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Cell cycle progression following naïve T cell activation is independent of Jak3/γc cytokine signals[1]

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Abstract

T cell proliferation following activation is an essential aspect of the adaptive immune response. Multiple factors, such as TCR signaling, costimulation, and signals from cytokines each contribute to determine the magnitude of T cell expansion. In this report, we examine in detail the role of Jak3/γc-dependent cytokines in promoting cell cycle progression and proliferation of naïve T cells. Using naïve $CD4^+$ T cells from Jak3-deficient mice and wild type $CD4^+$ T cells treated with a small molecule inhibitor of Jak3, we find that these cytokine signals are not required for proliferation; instead they are important for the survival of activated T cells. In addition, we show that the percentage of cells entering the cell cycle and the percentage of cells in each round of cell division are comparable between Jak3-deficent and wild-type T cells. Further, cell cycle progression and the regulated expression of key cell cycle proteins are independent of Jak3/γc cytokine signals. These findings hold true over a wide range of TCR signal strengths. However, when CD28 costimulatory signals, but not TCR signals, are limiting, Jak3-dependent cytokine signals become necessary for the proliferation of naïve T cells. As CD28 signaling has been found to be dispensable for autoreactive T cell responses, these data suggest the potential for interfering with autoimmune T cell responses by inhibition of Jak3 signaling.

Introduction

T cell proliferation is essential for mounting an effective adaptive immune response. A key element of proliferation is the entry of cells into the cell cycle, a complex process that is tightly controlled by the ordered expression of cyclins, the activation of cyclin-dependent kinase (Cdk) enzymatic activity and the subsequent phosphorylation of relevant substrates. The first cyclin expressed during the G1 phase is a D-type cyclin, which is a rate-limiting factor for cell cycle progression from the G1 to the S phase. The induction of cyclin E occurs at the late G1 restriction point, and cyclin A is expressed at S phase entry (1). The activity of Cdks is stimulated by cyclins and inhibited by cyclin-dependent kinase inhibitors (CDKI), such as p27kip1. Cyclin/Cdk complexes phosphorylate the retinoblastoma (Rb) gene product, leading to the activation of the E2F transcription factor, which is required for the transcription of S phase genes.

T cell proliferation is induced following stimulation of the T cell receptor (TCR) and costimulatory molecules; in addition, cytokines such as IL-2 and IL-4, that signal through receptors sharing the common γ (γc) chain, have been shown to promote lymphocyte

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proliferation (2). Among these, IL-2 has long been recognized as the most potent T cell growth factor (3). *In vitro* studies have shown that IL-2 very efficiently promotes the growth of antigen-activated T cells (4,5). Antigen- or mitogen-induced T cell proliferation *in vitro* can be substantially inhibited using monoclonal antibodies specific for IL-2 or the IL2R, suggesting that IL-2 is an essential element in T cell proliferation (6-8). In later studies, it was found that IL-2 promotes the transit of T cells through G1 to S phase of the cell cycle by up-regulating cyclin D2, cyclin D3, cyclin E and E2F, and down-regulating p27kip1 (9-12). Based on these findings, among others, the consensus view is that TCR and CD28 stimulation induce quiescent T cells to leave G0 and enter the G1 phase of the cell cycle (13); in addition, these signals induce the expression of the high-affinity IL-2 receptor and stabilize the IL-2 message, rendering the cells competent for IL-2-driven proliferation.

Recent studies performed in intact animals have challenged this view and demonstrated IL-2- or γc cytokine-independent T cell expansion *in vivo*. When adoptively-transferred, IL-2-deficient or IL-2R-deficient DO11.10 T cells challenged with OVA peptide underwent comparable expansion compared to wild type T cells (14,15). Similarly, after correcting the autoimmune defect in IL-2Rβ-deficient mice by selective expression of IL-2Rβ in the thymus, IL-2R $\beta^{-/-}$ T cells also showed normal expansion during both primary and secondary immune responses (16). Finally, Di Santo and colleagues reported that naïve γc chain-deficient T cells proliferate robustly in response to antigenic stimulation in *vivo* (17). Together, these results indicate that γc cytokine signals are not absolutely required *in vivo* for T cell proliferation.

Several *in vitro* studies also suggest that T cell proliferation can occur in an IL-2-independent manner. For instance, except under conditions of suboptimal stimulation, IL-2 or IL-2R antibody blockade cannot completely inhibit T cell proliferation (18,19). Further, IL-2- or IL-2R-deficient T cells can be induced to proliferate in response to specific antigens or mitogens, although the proliferation is generally reduced compared with that of control T cells (20-23). Finally, several studies have suggested that TCR plus CD28 stimulation controls cell cycle progression independently of IL-2. Using IL-2 or IL-2R blocking antibodies, or IL-2-deficient cells, these studies indicated that TCR/CD28 engagement could promote T cell proliferation by inducing the expression of cyclin D and cyclin E, enhancing the transcriptional activity of E2F, and down-regulating the inhibitory function of p27kip1 (24-30).

However, there are a number of caveats with these studies that have hampered the general acceptance of the view that T cell proliferation does not require IL-2 or other γc cytokine signals. First, the failure to completely block T cell proliferation with IL-2 or IL-2R antibodies may reflect the lower affinity of these interactions relative to the affinity of IL-2 for its receptor. Second, several of these studies were performed using tumor cell lines or anergic T cells, which may not reflect the requirements of primary naive T cells. A similar concern applies to the studies using IL-2- or IL-2R-deficient T cells, as these cells are also not naïve T cells (14). Finally, these *in vitro* studies did not rule out the possibility that T cell proliferation was being induced by γc cytokines other than IL-2; although IL-2 is the main γc cytokine that is secreted when T cells are initially activated *in vitro*, IL-4 and IL-21 are also produced by activated T cells and can promote T cell proliferation (31-33). Therefore, none of these studies conclusively demonstrated that naïve T cell proliferation was γc cytokine-independent.

In this report, we investigated the requirement of γc cytokines in the proliferation and cell cycle control of primary naïve T cells *in vitro*. We analyzed the proliferation of naïve $CD4^+$ T cells from the mice lacking Janus kinase 3 (Jak3), a tyrosine kinase that is exclusively associated with γc chain and is essential for signaling via all γc cytokine receptors (34-39). We complemented these experiments with analysis of wild type naïve $CD4^+$ T cells treated with a pharmacological inhibitor of Jak3. Together, these studies demonstrated that Jak3-dependent γc cytokine signals are not required for naïve primary $CD4^+$ T cell proliferation and cell cycle

regulation *in vitro*. We also show that when CD28 costimulatory signals are limiting, Jak3-dependent cytokine signals become necessary for the proliferation of naïve T cells. As CD28 signaling has been found to be dispensable for autoreactive T cell responses, these data suggest the potential for interfering with autoimmune T cell responses by inhibition of Jak3 signaling.

Materials and Methods

Mice and reagents

Jak3$^{-/-}$ mice (40) were backcrossed to C57BL/6 for ten generations. OTII-transgenic (41) and Bcl-2-transgenic (42) mice were purchased from Jackson laboratory (Bar Harbor, ME). *Jak3*$^{-/-}$ mice were crossed to OTII- and Bcl-2-transgenic mice to generate *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OTII+ Bcl-2-transgenic mice. All mice were maintained in pathogen-free conditions and used between 6-10 weeks of age. PS078507 was developed at Pharmacopeia, Inc. (Princeton, NJ). A stock solution (10 mM) was prepared by dissolving PS078507 in dimethylsulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO). All working compound solutions were made by serial dilutions in buffers or culture medium. DMSO was used as a vehicle control.

Cell preparation and activation

Thymocytes were harvested from Jak3$^{+/-}$ and Jak3$^{-/-}$ OT-II Bcl-2 mice, red blood cells were lysed, and cells were incubated with anti-CD4-PE (BD Pharmingen, San Diego, CA) and anti-CD8-APC (BD Pharmingen) antibodies. CD4 SP thymocytes were sorted by flow cytometry on a Mo-Flo sorter (Cytomation, Fort Collins, CO) to a purity of >98%. Splenocytes from Jak3$^{+/+}$ OTII-transgenic mice were incubated with anti-CD4 antibody-coated magnetic microbeads, and CD4^{+} T cells were purified by positive selection (Miltenyi, Auburn, CA) to a purity of >94%. T cells were stimulated with plate-bound anti-CD3 (1 μg/ml) plus anti-CD28 (4 μg/ml) antibodies (eBioscience, San Diego, CA), unless the concentrations were specified. T cells were cultured in 24-well plates at 1.2×10^6 cells/ml in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum, 2 mM glutamine, 100 iu/ml penicillin, 100 ug/ml streptomycin, 50 μg/ml geneticin, 2 uM β-mercaptoethanol, and 25 mM HEPES.

Analysis of cell proliferation by thymidine incorporation

Isolated CD4^{+} CD44^{lo} cells were plated out at a density of 1×10^5 cells /180 μl in triplicate in a 96-well flat-bottomed plate and stimulated with various concentrations of anti-CD3 and anti-CD28 or mitomycin C-treated C57BL/6 antigen presenting cells (APCs) plus the indicated concentrations of $OVA_{323-339}$. Forty-eight hours later, cells were pulsed with one microcurie of [3H]-thymidine for 18h, and harvested on a Tomtec Harvester 96 (Orange, CT). Thymidine incorporation was quantified on a Trilux microbeta counter (PerkinElmer, Wellesley, MA).

Analysis of cell proliferation with carboxyfluorescein diacetate succinimidyl ester (CFSE)

Isolated cells were washed once in PBS and resuspended at a density of 2.5×10^7 cells/ml in PBS. CFSE was added to a final concentration of 2.5 μM. The cell suspension was mixed thoroughly and placed at 37°C for 12 min and the reaction was terminated by adding RPMI 1640 with 10% fetal calf serum. The cells were then plated at 1.2×10^6 cells/ml in 24-well plates, stimulated with plate-bound anti-CD3 (1 μg/ml) and anti-CD28 (4 μg/ml) antibodies or $OVA_{323-339}$ peptide (1 μg) presented by mitomycin C-treated C57BL/6 APCs, in the absence or presence of the Jak3 inhibitor PS078507 (312nM) or IL-2 blocking antibodies (10 μg/ml of anti-IL-2, anti-CD25 and anti-CD122 antibodies; eBiosciences, Inc.). Three days later, the fluorescence of the cells was determined by flow cytometry.

Apoptotic analysis

Stimulated T cells were harvested, washed with cold PBS and resuspended in 1× BD Pharmingen™ Annexin V Binding Buffer to achieve a final concentration of 10.0×10^6 cells/ml. 5 μl of Annexin V-FITC and 7-AAD were added to 100 μl solution (~1×10^6 cells) and incubated at room temperature for 15 min in the dark. Apoptosis was analyzed by flow cytometry within 1h.

Cell cycle analysis by flow cytometry

One million naïve or activated T cells were washed with cold PBS and fixed overnight at -20° C in 95% ethanol. Cells were then pelleted, washed and resuspended in 1 ml PBS with the propidium iodide (PI) at a final concentration of 20 μg/ml, ribonuclease at 20 μg/ml and EDTA at 2mM. The cells were incubated at 37°C for 30 min in the dark. PI content was assessed by flow cytometry.

Immunoblot

Naïve cells or activated cells were harvested at the indicated time points, washed and lysed in RIPA buffer for 20 min on ice. The protein fraction was separated by centrifugation at 13,000 rpm for 10 min at 4°C and protein level was quantified with the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Proteins were separated on SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were immunoblotted with antibodies to cyclin D2 (Santa Cruz Biotechnology, Santa Cruz, CA), cyclin D3 (Santa Cruz Biotechnology), cyclin E (Santa Cruz Biotechnology), cyclin A (Santa Cruz Biotechnology), p27kip1 (Santa Cruz Biotechnology), Stat5 (Santa Cruz Biotechnology), PI-3-kinase p85 (Cell Signaling Technology, Danvers, MA), phospho-Stat5 (Cell Signaling Technology) and β-actin (BD Pharmingen) antibodies.

Statistical Analysis

Statistical analysis was performed using the two-tailed paired student's t test.

Results

Jak3-dependent cytokine signals are required for optimal T cell responses *in vitro*

To analyze the role of Jak3-dependent cytokine signals in T cell proliferation, we generated naïve T cells as described previously (43), by crossing *Jak3*$^{-/-}$ mice to the OT-II TCR transgenic mouse line, and then to a Bcl-2 transgenic line to improve the *in vivo* survival of the *Jak3*$^{-/-}$ T cells (hereafter referred to as *Jak3*$^{-/-}$ OT-II Bcl-2 mice). Previous studies have shown that IL-2-induced phosphorylation of Stat5 requires the activity of Jak3 (37). To confirm this result, we examined Stat5 phosphorylation in *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl-2 cells in response to IL-2 stimulation. As shown in Supplemental Figure 1, Stat5 phosphorylation in response to IL-2 stimulation is completely abolished in the absence of Jak3. Purified CD4$^+$ CD44^{lo} T cells from *Jak3*$^{+/-}$ or *Jak3*$^{-/-}$ OT-II Bcl-2 mice were then stimulated with varying concentrations of OVA peptide presented by mitomycin C-treated antigen presenting cells (APCs) from C57BL/6 mice (Fig. 1A) or anti-CD3 and anti-CD28 antibodies (Fig. 1B). As shown, *Jak3*$^{-/-}$ CD4$^+$ CD44^{lo} T cells were capable of proliferating in response to all stimuli, although the magnitude of the response was reduced in comparison to that of control cells. This reduced proliferation of *Jak3*$^{-/-}$ T cells could result from impaired T cell proliferation or impaired T cell survival, or both.

T cell survival, but not proliferation, is affected by Jak3-dependent cytokines

γc cytokines, especially IL-2, IL-4 and IL-7, promote T cell survival by up-regulating the anti-apoptotic factor Bcl-2 (44-47). In Jak3- or γc-deficient T cells, the expression of Bcl-2 is greatly decreased (17,38). To determine whether constitutive expression of Bcl-2 is sufficient to

reverse the survival defect of *Jak3*$^{-/-}$ T cells *in vitro*, we examined T cells for evidence of apoptosis. For these studies we used CD4^{+}CD8^{-} single-positive (CD4SP) thymocytes from *Jak3*$^{+/-}$ or *Jak3*$^{-/-}$ OT-II Bcl-2 mice as a source of homogeneous naïve T cells. Following 3 days of stimulation, cells from *Jak3*$^{-/-}$ mice showed a significantly higher degree of cell death compared to control cells (Fig. 2A). These findings indicate that Jak3-dependent cytokine signals normally induce a survival pathway that cannot be compensated for by constitutive expression of Bcl-2. Thus, in the absence of Jak3, the reduced level of ^{3}H-thymidine incorporation following T cell activation may be due to enhanced apoptosis, rather than impaired proliferation.

T cell proliferation can be assessed using the fluorescent dye, carboxyfluorescein succinimidyl ester (CFSE), providing a means of excluding dead cells in the population and of visualizing the proportion of cells representing each successive cell division cycle. As can be seen, after 3 days of stimulation with anti-CD3 and anti-CD28 antibodies, there was no significant difference in the percentage of divided cells between Jak3-deficient and Jak3-positive T cells, suggesting that Jak3-dependent cytokine signals do not regulate the proportion of cells entering the cell cycle (Fig. 2B, C). To examine this issue more carefully, we determined the percentage of cells in each round of cell division, and compiled the data from four independent experiments (Fig. 2D). This analysis confirmed that T cells lacking Jak3 showed comparable proliferative capacity to T cells expressing Jak3. Similarly, a comparison of the proliferation index as well as the division index between the two populations of T cells indicated no significant differences in the average number of divisions that cells of each genotype underwent. Taken together, these data indicate that Jak3-dependent cytokine signals are not required for naïve T cell proliferation *in vitro*, but are important in maintaining maximum T cell survival.

To confirm that IL-2 signals play no role in naïve T cell proliferation *in vitro*, we examined the consequences of adding exogenous IL-2 or blocking IL-2 in our experiments. As shown, addition of exogenous IL-2, or alternatively, addition of IL-2 blocking antibodies, did not alter the proliferation of wild-type naïve CD4^{+} T cells in response to either anti-CD3 and anti-CD28 antibody stimulation or $OVA_{323-339}$ peptide stimulation (Fig. 2E). In addition, blocking of *Jak3*$^{-/-}$ CD4^{+} T cells with IL-2 neutralizing antibodies had no effect on T cell proliferation (Fig. 2F).

Pharmacological inhibition of Jak3 impairs T cell survival, but not proliferation

To rule out the possibility that *Jak3*$^{-/-}$ T cells are developmentally abnormal, leading to their independence from Jak3-dependent cytokine signals for T cell proliferation, we examined the responses of *Jak3*$^{+/+}$ CD4^{+} T cells treated with a small molecule inhibitor of Jak3, PS078507 (43). The optimal concentration of PS078507 for Jak3 inhibition in murine peripheral CD4^{+} T cells was determined by examining IL-2-induced Stat5 phosphorylation (Fig. 3A). The effect of PS078507 on cell survival was then assessed following 3 days of *in vitro* stimulation. As shown, naïve *Jak3*$^{+/+}$ CD4^{+} T cells from OT-II transgenic mice displayed dramatically higher levels of apoptosis in the presence of the inhibitor, regardless of the expression of Bcl-2 (Fig. 3B). Consistent with the data shown above using *Jak3*$^{-/-}$ T cells, when cell proliferation was determined using CFSE dilution, *Jak3*$^{+/+}$ naïve CD4^{+} T cells treated with PS078507 showed a comparable proportion of divided cells compared to cells treated with vehicle alone (Fig. 3C). Further, the percentage of divided cells in each successive cell division cycle also showed no significant difference compared to controls, when averaged over four independent experiments (Fig. 3D). Together, these data indicate that cell survival, but not proliferation, is regulated by Jak3-dependent cytokine signals.

Cell cycle regulation is independent of Jak3 signaling

The precise function of TCR/CD28 signaling versus cytokine signaling in regulating naïve $CD4^+$ T cell proliferation *in vitro* remains ambiguous. To address this issue, we examined cell cycle progression of naïve T cells stimulated in the presence or absence of the Jak3 inhibitor, PS078507. *Jak3*$^{+/+}$ CD4+ T cells from OT-II transgenic mice were stimulated, harvested at a variety of timepoints, and analyzed for DNA content using propidium iodide (PI). The intensity of the PI signal is directly proportional to DNA content, with the rightmost peak on the histogram representing the cells in the G2/M phase, the leftmost peak showing the cells in the G0/G1 phase, and the area between the two peaks indicating the cells within the S phase (Fig. 4A). To compare cell cycle progression between samples, we calculated the percentage of live cells in each phase, and averaged the data from three independent experiments (Fig. 4B). This analysis indicated that CD4+ T cells lacking Jak3-dependent cytokine signals have indistinguishable cell cycle kinetics relative to cells receiving these cytokine signals ($P > 0.05$).

Cyclins and cyclin-dependent kinase inhibitors (CDKI) are the key regulators of cell cycle progression. The early stage of the G1 phase is regulated by D-type cyclins; in T cells, cyclin D2 is the first to be induced following activation, followed by cyclin D3 (48). To examine cell cycle progression at the molecular level, we determined whether TCR and CD28 stimulation could independently induce the expression of cyclin D3, in the absence of Jak3-dependent cytokine signals. As shown in Figure 5A, CD4 SP thymocytes from *Jak3*$^{-/-}$ OT-II Bcl-2 mice up-regulate cyclin D3 comparably to cells from control mice at day three of activation. Timepoint analysis further confirmed that the level of cyclin D2 increased at 48h and peaked at 96h after activation in both *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl-2 cells (Fig. 5A, lower panel). Whereas cyclin D2/D3 controls the entry of cells into the G1 phase, passage through the G1 restriction point into the late G1 stage requires the induction of cyclin E. When we examined cyclin E expression, we also saw no difference between *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl-2 cells at 48h or 96h post-activation (Fig. 5A).

To drive cell cycle progression, cyclins associate with cyclin-dependent kinases to form active holoenzymes. These holoenzymes are inhibited by CDKIs; specifically, p27kip1, which is constitutively expressed in resting naïve T cells, inhibits the activities of cyclinD2/cdk4/6 and cyclinE/cdk2 (27). Therefore, cell cycle progression depends on the down-regulation of p27kip1, in addition to the up-regulation of cyclins. When levels of p27kip1 were examined in activated *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl-2 cells, we found that p27kip1 was undetectable in both cell types by 3 days post-stimulation (Fig. 5A). Finally, we examined cyclin A, which is required for the cells to progress through S phase. Again, upregulation of cyclin A was comparable between *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ T cells (Fig. 5A). The upregulation of cyclin A and the loss of p27kip1 were confirmed with wild-type T cells stimulated in the presence of the Jak3 inhibitor, PS078507. Furthermore, an extensive timecourse indicated no differences in the kinetics of these changes with or without Jak3-dependent cytokine signals (Fig. 5B).

CD28 costimulation substitutes for cytokine signals to drive T cell proliferation

Our findings thus far indicated that Jak3-dependent cytokine signals are not required for T cell proliferation or cell cycle progression. However, it remained possible that cytokine-independent T cell proliferation requires strong TCR and/or CD28 stimulation. To examine whether Jak3-dependent cytokine signals are more essential under conditions of suboptimal T cell stimulation, we performed titration experiments in which the strength of TCR or CD28 stimulation was varied. As the anti-CD3 and anti-CD28 antibodies were titrated down, a substantial reduction in T cell proliferation was observed. The inhibition of Jak3 activity had no effect at any of the conditions tested, nor did addition of exogenous IL-2 (Fig. 6A). In a second set of experiments, the concentration of anti-CD28 antibody was fixed at 4 μg/ml and the anti-CD3 antibody was varied. As shown in figure 6A, T cell proliferation was largely

unaffected by this wide range of TCR stimulation conditions, and was also independent of Jak3 signaling. Finally, we fixed the concentration of anti-CD3 antibody at 5 μg/ml and varied the concentration of anti-CD28 antibody. In the absence of the Jak3 inhibitor, CD4+ T cells proliferated robustly in response to each stimulation condition. However, when CD28 stimulation was limiting, inhibition of Jak3-dependent cytokine signaling led to a marked inhibition of T cell proliferation (Fig. 6A).

To investigate this issue at the biochemical level, we examined the expression of cell cycle regulatory proteins by immunoblotting. Consistent with the CFSE analysis of T cell proliferation, under conditions of strong CD28 stimulation (4 μg/ml), blocking of Jak3 signals by a Jak3 inhibitor did not affect the expression of cyclin D3, cyclin E, cyclin A or the degradation of p27 (Fig. 6B, lanes 2 and 3). When CD28 stimulation was limiting (0.1 μg/ml), Jak3-sufficient cells were still able to up-regulate the expression of cyclin D3, cyclin E and cyclin A, and down-regulate p27 (Fig 6B, lane 4); in contrast, inhibition of Jak3-dependent cytokine signals under these conditions resulted in a dramatic decrease in the expression cyclin D3, cyclin E and cylin A and an increase in p27 expression (Fig 6B, lane 5). Similar results can be seen when comparing *Jak3*$^{+/-}$ to *Jak3*$^{-/-}$ OT-II Bcl-2 cells (Fig 6C). These results indicate that optimal CD28 stimulation can replace Jak3-dependent cytokine signals to drive T cell cycle progression, and further, that when CD28 costimulatory signals, but not TCR signals, are limiting, T cell proliferation and cell cycle progression become cytokine-dependent.

Discussion

Generation of a pool of daughter cells from a small number of naïve T cells is an essential step for the adaptive immune response against pathogens. Following clearance of an infection, the expanded effector cells are eliminated to prevent the pathological accumulation of these potent cells. Thus, both cell proliferation and cell death must be tightly controlled to maintain the integrity of the immune system. In this report, we demonstrate that Jak3-dependent cytokine signals are not required for naïve CD4+ T cell *in vitro* proliferation; instead, they are critical for cell survival. Optimal TCR/CD28 signaling, in the absence of cytokine signals, induces cell cycle progression by modulating cell cycle regulators, such as cyclins and p27kip1. However, we find that under suboptimal stimulation conditions, CD28 signaling is critical in promoting IL-2-independent T cell proliferation.

T cell proliferation is generally accompanied by cell death, an essential aspect of preventing the over-expansion of immune cells. However, during a productive immune response, the proliferating T cells must be temporarily protected from cell death. The role of γc cytokines in promoting the survival of resting T cells, and in maintaining the population of naïve T cells *in vivo*, is well established (49). However, evidence demonstration that activated T cells can be killed by cytokine withdrawal also points out the key role of cytokine signals, in particular γc cytokine signals, in promoting the survival of activated T cells (50). This latter role of γc cytokine signals does not appear to be mediated solely through regulation of Bcl-2. Although constitutive expression of Bcl-2 leads to the accumulation of nearly normal numbers of naïve resting CD4^{+} T cells in Jak3$^{-/-}$ or γc$^{-/-}$ mice (43,49), Bcl-2 expression only partially reverses the impaired survival of activated Jak3$^{-/-}$ T cells.

These data suggest that γc cytokine signals differentially regulate the survival of naïve versus activated CD4^{+} T cells. In this regard, a subgroup of Bcl-2 family proteins, the Bcl-2 homology (BH) 3-only proteins, play an essential role in activated T cell death (51,52), leading to the conclusion that it is the ratio of BH3-only proteins (pro-apoptotic) to Bcl-2-like proteins (anti-apoptotic) that determines whether activated T cells live or die (50). In a second mechanism, the NFκB-regulator/coactivator Bcl-3 has also been implicated in activated T cell death (53, 54). Thus, Jak3-dependent cytokine signals acting in the first several days following T cell

activation may prevent the upregulation of BH3-only proteins or induce the expression of Bcl-3.

While IL-2 has been shown to be important for many aspects of the immune response, including Th1, Th2, and Th17 differentiation (19,43,55), regulatory T cell development (56,57), as well as CD4 and CD8 cell memory generation and maintenance (58-61), the issue of whether IL-2 is required for T cell proliferation has been a long-standing controversy. Initial experiments characterizing IL-2 demonstrated that this cytokine functions as a T cell growth factor, leading to the conclusion that IL-2 is crucial for T cell proliferation (3-5). More recent studies clearly indicate that T cells are capable of proliferating in the absence of IL-2R signals. However, the proliferative responses of IL-2- or IL-2R-deficient cells to antigen stimulation are highly variable among experiments. Some experiments show that these responses are only 5-10% of control T cells (20,62,63). Other experiments demonstrate that the proliferative responses of T cells lacking IL-2 signals are 50% of control T cells, and that the replication of these cells is limited to only 2-3 rounds of cell division (23).

In contrast to these earlier findings, our studies indicate that naïve T cell proliferation occurs independently of all γc cytokine signals, including IL-2. Further, we show that T cells lacking IL-2 signals are capable of undergoing at least 7 rounds of cell division *in vitro*. The discrepancy between our results and those reported previously is likely due to two factors. First, the traditional method used to measure T cell proliferation is by tritiated-thymidine incorporation. As this technique cannot distinguish between reduced proliferation and increased cell death, it is likely that the proliferative responses of T cells in the absence of IL-2 were underestimated in earlier studies. Second, some previous studies were performed with mixtures of naïve T cells and activated/memory like T cells. Since activated/memory like T cells may be more dependent on IL-2 for clonal expansion, this may also have led to conclusions not entirely applicable to naïve CD4+ T cells.

Since IL-2 is not the sole cytokine secreted by activated T cells, we expanded our study to simultaneously assess the role of all γc cytokines in T cell proliferation by targeting the downstream kinase, Jak3, required for all of these receptor signaling pathways. A previous study had indicated that γc-deficient $CD4^+$ T cells could be activated and proliferate *in vivo* following antigenic stimulation (17). Our results, that naïve CD4+ T cell proliferation is independent of cytokines, are consistent these in vivo findings, as well as with a recent paper from Di Santo and colleagues, who reported robust *in vitro* proliferation of $\gamma c^{-/-}$ T cells (49). These data indicated that signaling through the TCR plus CD28 is sufficient to induce T cell proliferation. However, this latter study limited their analysis to examining CFSE profiles of T cells at day 4-6 post-activation. No examination at early timepoints after activation, no cell cycle analysis, no biochemical analysis of cell cycle regulators, and no assessment of the differential roles of TCR versus CD28 signaling were included in this former study. Thus we have extended these previous findings with a more thorough examination of cell cycle progression in the absence of Jak3-dependent cytokine signals, and have also discovered an important role for cytokines when costimulatory signals are limiting.

Past studies using IL-2 and IL-2R blocking antibodies or IL-2-deficient cells, have shown that TCR/CD28 stimulation directly promotes the upregulation of cyclins and the downregulation p27kip1. However, in these studies it was difficult to determine whether the blocking antibodies were able to completely inhibit IL-2R signaling, and in addition, activated T cells secrete other γc cytokines that may contribute to the regulation of cell cycle proteins. Therefore, we examined this issue using two approaches, each of which leads to complete inhibition of all γc cytokine signaling pathways. Our results indicate that a genetic deficiency in Jak3, or pharmacological inhibition of Jak3, have no effect on cell cycle progression following optimal stimulation of the TCR plus CD28. Upregulation of cyclin D2, cyclin E and cyclin A, as well

as downregulation of P27kip1, are indistinguishable in the presence versus the absence of cytokine signaling. This conclusion was also confirmed by a timecourse of cell cycle analysis quantitating the progression of cells through each phase of the cell cycle.

When antigen-presenting cells encounter pathogenic microorganisms, they up-regulate the expression of B7 to induce T cell responses (64,65). When T cells are stimulated under these conditions, their proliferation is γc cytokine-independent. In our studies, this cytokine-independent proliferation was observed over an extensive dynamic range of TCR signaling. However, in the course of spontaneous autoimmune diseases, such as the type I diabetes observed in nonobese diabetic (NOD) mice, CD28 costimulatory signaling has been shown to be dispensable for autoreactive T cell responses; furthermore, in this system, the lack of CD28 signaling results in the exacerbation of disease (66,67). While this latter phenomenon results from impaired regulatory T cell function, these findings also confirm that T cell activation, expansion, and effector cell differentiation can occur in a pathophysiological setting in the absence of CD28 costimulation. As our studies reveal that CD28-independent T cell proliferation is, instead, reliant on Jak3-dependent cytokine signals, these data suggest the potential for interfering with autoimmune T cell responses by inhibition of Jak3 signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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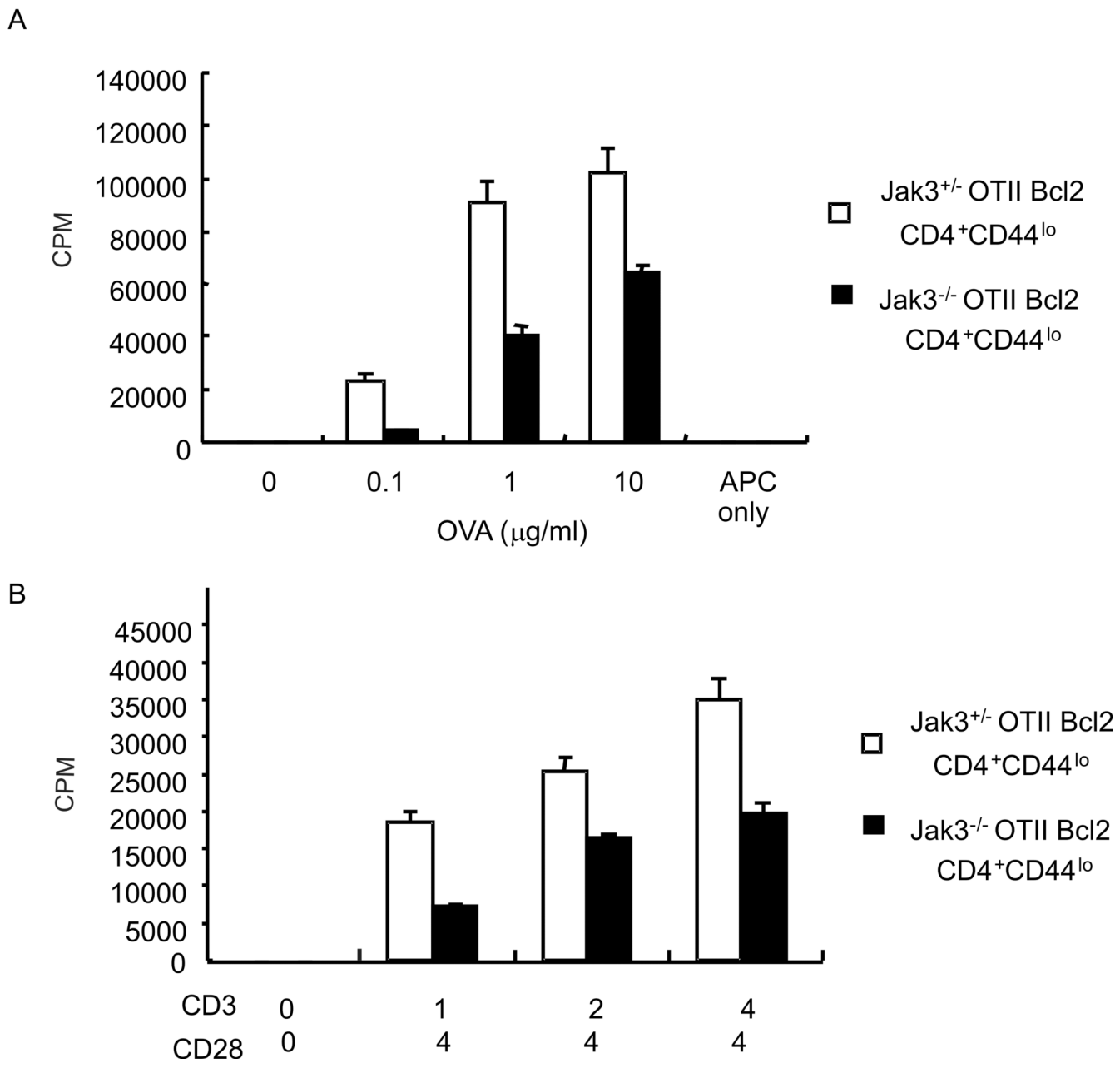

FIGURE 1. CD4$^+$ T cell proliferation appears reduced in the absence of Jak3-dependent cytokine signals

CD4$^+$ CD44^{lo} T cells were sorted from *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl-2 mice and stimulated with either mitomycin C-treated C57BL/6 APCs plus the indicated concentrations of $OVA_{323-339}$ (A) or various concentrations of anti-CD3 and anti-CD28 antibodies (B) for 48h, then pulsed with [3H]-thymidine for the final 18h. Cell proliferation was measured by [3H]-thymidine incorporation. Data represent the mean ± SE of the triplicate reactions. Statistically significant differences were seen between *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ cells. The significant level is $P < 0.05$.

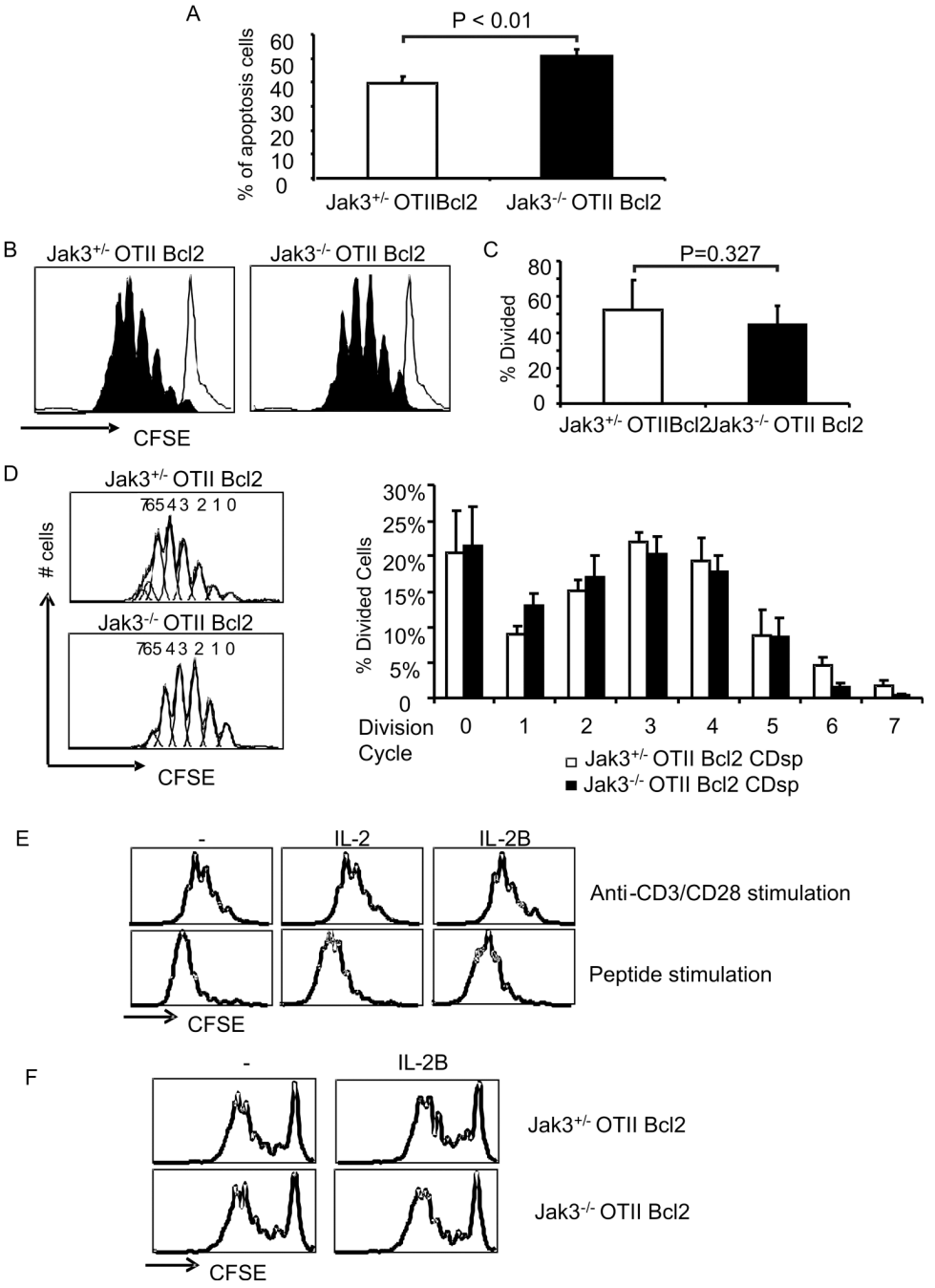

FIGURE 2. Jak3-dependent cytokine signals affect T cell survival but not proliferation

A, Purified CD4SP thymocytes from *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl-2 mice were stimulated with anti-CD3 (1 μg/ml) plus anti-CD28 (4 μg/ml) antibodies. Three days later, cells were stained with AnnexinV and 7-AAD, and analyzed by flow cytometry. Apoptotic cells were identified as AnnexinV+ 7-AAD+.

(*B,C,D*) CD4SP thymocytes were isolated from *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl-2 mice, labeled with CFSE, stimulated with anti-CD3 (1 μg/ml) plus anti-CD28 (4 μg/ml) antibodies, and analyzed by flow cytometry. *B*, representative histograms show the degree of CFSE dilution in *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl-2 T cells. *C*, Percentage of *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl-2 T cells that underwent cell division; data represent the mean ± SE from four independent

experiments. *D*, Histograms were analyzed using the proliferation platform of the FlowJo software to estimate the percentage of cells in each round of cell division; representative data are shown above. The graph depicts the mean ± SE using data from four independent experiments. No significant differences were seen between *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ cells.
E, CD4^{+} T cells were isolated from *Jak3*$^{+/+}$ OTII transgenic mice, labeled with CFSE, and stimulated with anti-CD3 (1 μg/ml) plus anti-CD28 (4 μg/ml) antibodies or 1 μg of $OVA_{323-339}$ peptide presented by mitomycin C-treated C57BL/6 APCs (peptide stimulation). Cultures were supplemented with nothing (-), exogenous recombinant IL-2 (5 μg/ml; IL-2) or IL-2 blocking antibodies (10 μg/ml each of anti-IL-2, anti-CD25, and anti-CD122 antibodies; IL-2B). Three days later, cell proliferation was analyzed by flow cytometry.
F, CD4SP thymocytes were isolated from *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl-2 mice, labeled with CFSE, stimulated with anti-CD3 (1 μg/ml) plus anti-CD28 (4 μg/ml) antibodies, in the absence (-) or presence of IL-2 blocking antibodies (10 μg/ml each of anti-IL-2, anti-CD25 and anti-CD122 antibodies; IL-2B). Four days later, cell proliferation was analyzed by flow cytometry.

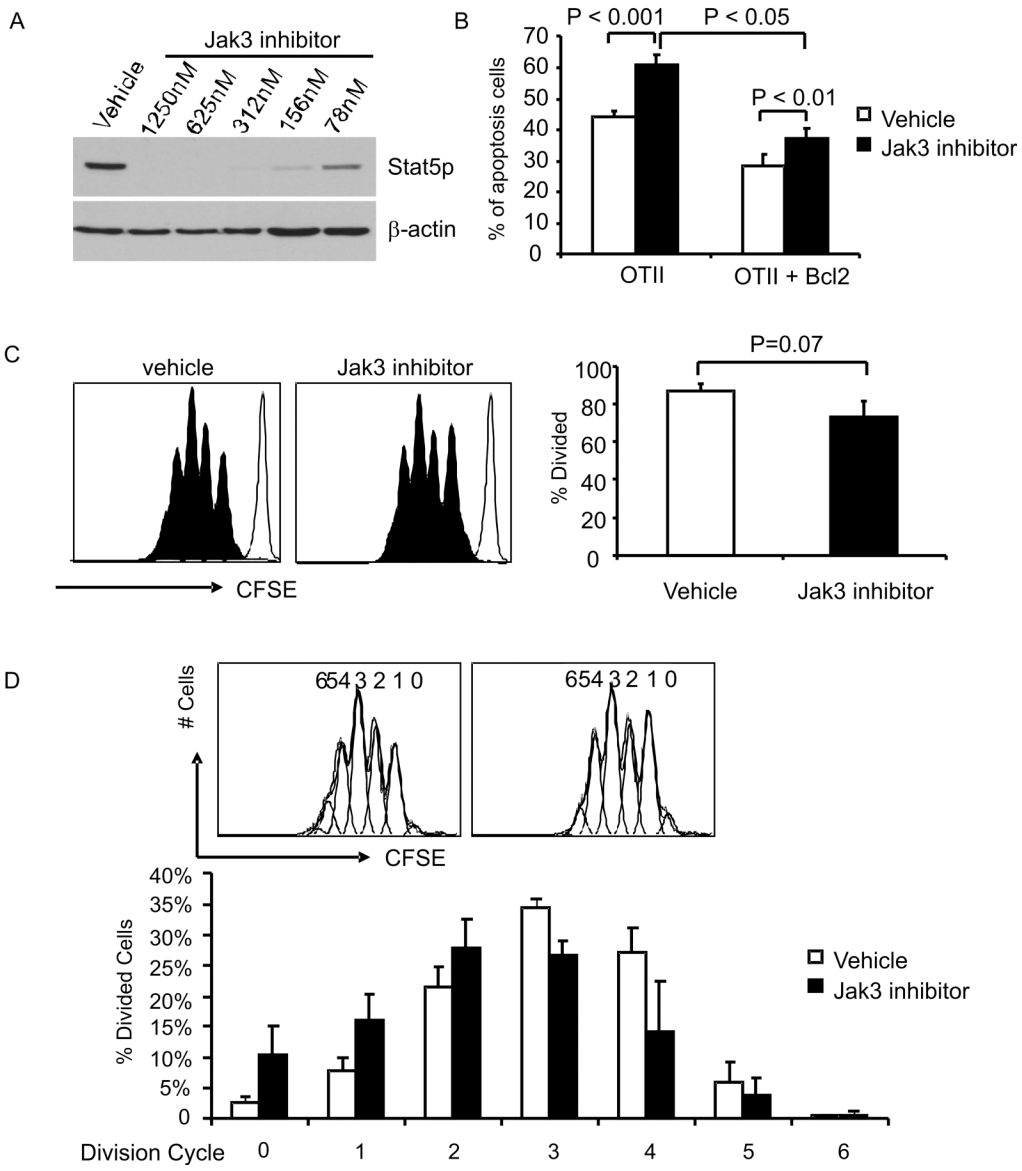

FIGURE 3. CD4$^+$ T cell survival but not proliferation is reduced by pharmacological inhibition of Jak3 activity

A, CD4$^+$ T cells were isolated from *Jak3*$^{+/+}$ mice, stimulated with anti-CD3 and anti-CD28 antibodies for 2 days, rested for 4 hours, incubated with vehicle alone or serial-diluted PS078507 for 30 minutes, then stimulated with IL-2 (50 ng/ml) for 15 minutes. Cell lysates were prepared and immunoblotted for pStat5 and β-actin.

B, Purified CD4$^+$ splenocytes from OT-II-transgenic or OT-II Bcl-2-transgenic mice were stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (4 μg/ml) antibodies for 3 days in the presence of vehicle alone or PS078507 at 312 nM, stained with AnnexinV and 7-AAD, and analyzed by flow cytometry. Apoptotic cells were identified as AnnexinV+ 7-AAD+.

(*C, D)* Purified CD4$^+$ splenocytes from OT-II-transgenic mice were labeled with CFSE and stimulated with anti-CD3 and anti-CD28 antibodies for 3 days in the presence of vehicle alone or PS078507 at 312 nM, and then analyzed by flow cytometry. *C*, Histograms show CFSE fluorescence. The bar graph indicates the percentage of T cells undergoing division in the absence or presence of PS078507; data show the mean ± SE compiled from four independent

experiments. *D*, Histograms were analyzed using the proliferation platform of the FlowJo software to estimate the percentage of cells in each round of cell division; representative data are shown above. The graph depicts the mean ± SE using data from four independent experiments. No significant differences were seen between cells stimulated in the presence or absence of the Jak3 inhibitor.

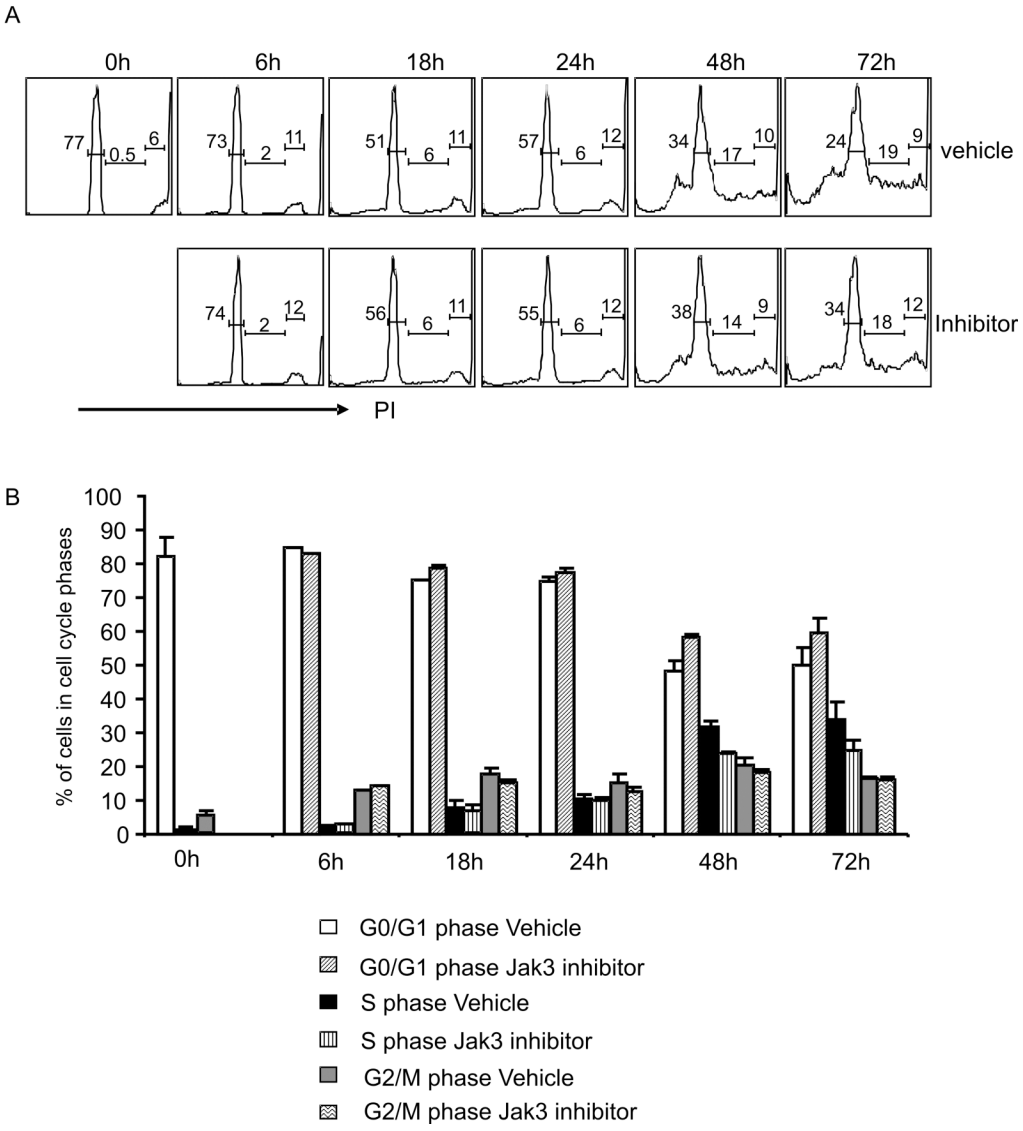

FIGURE 4. Cell cycle progression is not affect by Jak3 inhibition

Purified $CD4^+$ splenocytes from OT-II-transgenic mice were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of vehicle alone or PS078507 at 312 nM. At the indicated time points, cells were harvested, fixed, stained with propidium iodide (PI) and analyzed by flow cytometry. *A*, Histograms represent the DNA content of the cells after stimulation in the presence of vehicle or PS078507. *B*, Percentage of cells in G0/G1, S and G2/M phase is indicated for each timepoint; data represent the mean ± SE compiled from three independent experiments. No significant differences were seen between cells stimulated in the absence versus the presence of the Jak3 inhibitor.

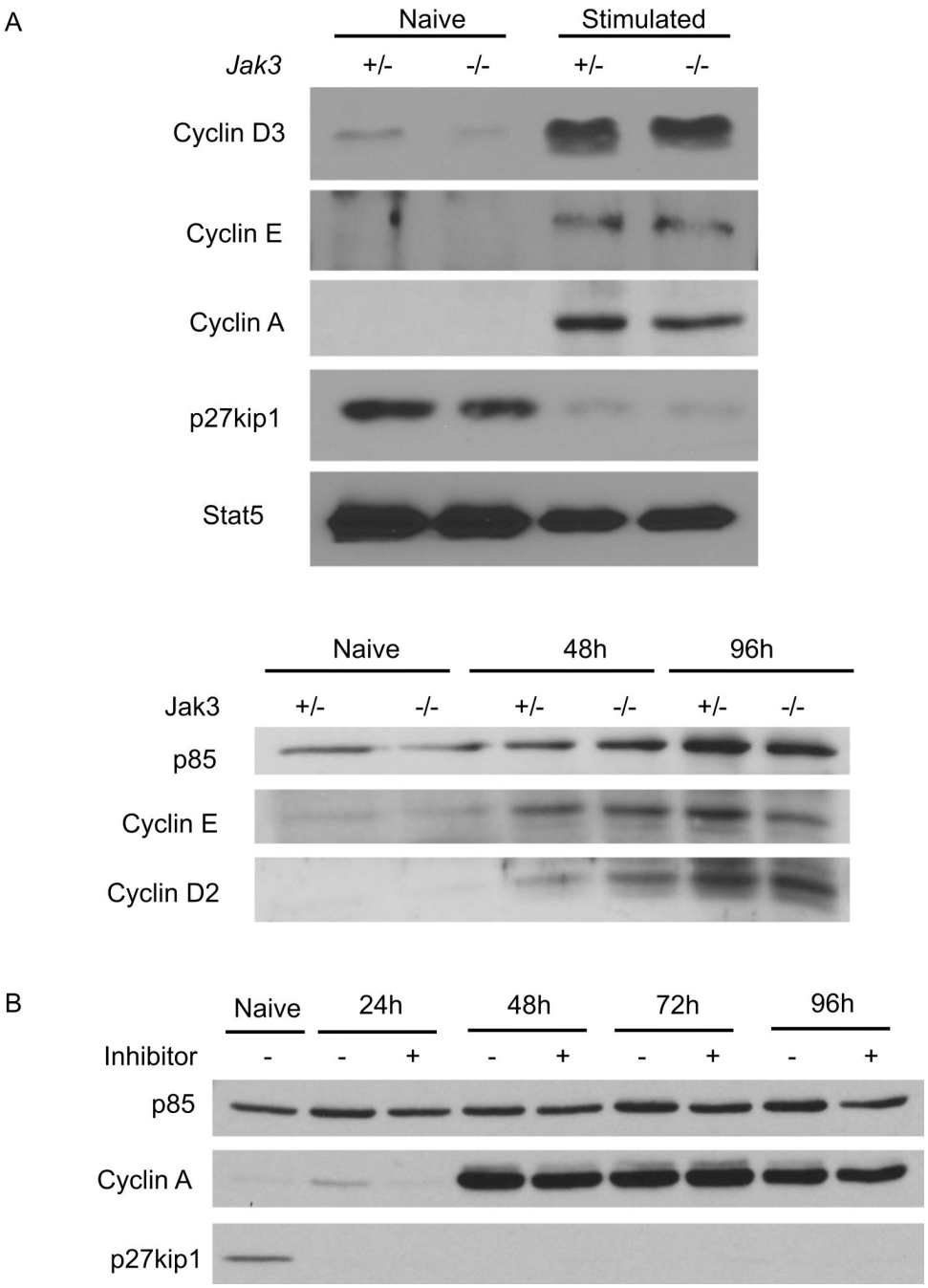

FIGURE 5. Cell cycle proteins are regulated normally in the absence of Jak3-dependent cytokine signals

A, Purified CD4SP thymocytes from *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl-2 mice were stimulated with anti-CD3 and anti-CD28 antibodies for 3 days (upper panel) or for indicated times (lower panel). Total cell lysates were prepared and immunoblotted for cyclin D3, cyclin D2, cyclin E, cyclin A, p27kip1, PI3-kinase p85, and total Stat5. Total Stat5 and PI3-kinase p85 are shown as loading controls.

B, Purified CD4^{+} splenocytes from OT-II-transgenic mice were stimulated with anti-CD3 and anti-CD28 antibodies in the presence or absence of PS078507 at 312 nM. At the indicated time

points, cells were harvested and total cell lysates were prepared and innunolotted for p85 (loading control), cyclin A, and p27kip1.

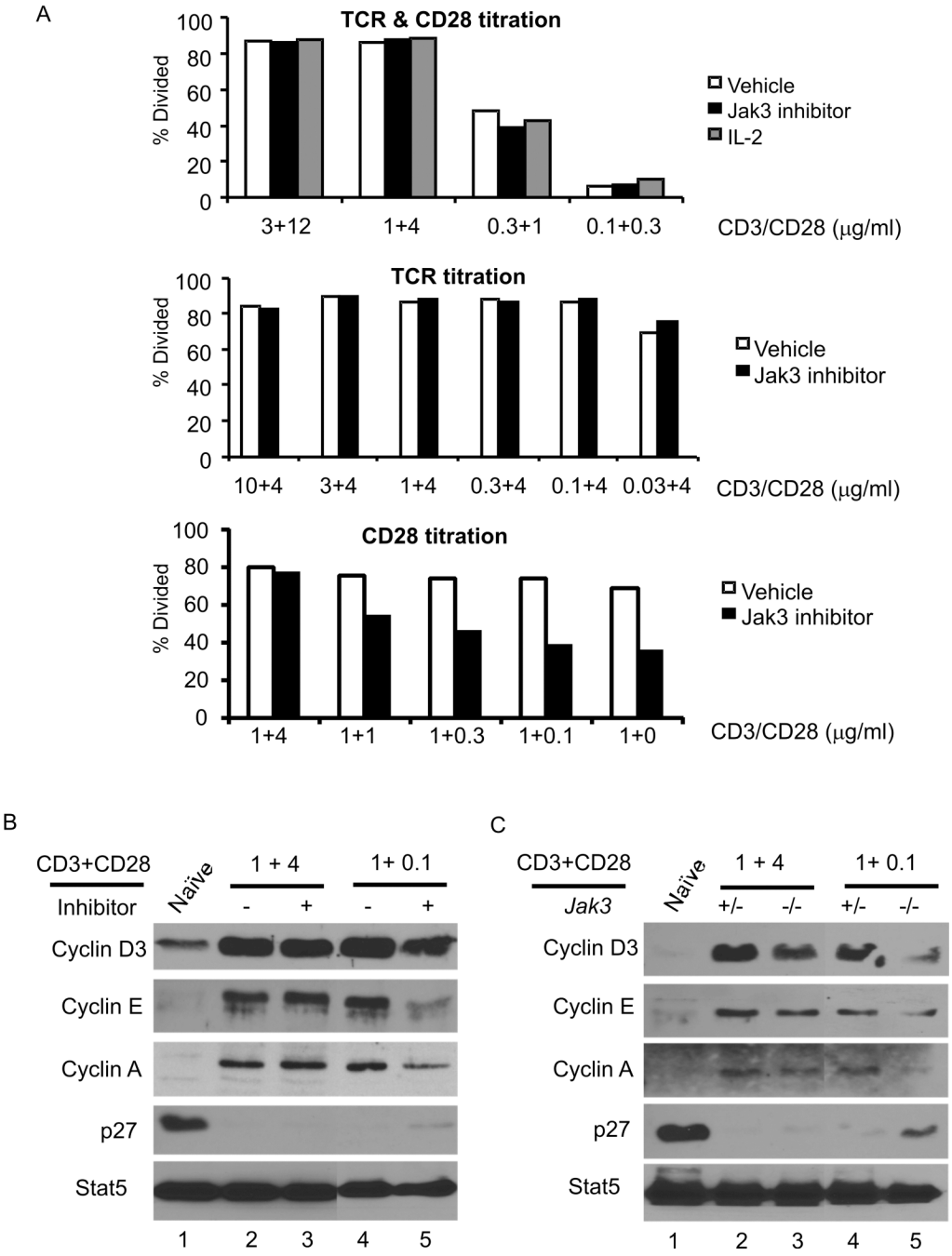

FIGURE 6. Jak3-dependent cytokine signals are required for T cell proliferation when CD28 costimulation is suboptimal

A, Purified CD4$^+$ splenocytes from OT-II-transgenic mice were labeled with CFSE and stimulated with indicated concentrations of anti-CD3 and anti-CD28 antibodies in the presence of vehicle alone or PS078507 at 312 nM, and then analyzed by flow cytometry. In the upper panel, recombinant IL-2 was added at 10 ng/ml. Bar graphs show the percentage of T cells that divided without or with the treatment of the inhibitor. Bar graphs represent one of the two independent experiments.

(B,C) Purified CD4$^+$ splenocytes from *Jak3*$^{+/+}$ OT-II-transgenic mice *(B)* or CD4SP thymocytes from *Jak3*$^{+/-}$ and *Jak3*$^{-/-}$ OT-II Bcl-2 mice *(C)*, were stimulated with the indicated

concentrations of anti-CD3 and anti-CD28 antibodies in the presence of vehicle alone or PS078507 at 312 nM. Cells were harvested after 3 days, total cell lysates were prepared and immunoblotted for cyclin D3, cyclin E, cyclin A and p27kip1 and total Stat5 (loading control). Freshly-isolated naïve cells are shown for comparison.