

The Pim kinases control rapamycin-resistant T cell survival and activation

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Although Pim-1 or Pim-2 can contribute to lymphoid transformation when overexpressed, the physiologic role of these kinases in the immune response is uncertain. We now report that T cells from Pim-1^{-/-}Pim-2^{-/-} animals display an unexpected sensitivity to the immunosuppressant rapamycin. Cytokine-induced Pim-1 and Pim-2 promote the rapamycin-resistant survival of lymphocytes. The endogenous function of the Pim kinases was not restricted to the regulation of cell survival. Like the rapamycin target TOR, the Pim kinases also contribute to the regulation of lymphocyte growth and proliferation. Although rapamycin has a minimal effect on wild-type T cell expansion in vitro and in vivo, it completely suppresses the response of Pim-1^{-/-}Pim-2^{-/-} cells. Thus, endogenous levels of the Pim kinases are required for T cells to mount an immune response in the presence of rapamycin. The existence of a rapamycin-insensitive pathway that regulates T cell growth and survival has important implications for understanding how rapamycin functions as an immunomodulatory drug and for the development of complementary immunotherapeutics.

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Abbreviations used: BrdU, bromo-deoxyuridine; PI, propidium iodide; SOCS, suppressor of cytokine signaling; STAT, signal transducers and activators of transcription; TSST, toxic shock syndrome toxin.

The murine immune system maintains a pool of lymphocytes that respond to antigenic challenge with rapid growth and proliferation. Naive T cells compete in vivo for exogenous factors including the cytokines IL-4 and IL-7 and for MHC-dependent proliferative signals (1, 2). Once T cells encounter antigen, their size increases dramatically (blastogenesis) as they prepare for clonal expansion and the acquisition of an effector phenotype (3). The ligation of cytokine or antigen receptors promotes T cell growth and survival in part by activating the effector enzymes of the PI3K pathway, the kinases Akt and TOR (4). Mice expressing an activated Akt transgene have increased numbers of peripheral T cells that manifest enhanced resistance to apoptotic stimuli in vitro and this effect correlates with the Akt-dependent activation of TOR (5–7). T cells from Akt transgenic mice are larger and show enhanced proliferation in response to mitogens (8, 9). However, Akt-deficient animals (10, 11) and mice treated with the TOR inhibitor rapamycin (12) mount a normal primary immune response. These data suggest that alternate pathways exist that can promote lymphocyte growth and survival in a PI3K/Akt/TOR-independent manner.

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IL-4, IL-7, IL-2, and TCR ligation activate members of the signal transducers and activators of transcription (STAT) family to promote expression of prosurvival molecules including the Pim family of oncogenic serine/threonine kinases. Pim-1, Pim-2, and Pim-3 are novel components of the transcriptional response to cytokine or antigen receptor ligation (13) and their function is regulated primarily at the level of expression (14). *Pim-1* and *pim-2* are expressed in most hematopoietic cells whereas *pim-3* expression is highest in brain, kidney, and mammary tissue (15). Several Pim targets have been identified and include the proapoptotic protein Bad (14, 16), members of the suppressor of cytokine signaling (SOCS) family (17, 18), the translational repressor eIF-4E binding protein 1 (4E-BP1; 14) and the transcription factor Myb (19). Pim-1 and Pim-2 transgenes can promote growth and survival of hematopoietic cell lines (14, 17, 20).

We now report that Pim-1 and Pim-2 are essential components of an endogenous pathway that regulates T cell growth and survival. Pim-1 up-regulation occurred rapidly after cytokine treatment or mitogenic stimulation and high levels of Pim-2 were observed several hours later. T cells from Pim-1^{-/-}Pim-2^{-/-} mice responded to cytokine- or antigen receptor-ligation comparably to cells from wild-type

littermates. However, rapamycin treatment eliminated the ability of IL-4 and IL-7 to promote the survival of Pim-1^{-/-} Pim-2^{-/-} but not wild-type T cells. This correlated with the failure of Pim-1^{-/-} Pim-2^{-/-} T cells to maintain the phosphorylation-dependent inactivation of the Bcl-2-related protein Bad in the presence of rapamycin. Rapamycin also blocked the mitogen-induced activation of Pim-1^{-/-} Pim-2^{-/-} T cells at an early stage of blastogenesis before the up-regulation of surface activation markers and cell cycle entry. Pim deficiency enhanced the effect of rapamycin in vivo and prevented superantigen-induced T cell activation and expansion. The identification of Pim-1 and Pim-2 as required components of a rapamycin-insensitive pathway that regulates lymphocyte growth and survival suggests that the Pim kinases might serve as attractive targets for the development of novel immunotherapeutic regimens.

RESULTS

Pim-1 and Pim-2 are induced by prosurvival signals

In contrast to most kinases implicated in T cell responses, Pim-1 and Pim-2 were undetectable in nonstimulated T cells. Pim-1 and Pim-2 expression was not observed in murine T cells ex vivo or after 12 h of culture without added cytokine (Fig. 1 A). However, when the T cells were cultured in the presence of IL-4 or IL-7, Pim-1 protein was detected at 3 h and Pim-2 by 12 h. The dissimilarity in the kinetics of their expression suggested that Pim-1 and Pim-2 might play independent or sequential roles in the response to prosurvival or proliferative signals. However, mice deficient in Pim-1 (hereafter referred to as Pim-1^{-/-}2^{+/+}), Pim-2 (Pim-1^{+/+}2^{-/-}), or both kinases (Pim-1^{-/-}2^{-/-}) showed no obvious differences when compared with Pim-1^{+/+}2^{+/+} littermates with respect to thymus size or thymocyte or peripheral T cell distribution (unpublished data) as described previously by Mikkers et al. (15).

Given recent demonstrations that Pim transgenes can promote cell survival, the response of T cells from Pim-1^{-/-}2^{-/-} animals to the prosurvival cytokines IL-4 and IL-7 was examined. Splenic T cells from Pim-1^{-/-}2^{-/-} or Pim-1^{+/+}2^{+/+} littermates cultured in vitro in the absence of added cytokines showed identical survival kinetics (Fig. 1 B). IL-4 and IL-7 addition strongly promoted T cell survival in both Pim-1^{+/+}2^{+/+} and Pim-1^{-/-}2^{-/-} T cells. Thus, either endogenous Pim-1 or Pim-2 play no role in the regulation of T cell survival or alternate survival pathways are activated by cytokine treatment.

IL-4 and IL-7 treatment of naive T cells also activated the central antiapoptotic effector of the PI3K pathway, Akt, as measured by the induction of Akt phosphorylation at Serine 473 (Fig. 2 A). TOR is one key mediator of Akt-dependent signal transduction (21). The TOR inhibitor rapamycin can suppress Akt-dependent cell survival (14, 22). Therefore, we examined the ability of rapamycin to affect T cell viability after cytokine treatment. As one readout of TOR activity, the phosphorylation status of the TOR substrate p70 S6 kinase (p70S6K) and its primary target, the S6

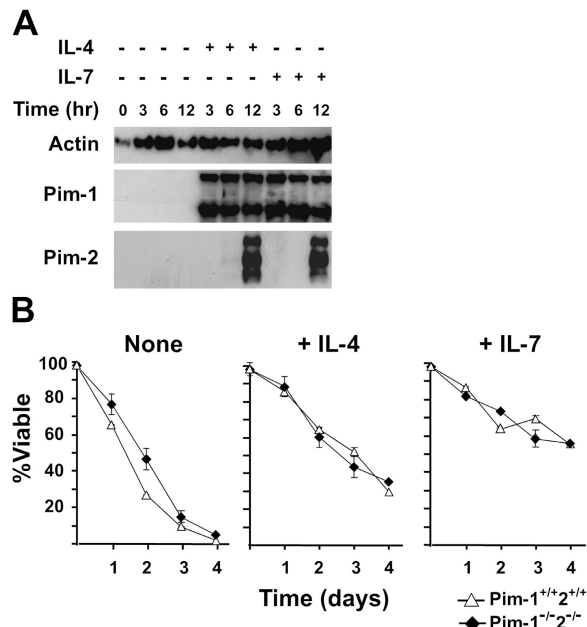


Figure 1. IL-4- and IL-7-dependent survival is intact in Pim-deficient T cells. (A) C57BL/6 splenic T cells were cultured without cytokine (-), with IL-4 (+), or IL-7 (+) for 3, 6, or 12 h (hr). Control was prepared from nonstimulated cells at the start of the culture period (0 h). Total cell lysates were serially probed for the expression of β -actin (Actin) as a loading control, Pim-1, or Pim-2 by Western blot. Due to alternative translational initiation, a single murine *pim-1* transcript gives rise to two proteins of 34 (bottom band) and 44 kD (top band). Murine *pim-2* encodes three isoforms of 34 (bottom band), 37 (middle band), and 40 kD (top band). The results shown are representative of three independent experiments. (B) T cells from Pim-1^{+/+}2^{+/+} and Pim-1^{-/-}2^{-/-} littermates (key) were cultured without cytokine (None), IL-4 (+ IL-4), or IL-7 (+ IL-7) and the percent of viable cells measured daily for 4 d. Error bars represent standard deviation of the mean of triplicate samples. Results have been reproduced in five additional experiments.

ribosomal subunit, was first assessed. IL-4 or IL-7 addition increased total p70S6K and S6 expression and phosphorylation relative to nonstimulated cells (Fig. 2 B). The addition of rapamycin prevented cytokine-dependent p70S6K and S6 phosphorylation (Fig. 2 B). Despite this observation, rapamycin had no effect on the ability of these cytokines to promote T cell viability (Fig. 2 C), suggesting that TOR might also be dispensable for the T cell response to prosurvival cytokines. The cytokine-dependent induction of Pim-1 and Pim-2 by IL-4 or IL-7 was not affected by rapamycin treatment (Fig. 2 D).

Endogenous levels of Pim-2 confer rapamycin-resistant T cell survival

IL-4 and IL-7 activate the Pim kinases and Akt/TOR but neither pathway is absolutely required for cytokine-dependent survival. Reasoning that the disruption of both pathways might have a more significant effect, we assessed cytokine-induced survival in wild-type, Pim-1^{-/-}2^{+/+}, Pim-1^{+/+}2^{-/-}, and Pim-1^{-/-}2^{-/-} T cells treated with rapamycin. Spontaneous apoptosis of T cells from all four genotypes was

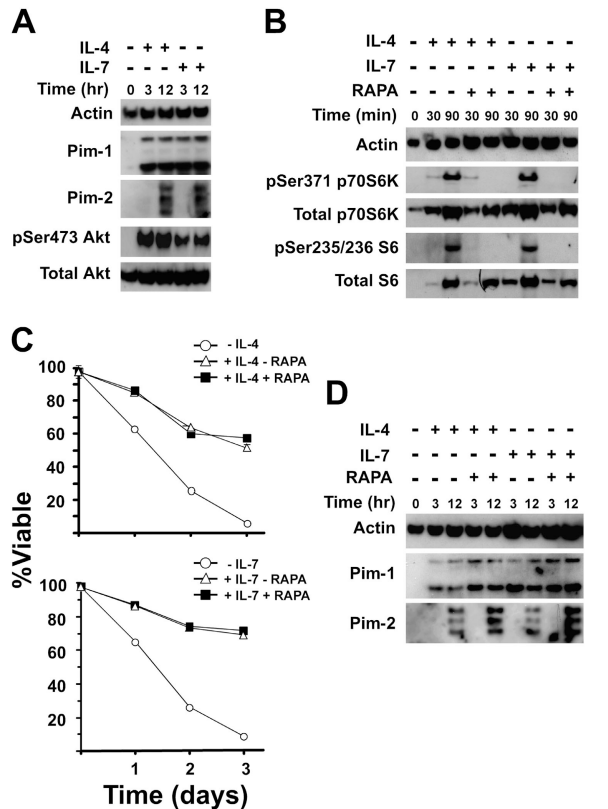


Figure 2. IL-4 and IL-7 promote rapamycin-independent survival. (A) Lysates were prepared from nonstimulated C57BL/6 T cells (0 h) or after 3 or 12 h of IL-4 (+) or IL-7 (+) treatment and serially probed for actin, Pim-1, Pim-2, phospho-Serine 473-Akt (pSer473 Akt), and Total Akt. Results shown were reproduced in four additional experiments. (B) C57BL/6 T cells were preincubated in the presence (+) or absence (-) of 50 nM rapamycin (RAPA) for 1 h before stimulation with IL-4 (+) or IL-7 (+) for 30 or 90 min. Control lysate was from nonstimulated T cells (time 0). Lysates were serially probed for actin, phospho-serine 371-p70 S6 Kinase (pSer371 p70S6K), Total p70S6K, phospho-Serine 235/Serine 236-S6 (pSer235/236 S6), and Total S6 protein by Western blot. Results shown represent two experiments. (C) Viability was measured daily for 3 d in Pim-1^{+/+}2^{+/+} T cells cultured without treatment, cytokine - RAPA, or cytokine + RAPA (keys). (Top) IL-4. (Bottom) IL-7. Error bars represent standard deviation of the mean of triplicate samples and summarize six individual experiments. (D) Pim-1 and Pim-2 induction are rapamycin resistant. C57BL/6 T cells were stimulated with IL-4 (+) or IL-7 (+) in the presence (+) or absence (-) of 50 nM RAPA for 3 or 12 h. Control was prepared from nonstimulated T cells (time 0). Lysates were serially probed for actin, Pim-1, and Pim-2.

suppressed by IL-4 or IL-7 (Fig. 3 A). Although rapamycin had no effect on cytokine-dependent survival of wild-type and Pim-1^{-/-}2^{+/+} T cells, rapamycin treatment suppressed the ability of T cells from Pim-1^{+/+}2^{-/-} or Pim-1^{-/-}2^{-/-} mice to survive in response to IL-4 (Fig. 3 A). Pim-1^{+/+}2^{-/-} and Pim-1^{-/-}2^{-/-} T cells also failed to survive in response to IL-7 in the presence of rapamycin. These phenotypes could not be explained by decreased IL-4 or IL-7 receptor expression or increased levels of the death receptor Fas in rapamycin-treated T cells relative to control Pim-1^{+/+}2^{-/-} or Pim-1^{-/-}2^{-/-} T cells (unpublished data).

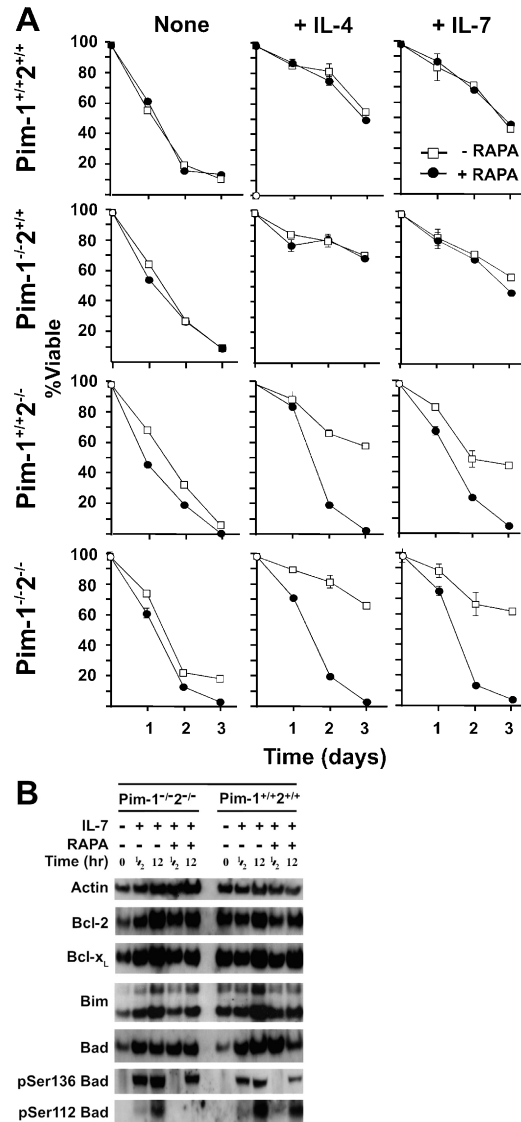


Figure 3. Pim-2 deficiency confers rapamycin sensitivity. (A) Viability was measured daily for 3 d in splenic T cells from Pim-1^{+/+}2^{+/+}, Pim-1^{-/-}2^{+/+}, Pim-1^{+/+}2^{-/-}, and Pim-1^{-/-}2^{-/-} littermates cultured without cytokine (None), + IL-4, or + IL-7 in the presence (+) or absence (-) of 25 nM RAPA (key). Error bars represent standard deviation of the mean of triplicate samples. Data have been reproduced in eight additional experiments. (B) T cells from female Pim-1^{-/-}2^{-/-} and Pim-1^{+/+}2^{+/+} littermates were stimulated with IL-7 (+) in the presence (+) or absence (-) of 50 nM RAPA and lysates prepared at 1/2 and 12 h. Control was from nonstimulated cells prepared at the start of the experiment (0 h). Lysates were serially probed for the expression of actin, Bcl-2, Bcl-x_L, Bim, Bad, phospho-serine 136 Bad (pSer136 Bad), and phospho-serine 112 Bad (pSer112 Bad). For Bim, the upper band corresponds to Bim_{EL} at ~23 kD and the lower to Bim_L at 16 kD. Results have been reproduced in three independent experiments.

Cytokines promote cell survival in part by affecting the expression and function of members of the Bcl-2 protein family. Survival can be potentiated by enhanced Bcl-2 and Bcl-x_L expression and their antiapoptotic function can be suppressed by association with the BH3-only, proapoptotic

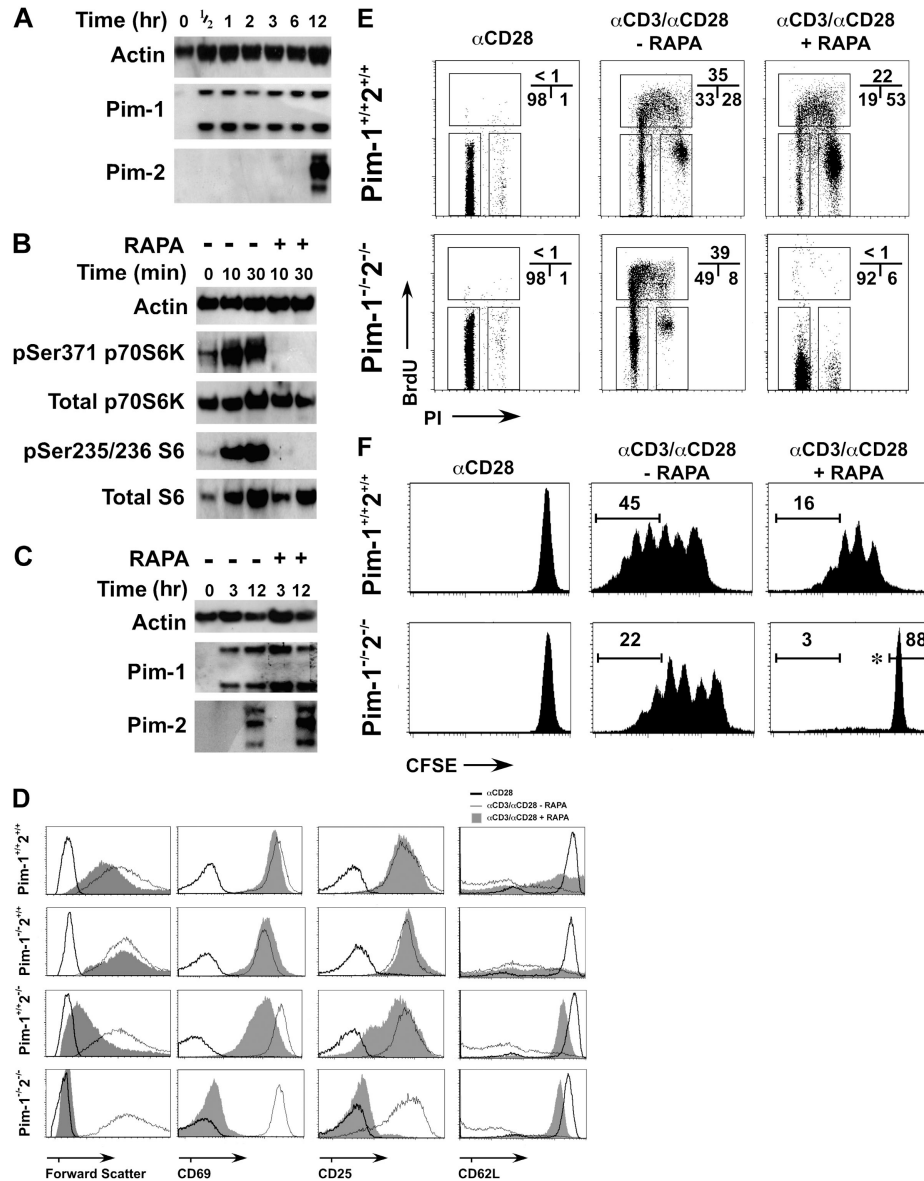


Figure 4. The Pim kinases confer rapamycin-resistant T cell blastogenesis. (A) C57BL/6 splenic T cells were activated by α CD3/ α CD28 and lysates were prepared after 0, 1/2, 1, 2, 3, 6, and 12 h of stimulation and serially probed for the expression of actin, Pim-1, and Pim-2 proteins by Western blot. (B) Activation-induced p70S6K and S6 phosphorylation is rapamycin sensitive. C57BL/6 splenic T cells were preincubated for 1 h in the presence (+) or absence (-) of 50 nM RAPA and activated with α CD3/ α CD28 for 0, 10, and 30 min. Lysates were probed for actin, phospho-serine 371-p70 S6 kinase (pSer371 p70S6K), total p70S6K, phospho-serine 235/serine 236-S6 (pSer235/236 S6), and total S6 expression. (C) In the experiments described in B, Western blots were also performed with lysates from rapamycin-treated and control cells after 3 and 12 h of activation and probed for actin, Pim-1, and Pim-2 expression. (D) Splenic T cells from Pim-1^{+/+}2^{+/+}, Pim-1^{-/-}2^{+/+}, Pim-1^{+/+}2^{-/-}, and Pim-1^{-/-}2^{-/-} mice were activated by α CD3/ α CD28 in the absence (-) or presence (+) of

proteins Bim and Bad (2). High levels of Bcl-2, Bcl-x_L, Bim, and Bad were evident in IL-7-treated Pim-1^{+/+}2^{+/+} and Pim-1^{-/-}2^{-/-} T cells in the presence and absence of rapamycin (Fig. 3 B). The proapoptotic function of Bad can be

25 nM RAPA (key). Control cells were cultured with α CD28 only (α CD28). After 2 d of culture, forward scatter, and surface CD69, CD25, and CD62L expression was assessed. (E) Cell cycle was assessed in the Pim-1^{+/+}2^{+/+} and Pim-1^{-/-}2^{-/-} T cells described in D by BrdU incorporation analysis. The percentage of cells in the G₁ (2N-PI⁺BrdU⁻; bottom left), S (PI⁺BrdU⁺; top), and G₂M (4N-PI⁺BrdU⁻; bottom right) phases of the cell cycle is shown (insets). Results shown are representative of six experiments. (F) 5-(and 6)-carboxyfluorescein diacetate, succinimidyl ester-labeled input T cells from Pim-1^{+/+}2^{+/+} or Pim-1^{-/-}2^{-/-} mice were activated for 4 d in the presence (+) or absence (-) of 25 nM RAPA. Control cells were cultured with α CD28 only for 2 d (α CD28) and showed no decay in fluorescence relative to input cells at day 0. Numbers above the leftmost brackets represent the percent of cells that had divided more than three generations. For Pim-1^{-/-}2^{-/-} cells, the majority of the input population failed to divide (*). Data are representative of three experiments.

suppressed by its phosphorylation at multiple residues including serines 112 (Ser112) and Ser136 (23). The pattern of Bad phosphorylation at Ser136 in response to IL-7 and rapamycin treatment was identical when Pim-1^{-/-}2^{-/-} were

compared with Pim-1^{+/+}2^{+/+} T cells (Fig. 3 B). A dissimilar pattern of Ser112 phosphorylation was observed. Whereas IL-7-induced Ser136 phosphorylation was detected after 30 min of cytokine treatment, Bad phosphorylation at Ser112 was not observed until 12 h. This pattern overlapped with that of Pim-2 induction in response to IL-7 (Figs. 1 A and 2 D). Further, IL-7-induced Ser112 phosphorylation was resistant to rapamycin in Pim-1^{+/+}2^{+/+} T cells but not in Pim-1^{-/-}2^{-/-} cells (Fig. 3 B). Thus, the Pim kinases contribute to the regulation of cell survival in part through regulating the phosphorylation of Bad.

The Pim kinases are required for rapamycin-resistant T cell activation

In addition to its role in Akt-dependent cell survival (22), TOR is considered a critical regulator of protein translation and cell growth (24). Since CD28 ligation has been reported to activate Akt in T cells and might therefore promote TOR-dependent translation (25), we examined the relative contributions of endogenous Pim-1, Pim-2 and TOR to T cell growth after mitogenic stimulation with anti-CD3/anti-CD28 antibodies (α CD3/ α CD28). α CD3/ α CD28 treatment induced a pronounced increase in p70S6K and S6 phosphorylation that could be suppressed by rapamycin (Fig. 4 B). α CD3/ α CD28 treatment up-regulated Pim-1 expression in T cells within 30 min and Pim-2 by \sim 12 h (Fig. 4 A). Activation-induced Pim kinase expression was rapamycin resistant (Fig. 4 C).

T cells increase in size before their entry into the S phase of the cell cycle. Consistent with this, the mean forward scatter of T cells had more than doubled 2 d after α CD3/ α CD28 treatment (Fig. 4 D). Blastogenesis was accompanied by increased surface expression of the activation markers CD69 and CD25 and CD62L down-regulation. All of these parameters were nearly identical when α CD3/ α CD28-treated T cells from Pim-1^{+/+}2^{+/+}, Pim-1^{-/-}2^{+/+}, Pim-1^{-/-}2^{-/-}, or Pim-1^{+/+}2^{-/-} mice were compared (Fig. 4 D). Rapamycin had little effect on α CD3/ α CD28-induced blastogenesis and CD69 or CD62L expression in Pim-1^{+/+}2^{+/+} or Pim-1^{-/-}2^{+/+} T cells (Fig. 4 D) even when added at 200 nM (unpublished data). In contrast, rapamycin treatment suppressed α CD3/ α CD28-induced blastogenesis in Pim-1^{+/+}2^{-/-} T cells and completely prevented activation-induced blastogenesis in Pim-1^{-/-}2^{-/-} T cells. Pim-1^{-/-}2^{-/-} T cells also failed to up-regulate surface CD69 and CD25 expression and decrease CD62L when stimulated by α CD3/ α CD28 in the presence of rapamycin. For both Pim-1^{-/-}2^{-/-} and Pim-1^{+/+}2^{-/-} T cells, the addition of IL-2 had no effect on the rapamycin-dependent suppression of cell growth (unpublished data).

In the absence of stimulation, >98% of T cells were in the G₀/G₁ phase of the cell cycle as assessed by bromo-deoxyuridine (BrdU) incorporation (Fig. 4 E) and did not proliferate in culture (Fig. 4 F). After 2 d of α CD3/ α CD28 treatment, Pim-1^{+/+}2^{+/+} T cells were distributed across the cell cycle with approximately one-third of the population in

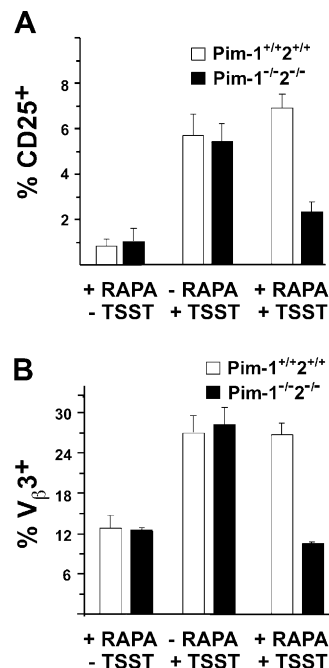


Figure 5. Pim-1 and Pim-2 are required for rapamycin-resistant T cell expansion and activation in vivo. (A) 6–8-wk-old Pim-1^{+/+}2^{+/+} or Pim-1^{-/-}2^{-/-} mice (key) were pretreated with RAPA (+ RAPA) or no drug (– RAPA) before TSST (+ TSST) or vehicle (– TSST) injection. The percent of CD25⁺ peripheral T cells (gated on live Thy1⁺ cells) is shown. Error bars represent the standard error from the mean of 3 to 12 individuals in each group. (B) The percent of the T cells described in A that were positive for TCR V β 3 is also shown.

each of G₁, S, or G₂M phases. At 4 d, all input Pim-1^{+/+}2^{+/+} T cells had divided at least once and 45% of the population had undergone three or more rounds of division (Fig. 4 F). Both rapamycin treatment and Pim deficiency alone had modest effects on cell cycle entry and proliferation after α CD3/ α CD28 treatment relative to control. However, the combined effect of suppressing both pathways was striking. No T cells derived from Pim-1^{-/-}2^{-/-} mice had entered the S phase of the cell cycle 2 d after α CD3/ α CD28 treatment in the presence of rapamycin (Fig. 4 E). Close to 90% of input Pim-1^{-/-}2^{-/-} T cells failed to divide in the presence of rapamycin even 4 d after α CD3/ α CD28 stimulation (Fig. 4 F).

Pim kinase deficiency enhances rapamycin action in vivo

The deletion of Pim-1 and Pim-2 sensitized T cells to TOR inhibition in vitro. This raised the possibility that the in vivo immunosuppressive action of rapamycin might be enhanced in Pim-deficient animals. Superantigens such as the toxic shock syndrome toxin (TSST) have been widely used to study activation and proliferation of T cells in vivo. Both Pim-1^{+/+}2^{+/+} and Pim-1^{-/-}2^{-/-} animals treated with TSST for 2 d contained sixfold more CD25⁺ T cells (Fig. 5 A) and a higher proportion of TSST-reactive V β 3⁺ cells (Fig. 5 B) relative to vehicle-treated littermates. In Pim-1^{+/+}2^{+/+} animals, rapamycin did not affect these parameters. In contrast, rapamycin treatment of Pim-1^{-/-}2^{-/-} mice suppressed TSST-induced T cell activation (Fig. 5 A). Further,

$V\beta 3^+$ T cell expansion was completely suppressed by rapamycin in TSST-treated Pim-1^{-/-}2^{-/-} mice (Fig. 5 B).

DISCUSSION

We report that Pim-1 and Pim-2 are regulators of rapamycin sensitivity in T cells. Although mature T cells developed normally in Pim-deficient animals, T cells from Pim-1^{+/+}2^{-/-} and Pim-1^{-/-}2^{-/-} mice were unable to respond to pro-survival cytokines in the presence of the drug. The data suggest that endogenous Pim-2 is the primary mediator of rapamycin-resistant cell survival and that Pim-1 expression alone is insufficient to promote prolonged rapamycin-resistant survival. Rapamycin also blocked α CD3/ α CD28-induced T cell activation at early blastogenesis before cell cycle entry in Pim-1^{-/-}2^{-/-} but not wild-type T cells. Despite its clinical use in delaying transplant rejection, the precise basis by which rapamycin functions as an immunosuppressant is unclear. This report suggests that rapamycin does not inhibit T cell growth, proliferation or survival because endogenous Pim-1 and Pim-2 can maintain the survival and growth of rapamycin-treated T cells. Therefore, despite the established clinical efficacy of rapamycin, it remains at best a partial immunosuppressant *in vivo* and *in vitro*. Complete immunosuppression requires the concomitant inhibition of Pim kinase activity.

Our work suggests that endogenous Pim kinase expression is an important determinant of rapamycin sensitivity. The data help to explain some of the confusing results that have been reported concerning the effect of rapamycin treatment in lymphocyte activation and survival. Although rapamycin was originally described as an inhibitor of cell proliferation that acts by inducing arrest at the G₁ phase of the cell cycle (26), subsequent data suggest that its antiproliferative effects may be stimulus or cell type specific. For example, rapamycin treatment can cause thymic atrophy in mice and rats (27–29). However, rapamycin has only a modest effect on the survival of peripheral T cells *in vivo* (29). In activated T cells, rapamycin has been reported to inhibit IL-2R up-regulation and IL-2-dependent proliferation in some studies (26, 30–32) but not others (33–35). Recent work suggests that while some CD4-dependent immune responses are rapamycin sensitive (36, 37), CD8⁺ T cell function is largely rapamycin resistant (29, 36, 38, 39). It is tempting to speculate that the Pim kinases might account for the residual immune function observed in the presence of rapamycin in these studies.

Pim-1 and Pim-2 do not contribute equally to the phenotypes we have observed. Pim-2 was induced much later than Pim-1 and appeared to play a more substantial role in the regulation of cytokine and mitogen-induced survival and growth. Nevertheless, endogenous Pim-1 does appear to partially compensate for Pim-2 deficiency in mitogen-induced T cell growth. Because the combined deficiency of Pim-1 and Pim-2 eliminated cytokine-dependent cell survival and α CD3/ α CD28- and superantigen-induced T cell activation in the presence of rapamycin, Pim-3 seems un-

likely to contribute to the rapamycin-insensitive growth of T lymphocytes. Recently, animals deficient in all three Pim kinases have been reported. Although these mice show reduced body size, their lymphocytes display only mild defects in cytokine-induced proliferation (15).

The molecular basis of Pim kinase-dependent lymphocyte survival is explained in part through regulation of the proapoptotic Bcl-2-related protein Bad. Pim-2-deficient T cells die subsequent to pro-survival cytokine treatment in the presence of rapamycin despite continued Bcl-2 and Bcl-x_L expression. It is well appreciated that Bcl-2 and Bcl-x_L function are antagonized in part by their heterodimerization with Bad and that this interaction is controlled by Bad phosphorylation at multiple residues, including Ser112, Ser136, and Ser155 (23). Phosphorylated Bad is retained in the cytoplasm by the 14-3-3 proteins (23). The relative roles played by each of these residues in regulating the ability of Bad to antagonize Bcl-2 and Bcl-x_L function remains controversial. Many studies suggest that Akt is one of the primary kinases that phosphorylates Bad at Ser136 (40, 41), in part because Ser136 phosphorylation is sensitive to PI3K inhibitors (41, 42). Another group has reported that the kinetics of Ser112 and Ser136 phosphorylation in response to IL-7 treatment differ and that only the former occurs in a PI3K-independent manner (42). Recently, Chiang et al. showed that the PP2A-dependent dephosphorylation of Bad at Ser112 is required for Ser136 dephosphorylation, the dissociation of Bad from 14-3-3, and apoptosis (43). The authors proposed that Ser112 served as the “gatekeeper” of Bad function. Although kinases in addition to Pim-2 can phosphorylate Bad at Ser112 *in vitro* (41, 44, 45), the present data suggests that Pim-2 plays a primary role in regulating Bad phosphorylation at Ser112 in T cells.

In summary, Pim kinase deficiency greatly enhances the immunosuppressive action of rapamycin *in vivo* and *in vitro*. Thus, inhibitors of the Pim kinases in combination with rapamycin would be expected to induce a greater degree of immunosuppression as compared with either drug alone. Analogous to the rapamycin-dependent inhibition of TOR, the pharmacological inhibition of the Pim kinases might have immunomodulatory benefits on its own. The observation that the germline deletion of Pim-1 and Pim-2 has a modest phenotype outside of the hematopoietic system and that, aside from its effects on T cells, rapamycin is tolerated well in Pim-1^{-/-}2^{-/-} animals, suggests that these possibilities should be explored.

MATERIALS AND METHODS

Mice. Pim-1^{+/+}2^{+/+}, Pim-1^{-/-}2^{+/+}, Pim-1^{+/+}2^{-/-}, and Pim-1^{-/-}2^{-/-} animals were generated from Pim-1^{+/+}Pim-2^{+/-} mice, a gift of Paul Rothman, Columbia University, New York, NY (17). Genotyping by PCR with tail snip DNA was performed as described previously (17). C57BL/6 mice were purchased from Jackson ImmunoResearch Laboratories. Unless otherwise indicated, all experiments were performed on male littermates at 4 to 10 wk of age. In all experiments, mice were injected IP. Mice were bred and maintained at the University of Pennsylvania in accordance with the Institutional Animal Care and Use Committee guidelines.

Antibodies and Western blots. Mouse anti-Pim-2 1D12, mouse anti-Pim-1 19F7, and goat anti-actin I-19, and goat anti-mouse IgG, bovine anti-goat IgG, and goat anti-rabbit IgG coupled to horseradish peroxidase were purchased from Santa Cruz (Santa Cruz Biotechnology, Inc.). Hamster anti-mouse Bcl-2 3F11, rabbit anti-mouse Bim, and anti-hamster IgG-HRP were purchased from BD Biosciences. Rabbit anti-mouse phosphoserine 371 p70S6K, rabbit anti-mouse phospho-Ser235/Ser236 S6 ribosomal protein, rabbit anti-phospho Ser473 Akt, rabbit anti-phospho Ser136 Bad, rabbit anti-phospho Ser112 Bad, rabbit anti-phospho Ser155 Bad, rabbit anti-S6 ribosomal protein, rabbit anti-Akt, and rabbit anti-Bad were purchased from Cell Signaling. Preparation and quantitation of whole cell extracts lysed in PBS + 1% NP-40 supplemented with protease (Roche) and phosphatase inhibitor cocktails I and II (both from Sigma-Aldrich) and Western blotting as described previously in detail (14). 50–100 μ g of protein was resolved on a 4–12% or 10% NuPage bis-tris polyacrylamide gels and transferred to nitrocellulose as directed (Invitrogen). Blots were blocked in PBS containing 10% milk and 0.2% Tween-20 (both from GIBCO BRL), incubated in primary antibody in 5% milk overnight at 4°C and washed several times for 15 min each in PBS + 0.2% Tween. Target protein was visualized by incubation with a species appropriate HRP-conjugated secondary antibody at room temperature for 1 h and ECL-Advance chemiluminescent reagent (Amersham Bioscience). For serial Western blotting, membranes were stripped by incubating for 30 min at 65°C in PBS + 1% SDS and 100 μ M β -mercaptoethanol followed by six washes in PBS + 0.2% Tween-20.

Thymocyte and T cell preparation, activation, and flow cytometric analyses. Thymuses and spleens were pooled from three to five animals. Live thymocytes were enriched by Ficoll centrifugation as directed by the manufacturer (Amersham Biosciences) and then resuspended at 10⁶/ml in DMEM supplemented with 10% FCS, penicillin/streptomycin/fungizone, 100 μ M β -mercaptoethanol, 2 mM L-glutamine, 100 μ M nonessential amino acids, and 10 mM Hepes buffer (all from GIBCO BRL). Flow cytometric analysis of thymocytes was performed using a FACSCalibur (BD Biosciences) and FITC rat anti-mouse Thy1.2 53-2.1 or FITC anti-rat Thy1.1 OX-7, PE anti-mouse CD4 L3T4 and APC anti-mouse CD8a 53.6-7 antibodies (all from BD Biosciences). Total thymocyte numbers were calculated by Trypan blue exclusion. Splenic T cells were enriched to ~95% purity (as assessed by staining with PE rat anti-mouse Thy1.2 30-H12 or PE anti-rat Thy1.1 OX-7 antibodies) using the StemStep T Cell Purification kit as directed by the manufacturer (Stem Cell Technologies Inc.). Lysates were then immediately prepared from a fraction of each thymocyte or T cell preparation (time 0). In some Western blotting experiments, half of each cell preparation was preincubated in 50 nM of rapamycin (Sigma-Aldrich) dissolved in methanol, or the vehicle control, for 1 h at 37°C before addition of cytokines or in vitro activation. For some flow cytometric analyses, T cells were preincubated in 25 nM rapamycin or vehicle in an identical manner and fresh rapamycin was added at day 2 of the experiment. For cytokine treatment of naive T cells, cells were cultured at 5 \times 10⁶/ml in vitro with the addition of 10 ng/ml of recombinant mouse IL-4 or 10 ng/ml of recombinant mouse IL-7 (both from BD Biosciences) for the times indicated. Viability was assessed daily by flow cytometric analysis of the exclusion of propidium iodide (PI). Flow cytometric analysis was also performed with FITC Thy1.1 or Thy1.2, biotin hamster anti-mouse Fas Jo.2, PE rat anti-mouse IL-7R SB/199, rat anti-mouse IL-4R mIL4R-M1, and FITC rat anti-mouse IgG (all from BD Biosciences). Another set of cells were activated in one of two ways, either by the addition of 10 ng/ml PMA and 250 ng/ml ionomycin (both from Sigma-Aldrich) or by antibody-mediated TCR and CD28 cross-linking as follows: 24 well plates were preincubated at 4°C overnight with 1 μ g/ml of both anti-Armenian and anti-Syrian Hamster IgG antibodies (Jackson ImmunoResearch Laboratories, Inc.) in PBS, washed three times in PBS and then incubated with 1 μ g/ml hamster anti-mouse CD3 ϵ 145-2C11 and 2 μ g/ml hamster anti-mouse CD28 37.51 in PBS at 37°C for 1 h and cells added at 5 \times 10⁶/ml. Control cells were cultured in an identical manner except anti-CD3 was

omitted from the culture preparation. For T cell activation studies, live cells were enriched by Ficoll centrifugation after 2 d of culture and activation status was assessed via analysis of mean forward scatter and using FITC anti-mouse CD69 H1.2F3, APC rat anti-mouse CD25 PC61, and PE anti-mouse CD62L MEL-14 (all antibodies from BD Biosciences). A fraction of each preparation was also resuspended in medium and incubated for 2 h at 37°C with 10 μ M BrdU (Calbiochem). Cells were then fixed in cold 70% ethanol and stained with mouse anti-BrdU 3D4 and FITC rat anti-mouse IgG (both antibodies from BD Biosciences) according to the manufacturer's protocol and resuspended in PI + 1 μ g/ml RNase A before FACS analysis. In a series of parallel experiments, T cells were suspended in PBS and labeled after a 5-min incubation with 5 μ M 5-(and 6-)carboxyfluorescein diacetate, succinimidyl ester (Molecular Probes) as directed by the manufacturer and then activated by TCR and CD28 cross-linking in the presence of 50 nM rapamycin. Fresh rapamycin or vehicle was added at day 2 of culture. In some cases, recombinant murine IL-2 (BD Biosciences) was added.

In vivo rapamycin and superantigen treatment. For assessment of total thymocyte numbers, animals were given daily doses for 3 d of 3 mg/kg rapamycin in water. Animals were then killed and total thymocytes counted by Trypan blue exclusion. Control animals were given water only. For the superantigen-induced activation experiments, animals were treated with 3 mg/kg rapamycin daily for 3 d. On day 2, mice were given 50 mg of D-galactosamine hydrochloride (Sigma-Aldrich) in PBS, followed by 100 μ g of TSST (Sigma-Aldrich) in PBS 30 to 60 min later. On day 4, spleen and LN cells from individual animals were pooled, live lymphocytes enriched by Ficoll centrifugation, and then assessed by flow cytometric analysis with PE anti-Thy1, APC anti-CD25, biotinylated anti-mouse V β 3 T cell receptor KJ25, anti-mouse V β 4 KT4, anti-mouse V β 7 TR10, and anti-mouse V β 11 RR3-15 antibodies, and then FITC-streptavidin (all antibodies from BD Biosciences).

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