Cutting edge: abortive proliferation of CD46-induced Tr1-like cells due to a defective Akt/Survivin signaling pathway.

Grégory Meiffren, Monique Flacher, Olga Azocar, Chantal Rabourdin-Combe, Mathias Faure

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Running Title : **Deficiency of Akt activation drives Tr1 abortive proliferation**

Grégory Meiffren, Monique Flacher, Olga Azocar, Chantal Rabourdin-Combe and Mathias Faure

Inserm U503 – IFR128 Biosciences Lyon Gerland – UCBLyon1

21, Avenue Tony Garnier

69365 Lyon cedex 7, France

Corresponding author:

Dr. Mathias Faure

phone : +33 4 37 28 23 77

fax : +33 4 37 28 23 41

e-mail : faure@cervi-lyon.inserm.fr

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ABSTRACT

T-regulatory cell 1 (Tr1) are low proliferating peripherally-induced suppressive T cells. Engaging CD3 and CD46 on human $CD4^+$ T cells induces a Tr1-like phenotype. Here, we report that human Tr1-like cells do not sustain proliferation over time. The weak proliferation of these cells results first from their inability to sustain expression of various cell cycle-associated proteins, to efficiently degrade the inhibitor of cell cycle progression p27/Kip1, and, as a consequence, in their accumulation in the G0/G1 phase. Second, the reduced proliferation of Tr1-like cells results from their increased sensitivity to death as they divide, through a mechanism that is neither Fas-mediated nor Bcl2/Bcl-xL-related. Both properties, impaired cell cycle and death sensitivity, are explained by a specific defective activation of Akt which impairs the expression of Survivin. Thus, our results show that CD3/CD46-induced Tr1-like cells die through a process of abortive proliferation.

INTRODUCTION

Regulatory T cells are potent cells that inhibit self-antigen and innocuous-foreign antigen-specific adaptive immune effectors (1). Disrupting their functional activity often results in clinical pathologies. Moreover, pathogens and tumor cells can subvert immune responses by deviating regulatory T cells to their benefit (1, 2). Therefore, identifying what triggers the generation of regulatory T cells and understanding their biology appear to be required to struggle certain diseases and/or to efficiently use them as therapeutic tools (3).

Among regulatory T cells, the peripherally-induced Tr1 cells were shown to control inflammation and allergy, to induce transplantation tolerance and to suppress immune responses to pathogens both in mice and humans (4-7). Tr1 cells are characterized by their production of IL-10 and IFN-γ, their inability to secrete IL-2 and IL-4 and their poor proliferative potential (4). Tr1 cells were differentiated *in vivo* from mice injected with antigen-pulsed IL-10-treated DC (8) and *in vitro* from $CD4^+$ T cells cultured with their cognate antigen in the presence of IL-10 both in mice and human (4). Recently, it has been reported that the minimal engagement of the TCR and CD46 induces human $CD4^+$ T cells with a Tr1-like phenotype, able to suppress T cell proliferation through the production of IL-10 (9). CD46 is a complement regulator that we initially described for its potential to co-stimulate T cells (10, 11). Nevertheless, unlike Tr1 cells, CD3/CD46-induced Tr1-like cells were described to exhibit a strong and prolonged proliferation (9).

Using low concentration of anti-CD3 to better assess for the co-stimulatory role of CD46, we differentiated weakly proliferating human Tr1 cells. We report here that the poor proliferation of CD3/CD46-induced Tr1 cells results from a defect in cell cycle

progression and an increased sensitivity to cell death, both mechanisms being explained by a defective Akt pathway.

MATERIALS AND METHODS

Cells and antibodies

Human $CD4^+$ T cells (>95% purity) were purified from blood donors (EFS Lyon, France) as described (10) and cultured in complete RPMI. For cell culture, the mAbs used were: anti-CD3, (OKT3, 1μg/ml, otherwise indicated); anti-CD46, (20.6, 10μg/ml); anti-CD28, (CD28.2, 10μg/ml) (all Abs purified in our laboratory); anti-CD95, (ZB4, 1μg/ml); isotype control (10μg/ml) (both from Beckman-Coulter Immunotech, Marseille, France). For FACS analysis, anti-CD25-PE, anti-CD69-FITC and anti-CD71-FITC were from Beckman-Coulter. For western blot, the Abs used were: anti-Survivin (D-8), and the rabbit polyclonal Abs specific for Akt, p27/Kip1, Cyclin A, Cyclin E and Cyclin-dependent-kinase-2 (Cdk2) (all from Santa Cruz Biotech. Inc., CA US); anti-Cyclin D1 (DCS6) and anti-phospho-Akt, (193H12) (both from Cell Signaling Technologies, Beverly, MA, US); anti-phospho-Erk1/2 (12D4) and the rabbit polyclonal Abs specific for Erk1/2 (both from Upstate Biotechnology, Lake Placid, NY) and anti-actin (Sigma-Aldrich, St Quentin Fallavier, France).

Cell stimulation, proliferation and cytokine production analysis

Naive $CD4^+$ T cells were cultured on plates coated with the indicated mAbs, at a concentration of $1x10^6$ T cells/ml. When indicated, 10 U/ml of recombinant human IL-2 was added (a gift from Dr. Demetrier-Caux). ^{3}H-Thymidine incorporation assays were as described (10). Cytokines were quantified from the supernatant of 3 days stimulated T cells by ELISA according to manufacturer's instructions: IL-2, TNF-α and IL-8 (R&D Systems, Lille, France), IL-4, IL-5, INF-γ and IL-13 (Pierce Perbio, Rockford, IL, US), IL-10 and TGF-β (Bender, Vienne, Austria).

Flow cytometry

For cell death assessments, T cells were labeled with 67nM of ToPro-3 (Molecular Probes, Leiden, The Netherlands). To determine cell division numbers, $2x10^7$ T cells/mL (RPMI 2%FCS) were kept at 37°C for 13 min after addition of 0,5M CFSE (Molecular Probes), washed 3 times in cold RPMI 10%FCS and cultured for various periods of time before being analyzed by FACS. For cell cycle analysis, $0,5x10^6$ $CD4^+$ T cells were labeled 30min at room-temperature with 20µM 7-Aminoactinomycin D (7-AAD) in 500µL of 0,03% saponin/NASS Buffer (100mM phosphate-citrate buffer pH=6, 150mM NaCl, 5mM EDTA, 0,5% BSA). Cells were kept on ice 5min and 5µM PY (Sigma) was added prior analysis.

Western blot analysis

T cells were lysed in ice-cold buffer, the protein concentration from each lysate determined (Micro-BCA kit, Pierce Perbio) and 50 µg of proteins were analyzed by SDS-PAGE and Western blotting as described (11).

Real-time RT-PCR

One µg of mRNA was extracted from cultured T cells with Trizol (Invitrogen, Carlsbad, CA) and reverse transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen), 80pg of random hexamer primers (Promega, Charbonnières, France) and 150ng of oligo$(dT)_{12-18}$ (Invitrogen). Real-Time PCR was performed with Platinium SYBR Green qPCR Supermix UDG (Invitrogen) on an Applied Biosystems GeneAmp 7600 thermocycler. The TATA-box Binding Protein (TBP) was used as housekeeping gene for mRNA normalization. The specificity of amplification was checked after each run and for each sample with a melting curve.

The TBP-specific primers were: F-5'-TGCTCATACCGTGCTGCTATCTG-3' and R-5'-TTCTCCCTCAAACCAACTTGTCAAC-3'. Bcl-2 and Bcl-xL primers were a gift from Dr. N. Bonnefoy-Berard. Primers amplification efficiencies were 1.90 for TBP, 1.94 for Bcl-2 and 1.96 for Bcl-xL.

RESULTS AND DISCUSSION

Weakly proliferating CD3/CD46-differentiated Tr1-like cells

We previously reported that CD46 provides a co-stimulatory signal for human T cells (10). It was subsequently described that, in addition, CD46 favors the differentiation of human Tr1-like regulatory cells (9). To allow for an efficient assessment of the role of CD46 co-stimulation in Tr1 cell differentiation, we stimulated freshly purified naive human $CD4^+$ T cells with an immobilized non-saturating concentration of anti-CD3 (1μg/ml) in combination with anti-CD46 (Fig. 1A left panel). Such CD3/CD46-stimulated T cells displayed a weak proliferation that was reminiscent to the Tr1 cell phenotype. On the contrary, as previously reported (9), higher concentrations of anti-CD3 did allow for a more sustained proliferation of CD3/CD46-stimulated compared to CD3/CD28-stimulated (control effector) T cells (Fig. 1A).

We then ensured that using a limiting concentration of anti-CD3 induced functional Tr1 cells. As shown in Fig. 1B low proliferating CD3/CD46-stimulated $CD4^+$ T cells produced indeed the described phenotype of Tr1 cells (4, 9, 12). They produced IL-10, IFN-γ and IL-5, but neither IL-2 nor IL-4. However, they did not produce TGF-β, a cytokine produced at low levels when saturating concentrations of anti-CD3 were used (9). Moreover, low proliferating CD3/CD46-stimulated $CD4^+$ T cells produced higher concentrations of IL-8 than CD3-stimulated $CD4^+$ T cells (0.45 ± 0.07 ng/ml versus 0.19 ± 0.03 ng/ml, respectively), but equivalent IL-13 (0.4 ng/ml versus 0,38ng/ml, respectively) and no significant levels of IL-6 and TNF-α were produced (data not shown.

Finally the supernatant of weakly proliferating CD3/CD46-stimulated T cells efficiently suppressed the proliferation of CD3/CD28-stimulated $CD4^+$ T cells in a dose dependant manner, whereas the supernatant of CD3-stimulated T cells did not

(Fig. 1C), confirming the regulatory phenotype of these cells. Altogether, these results clearly demonstrate that CD46 is a sufficient co-signal for the differentiation of weakly proliferating human suppressive Tr1 cells.

Cell cycle defect of proliferating Tr1-like cells

We reasoned as if the poor proliferation of Tr1-like cells could result from their inability to sustain cell division. A FACS analysis of CFSE-labeled stimulated naive $CD4^+$ T cells revealed that both CD46 and CD28 co-stimulated T cells started to divide by day 3 with a similar kinetic (Fig. 2A). However, by day 5 of stimulation Tr1-like cells hardly performed more than 4 cycles of division (5% divided ≥5 times) compared with control effector T cells (21% divided ≥5 times) (Fig. 2A).

The incompetence of Tr1-like cells to sustain cell division might result from a cell-intrinsic deficiency in signal(s) controlling the progression of the cell cycle. Therefore, we analyzed the expression/down regulation of proteins associated with cell cycle in CD3/CD46-stimulated T cells. Three days post stimulation, CD3/CD46-differentiated Tr1-like cells up-regulated the expression of the cyclins A, D1 and E and of Cdk2 to a similar extend than CD3/CD28-stimulated $CD4^+$ T cells. However, by day 5, CD3/CD46-stimulated T cells failed to sustain the expression of the cyclins (Fig. 2B).

We then examined the down regulation of the inhibitor of cell cycle progression p27/Kip1. Whereas CD28-co-stimulated T cells degraded p27/Kip1 as soon as day 1 post activation and maintained such degradation up to day 5, CD46-co-stimulated T cells only partially down regulated p27/Kip1 mainly on day 3 (Fig. 2B). Since the degradation of p27/Kip1 is required for cells to progress from the G0/G1 phase to the S phase of the cell cycle (13), we wondered if CD3/CD46-stimulated T cells might

have a defect in cell cycle progression. Staining cells with 7-AAD and PY revealed that by day 3 of culture CD3/CD46-stimulated T cells accumulated in the G0/G1 phase more abundantly than CD3/CD28-stimulated T cells (Fig. 2C). This result indicates that the lack of sustained proliferation of CD3/CD46-generated Tr1-like cells is due, at least partially, to a G0/G1 blockage in their cell cycle progression.

Cell death sensitivity of dividing Tr1-like cells due to abortive proliferation

In addition to a cell-intrinsic defect in cell cycle progression, the lack of sustained Tr1-like cell proliferation could result from an increased sensitivity of these cells to death. Therefore, we examined the survival of proliferating CD3/CD46-stimulated T cells by determining the rate of dead cells by ToPro-3 staining after 3, 4 and 5 days of culture. Until day 4, $CD4^+$ T cells displayed comparable level of apoptosis whatever the nature of the stimulation, CD3, CD3/CD28 or CD3/CD46 (Fig. 3A). However, by day 5 more than 30% of the CD3/CD46-stimulated cells were apoptotic whereas less than 20% of cells in all other conditions of culture were ToPro-3^+. We then asked if the sensitivity to death of Tr1-like cells correlated with their proliferation since Tr1 are low proliferating cells. Indeed, the most the 5 days CD3/CD46-stimulated T cells divided, the most they underwent apoptosis with more than 40% of the cells that divided 5 times that were ToPro-3^+ (Fig. 3B). On the contrary, whatever the number of division considered, we constantly observed about 20% of CD3/CD28-stimulated T cells that were ToPro-3^+.

Since Tr1-like cells do not produce IL-2, it was possible that CD3/CD46-stimulated T cells died because of the absence of a survival signal provided by IL-2. However, adding IL-2 to the CD3/CD46-stimulated T cell culture only marginally rescued differentiated Tr1-like cells from death (Fig. 3C). Therefore, the defective

proliferation of Tr1-like cells is due not only to a limited potential of proliferation but also to an increased sensitivity to cell death.

Likewise, proliferating T cells may become sensitive to death through a Fas/FasL-mediated mechanism (14). However, preventing the interaction between Fas and FasL with a blocking anti-Fas mAb did not rescue CD3/CD46-stimulated $CD4^+$ T cells from death (Fig. 3D).

Finally, the death of CD3/CD46-stimulated T cells could result from a defect of Bcl-2 and/or Bcl-xL expression, two anti-apoptotic genes crucial for T cell survival (15). However, as revealed by real-time RT-PCR analysis from 3 days stimulated T cells, CD46 and CD28 co-stimulation up-regulated the expression of Bcl-xL mRNA to an equivalent extend, above the one observed from CD3-alone-stimulated T cells (Fig. 3E). Moreover, the up-regulation of the Bcl-2 mRNA induced in CD3-stimulated T cells was neither impaired nor increased when cells were co-stimulated with either CD46 or CD28. Thus, proliferating Tr1-like cell death is not related to a defect of expression of either Bcl-2 or Bcl-xL. Altogether our results strongly suggest that the CD3/CD46-differentiated Tr1-like cells die because of an abortive proliferation mechanism.

Defective expression of the Akt/Survivin pathway in Tr1-like cells

The differences in terms of proliferation and survival between Tr1-like cells and effector T cells, evoked us that an important intracellular signaling pathway might be impaired in CD3/CD46-stimulated naive T cells. The PI(3)K/Akt pathway appeared to be a privileged target since Akt activation is crucial in CD28 co-stimulation dependant T cell proliferation and survival (16, 17). We looked for Akt activation by western blot using an activated-Akt specific mAb. Very interestingly, only marginal

activation of Akt was detected from lysate of T cells stimulated for 3 days with CD3 and CD46 (Fig. 4A). On the contrary, as expected Akt was heavily activated in 3 days CD3/CD28-stimulated T cells and such activation was strongly maintained until day 5. The defective Akt phosphorylation pathway in CD46-induced Tr1 cells was specific since the Erk1/2 MAPK pathway was equivalently phosphorylated from 3 day CD3/CD46- and CD3/CD28-stimulated naive $CD4^+$ T cells (Fig. 4A). Moreover, although the PI(3)K/Akt pathway was required for CD3/CD46-activated T cell proliferation, the sensitivity of such cells to PI(3)K-specific inhibitors, wortmannin and LY2940002 , was much more exacerbated compared to CD3/CD28-stimulated T cells and close to CD3-activated T cells, confirming the weak Akt activation in Tr1-like cells (Fig. 4B and data not shown). On the contrary, CD3/CD46-stimulated $CD4^+$ T cells were as sensitive as CD3/CD28-stimulated $CD4^+$ T cells towards the MEK/Erk1/2 specific inhibitor U0126 (Fig. 4C).

In murine T cells, the activation of the Akt kinase regulates the expression of Survivin, a gene recently described to be crucial to maintain murine CD3/CD28-stimulated $CD4^+$ T cell division over time and to antagonize T cell apoptosis (18). We therefore assessed if an absence of Survivin expression might explain the sensitivity of the Tr1-like cells to death. First, we found that, as in mouse T cells, the co-stimulation of human $CD4^+$ T cells with CD3/CD28 did induce the expression of the Survivin protein by day 3 (Fig. 4D). Second, CD3/CD46-stimulated T cells did not up regulate the expression of Survivin over the level observed from CD3-stimulated T cells. Therefore, the impairment of CD46 to allow the induction of expression of Survivin probably explains why Trl-like cells have a limited proliferative potential and exhibit an increased potency to death.

The present work demonstrate that human CD3/CD46-induced Tr1 cells undergo abortive proliferation caused by a defect of cell cycle progression associated with a death of proliferating cells. The identification of a defective Akt pathway in human Tr1-like cells explain this particular phenotype and highlights several biological characteristics of these regulatory T cells. First, it explains why Tr1-like cells are incompetent at producing IL-2. This cytokine is produced by T cells when both Akt and ERK signaling pathways are activated (19), however we and others reported that the ERK pathway is fully activated in CD3/CD46-stimulated T cells (Fig. 4A, 11, 20). Second, the Akt pathway is required to optimally degrade p27/Kip1 (16), an inefficient event of Tr1-like cells, that is necessary for an efficient T cell proliferation. Third, the Akt pathway is crucial for the antigen-induced T cell survival and regulates the Survivin expression (17, 18), two processes that we show here to be defective in Tr1-like cells. Since Bcl-xL induction requires Akt activation it is possible that, in CD3/CD46-stimulated naive $CD4^+$ T cells, the weak Akt activation is sufficient to induce Bcl-xL expression but not to sustain proliferation and Survivin expression.

The nature of the signals involved in Tr1 differentiation *in vivo* still remains elusive and might be of circumstances. By engaging CD46, at least one self (complement C3b factor) and one nonself (streptococcal M protein) molecules have been shown to induce Tr1 cells (9, 21). Whatever the nature of the ligand engaging CD46 is, our results describe a mechanism that might be crucial to control the functional activity of the peripherally-CD46-induced Tr1 cells in order to limit bystander incongruous immunosuppression. Indeed, this control could be achieved by the elimination of activated Tr1 cells through a process of abortive proliferation.

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Disclosures

The authors have no financial conflict of interest.

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Footnotes

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Abbreviation

Tr1: T-regulatory cell 1.

FIGURE LEGENDS

Figure 1: CD3/CD46 co-ligation induces weakly proliferating functional Tr1-like cells. (A) Naive human $CD4^+$ T cells were stimulated with the indicated concentration of anti-CD3 mAbs alone or in combination with 10µg/ml of either anti-CD46 or anti-CD28. T cell proliferation was measured by ^{3}H-thymidine incorporation up to 5 days of culture. (B) $CD4^+$ T cells were stimulated 3 days with anti-CD3 (1µg/ml) mAbs alone or in combination with 10µg/ml of either anti-CD46 or anti-CD28 and the culture supernatants were collected for cytokine quantification by ELISA. (C) $CD4^+$ T cells were cultured with anti-CD3 (1µg/ml) + anti-CD28 (10µg/ml) mAbs in the presence of medium (Δ) or of serial dilutions of supernatants of 3 days CD3 (□) or CD3+CD46 (●) stimulated T cells. Proliferation was measured by ^{3}H-thymidine incorporation after 3 days of culture. (** P<0,001 and * P<0,01). Results are representative of 2 (C) to 3 (A-B) independent experiments. When indicated, the ±SE is from means of triplicates of one out of 2 or 3 independent experiments.

Figure 2: Accumulation of CD3/CD46-activated $CD4^+$ T cells in the G0/G1 phase. (A) CFSE-labeled naive human $CD4^+$ T cells were activated as indicated and proliferation was analyzed by FACS at day 3, 4 and 5 of culture. (B) $CD4^+$ T cells were activated with the indicated mAbs. At the mentioned time point, cells were lysed and 50µg of proteins for each condition were analyzed by SDS-PAGE and western blotted for the indicated proteins. (C) $CD4^+$ T cells were activated as indicated for 3 days. Cells were then stained with 7-AAD and Pyronin Y (PY) and analyzed by FACS. Percentage of cells in each cell cycle phases is given. Results are representative of 3 independent experiments.

Figure 3: CD3/CD46 co-ligation induces abortive proliferation in $CD4^+$ T cells. (A) Naive $CD4^+$ T cells were stimulated as indicated for 3, 4 or 5 days, stained with ToPro-3 and the % of ToPro-3^+ cells was determined by FACS. (B) $CD4^+$ T cells were CFSE-labeled and activated as indicated. At day 5, cells were stained with ToPro-3 and analyzed by FACS to determine the % of ToPro-3^+ cells according to the number of cell division. (C) Cells were analyzed as in (B) but 10U/ml of recombinant IL-2 was added during cultures. (D) CFSE-labeled $CD4^+$ T cells were stimulated with the indicated mAbs in the presence or not of the blocking anti-Fas mAb ZB4. After 5 days, cells were labeled with ToPro-3 to determine the % of dead cell per cell division. (E) $CD4^+$ T cells were activated as indicated for 3 days before mRNA extraction. Bcl-2 or Bcl-xL mRNA expression was determined by real-time RT-PCR. The relative mRNA levels were normalized to the TATA box binding protein (TBP) gene. (** $P<0,001$ and * $P<0,01$). Results are representative of one out of three independent experiments. When indicated, the ±SE is from means of triplicates of one out of 3 independent experiments.

Figure 4: Defective Akt activation and Survivin expression in CD3/CD46 co-stimulated T cells. (A) Naive $CD4^+$ T cells were activated up to 5 days as indicated. At each time point, cells were lysed and 50µg of proteins were analyzed as in Fig. 2B. Akt phosphorylation (p-Akt), Erk1/2 phosphorylation (p-Erk) and total Akt, Erk1/2 and Actin were assessed by western blot. (B and C) $CD4^+$ T cells were stimulated with the indicated mAbs in the presence of serial concentrations of Wortmannin (B) or U0126 (C) and by day 3 T cell proliferation was measured by ^{3}H-thymidine incorporation. For each point, the % of inhibition of proliferation was determined as follow: 100 - (cpm with inhibitor x 100 / cpm without inhibitor). (D) Survivin

expression was determined as in (A). Results are representative of 3 independent experiments.

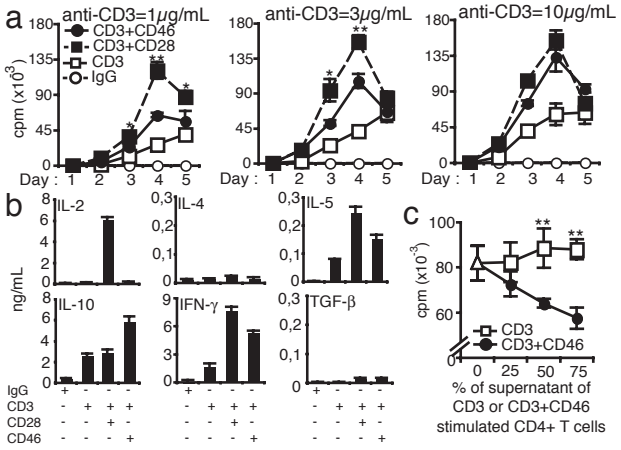

Figure 1

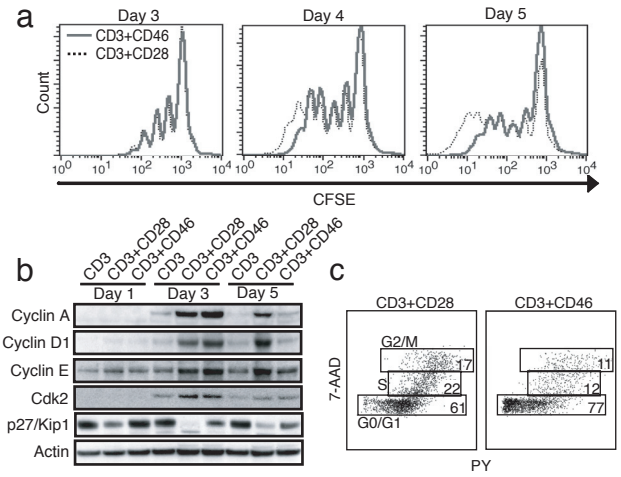

Figure 2

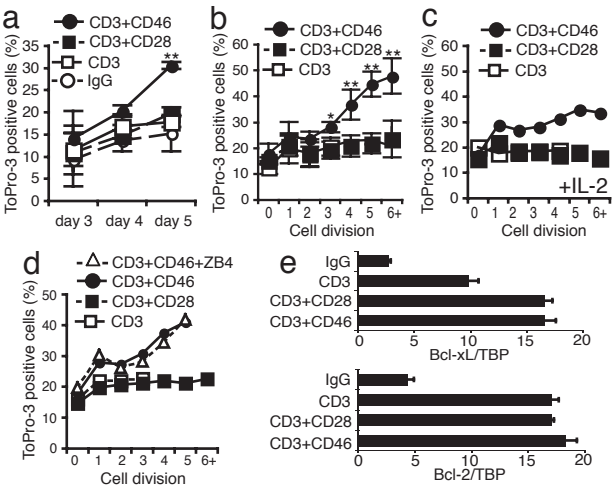

Figure 3

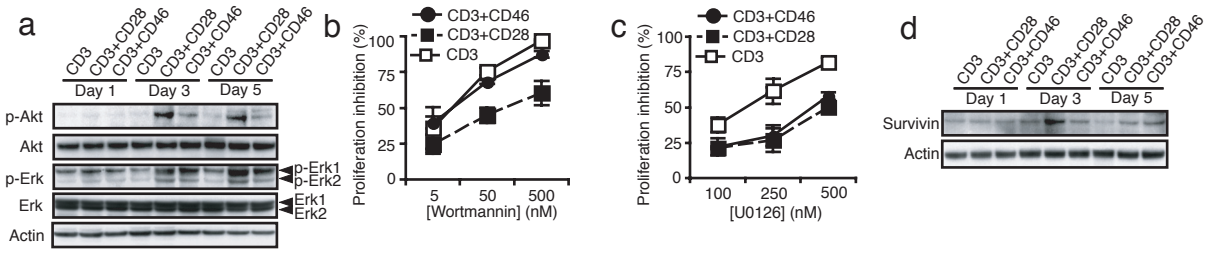

Figure 4