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Supplementary Information for

T cells selectively filter oscillatory signals on the minutes timescale

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This PDF file includes:

Figures S1 to S5

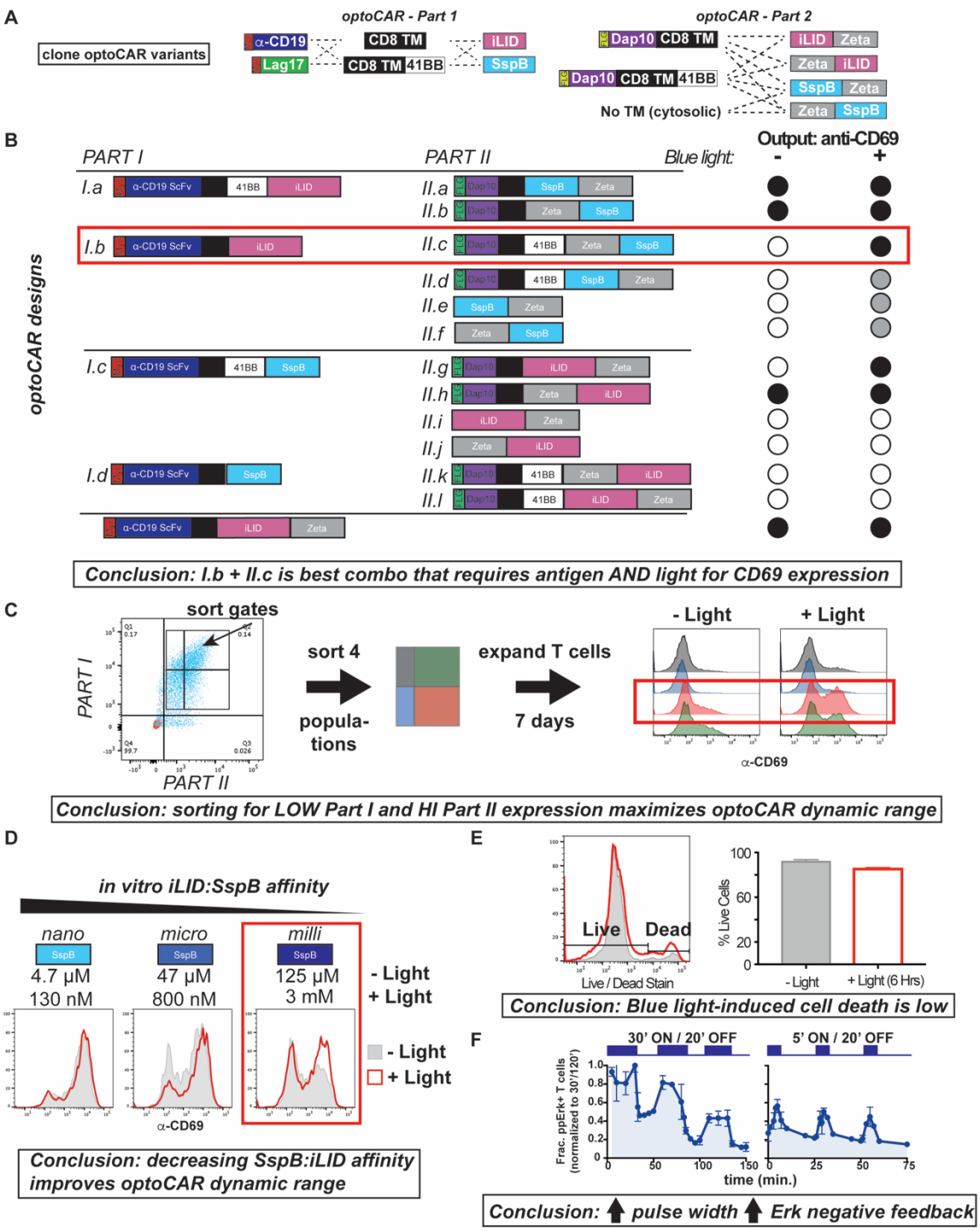

Fig. S1. Development and characterization of the optoCAR. (A) A small library of optoCAR Parts I and II were cloned by amplifying different modular domains via polymerase chain reaction and subsequent ligation via In-Fusion. (B) Combinations of Part I and Part II were co-expressed in CD4+ primary human T cells and assessed for efficacy via flow cytometry measurement of CD69 expression. Open circles indicate low (~5-10% positive) CD69 activation, gray filled circles indicate intermediate (~10-20% positive) CD69 activation, and black filled circles indicate high (>40% positive) CD69 activation. The *I.b* + *II.c* and *I.c* + *II.g* combinations both resulted in adequate dynamic range and maximal activation; *I.b and II.c* were chosen in order to completely separate ligand-binding and signaling on separate polypeptides. (C) optoCAR T cells were further sorted for

low Part I and high Part II expression in order to further minimize background activation. (D) The lowest affinity *milli* SspB peptide was also cloned into *II.c* in order to further minimize background activation. (E) CD4+ optoCAR T cells were stained with Live/Dead stain and assessed after 6 hours of stimulation with ~4 mW/cm^2 470 nm light. Blue light exposure resulted in the death of ~5% more cells (~85% vs ~90%). (F) Increasing the pulse width interval while keeping the off interval constant accelerates the decline in Erk Thr202/Tyr204 phosphorylation. Here the response curve also seen in Fig. 1F is rescaled to the maximal activation observed in response to the 30' ON / 20' OFF pulse train.

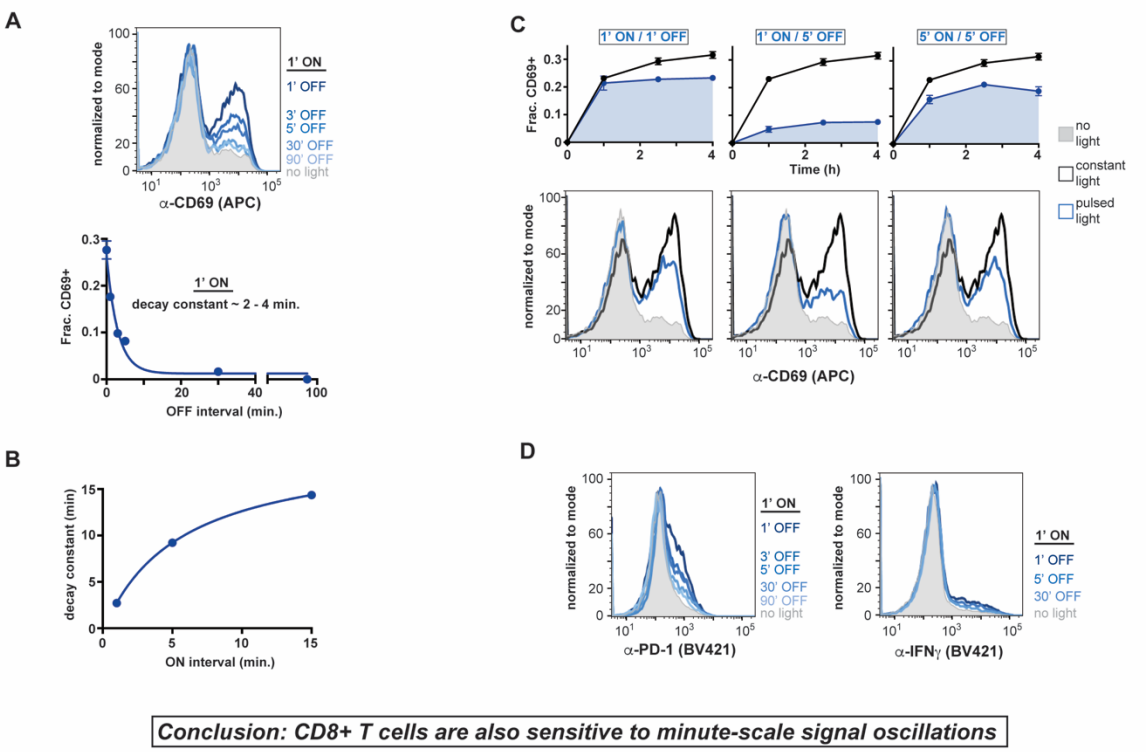

Fig. S2. CD69, PD-1, and IFNγ responses to oscillating optoCAR signals in CD8+ primary human T cells. (A) (Top) Flow cytometry histograms of CD8+ primary human T cells stained with an α-CD69 antibody after stimulation with 10.5 hours of optoCAR pulse trains with 1' ON intervals and OFF intervals ranging from 1' to 90'. Lighter shades of blue indicate longer OFF intervals. Shaded gray indicates no light negative control. (Bottom) The fraction of CD69+ T cells is plotted vs. OFF interval. The curve fits an exponential with a half-life of 2-4 minutes (95% confidence interval). (B) The half-life of similar curves for 1', 5', and 15' ON intervals is plotted against the ON interval duration. In general the OFF period required to maintain half-maximal CD69 activation increases non-linearly as the ON interval is increased from one to 15 minutes. The half-life of the analogous curve corresponding to 40' ON intervals was undetermined from a fit to the current data. (C) A CD69 expression response pattern to 1'/1', 1'/5', and 5'/5' pulse trains similar to that seen in CD4+ T cells is also observed in CD8+ T cells. (D) Increasing the off interval of 1' ON pulse trains similarly decreases both PD-1 and IFNγ expression levels in CD8+ primary human T cells. Error bars are SEM, n = 2; representative of at least two independent experiments.

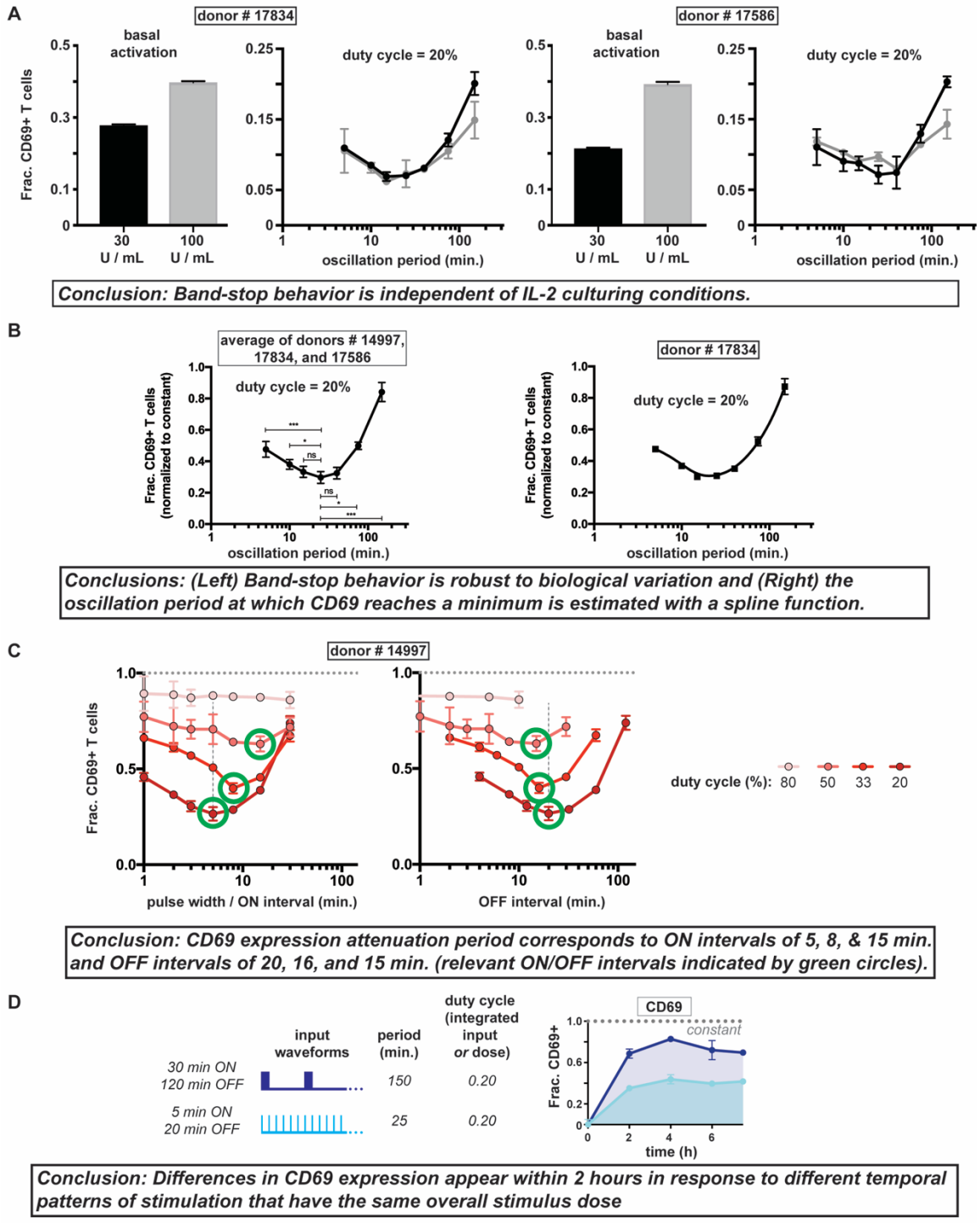

Fig. S3. CD4+ T. cell CD69 expression in response to oscillation period and supplemental IL-2. (A) CD4+ primary human T cells from two additional donors (#17834 and 17586) were cultured in 30 U/mL and 100 U/mL IL-2 for ten days. Immediately prior to optogenetic stimulation the media was switched to human T cell culture medium with no supplemental IL-2, and CD69 expression levels were then measured after 22 hours of optoCAR stimulation. Basal CD69 expression levels were higher in the optoCAR T cells cultured in 100 U/mL than 30 U/mL IL-2. The degree of CD69 induction – i.e., the fraction of CD69+ T cells after the relevant basal expression levels are substracted – as a function of oscillation period was similar in the samples treated with 100 U/mL

vs. 30 U/mL IL-2, except at the highest oscillation period (150 minutes). (B) (*Left*) Data averaged from three healthy human donors demonstrate that the CD69 response to oscillation period is robust to biological variability. Average values and error bars plotted represent the average and standard deviation of data from three healthy human donors. (*Right*) The oscillation period at which CD69 expression reaches a minimum was estimated to be 25.8. +/- 4.6 min. by fitting data for each individual donor to a spline function and calculating the minimum of the spline function. The error in the minimum represents the standard deviation of the average from experiments performed with three healthy human donors. (C) The CD69 response to oscillation period for donor #14997 as seen in Fig. 3 are re-plotted vs. ON interval and OFF interval. The local minimum corresponds to ON intervals of 5, 8, and 15 minutes and OFF intervals of 20, 16, and 15 minutes, as indicated by the green circles. (D) CD69 expression was assessed at several timepoints after stimulation with 30’ ON / 120’ OFF and 5’ ON / 20’ OFF pulse trains. Fewer T cells exposed to 5’ ON / 20’ OFF pulse trains expressed CD69 than those exposed to 30’ ON / 120’ OFF pulse trains even within a few hours of stimulation, despite each pulse train having identical duty cycles or integrated doses.

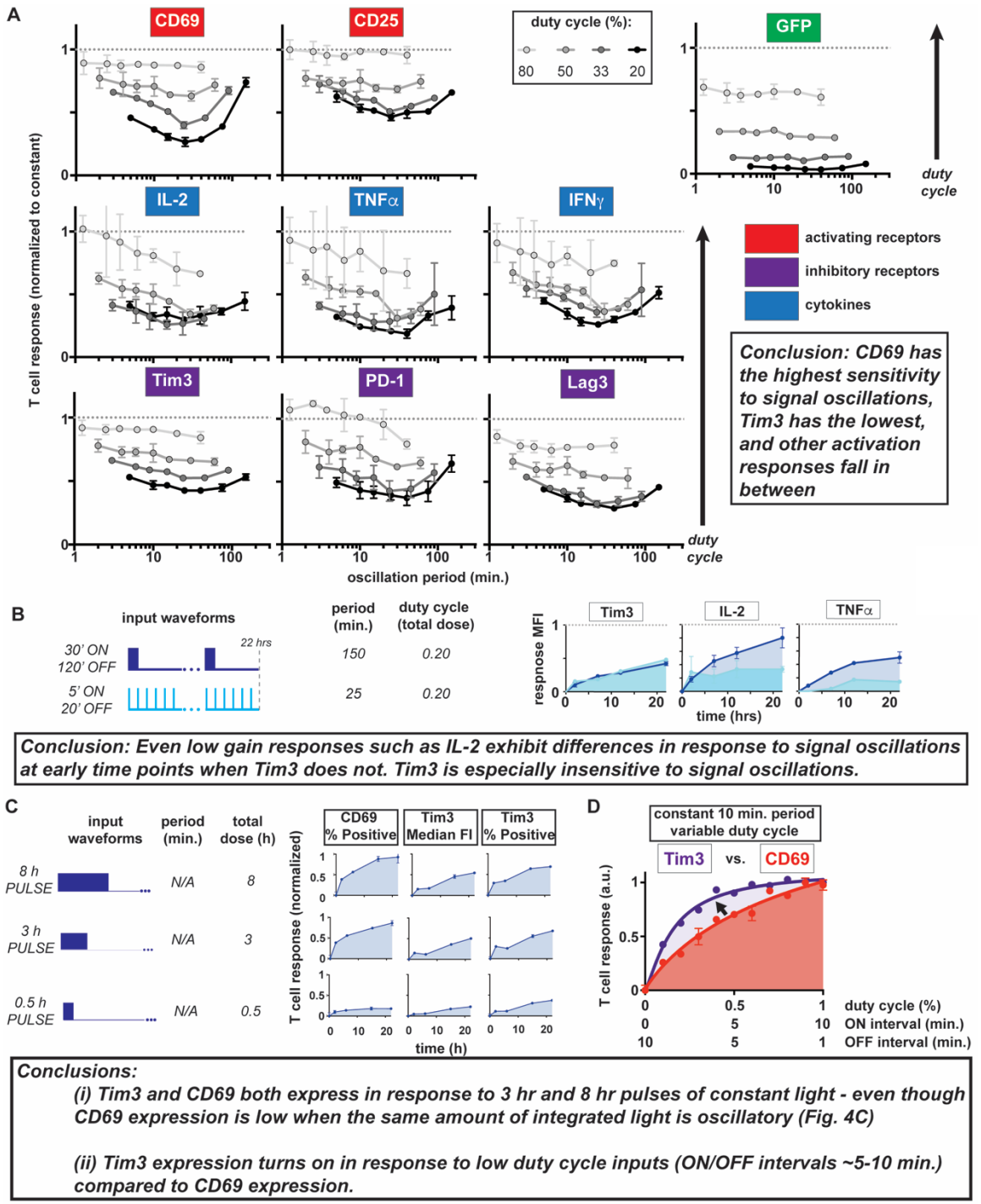

Fig. S4. Characterization and comparison of CD69, Tim3, IFNγ, IL-2, TNFα, CD25, PD-1, Lag-3, and GFP frequency and duty cycle responses. (A) Expression of Lag-3, CD25, PD-1, IL-2, TNFα, and IFNγ and the internalization of GFP ligand as a function of both oscillation period and duty cycle. (B) Tim3, IL-2, and TNFα expression was assessed at several timepoints after stimulation with 30' ON / 120' OFF and 5' ON / 20' OFF pulse trains. Tim3 is insensitive to oscillation period compared to all other T cell activation responses. (C) CD69 expression (% positive T cells) and Tim3 expression (Median Fluorescence Intensity and % positive T cells) were assessed over a 22 hour period in response to 8 hour, 3 hour, and 0.5 hour pulses of constant light stimulation. CD69 expression is similar in response to 3 hr and 8 hr impulses of light, despite differences when

the same amount of integrated signal is periodic with 1'/1' vs 1'/5' pulse trains. Further, one thirty-minute pulse of light is insufficient to induce a high level of CD69 upregulation. (D) Tim3 expression reaches a maximum at a lower duty cycle than CD69 expression; i.e., Tim3 expression reaches a maximum expression level with a shorter ON interval and a longer OFF interval than CD69 expression.

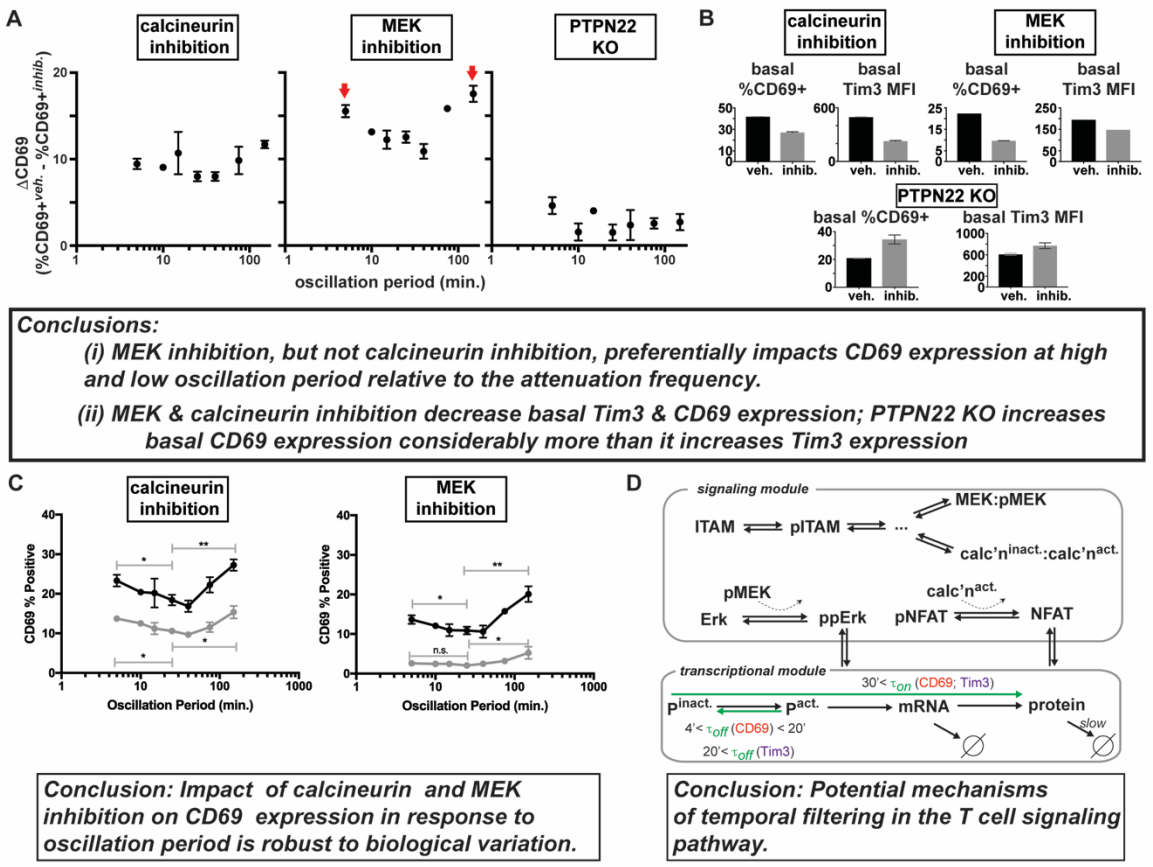

Fig. S5. Studying the effect of T cell signaling perturbation on CD69 and Tim3 expression responses to oscillation period. (A) The difference in the induction of CD69 expression (i.e., the degree CD69 expression after the relevant basal levels are subtracted) without (*vehicle*) and with (*inhib.*) various signaling perturbations. Calcineurin and MEK inhibition each decrease CD69 induction by ~10-15%, but MEK inhibition decreases CD69 selectively on either side of the attenuation frequency. PTPN22 KO increases the degree of CD69 induction at all oscillation periods, but only minimally. (B) Inhibition of calcineurin and MEK resulted in lower basal expression levels of CD69 and Tim3. PTPN22 KO resulted in higher basal expression levels of CD69 and Tim3. SEM, n = 2, representative of 2-4 independent experiments. (C) The effects of MEK and calcineurin inhibition are robust to biological variation. The error represents the standard deviation of the average from experiments performed with two healthy human donors. (D) Filtering of dynamic signals could result from mass action kinetics downstream of Erk activation. For example, even though CD69 and Tim3 expression are both regulated by Erk, differences in promoter transition kinetics from an active ($P^{activ.}$) to an inactive ($P^{inactiv.}$) state could drive differences in CD69 and Tim3 expression in response to the same temporal stimulation patterns. Here we have speculated that the off kinetics follow the OFF periods that lead to the dip in CD69 expression as seen in Figure 3. Above a threshold "ON" duration (between 30' and 180' for CD69, see Fig. S4C), T cells commit to CD69 expression.