Supplementary Figures

Supplementary Figure S1. VIP induces hyporesponsiveness on T cells. A. VIP decreases the proliferative response of human T cells activated by different stimuli. Purified primary human T cells were activated with phytohemagglutinin (PHA, 1 μg/ml), the superantigen staphylococcal enterotoxin B (SEB, 1 ng/ml), or anti-CD3/CD28 mAb-coated beads (CD3/CD28) in the absence (control) or presence of 10^{-7}M VIP. Proliferation was determined after 4 days by incorporation of ^{3}H-thymidine. Results represent the mean±SD of 4 experiments performed in duplicate. *p<0.01 vs. control activated cells in the absence of VIP. **B.** VIP primed human T cells do not respond to secondary restimulation. Human T cells were activated with anti-CD3/CD28 beads in the absence or presence of VIP (10^{-7}M) for 4 days. Cells were harvested and rested for 2 days. Recovered viable cells were incubated in a secondary culture with medium (none), anti-CD3 mAb (2 μg/ml), IL-2 (50 U/ml) or anti-CTLA4 mAb (10 μg/ml) and the proliferation determined after 4 days by ^{3}H-thymidine incorporation. Results show mean±SD of 4 experiments performed in duplicate. *p<0.01 vs. CD3/CD28-activated control cells.

Supplementary Figure S2.- VIP induces a rapid and sustained expression of CTLA4, but not of PD-1 or BTLA, on activated T cells. Human T cells were cultured with medium alone (unstimulated) or activated with anti-CD3/CD28 beads in the absence or presence of VIP (10^{-7}M). At different times after stimulation, the expression of CTLA4 (**A**) and PD-1 (**B**) was determined by flow cytometry in gated $CD4^{+}$ cells and expressed as the mean fluorescence intensity (MFI). Results represent the mean±SD of 3-4 experiments performed in duplicate. *p<0.01 vs. CD3/CD28-activated cells. Histograms correspond to 96h. **C.** BTLA gene expression was determined by Northern blot at 48h. Blot is representative of three experiments.

Supplementary Figure S3.- VIP induces suppressive functions on $CD4^{+}CD25^{-}$ T cells. $CD4^{+}CD25^{-}$ T cells isolated from donor A were CD3/CD28-activated without ($T_{control}$) or with VIP

(T_{VIP}) for 96h. After a 48h rest period, the suppressive potential of the recovered cells (suppressor $T_{control}$ or T_{VIP}) was determined by adding increasing numbers of $T_{control}$ or T_{VIP} to co-cultures of responder T cells (donor A) and allogeneic mDCs (donor B). Proliferation of responder T cells was determined after 4 days. Black bar represents background proliferation of unstimulated responder T cells. *$p<0.001$ vs. $T_{control}$.

Supplementary Figure S4.- VIP-induced $CD25^{high}$ T cells are noncycling inactivated cells and do not respond to allogeneic restimulation. $CD4^+CD25^-$ T cells isolated from donor A were CFSE-labeled and activated with CD3/CD28-beads in the absence ($T_{control}$) or presence of VIP (T_{VIP}) for 96h. $T_{control}$ and T_{VIP} were analyzed in the $CD4^+$ T cell fraction for CD25 expression by flow cytometry. The subsets formed ($CD25^{negative}$, $CD25^{intermediate}$ and $CD25^{high}$) were sorted by flow cytometry. **A.** The expression of CD69 and CD62L, as well as the expression of FoxP3 on the $CFSE^{high}$ (noncycling cells) and $CFSE^{mild/low}$ (cycling cells), was determined on the sorted T_{VIP} $CD25^{high}$ cells by FACS. **B.** The sorted $CD25^{negative}$, $CD25^{intermediate}$ and $CD25^{high}$ subpopulations were restimulated with allogeneic mDCs (donor B) in the absence (upper panel) or presence of IL-2 (lower panel). Naïve $CD4^+CD25^-$ T cells were used as controls. Proliferation was determined after 3 days of restimulation. n=3, in duplicate.

Supplementary Figure S5.- Quantitative analysis of the expression of cyclins and cdks on VIP-treated activated T cells. A. Effect of VIP on the expression of G1 cyclins, cdk and cdki proteins. Human T cells were CD3/CD28-activated without or with VIP (10^{-7}M) for different time intervals. Cell lysates were subjected to Western blot analysis for cyclins, cdks, $p27^{kip1}$, phosphorylated pRB and expressed as relative densitometric units normalized for the expression of actin in each sample. **B.** IL-2 partially reverses VIP effects on cyclins and $p27^{kip1}$. Human T cells were CD3/CD28-activated without or with VIP (10^{-7}M) and IL-2 (50 U/ml) for 72h. Western blot analysis was performed in cell lysates for cyclins and $p27^{kip1}$ and expressed as densitometric units relative to

Zap70 (zeta chain associated protein kinase). **C.** VIP inhibits cyclins/cdk activity. Human T cells were CD3/CD28-activated without or with VIP (10^{-7}M) for 36h. Cell lysates were immunoprecipitated with Abs against cyclin D2, cyclin D3, cyclin E, cdk2 or cdk4. Cdk4-cyclin D2/D3 and Cyclin E-cdk2 kinase activities were measured using pRB-GST and histone H1 as substrates, respectively, and expressed as relative densitometric units to those obtained on unstimulated cells. n=3. *p<0.001 vs. CD3/CD28-stimulated control samples. #p<0.05.

Supplementary Figure S6.- VIP increases interaction of $p27^{kip1}$ to cyclin-cdk complexes and retains $p27^{kip1}$ in the nucleus of activated T cells. Primary human T cells were cultured with medium (unstimulated) or activated with anti-CD3/CD28 mAbs-coated beads in the absence or presence of VIP (10^{-7}M). **A.** Cell lysates obtained after 24h culture were immunoprecipitated with Abs against cyclin D2, cyclin E, cdk2 or cdk4, and analyzed by Western blot for cyclins, cdk and $p27^{kip1}$. Interactions of $p27^{kip1}$ with each cyclin and cdk were expressed as arbitrary densitometric units (n=3). **B.** After 48h of culture, nuclear and cytoplasmic protein extracts were analyzed by Western blotting for laminin B (control for nuclear protein), β-tubulin (control for cytoplasmic protein) and $p27^{kip1}$. Results are expressed as densitometric units relative to total laminin B or β-tubulin (n=3). *p<0.001 vs. CD3/CD28-treated control samples.

Supplementary Figure S7.- Effect of VIP on various transduction pathways, transcription factors and cdk inhibitors. A. VIP does not affect the expression of the cdki $p16^{ink4a}$ and $p21^{cip1}$. Human T cells were incubated with medium (unstimulated) or activated with anti-CD3/CD28-coated beads in the absence or presence of VIP (10^{-7}M) for different time intervals (24h and 48h). Cell lysates were subjected to Western blot analysis for $p16^{ink4a}$ and $p21^{cip1}$. **B.** VIP does not affect the activation of p38 MAPK and JNK. Cell lysates obtained after 3h of cultures were subjected to Western blot analysis for the expression of phosphorylated and total p38 and JNK. **C.** VIP does not affect intracellular calcium fluxes on activated T cells. Human T cells were loaded with Indo-1 (2

μM) for 45 min at 37°C. After two rounds of washing, cells were rested on ice for at least 15 min and then equilibrated at 37°C for 10 min. Thereafter, changes in intracellular calcium with time were monitored using a flow cytometer with the following protocol: during the first 2 min, unstimulated cells were studied for evaluation of baseline activity; then, anti-CD3/CD28-beads were added in the absence (black line) or presence of VIP (red line), and cells were studied for another 6 min. The calcium ionophore ionomycin (2 μM) was used as a control. **D.** VIP does not alter the activation of the ζ chain associated to TCR signaling. Human T cells were incubated with medium (unstimulated) or activated with anti-CD3/CD28-coated beads in the absence or presence of VIP. After 5 min, cell extracts were isolated and subjected to immunoprecipitation of the ζ chain; thereafter, phosphotyrosine content and expression of the ζ chain was assessed in the immunoprecipitates. **E.** VIP does not affect activation of $p56^{lck}$. The active form of $p56^{lck}$ was determined in cell extracts isolated after 5 min of culture by analyzing by immunoblotting the phosphorylated form (Tyr394) of lck. **F.** VIP does not affect the kinase activity of $p59^{fyn}$. Cell lysates obtained after 5 min of culture were immunoprecipitated with anti-human $p59^{fyn}$ Abs and subjected to immune complex kinase assay using enolase as an exogenous substrate. **G.** VIP does not affect the IL-2-induced STAT5-mediated signaling. Human T cells were incubated with medium (unstimulated) or activated with anti-CD3/CD28-coated beads with or without IL-2 in the absence or presence of VIP (10^{-7}M). After 10 min, cells were analyzed by flow cytometry for the expression of intracellular phospho-STAT5. After 30 min, nuclear extracts were isolated and DNA finding of STAT5 was determined by EMSA. After 30 min, Akt kinase activity was determined by immunoprecipitation of cell lysates with an anti-Akt mAb followed by immunocomplex kinase assay in the presence of GSK-3 fusion protein as substrate and expressed as percentage of Akt activity of CD3/CD28-activated T cells. Activation of Ras was examined in the same cell lysates by pull-down assays using glutathione beads coated with Raf1RBD-GST, followed by Western blot with Ras-specific Abs, and expressed as densitometric units relative to total levels of Rap1. Results

are representative of 2-3 experiments. *p<0.001 vs. samples with CD3/CD28 plus IL-2. #p<0.001 vs. samples with IL-2.

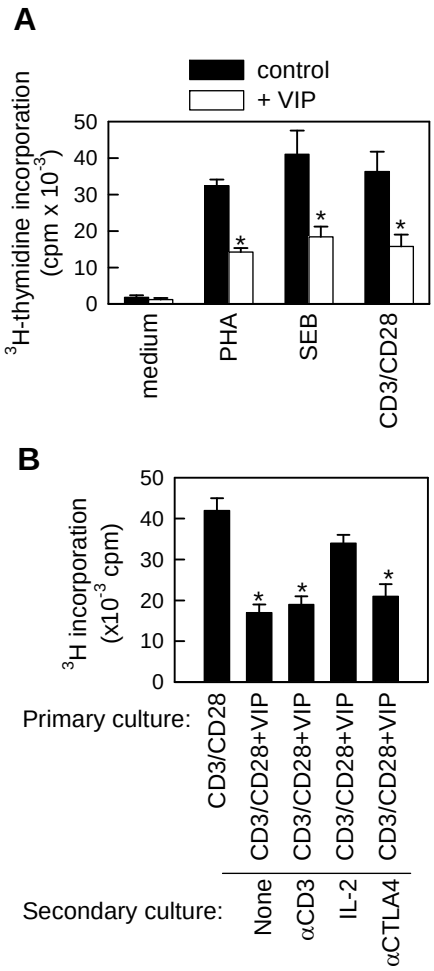
Supplementary Figure S8.- VIP inhibits IL-2 gene activation and transactivation dependent of NFkB, NFAT and AP1. Jurkat T cells were transiently transfected with reporter constructs of luciferase driven by either the IL-2 promoter/enhancer, AP1, NFAT or NFkB. Forty eight hours after transfection, cells were stimulated with anti-CD3/CD28 mAbs-coated beads (CD3/CD28) in the absence or presence of 10^{-7}M VIP. Luciferase activity was measured after 6h of culture. *p<0.001 vs. CD3/CD28-activated controls.

Supplementary Figure S9.- VIP mediates the suppressive effect by elevating cAMP levels. Human T cells were CD3/CD28-stimulated in the absence or presence of forskolin (10^{-6}M), 8-Br-cAMP (0.1 mM), or VIP (10^{-7}M) with or without H89 (50 ng/ml) or Rp-cAMP (0.1 mM). The expression and phosphorylation status of cyclins, cdk, $p27^{kip1}$, FOXO1, Akt, Raf1 and ERK1/2 and the activation of cyclin-cdk complexes and Ras were determined as detailed in Figures 5-8. Blots are representative of 3-4 experiments.

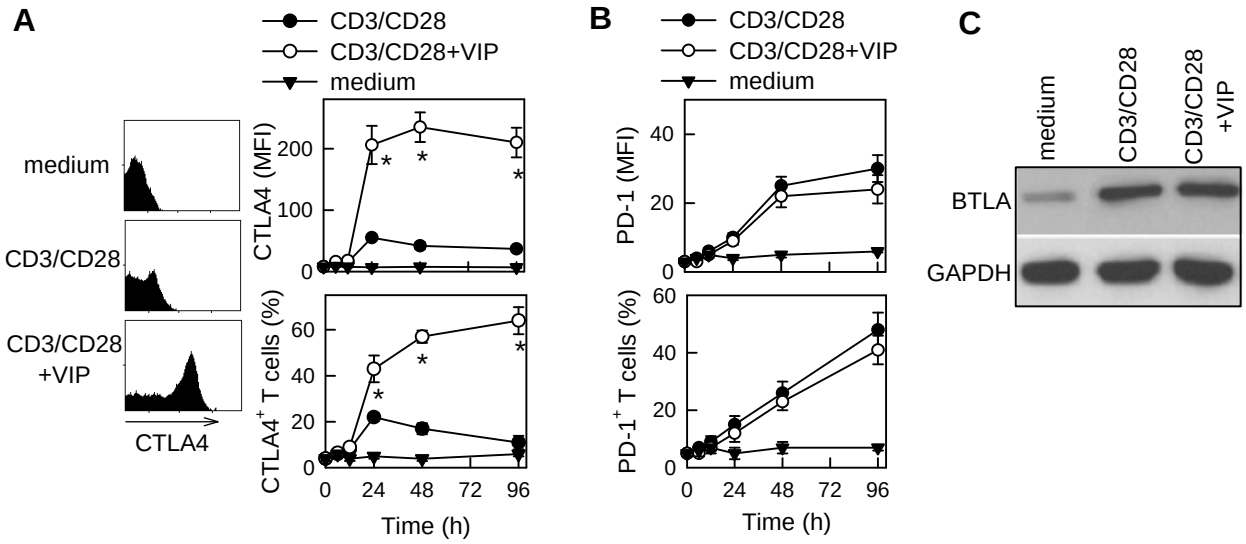
Supplementary Figure S10.- Potential mechanisms involved in the VIP induction of cell cycle arrest and regulatory functions on human T cells. Panel A: T cell signaling via TCR/CD3-CD28 costimulation activates various intracellular pathways that regulate entry into the cell cycle and progression through the G1 phase and IL-2 production. Signals through the Ras-Raf1-ERK MAPK and PI3K-PDK-Akt cascades cooperate to induce the synthesis and activation of cdk4, cdk6, cdk2 and D-type cyclins. The cyclin D-cdk4/6 complex is responsible for phosphorylation of pRb and activation of the first wave of E2F transcription factor early in the G1 phase, that triggers the synthesis of cdk2 and cyclin E. Active cyclin E-cdk2 complexes also phosphorylate pRb, resulting in a second wave of E2F activity, which induces genes involved in DNA replication and

progression through S phase. Activation of the cyclin E-cdk2 holoenzyme depends on the dissociation of the cdk inhibitor $p27^{kip1}$ from the complex. This is achieved by degradation of $p27^{kip1}$, usually initiated through phosphorylation by cdk2 and other kinases including Akt and ERK1/2. Furthermore, $p27^{kip1}$ can be regulated at the transcriptional level by the transcription factor FOXO1, which is sequestrated at the cytosol upon phosphorylation by Akt. Finally, $p27^{kip1}$ activity can also be diminished by sequestering it in cyclin D-cdk4 complexes, or by nuclear exclusion, which is preceded by phosphorylation of $p27^{kip1}$ by Akt. On the other hand, signal through Raf1-ERK pathway activates the transcription factor AP-1 that together with NFAT and NFkB translocate to nucleus and activate gene expression of IL-2. Once secreted, IL-2 acts as a mitogenic factor for T cells by signaling through IL-2 receptor and activating various pathways, including Akt, Ras-Raf1 and STAT5. **Panel B:** VIP binding to its receptor (VPAC1) increases cAMP and activates PKA, which could regulate T cell cycle and activation at multiple levels. First, VIP downregulates the PI3K-Akt pathway in two ways: by directly inhibiting PI3K activity and by inducing the dephosphorylation/inactivation of Akt by the phosphatase PP2A. Consequently, Akt-mediated phosphorylations of $p27^{kip1}$ and FOXO1 are blocked, and then, FOXO1-induced $p27^{kip1}$ gene expression could be promoted as well as the $p27^{kip1}$ binding and inactivation of the cyclin E-cdk2 complexes. Inhibition of Akt activity and of cyclin D3 synthesis by VIP could also affect the activity of cdk4-cyclin D complexes. The regulation of both early and late events on G1 phase results in an inefficient progression to S phase. Second, VIP inhibits signaling through the Ras-Raf1-MEK1-ERK1 cascade by decreasing Ras activity and also by impairing Raf1-Ras interaction probably by phosphorylating Raf1 in two Ser residues. This could deactivate AP-1 and reduce its binding to the IL-2 promoter. Moreover, VIP decreases the nuclear translocation of NFkB and NFAT. Consequently, IL-2 gene transcription is compromised, something that could contribute to a hyporesponsive state. IL-2 can partially bypass the suppressive effect of VIP on cell cycle, because although VIP impairs IL-2-mediated Akt and Ras signaling, it fails to block STAT5-mediated activation of cell cycle players (not depicted in the diagram). Third, VIP increases the expression of

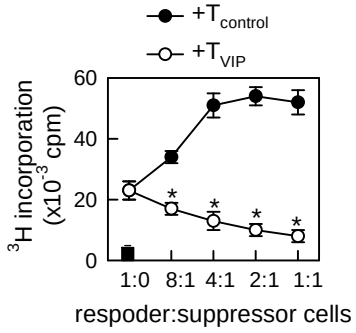
both soluble and membrane forms of CTLA4, which are critically involved on the induction on the expression of FoxP3 and on the regulatory activity of the VIP-converted T_{reg}.



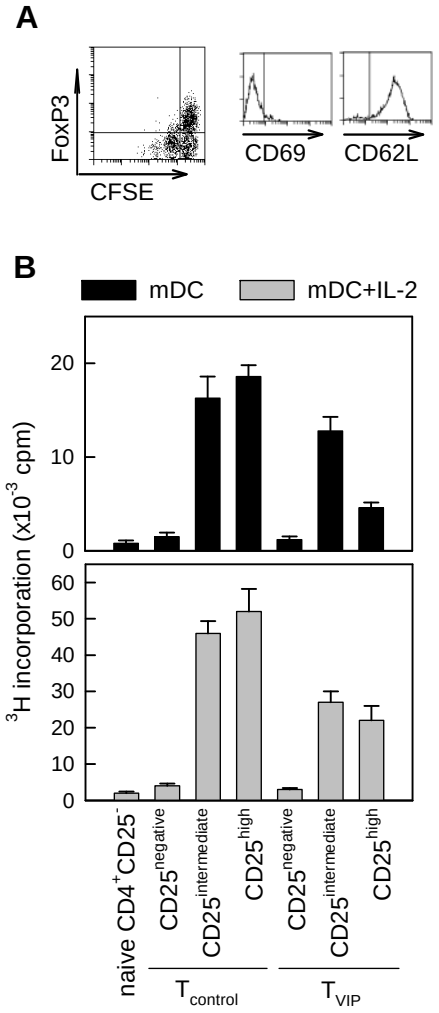
Supplementary Figure S1



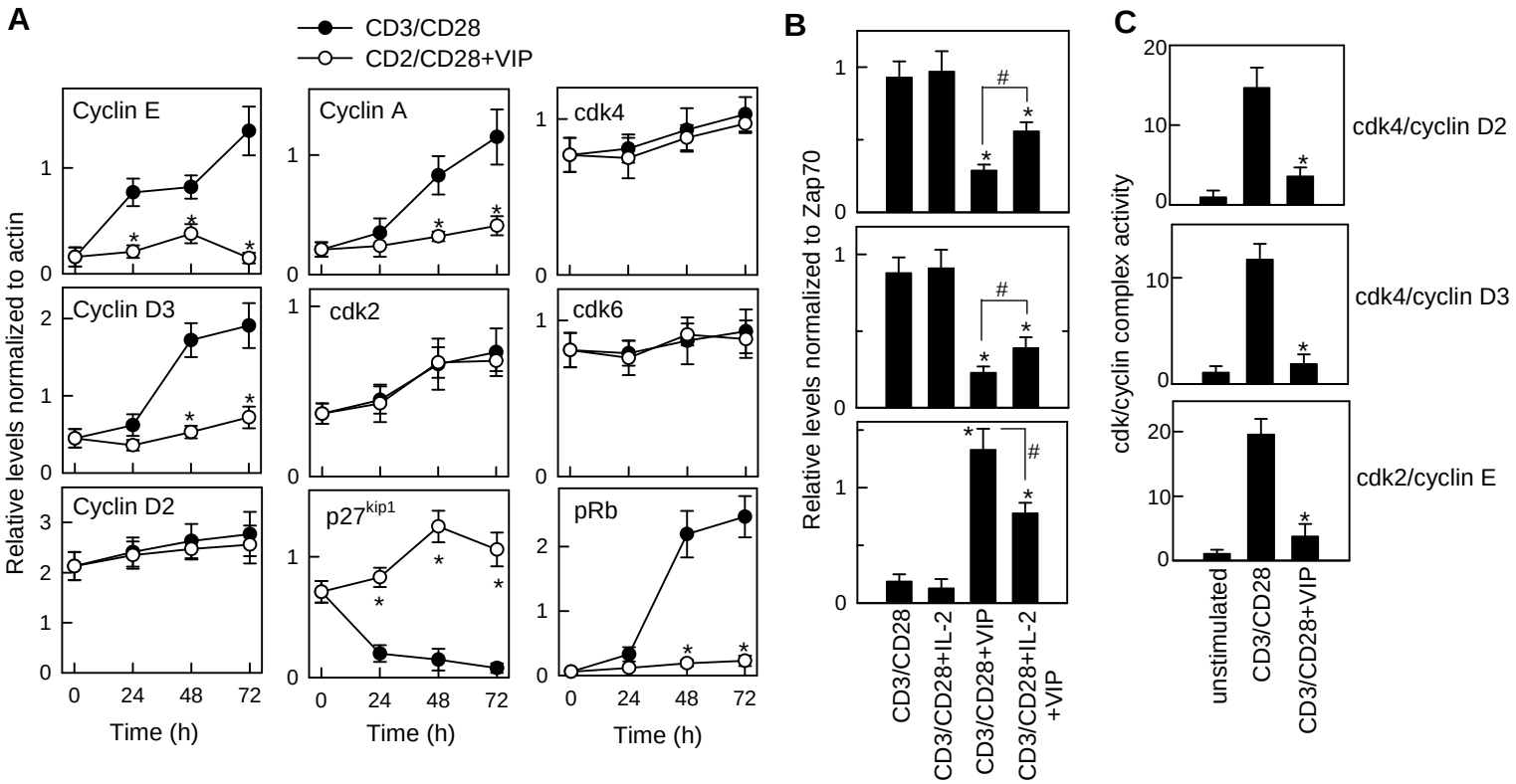
Supplementary Figure S2



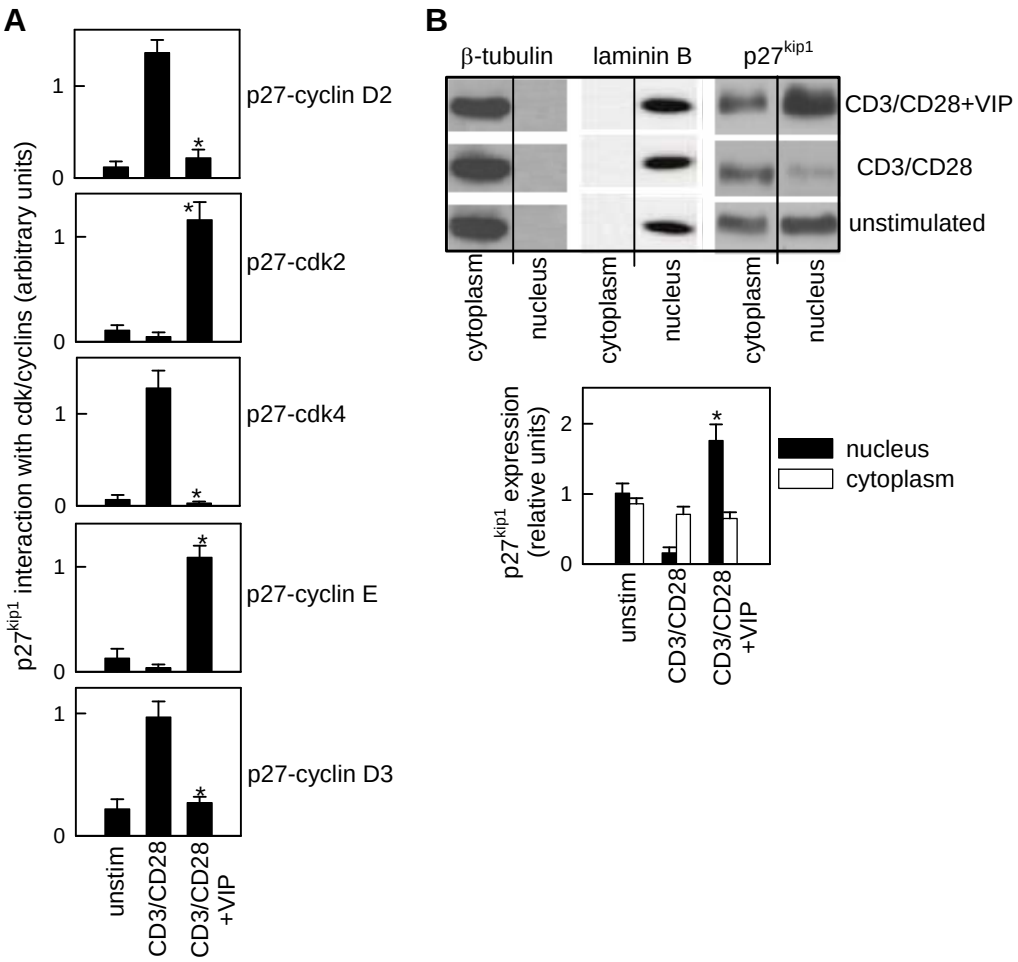
Supplementary Figure S3



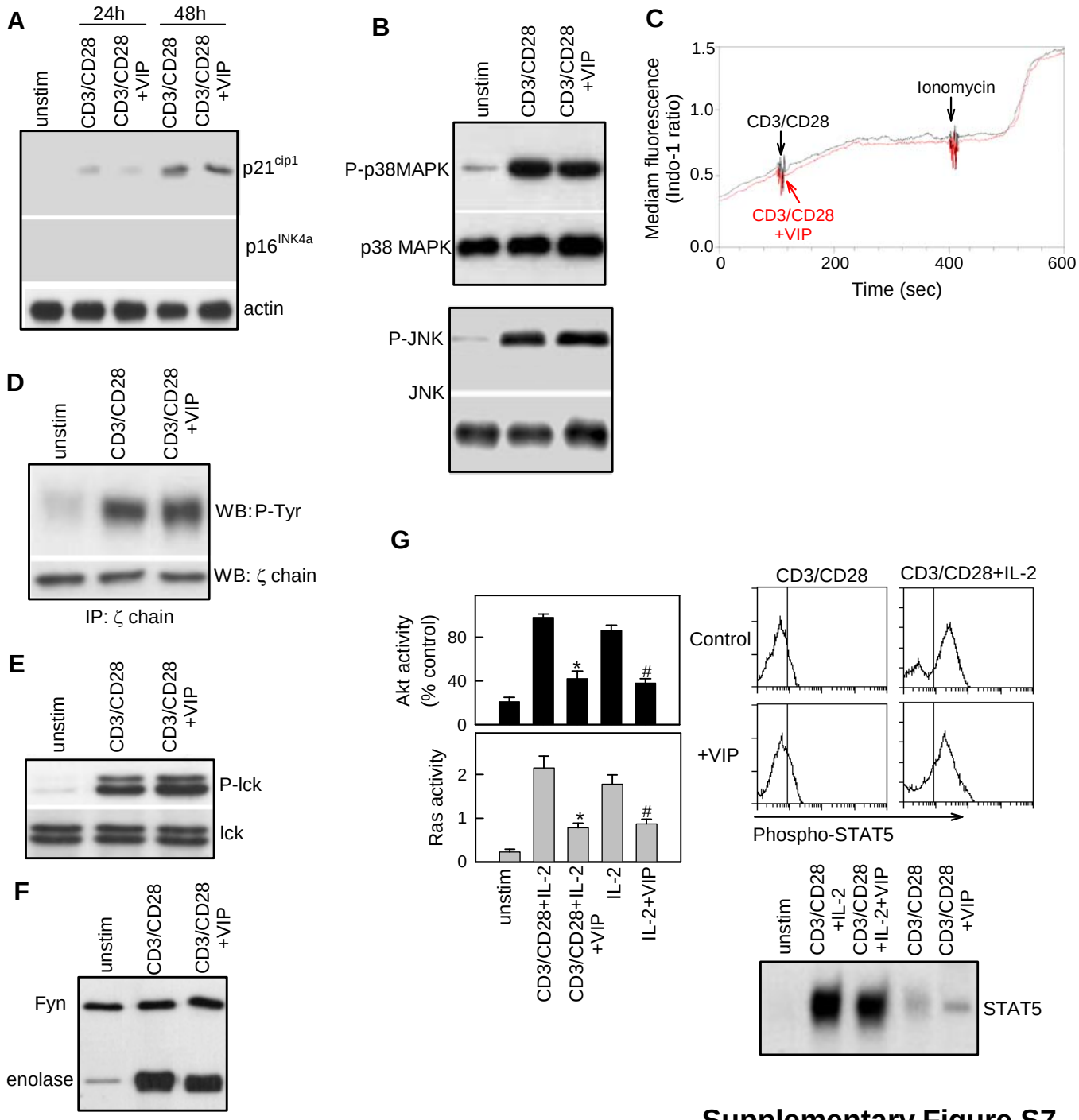
Supplementary Figure S4



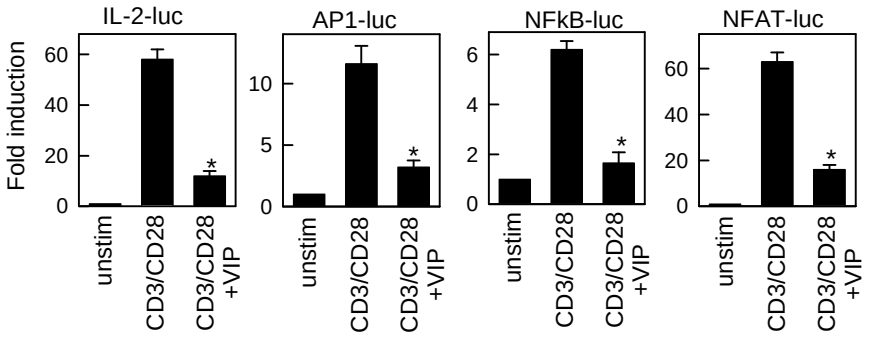
Supplementary Fig. S5



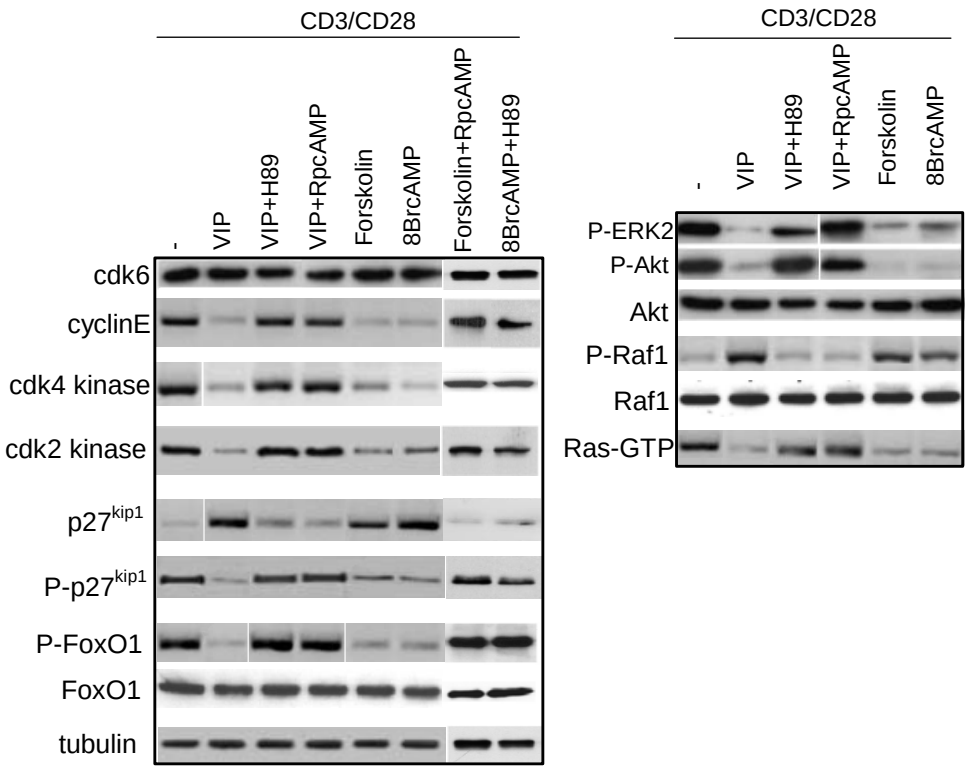
Supplementary Figure S6



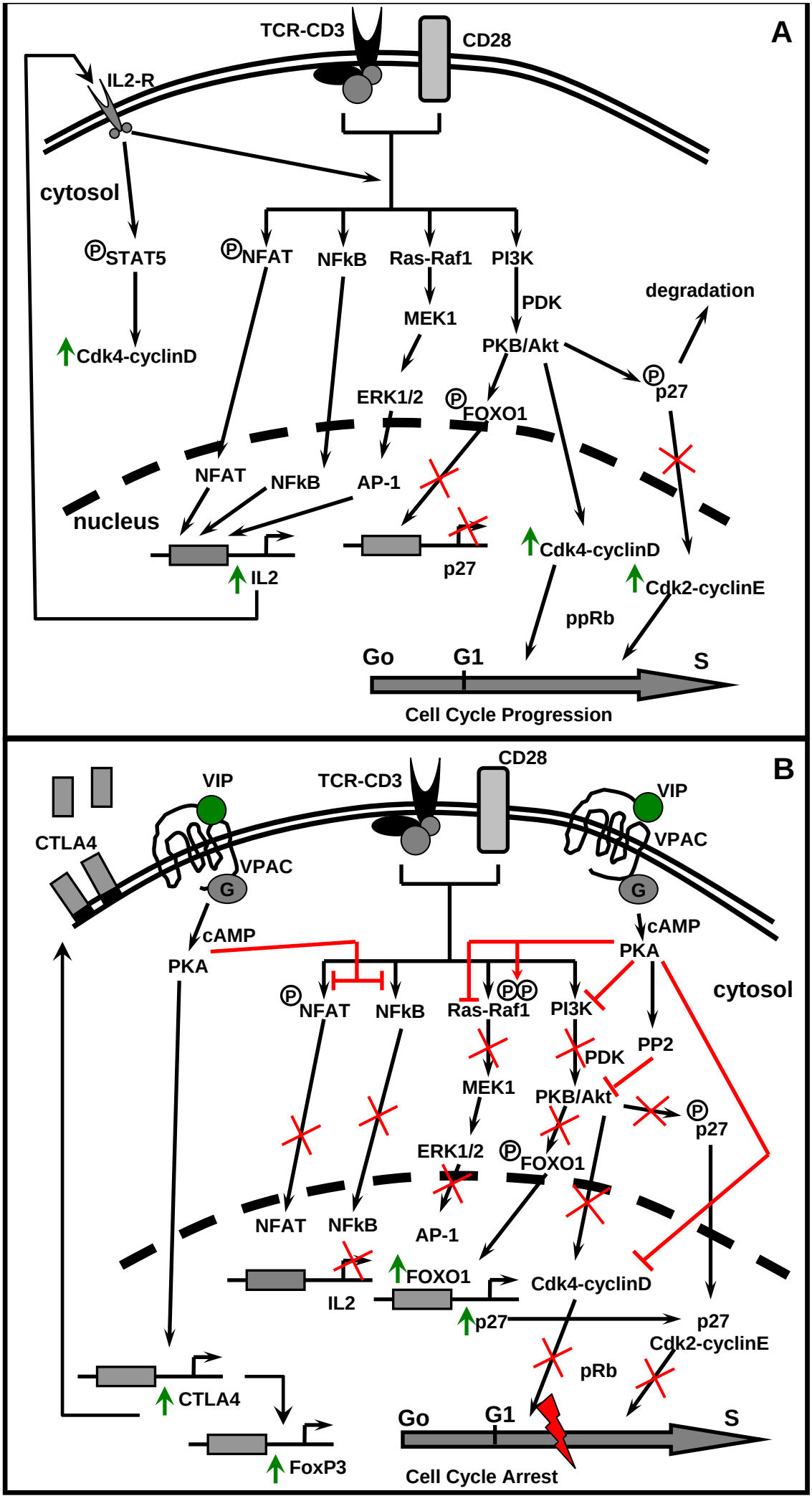
Supplementary Figure S7



Supplementary Figure S8



Supplementary Figure S9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FOXO1
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Cell Cycle Progression
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