Supplementary Information

LYP inhibits T cell activation when dissociated from CSK

Torkel Vang[1,3], Wallace H. Liu[1,9], Laurence Delacroix[4,9], Shuangding Wu[1], Stefan Vasile[2], Russell Dahl[2], Li Yang[2], Lucia Musumeci[4], Dana Francis[5], Johannes Landskron[3], Kjetil Tasken[3], Michel L. Tremblay[6], Benedicte A. Lie[7], Rebecca Page[5], Tomas Mustelin[1,8], Souad Rahmouni[4], Robert C. Rickert[1], and Lutz Tautz[1]*

[1]Infectious and Inflammatory Disease Center and [2]Conrad Prebys Center for Chemical Genomics, Sanford-Burnham Medical Research Institute, La Jolla, California, 92037, USA.
[3]The Biotechnology Centre of Oslo, University of Oslo, 0317 Oslo, Norway.
[4]Immunology and Infectious Diseases Unit, GIGA-R, Liège University, 4000 Liège, Belgium.
[5]Departments of Molecular Biology, Cell Biology, and Biochemistry, Brown University, Providence, Rhode Island 02912, USA.
[6]Goodman Cancer Research Centre, McGill University, Montreal, Quebec H3A 1A3, Canada.
[7]Department of Medical Genetics, University of Oslo and Oslo University Hospital, 0424 Oslo, Norway.
[8]Current address: MedImmune LLC, Gaithersburg, Maryland, 20878, USA.
[9]These authors contributed equally to this work.

*To whom correspondence may be addressed: Lutz Tautz, Infectious and Inflammatory Disease Center, Sanford-Burnham Medical Research Institute, La Jolla, California, 92037; Phone: 858-646-3100 x3640; E-mail: tautz@burnham.org.

Supplementary Methods

***In silico* docking studies.** The ICM docking algorithm was applied as part of the ICM Pro software package (version 3.7, Molsoft, L.L.C.), and calculations were run on a Mac Pro workstation. The protein structures were converted into ICM objects, charges were assigned, orientations of side chain amides were corrected, and hydrogens were added with their positions optimized by energy minimization using the MMFF force field. The receptor site was defined as an 8 Å radius about the catalytic cysteine 227. 3D coordinates of the compounds were generated and charges were assigned using the implemented compound preparation tool.

Cytotoxicity Assay. HeLa cells were pre-treated in the absence of serum for 45 min at 37°C with various concentrations of compound **1** in DMEM (25,000 cells per well of a 96 well plate). Serum was then added to a final concentration of 10% and cells were cultured for 2 d. Cell viability was evaluated using the MTT assay kit according to the manufacturer's recommendations (Roche). Formazan crystals formed by metabolic active cells were quantified using a multiwell spectrophotometer and absorbance was determined at 595nm (test wavelength) from which absorbance at 690nm (reference wavelength) was subtracted. The percentage of viable cells was calculated relative to the vehicle control (DMSO at 0.5%). Jurkat Tag T cells (50,000 cells per well) and PBMC (200,000 cells per well) were treated with compound **1** in complete growth medium for 48 h and the percentage of viable cells was determined as described above for HeLa cells.

HEK cell transfection, FACS analysis, and cell lysis. Lipofectamine 2000 and the manufacturer's recommended protocol (Invitrogen) were used for transfection of human embryonic kidney (HEK) cells. For FACS analysis, transfected HEK cells were harvested, fixed, permeabilized, stained with fluorescently labeled antibodies and analysed on a BD FACS Canto II. For analysis of transfected HEK cells with immunoblotting, cells were lysed in ice-cold standard lysis buffer (50 mM HEPES pH 7.4, 100 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM PMSF, 5 mM sodium orthovanadate, 50 mM NaF) containing n-octyl-β-D-glucoside (50 mM). Later, post-nuclear supernatants were subjected to SDS-PAGE and immunoblotting with the indicated antibodies.

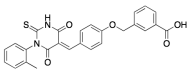
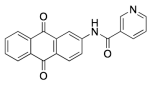
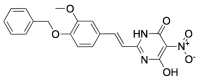
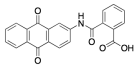
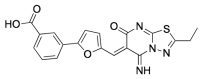
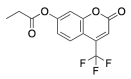
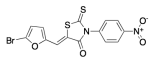
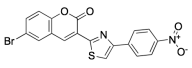
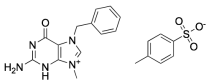
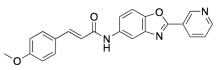
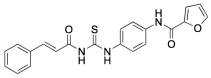
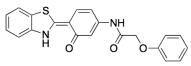
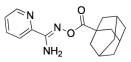
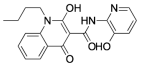
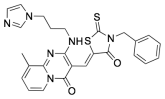
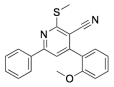
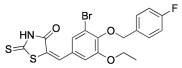
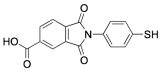
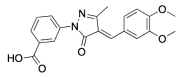
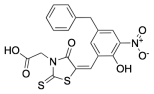
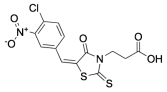
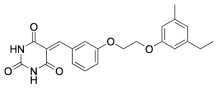
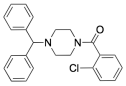
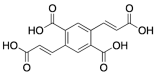
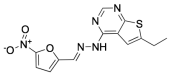
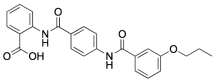
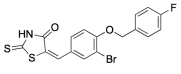
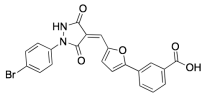
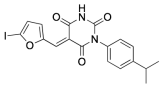
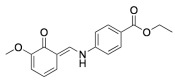
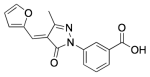
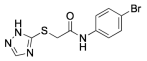
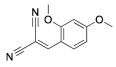
Supplementary Results

Supplementary Table 1. Small molecule screening data

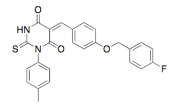
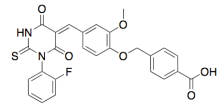
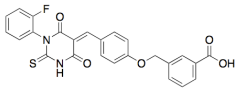
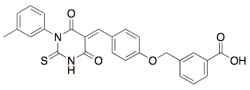
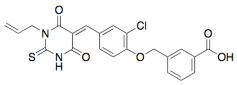
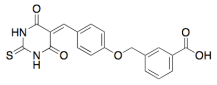
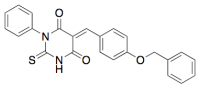
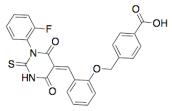
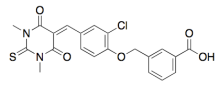
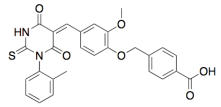
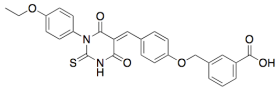
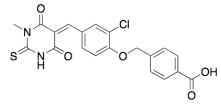
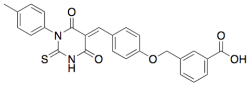
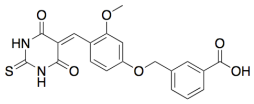
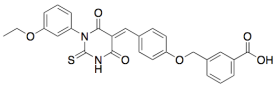
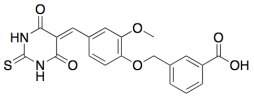
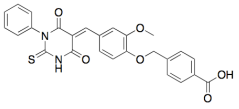
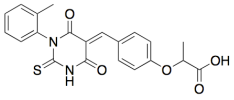
Category	Parameter	Description
Assay	Type of assay	*In vitro* assay
	Target	LYP (PTPN22, UniProt Q9Y2R2)
	Primary measurement	Detection of phosphatase activity
	Key reagents	3-O-methylfluorescein phosphate (OMFP, Sigma)
	Assay protocol	50,000 drug-like molecules from the DIVERSet™ library (ChemBridge) were screened in a 384-well format *in vitro* assay. Compound working concentration was 0.025 mg/mL in 5% DMSO. Each reaction (25 μL) contained 6.5 nM LYP, 60 μM OMFP (K_m value for LYP), 0.005 mg/mL compound, 20 mM Bis-Tris pH 6.0, 1 mM dithiothreitol, 1% PEG8000, and 1.6% DMSO. Reactions were initiated by addition of OMFP after preincubating the enzyme with compounds for 10 min at room temperature. After 60 min reactions were quenched by addition of 25 μL 0.2 M NaOH, and the OMFP hydrolysis was determined by measuring the fluorescence intensity of the dephosphorylated product 3-O-methylfluorescein (excitation 485 nm, emission 528 nm) using an EnVision 2103 reader (Perkin Elmer). Nonenzymatic hydrolysis of substrate was corrected by measuring control samples not containing enzyme. Other controls included a positive control (1 mM vanadate added) and a negative control (no compound added). The ratio of inhibition compared to the negative control was determined for each compound.
	Additional comments	See also: Tautz, L. & Mustelin, T. Strategies for developing protein tyrosine phosphatase inhibitors. *Methods* **42**, 250-260 (2007).
Library	Library size	50,000 compounds
	Library composition	DIVERSet™ of drug-like molecules
	Source	ChemBridge, Inc.
	Additional comments	
Screen	Format	384-well
	Concentration(s) tested	0.005 mg/mL compound, 1.6% DMSO
	Plate controls	Nonenzymatic hydrolysis control (no enzyme), positive control (1 mM vanadate), negative control (no compound).
	Reagent/ compound dispensing system	Multidrop Combi Bulk Reagent Dispenser (Thermo

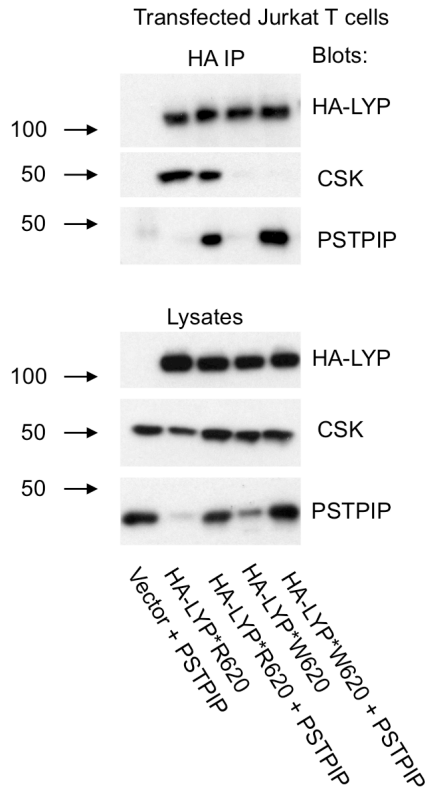
		Scientific)
	Detection instrument and software	EnVision 2103 reader (Perkin Elmer), CBIS software for analysis
	Assay validation/QC	Z' value was 0.86, signal-to-background ratio was 9.3
	Correction factors	
	Normalization	
	Additional comments	The screening was performed at the Conrad Prebys Center for Chemical Genomics, Sanford-Burnham Medical Research Institute, La Jolla, CA.
Post-HTS analysis	Hit criteria	Compounds with >50% inhibition were cherry-picked and re-screened in a 10-point dose-response ($5x10^{-3}$ mg/mL to $9.8x10^{-6}$ mg/mL).
	Hit rate	712 primary hits (hit rate 1.4%), of which 190 compounds exhibited dose-dependent inhibition with IC_{50} values <0.005 mg/mL (hit rate 0.38%).
	Additional assay(s)	Hits with dose-dependent inhibition of LYP with IC_{50} values <0.005 mg/mL were compared to hits from screenings with the PTPs HePTP, VHR, and MKP-3, all of which were carried out under similar conditions. Common hits between LYP and either HePTP, VHR, or MKP-3 were filtered out for non-selective inhibition. The datasets for HePTP, VHR, and MKP-3 were retrieved from the PubChem website (http://pubchem.ncbi.nlm.nih.gov/), BioAssay IDs 521 (HePTP), 1992 (VHR), and 425 (MKP-3). 33 hits selectively inhibited LYP.
	Confirmation of hit purity and structure	33 selective hits were repurchased as powder from ChemBridge. Identity and purity was verified by NMR and LC/MS analysis.
	Additional comments	

Supplementary Table 2. 33 selective HTS hits, sorted by their IC_{50} value for LYP.

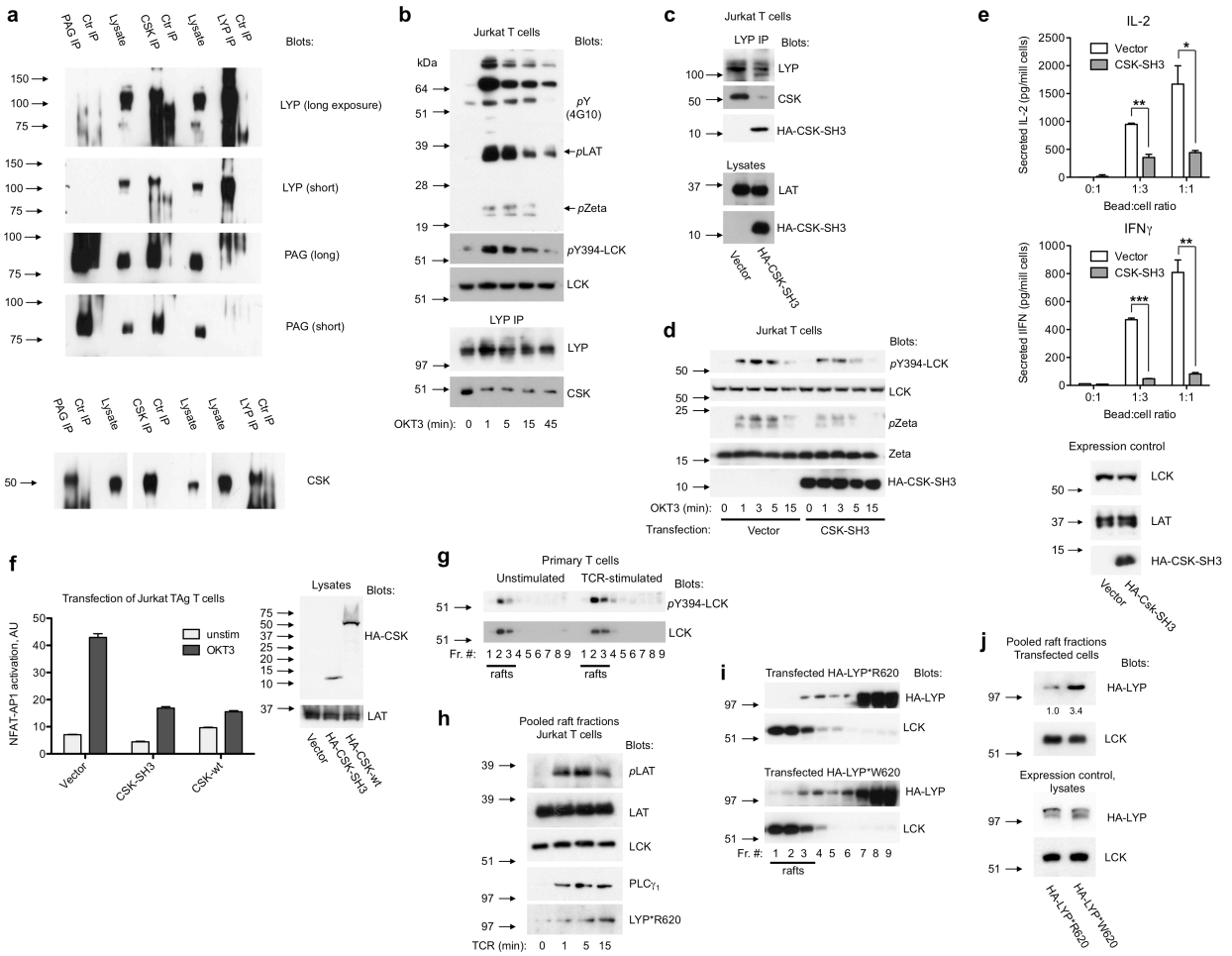
#	Structure	Mol Weight	IC50, μM	#	Structure	Mol Weight	IC50, μM	#	Structure	Mol Weight	IC50, μM
1		472.51	0.508	12		328.32	3.29	23		395.37	7.46
2		371.34	0.598	13		394.4	3.63	24		286.2	7.62
3		411.25	0.671	14		429.24	3.70	25		256.28	7.76
4		371.39	1.41	15		391.44	3.93	26		376.43	7.97
5		299.37	1.94	16		353.37	4.36	27		516.64	9.35
6		332.42	2.04	17		468.36	4.85	28		299.3	9.39
7		366.37	2.27	18		430.45	6.06	29		372.8	9.47
8		394.42	2.44	19		390.91	6.32	30		306.22	10.91
9		317.32	2.80	20		418.44	6.83	31		424.31	11.15
10		453.24	2.96	21		450.23	7.31	32		299.32	12.19
11		296.28	3.10	22		313.17	7.44	33		214.22	15.12

Supplementary Table 3. Thiobarbituric acid analogs of compound **1** (LTV-1) and their IC_{50} values for LYP.

#	Structure	Mol Weight	IC50, μM	#	Structure	Mol Weight	IC50, μM
34		446.11	2.74	**43**		506.09	17.0
35		476.08	3.04	**44**		472.11	17.7
36		456.05	3.88	**45**		382.06	28.4
37		414.10	5.74	**46**		476.08	31.7
38		444.05	11.2	**47**		502.12	33.7
39		502.12	11.8	**48**		430.04	50.1
40		472.11	12.1	**49**		412.07	70.4
41		502.12	12.4	**50**		412.07	87.2
42		488.10	14.2	**51**		410.09	98.0

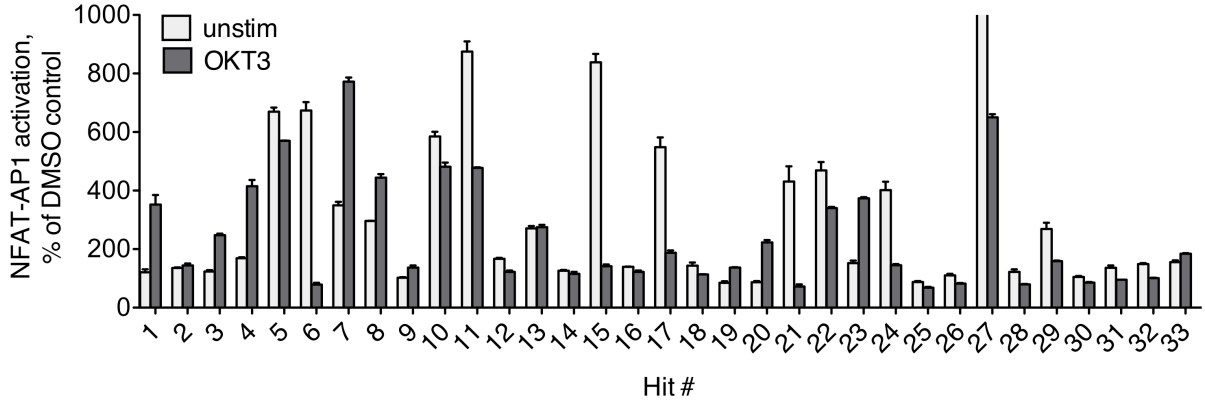


Supplementary Figure 1. The R620W mutation in LYP results in loss of CSK binding but not PSTPIP binding. Jurkat TAg T cells were co-transfected with various combinations of plasmids encoding HA-tagged LYP*R620, HA-tagged LYP*W620 and PSTPIP, empty vector was used as control plasmid. Later, cell lysates were prepared and subjected to immunoprecipitation with anti-HA antibodies, followed by immunoblotting with the indicated antibodies. Immunoblots of whole cell lysates are also shown.

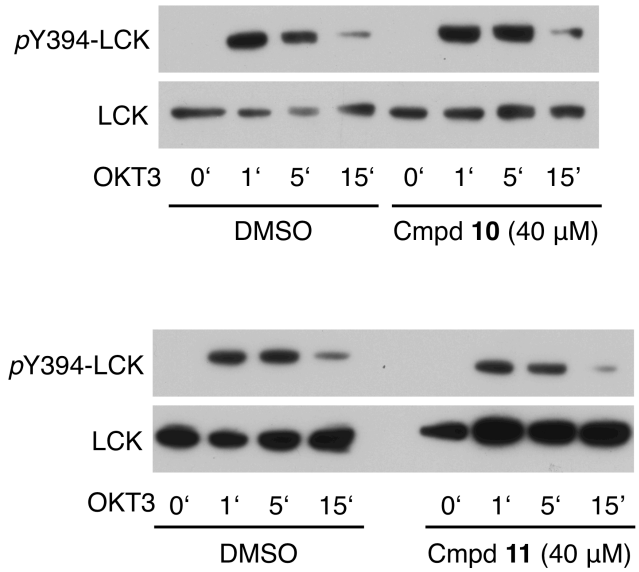


Supplementary Figure 2. Dissociation of the LYP/CSK complex is necessary for recruitment of LYP to lipid rafts and down-modulation of TCR-mediated signaling. (**a**) Lysates prepared from resting human T cells (LYP*R620 homozygous) were subjected to immunoprecipitation with the indicated antibodies or isotype controls, followed by immunoblotting with the given antibodies. For LYP and PAG blots, both long and short exposures are shown. There are several panels with CSK blots since CSK co-migrates with immunoglobulin G heavy chain, and therefore, antibodies used for immunoprecipitation and immunoblots must be from different species. (**b**) TCR time course (OKT3) with Jurkat TAg T cells, thereafter, prepared lysates and LYP immunoprecipitates were analyzed with the indicated antibodies. (**c**) Jurkat TAg T cells were transfected with empty vector or a plasmid encoding HA-tagged CSK-SH3, followed by immunoprecipitation of LYP and immunoblotting. Immunoblots of whole cells lysates are also shown. (**d**) Jurkat TAg T cells were transfected with empty vector or a plasmid encoding HA-tagged CSK-SH3. Later, cells were TCR-stimulated (OKT3) for the

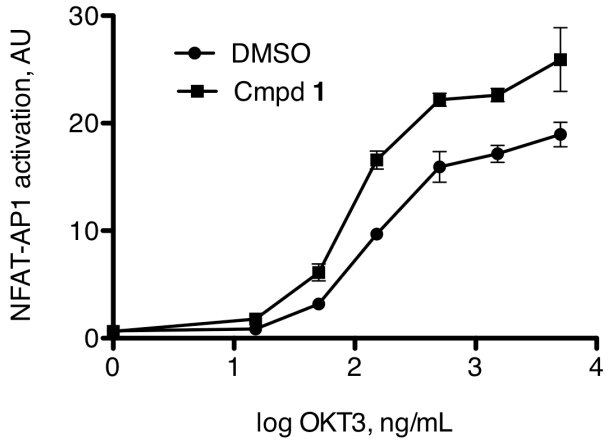
indicated periods of times and prepared lysates were subjected to immunoblotting with the specified antibodies. (**e**) Human T cells (LYP*R620 homozygous) were transfected with empty vector or a plasmid encoding HA-tagged CSK-SH3, followed by TCR stimulation with anti-CD3/anti-CD28 coated beads (different bead:cell ratios, three independent cultures for each condition) for 20h. IL-2 and IFNγ cytokines were measured in the collected supernatants. Data are presented as average ± SD, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Immunoblots verifying expression are also shown. (**f**) Jurkat TAg T cells were cotransfected with plasmids encoding firefly luciferase (under control of a promoter containing NFAT and AP1 sites) and *Renilla* luciferase (containing a null promoter) in addition to empty vector or plasmids encoding either HA-tagged wild-type CSK or HA-tagged CSK-SH3. Later, cells were stimulated through the TCR (OKT3) for 6h. Subsequently, dual luciferase assays were performed, and the level of NFAT-AP1 activation for each sample was calculated as the ratio between firefly and *Renilla* luciferase. Data are presented as average ± SD (triplicate measurements). Blots verifying expression of transfected wild-type CSK and CSK-SH3 are also shown. (**g**) Purified human T cells (LYP*R620 homozygous) were TCR-stimulated (CD3 cross-linking for 2 min) or not, followed by homogenization and sucrose gradient centrifugation. The harvested fractions were then subjected to immunoblotting with the indicated antibodies. (**h**) Jurkat TAg T cells were TCR-stimulated (OKT3) for the indicated periods of times, followed by lysis and sucrose gradient centrifugation. The lipid raft containing fractions were then pooled and subjected to immunoblotting with the given antibodies. (**i**) Jurkat TAg T cells were transfected with plasmids encoding either HA-tagged LYP*R620 or HA-tagged LYP*W620. Later, cells were lysed and sucrose gradient centrifugation was performed. The harvested fractions were then analyzed with immunoblotting with the indicated antibodies. (**j**) Experiment as in (i), but after harvesting of fractions, the lipid raft containing fractions were pooled and analyzed with immunoblotting. The numbers below the upper panel represent the relative density of the HA-LYP*R620 and HA-LYP*W620 bands (with LCK amounts in the same samples used as control). Blots with whole cell lysates for expression control (collected before sucrose gradient centrifugation was initiated) are also shown. All data presented in this entire figure are representative of at least three experiments with similar results.



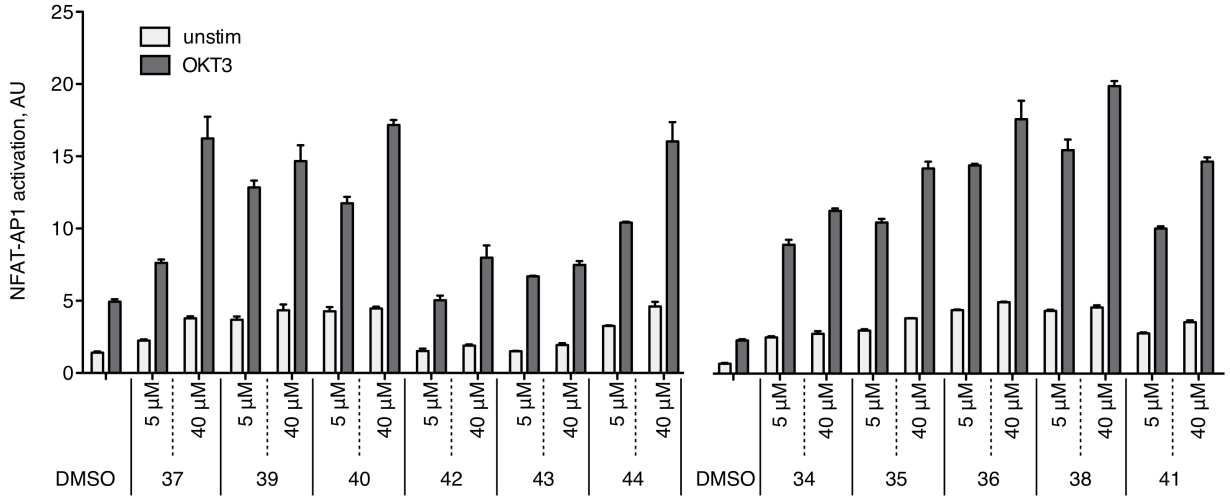
Supplementary Figure 3. T cell-based screening of HTS hits using a luciferase reporter assay. Jurkat TAg T cells cotransfected with plasmids encoding firefly luciferase (under control of a promoter containing NFAT and AP1 sites) and *Renilla* luciferase (containing a null promoter) were pretreated with compounds (40 μM) or DMSO and then stimulated through the TCR (OKT3). Dual luciferase assays were then conducted, and the level of NFAT-AP1 activation for each sample was calculated as the ratio between firefly and *Renilla* luciferase. All values given are relative to the DMSO control. Each sample was run in duplicate and is presented as average ± half range.



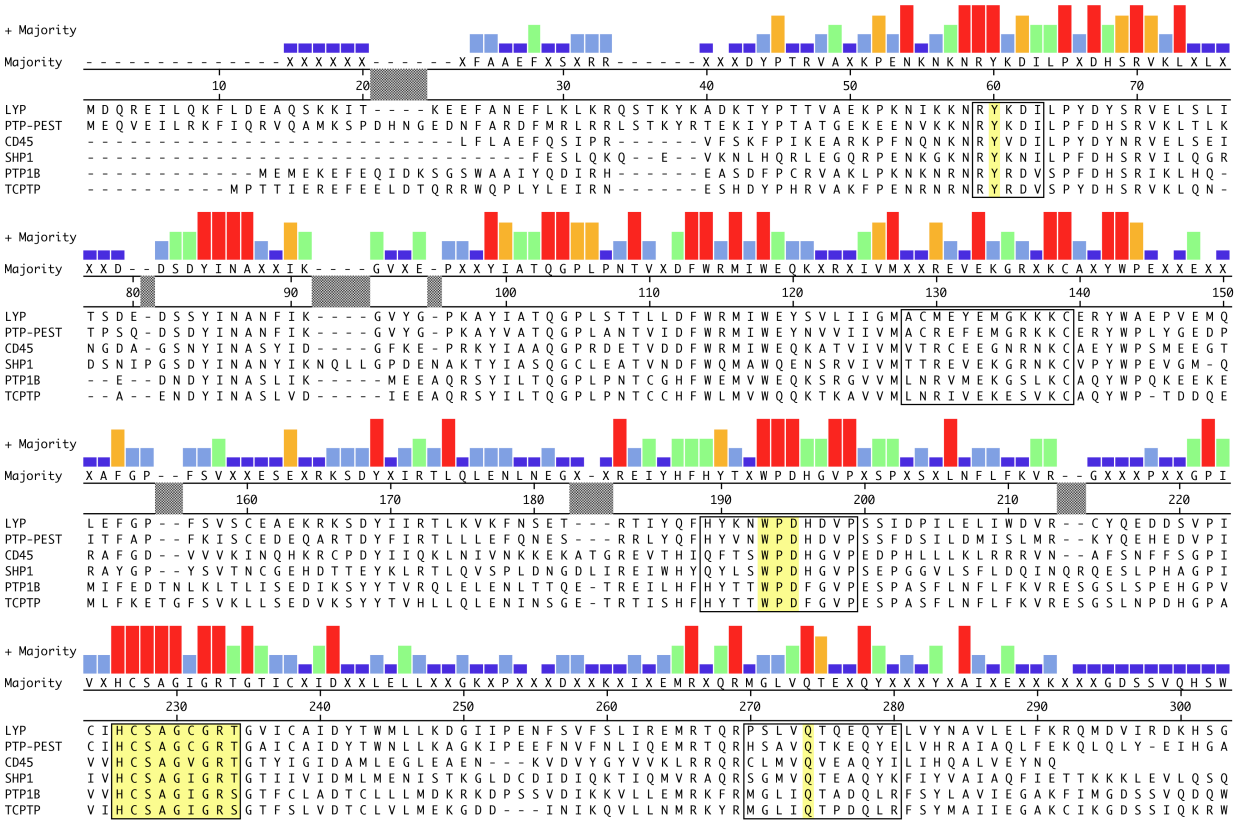
Supplementary Figure 4. TCR time courses with cells pretreated with compounds 10 or 11. Jurkat TAg T cells were pretreated with the indicated compounds (40 µM) or DMSO and then TCR stimulated (OKT3) for 0-1-5-15 min. Reactions were stopped by adding lysis buffer, and cell extracts were subjected to immunoblotting with the indicated antibodies.



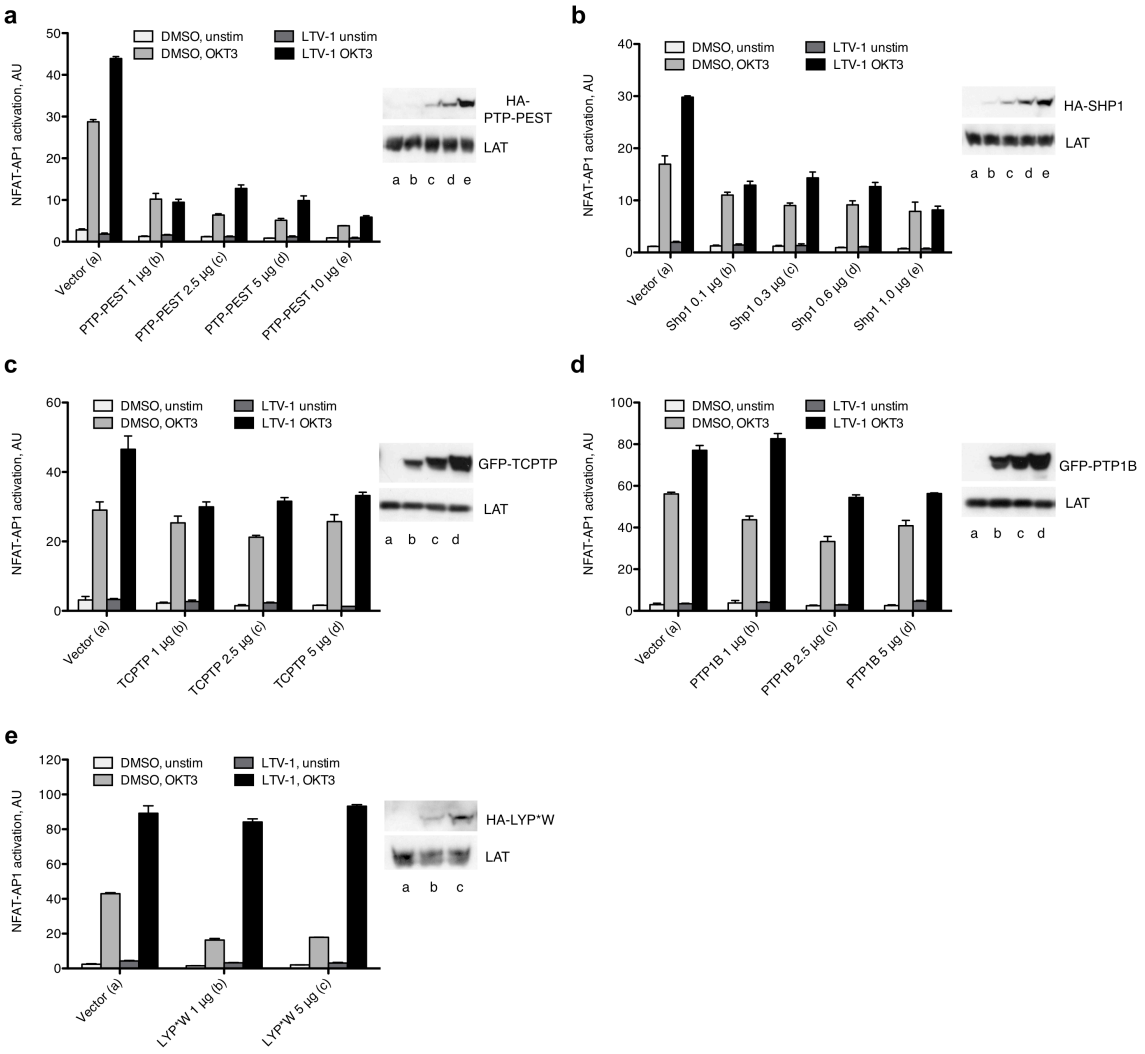
Supplementary Figure 5. Lower threshold for TCR signaling in cells pretreated with compound 1. Jurkat TAg T cells cotransfected with plasmids encoding firefly luciferase (under control of a promoter containing NFAT and AP1 sites) and *Renilla* luciferase (containing a null promoter) were pretreated with compound **1** (5 μM) or DMSO, followed by TCR stimulation with different OKT3 concentrations. Thereafter, dual luciferase assays were performed. Level of NFAT-AP1 activation for a sample was calculated as the ratio between firefly and *Renilla* luciferase for the same sample. Each sample was run in triplicate and is presented as average ± SD. The data are representative of 3 independent experiments.



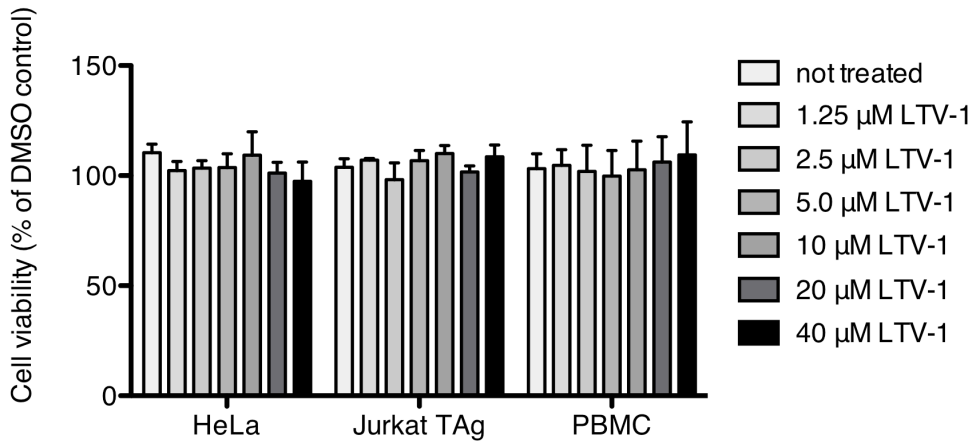
Supplementary Figure 6. T cell based screening of compound 1 (LTV-1) analogs using an NFAT-AP1 reporter assay. Jurkat TAg T cells, cotransfected with plasmids encoding firefly luciferase (under control of a promoter containing NFAT and AP1 sites) and *Renilla* luciferase (containing a null promoter), were pretreated with analog compounds (5 and 40 µM) or DMSO and then stimulated through the TCR (OKT3). Subsequently, dual luciferase assays were conducted, and NFAT-AP1 activation levels were calculated as the ratio between firefly and *Renilla* luciferase for the same sample. All values given are relative to the DMSO control. Each sample was run in triplicate and is presented as average ± SD.



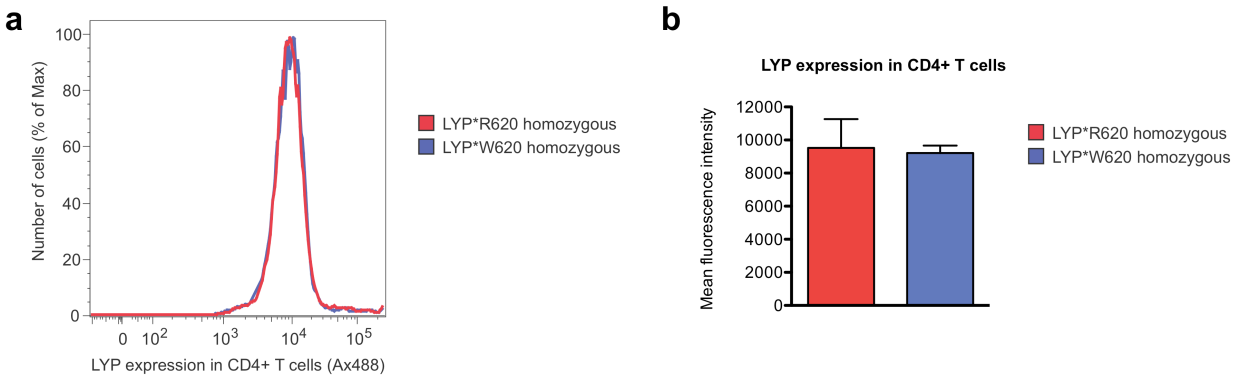
Supplementary Figure 7. Alignment (ClustalW) of the catalytic domains of LYP, PTP-PEST, SHP1, CD45, PTP1B, and TCPTP. Colored bars indicate consensus strength (red, most agreement; dark blue, least agreement). Numbering represents amino acid positions in LYP. PTP signature residues are colored in yellow, including Tyr60 of the phosphotyrosine recognition loop, the WPD motif of the WPD-loop, the PTP signature motif HCxxGxxRT/S of the P-loop, and Gln274 of the glutamine loop. Boxes indicate residues that form the active site in LYP.



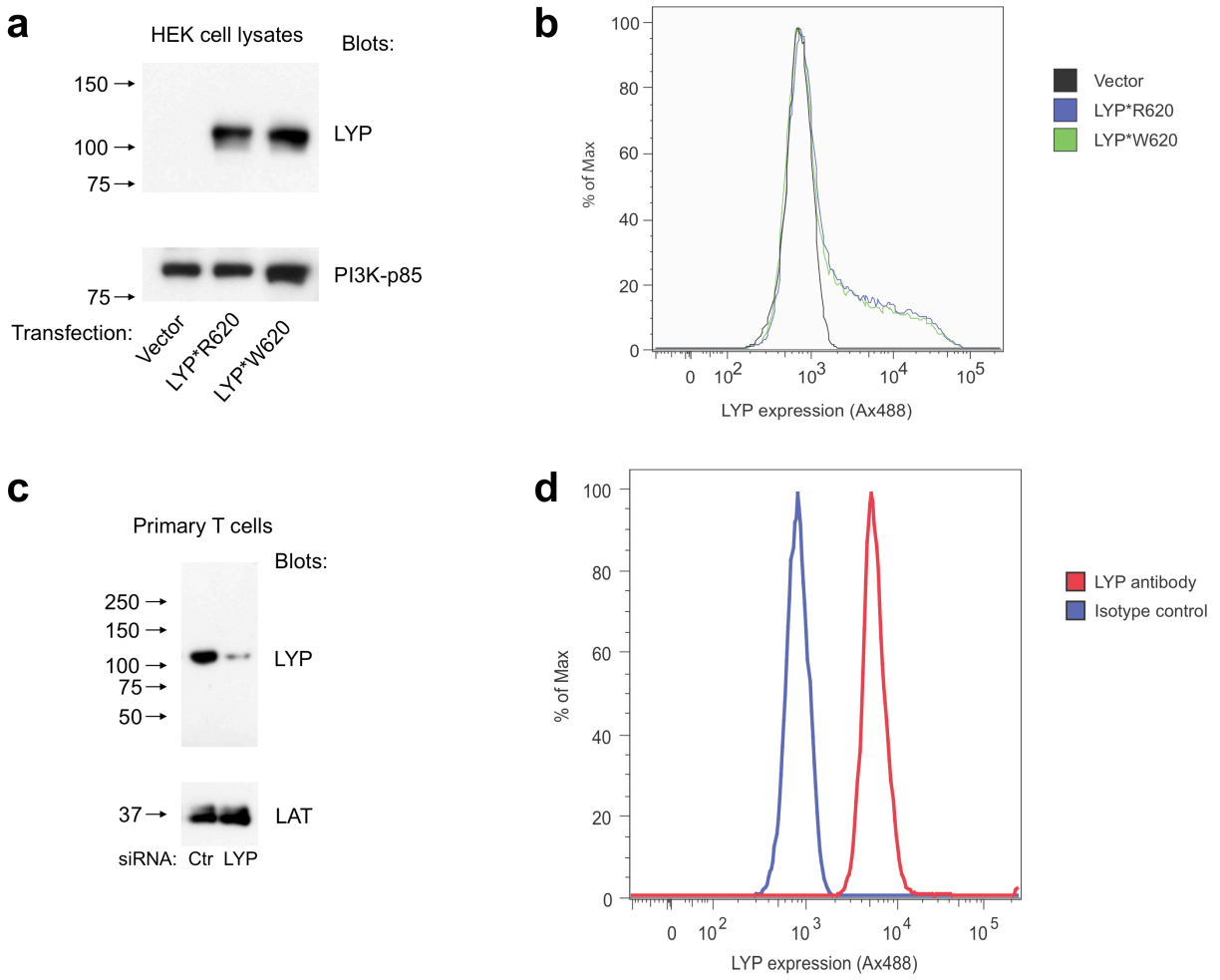
Supplementary Figure 8. Effects of LTV-1 on TCR signaling in cells overexpressing various PTPs. (**a**) Jurkat TAg T cells were cotransfected with plasmids encoding firefly luciferase (under control of a promoter containing NFAT and AP1 sites) and *Renilla* luciferase (containing a null promoter) in addition to empty vector and increasing amounts of a plasmid encoding HA-tagged PTP-PEST. Later, cells were pretreated with LTV-1 (5 µM) or DMSO, followed by TCR stimulation (OKT3). Subsequently, dual luciferase assays were performed, and the level of NFAT-AP1 activation for each sample was calculated as the ratio between firefly and *Renilla* luciferase. Each sample was run in triplicate and is presented as average ± SD. Blots showing expression levels of transfected PTP-PEST and amounts of LAT (loading control) are also presented. (**b-e**) Experiments as in (a), but with plasmids encoding HA-tagged SHP1 (**b**), GFP-tagged TCPTP (**c**), GFP-tagged PTP1B (**d**) or HA-tagged LYP*W620 (**e**) instead of PTP-PEST. All data are representative of 3 or more experiments.



Supplementary Figure 9. Cytotoxicity evaluation of LTV-1. HeLa cells, Jurkat TAg T cells, and PBMC were cultured for 48 h in the presence of increasing doses of compound **1** or with DMSO (0.5%). Cell viability was determined by MTT assay based on a colorimetric evaluation of metabolic active cells. The percentage of viable cells compared to the DMSO control is presented as the average ± SD of 3 individual experiments (HeLa, Jurkat TAg) or 3 different healthy donors (PBMC).



Supplementary Figure 10. Expression of LYP*R620 vs. LYP*W620 in human T cells. (**a**) Purified human $CD4^+$ T cells (homozygous for either LYP*R620 or LYP*W620) were stained with a LYP-specific antibody, followed by assessment of LYP expression levels by FACS. (**b**) Experiment as in (a), but data from 6 independent donors (3 for each genotype) are combined and presented as mean ± SD.



Supplementary Figure 11. Characterization of LYP antibodies. (**a**) HEK cells were transfected with empty vector or plasmids encoding either LYP*R620 or LYP*W620. Subsequently, cell extracts were subjected to SDS-PAGE and immunoblotting with LYP and PI3 kinase p85 (loading control) antibodies. (**b**) HEK cells transfected as in (a) were stained with LYP antibodies and fluorescently labeled secondary antibodies, followed by analysis of LYP expression on a BD Biosciences FACSCanto II. The transfection efficiency is relatively low; only the transfected (i.e. LYP-expressing) cells give higher signals than untransfected or vector-transfected (i.e. LYP-negative) cells, indicating that the LYP antibodies used specifically stain LYP during FACS analysis. (**c**) Cell extracts from primary T cells with or without LYP knockdown were subjected to SDS-PAGE and transferred to a PVDF membrane. The upper part of the PVDF membrane containing proteins with migration ranging from 45 to 250 kDa, was immunoblotted with LYP antibodies. Note that the LYP antibodies recognized only one band in

this molecular weight range, and this band was strongly reduced in cells where LYP had been knocked down. The lower part of the PVDF membrane containing proteins with migration <45 kDa was immunoblotted with LAT antibodies as a loading control. (**d**) Purified human $CD4^+$ T cells were fixed, permeabilized, and then stained with either LYP-specific antibodies or isotype control. Later, cells were stained with fluorescently labeled secondary antibodies, and LYP expression was analyzed on a BD Biosciences FACSCanto II.