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Supplementary Materials for

The costimulatory molecule CD226 signals through VAV1 to amplify TCR signals and promote IL-17 production by CD4$^+$ T cells

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

(available at www.sciencesignaling.org/cgi/content/full/11/538/eaar3083/DC1)

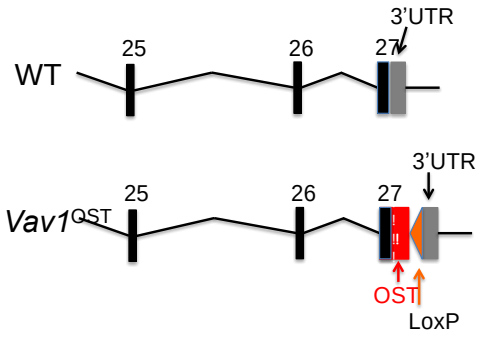

Fig. S1. Structure of the 3′ end of the wild-type *VAV1* allele and the *VAV1*^{OST} allele. Exons are shown as filled black boxes and numbered. The one-STrEP tag (OST, red) was inserted into the intron between exon 27 and the 3'UTR to generate the *VAV1*^{OST} allele. The loxP site retained in the allele after knock-in is shown in orange.

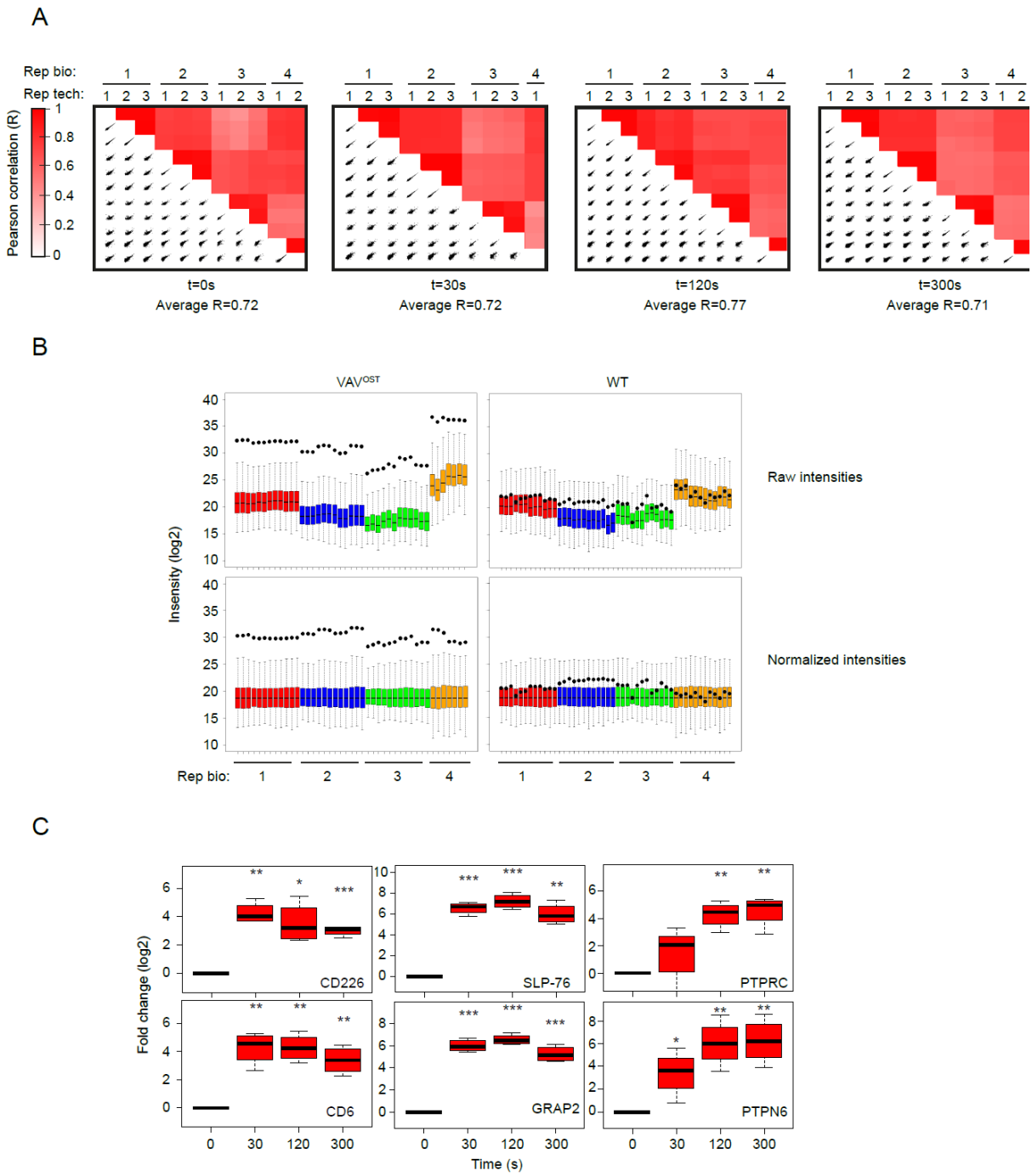

Fig. S2. Assessment of biological and technical variability across samples. Four independent biological experiments were performed, each of them involving four different conditions corresponding to no stimulation (t=0 sec) and 3 time points (t=30 sec, t=120 sec and t=300 sec) after pervanadate stimulation. For each sample, MS runs were performed in triplicates (for biological replicates 1-3) or in duplicates (for biological replicate 4). (**A**) The variability between samples corresponding to a given condition of activation was estimated by computing the Pearson correlation coefficient from log transformed raw intensities of all detected proteins for all pairs of technical and biological replicates (denoted as Rep. tech. and Rep. bio., respectively). For each duration of activation scatter plots of log transformed raw intensities for all pairs of technical and biological replicates are represented along with the corresponding Pearson correlation coefficients (R). The average Pearson correlation

coefficient across all pairs of technical and biological replicates (denoted as Average R) is also indicated for each time point. (**B**) Boxplots showing the medians and the dispersions of raw intensities or normalized intensities for the population of proteins identified in each MS analysis. Box-plots are depicted in a different colour for each biological experiment. (Note that samples from biological replicate 4 were specifically analysed on a Q-Exactive and not on a LTQ-Velos mass spectrometer, leading to increased absolute values of the measured protein raw intensities in this time series). Technical MS replicates are represented consecutively, and the boxplots are then represented in the order of the time-course experiment (0, 30, 120, 300 sec) in each biological replicate. Normalization of the data was performed by adjusting the median of all distributions. Intensities of VAV1 are displayed by black dots, showing reproducibility of the immunoprecipitation and the enrichment of the bait in Vav1 OST-purified samples compared to WT samples. (**C**) Boxplots showing the reproducibility across replicates of the relative dynamic association of CD226, CD6, SLP-76, GRAP2, PTPRC (CD45) and PTPN6 (SHP-1) with $VAV1^{OST}$. Kinetics are displayed as fold changes of normalized intensities of association relative to the unstimulated condition. For each time point, the box-plot illustrates the median and distribution of calculated fold change values across biological replicates. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

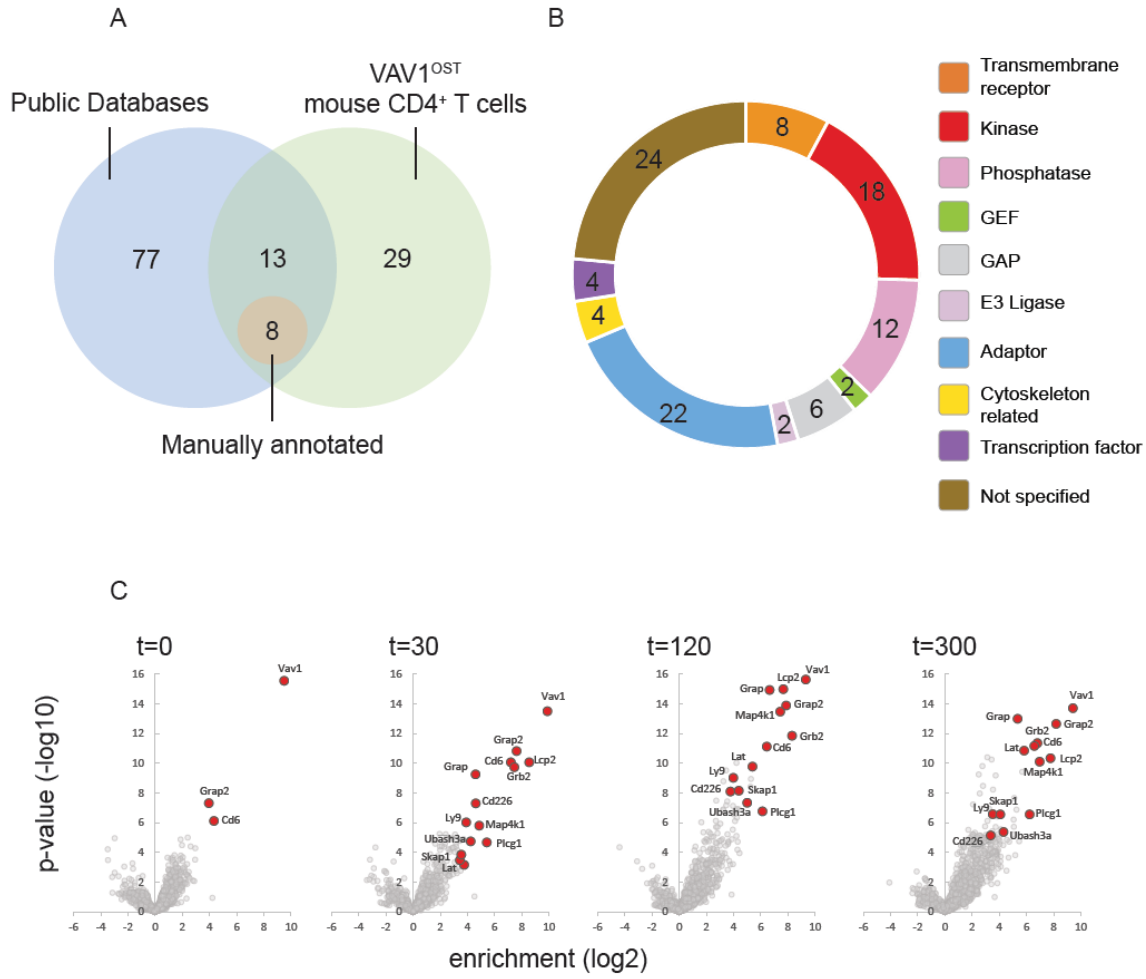

Fig. S3. The VAV1 interactome. (**A**) Venn diagram displaying the number of VAV1 partners found in public databases (Intact, Biogrid, MINT, and HPRD) and specifically identified in this study (*VAV1*^{OST} mouse CD4+ T cells). Manually annotated partners were also included in the analysis (Table SI). (**B**) Diagram showing the proportion (percentages) of Vav1^{OST} partners classified according to their functions. (**C**) Volcano plot representations of the VAV1^{OST} interactome before and after 30, 120, and 300 seconds of pervanadate stimulation. x- and y- axes represents the mean enrichment and p-value of the VAV1 partners in Vav1^{OST} pull-down compared to WT conditions. Data are representative of four independent experiments.

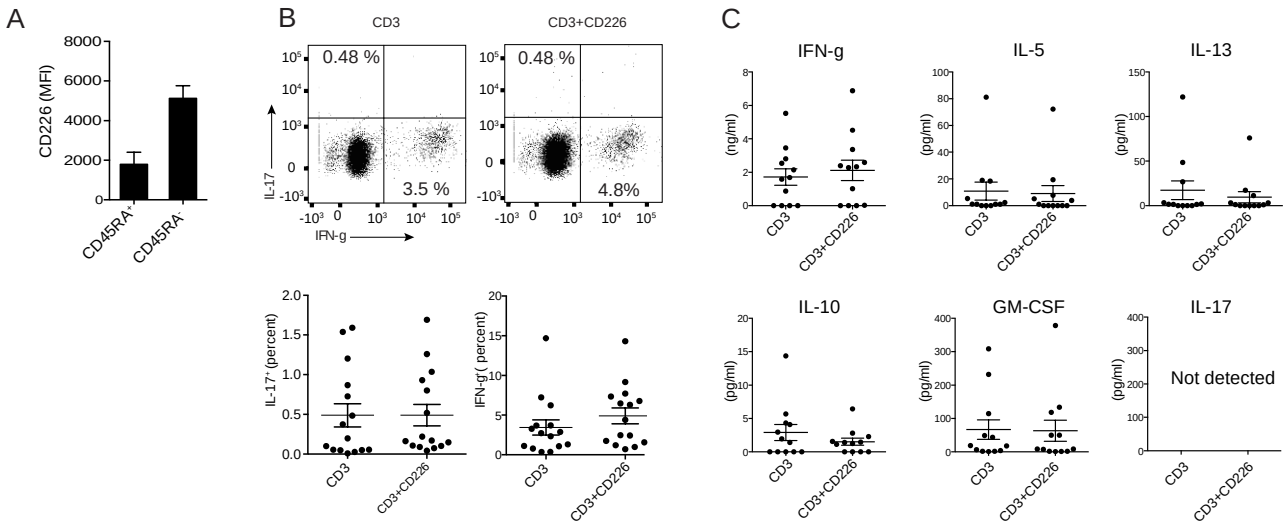

Fig. S4. The synergistic effect of CD3 and CD226 co-engagement is not observed in naïve CD4$^+$ T cells. (A) Flow cytometry analysis of CD226 expression on naïve (CD45RA+) and effector/memory (CD45RA-) CD4$^+$ T cells from healthy donors. (**B**) Intracellular staining for IL-17 and IFN-γ expression by human primary naïve CD4$^+$ T cells. Cells were stimulated with anti-CD3 antibody associated with 10 μg/ml of anti-CD226 (CD3+CD226) or IgG1 isotype control (CD3) antibodies for 5 days. Numbers represent the frequency of IL-17 single positive (upper left) and IFN-γ single positive cells (lower right). The graphs summarize the data obtained from 15 unrelated donors. (**C)** Cytokines secretion (IL-5, IL-10, IL-13, IL-17, GM-CSF and IFN-γ) was quantified in supernatants of human primary CD45RA$^+$CD4$^+$ T cells using cytometric beads array assay. Cells were stimulated with anti-CD3 antibody associated with 10 μg/ml of anti-CD226 (CD3+CD226) or IgG1 isotype control (CD3) antibodies for 5 days. Data are from 12 unrelated donors

Table S1. List of proteins associated with VAV1 in resting and pervanadate-activated $CD4^+$ T cells.

This table is provided as a separate Excel file. The table lists proteins identified as associating with VAV1 in the present study by affinity purification and mass-spectrometry. Each of the proteins is denoted by its Uniprot symbol (http://www.uniprot.org).