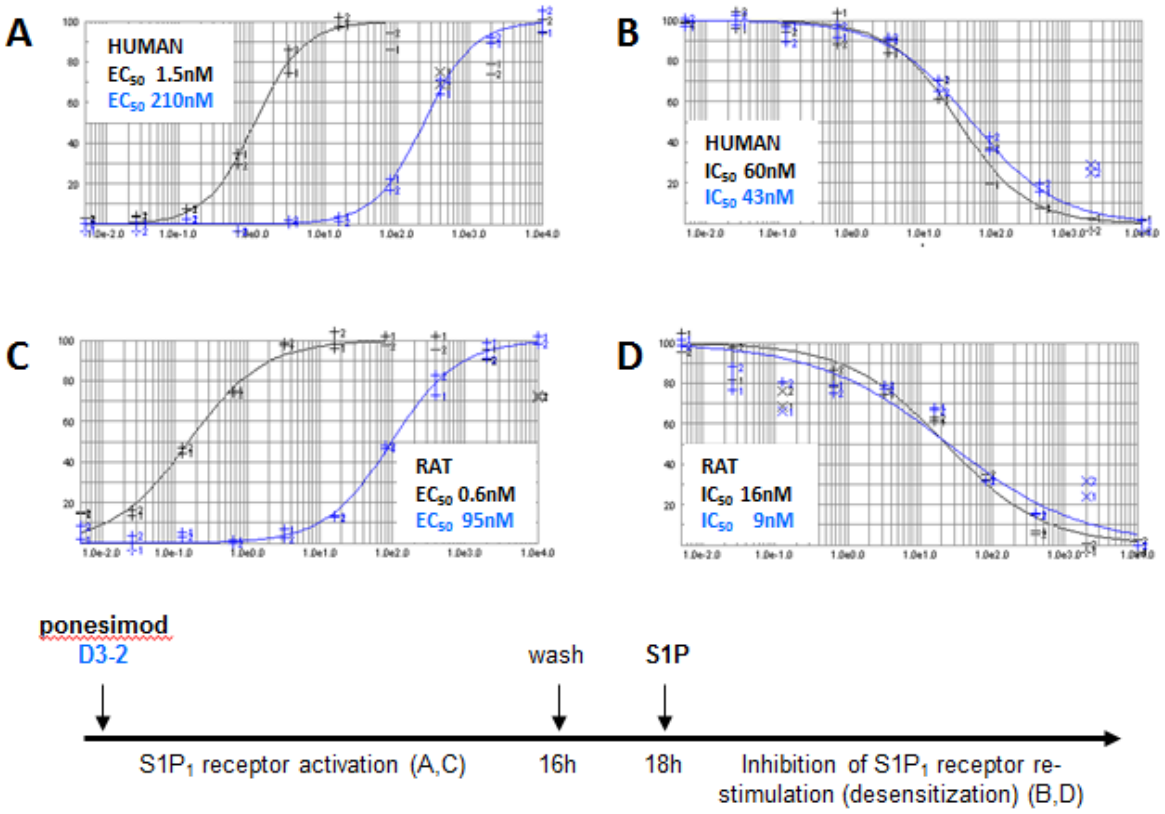


Supplemental Figure 2. β-arrestin recruitment and receptor internalization are functionally linked.

Correlation between EC_{50} determined in $S1P_1$ internalization (SNAP) and β-arrestin recruitment (Tango) assays (A). EC_{50} of ponesimod (B,C) and compound D3-2 (D,E) in SNAP-$S1P_1$ internalization (B,D) and β-arrestin Tango (C,E) in absence (black curves) and presence of PTX (blue curves).



Supplemental Figure 3. $S1P_1$ receptor degradation.
Immunoblotting with anti-$S1P_1$ and α/β-tubulin antibodies of lysates from Hela-SNAP-h$S1P_1$ cells treated for 16h with the indicated concentrations of $S1P_1$ agonists.



Supplemental Figure 4. Activation and desensitization of human and rat $S1P_1$ receptors. CHO-h$S1P_1$ cells were stimulated with increasing concentrations of ponesimod (black curves) and compound D3-2 (blue curves) and EC_{50} determined in EPIC assay (A). After 16 h incubation the compounds were removed, the cells washed and 2 h later re-stimulated with 50 nM S1P. Inhibition of S1P-induced response was expressed as desensitization IC_{50} (B). The same experiment performed on CHO-r$S1P_1$ cells with corresponding EC_{50} (C) and desensitization IC_{50} (D). Shown is one representative experiment. Values are geomeans (*n=6-12*)