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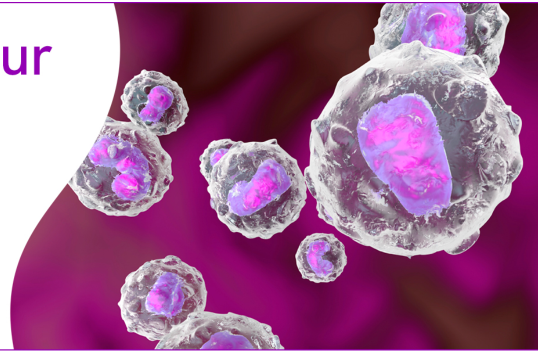

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https://doi.org/10.4049/jimmunol.169.2.714

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The Homeostasis But Not the Differentiation of T Cells Is Regulated by p27^{Kip1}[1]

Randy Shen and Mark H. Kaplan[2]

The cyclin-dependent kinase inhibitor p27^{Kip1} is a critical regulator of T cell proliferation. To further examine the relationship of T cell proliferation and differentiation, we examined the ability of T cells deficient in p27^{Kip1} to differentiate into Th subsets. We observed increased Th2 differentiation in p27^{Kip1}-deficient cultures. In addition to increases in CD4$^+$ and CD8$^+$ T cells, there is a similar increase in $\gamma\delta$ T cells in p27^{Kip1}-deficient mice compared with wild-type mice. The increase in Th2 differentiation is correlated to an increase of IL-4 secretion by CD4$^+$DX5$^+$TCR$\alpha\beta^+$CD62L^{low} T cells but not to increased expansion of differentiating Th2 cells. While STAT4- and STAT6-deficient T cells have diminished proliferative responses to IL-12 and IL-4, respectively, proliferative responses are increased in T cells doubly deficient in p27^{Kip1} and STAT4 or STAT6. In contrast, the increased proliferation and differentiative capacity of p27^{Kip1}-deficient T cells has no effect on the ability of STAT4/p27^{Kip1}- or STAT6/p27^{Kip1}-deficient CD4$^+$ cells to differentiate into Th1 or Th2 cells, respectively. Thus, while p27^{Kip1} regulates the expansion and homeostasis of several T cell subsets, it does not affect the differentiation of Th subsets. *The Journal of Immunology,* **2002, 169: 714–721.**

T cell proliferation and expansion are critical to an effective immune response. Stimulation of T cell proliferation occurs via signaling from the Ag receptor and cytokine receptors. Cytokine-stimulated expansion of T cells depends on the regulation of many factors, including p27^{Kip1}, a cyclin-dependent kinase inhibitor. Levels of p27^{Kip1} are decreased in response to both Ag receptor and cytokine signaling (1–4). Recently, gene-deficient and transgenic mice have provided direct evidence for the role of p27^{Kip1} in T cell proliferation. Both splenic and thymic T cell numbers are increased in p27^{Kip1}-deficient mice (5–7). T cells from p27^{Kip1}-deficient mice are hyperresponsive to the stimulation of cytokines, including IL-2, IL-4, and IL-12 (8). There is an increase of CD4$^+$ memory cells in p27^{Kip1}-deficient mice compared with wild-type mice, and the differences are increased following immunization (8). Transgenic mice that overexpress p27^{Kip1} have reduced T cell proliferation and diminished immune responses (9). These data support a central role for p27^{Kip1} in regulation of T cell homeostasis and immune responses.

The regulation of p27^{Kip1} levels within the cell occurs primarily posttranslation. It is still not entirely clear which cytokine-activated signaling pathways are responsible for regulation of p27^{Kip1}. Studies have demonstrated that mammalian target of rapamycin- and phosphatidylinositol 3-kinase-dependent pathways are important in reducing p27^{Kip1} levels in response to IL-2 (10). High p27^{Kip1} levels were correlated with the decreased proliferative response to IL-4 and IL-12 in STAT6- and STAT4-deficient T cells, respectively (11). Furthermore, an IRS-2-dependent pathway has recently been shown to be important for IL-4-stimulated reductions in p27^{Kip1} levels (12). Mice doubly deficient in STAT6 and p27^{Kip1} recover IL-4-stimulated proliferative responses (8), supporting the concept that STAT proteins may also be involved in regulation of p27^{Kip1} levels.

To further examine the role of p27^{Kip1} in cytokine-stimulated expansion and STAT-regulated proliferation, we have examined proliferative and differentiative responses in mice that are doubly deficient in p27^{Kip1} and either STAT4 or STAT6. STAT4/p27^{Kip1}-deficient and STAT6/p27^{Kip1}-deficient T cells recover, at least in part, IL-12- and IL-4-stimulated proliferation. In contrast, p27^{Kip1} deficiency has no effect on the ability of STAT4- or STAT6-deficient T cells to differentiate into Th1 or Th2 cells, highlighting the specificity of the role of p27^{Kip1} for proliferative responses. We also observed that there is an increase of IL-4-secreting p27^{Kip1}-deficient T cells. This did not correlate with increased percentages of $\gamma\delta$ T cells but did appear to be a product of CD4$^+$DX5$^+$ cells, which have a phenotype similar to NKT cells. Thus, this report defines a role for p27^{Kip1} in STAT4- and STAT6-dependent proliferative responses and in the homeostatic expansion of additional T cell subsets.

Materials and Methods

Mice

Generation of STAT6-deficient mice has been previously described (13). STAT6-deficient mice in this study were backcrossed 10 generations to the BALB/c genetic background. Generation of STAT4-deficient mice has been previously described, and mice in this study were backcrossed eight generations to the C57BL/6 genetic background (14). Mice deficient in p27^{Kip1} were backcrossed six generations to the C57BL/6 background and were provided by Dr. J. Roberts (Fred Hutchinson Cancer Research Center, Seattle, WA). STAT6/p27^{Kip1} double deficient mice and STAT4/p27^{Kip1} double deficient mice were generated by mating of F_1 mice heterozygous for both alleles.

Activation of cells and proliferation assay

Spleen cells or purified T cell populations were activated with 3 μg/ml plate-bound anti-CD3 (145-2C11) for 48–72 h as indicated in RPMI (15) at 2×10^6 cells/ml. Supernatants were recovered for ELISA analysis at the time points indicated. CD4$^+$ cells were positively selected using MiniMacs (Miltenyi Biotec, Auburn, CA) or sorted by flow cytometry as indicated. CD4$^+$CD62L$^+$, CD4$^+$DX5$^+$, CD8$^+$, and TCR$\gamma\delta^+$ cells were sorted by

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Received for publication January 9, 2002. Accepted for publication May 6, 2002.

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[1] This work was supported by National Institutes of Health Grant AI45515.

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flow cytometry. Activated T cells were purified over Histopaque (Sigma-Aldrich, St. Louis, MO) and used for subsequent analysis. For proliferation assays, anti-CD3-activated spleen cells were plated at 10^5 cells per microtiter well and incubated with the indicated concentrations of IL-12 or IL-4 (PeproTech, Rocky Hill, NJ). Wells were pulsed with 1 μCi [^{3}H]thymidine for the last 18 h of a 48-h incubation for cytokine stimulation, harvested, and counted in scintillation fluid. Cell numbers recovered after activation of wild-type and p27^{Kip1}-deficient mice are comparable and there are no significant differences in the levels of apoptosis in these two populations.

CFSE analysis

Following activation as described above, cells were labeled as described (16). Briefly, cells were washed in PBS and incubated for 10 min at room temperature with 10 μM CFSE with cells at a concentration of 10^7/ml. An equal volume of FCS was added and cells were washed several times. Cells were then washed and fixed immediately for FACS analysis or placed in culture at 5×10^5 cells/ml with 20 U/ml IL-2 (Roche, Indianapolis, IN) or 10 ng/ml IL-4. At each time point cells were removed from culture and fixed for FACS analysis.

Th cell culture

Th cell differentiation assays were performed as described (15). Briefly, MiniMacs-purified CD4$^+$ T cells (5×10^5 cells/ml) were activated with 3 μg/ml plate-bound anti-CD3 plus 1 μg/ml anti-CD28 (BD PharMingen, San Diego, CA). Cells were stimulated with no additional culture reagents (for unskewed conditions), with 10 ng/ml IL-4 plus 10 μg/ml anti-IFN-γ (R4-6A2) for Th2 conditions, or with 2 ng/ml IL-12 plus 10 μg/ml anti-IL-4 (11B11) for Th1 conditions. After 6 days in culture, cells were purified over Histopaque and restimulated with plate-bound anti-CD3 for 24 h. Supernatants were then harvested for ELISA analysis. Cultures were restimulated for only 6 h, with the last 4 h in the presence of 2 μM monensin, for intracellular cytokine analysis. Cell recovery from wild-type and p27^{Kip1}-deficient mice following differentiation were comparable.

Cytokine ELISAs

ELISA was used to detect levels of secreted IL-4, IL-5, and IFN-γ. All Abs used in ELISA were purchased from BD PharMingen, with the exception of 11B11, used as the capture Ab in the IL-4 ELISA. Streptavidin-alkaline phosphatase (Sigma-Aldrich) and p-nitrophenyl phosphate (Roche) were used for detection. Analysis was performed with an ELISA plate reader and software (Bio-Rad, Hercules, CA).

FACS analysis

Flow cytometric analysis was performed with either a FACScan or a FACSCalibur (BD Biosciences, Mountain View, CA). All Abs for staining and sorting were from BD PharMingen. These included Abs to FITC-labeled anti-CD3 (145-2C11), FITC- or PE-labeled anti-CD4 (RM4-4, RM4-5), PE-labeled anti-CD8 (53-6.7), FITC-labeled anti-TCRβ (H57-597), PE-labeled anti-TCRγδ (GL3), PE-labeled anti-CD49 (DX5), and FITC-labeled anti-CD62L (Mel-14). Intracellular cytokine analysis was performed using PE-labeled anti-IL-4 in a FACS buffer of PBS plus 2% BSA and 0.1% saponin, following staining of surface markers.

Complement depletion

Depletion of DX5$^+$ splenocytes or heat-stable Ag (HSA$^+$)[3] thymocytes was accomplished by incubating cells with purified DX5 or J11d hybridoma supernatant, respectively, on ice for 30 min, washing, and incubating with rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada) for 45 min at 37°C. Cells were then washed, purified over Histopaque, and plated as described above for T cell activation of differentiation.

Statistics

Statistics were performed using the SPSS statistical program (SPSS, Chicago, IL). A *t* test was used to determine significance.

Results

Enhanced generation of Th2 cells from p27^{kip1}-deficient T cell cultures

Because cell cycle progression has been linked to Th cell differentiation (16–18) and because we have shown that p27^{Kip1}-deficient T cells are hyperproliferative to cytokines (8), we wanted to explore the ability of p27^{Kip1}-deficient T cells to differentiate into Th subsets. To examine Th2 differentiation we stimulated wild-type and p27^{Kip1}-deficient CD4$^+$ T cells with anti-CD3 supplemented with 1 μg/ml anti-CD28, 10 ng/ml IL-4, and 10 μg/ml anti-IFN-γ. After 6 days in culture, cells were washed and restimulated with anti-CD3 alone. Supernatants were harvested after 24 h and tested for IL-4 levels by ELISA. Th2 cells generated from p27^{Kip1}-deficient mice secreted increased levels of IL-4 compared with wild-type cells (Fig. 1*A*). We also determined that p27^{Kip1}-deficient Th2 cultures secreted higher levels of IL-5 and IL-10, as well as IL-4, compared with wild-type cultures (Fig. 1*A* and data not shown), suggesting that there is an increase in Th2 populations secreting several Th2 cytokines. Because p27^{Kip1} regulates cell division, it was possible that cells committed to cytokine secretion during the differentiation process had expanded in culture. To test this we analyzed the Th2 cultures by intracellular cytokine staining. Fig. 1*B* demonstrates that there is a higher percentage of cytokine-secreting cells in p27^{Kip1}-deficient Th2 cultures than in wild-type cultures, although on a per cell basis the cells are not making more IL-4. It was still possible that higher IL-4 was a result of more cells committed to Th2 differentiation in p27^{Kip1}-

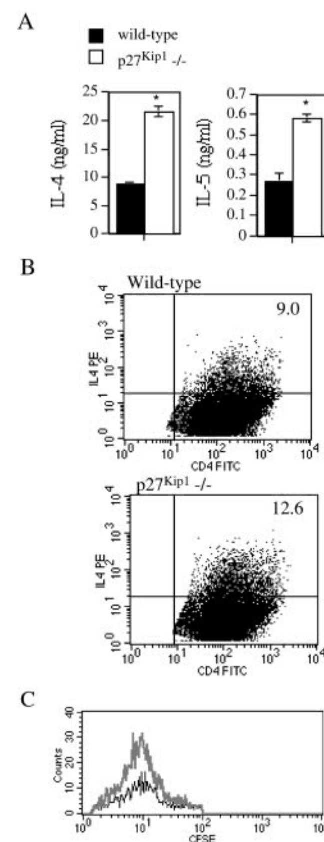

FIGURE 1. Th2 cells from p27$^{kip1-/-}$ mice secrete higher levels of cytokines. *A*, In vitro differentiated Th2 cells from wild-type and p27$^{kip1-/-}$ mice, generated by incubation for 6 days with 2 μg/ml anti-CD3, 1 μg/ml anti-CD28, 10 ng/ml IL-4, and 10 μg/ml anti-IFN-γ, were restimulated with anti-CD3. ELISA was performed with 24-h culture supernatants to determine levels of IL-4 and IL-5. *, Significantly different from wild type ($p < 0.05$). *B*, FACS analysis of IL-4 intracellular staining from Th2 cultures prepared as above. *C*, FACS analysis of anti-CD3-stimulated Th2 cells generated as above. Primary cells were stained with CFSE before differentiation. Histograms represent the CFSE staining of wild-type (solid line) and p27^{Kip1}-deficient (shaded line) cells that stained positive for IL-4 as in *B*.

[3] Abbreviation used in this paper: HSA, heat-stable Ag.

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deficient Th2 cultures rather than greater expansion of cells initially committed to the Th2 phenotype. To distinguish between these possibilities, we labeled $CD4^+$ T cells from wild-type and $p27^{Kip1}$-deficient mice with CFSE and differentiated them to Th2 cells as above. After 6 days in culture, cells were restimulated with anti-CD3 and stained for intracellular cytokine analysis of IL-4. We then examined the CFSE profile of $IL\text{-}4^+$ cells. Fig. 1*C* shows that, although there are clearly more IL-4-secreting cells in $p27^{Kip1}$-deficient cultures than in wild-type cultures, the CFSE profiles of these cells are almost identical. Furthermore, $p27^{Kip1}$-deficient cells do not acquire the IL-4-secreting phenotype after any fewer divisions than wild-type cells (data not shown). These data suggest that the higher Th2 cytokine secretion in $p27^{Kip1}$-deficient mice is not due to increased proliferation of differentiating cells but rather is due to an increase in the number of cells stimulated to undergo Th2 differentiation.

Enhanced IL-4 secretion from $p27^{Kip1}$-deficient primary T cells

One possible explanation for the increase in Th2 cells in $p27^{Kip1}$-deficient cultures is an increase in endogenous IL-4 secreted following anti-CD3 stimulation of primary cultures that may stimulate increased differentiation of uncommitted T cells to the Th2 phenotype. Analysis of supernatants from $p27^{Kip1}$-deficient spleen cells stimulated with anti-CD3 for 48 or 72 h demonstrated secretion of similar levels of IL-2 and IFN-γ (data not shown), but greatly increased levels of IL-4, compared with wild-type cultures (Fig. 2*A*). It is known that both $CD4^+$ and $CD8^+$ T cells are expanded in spleens from $p27^{Kip1}$-deficient mice (5, 6). To determine the source of IL-4 production, we sorted by flow cytometry the $CD4^+$ and $CD8^+$ T cell populations. IL-4 production was enriched in the $CD4^+$ population and $p27^{Kip1}$-deficient $CD4^+$ cells secreted higher amounts of IL-4 than wild-type $CD4^+$ cells (Fig. 2*A*). $CD8^+$ cells secreted minimal amounts of IL-4 in this assay.

We have previously documented that $p27^{Kip1}$-deficient mice have increased levels of $CD4^+$ cells that have a memory phenotype (8). To determine whether $CD4^+$ naive cells could produce significant amounts of IL-4, or whether it was the memory phenotype population, we purified $CD4^+CD62L^{high}$ cells from wild-type and $p27^{Kip1}$-deficient spleen and stimulated them with anti-CD3 as above. Fig. 2*B* demonstrates that naive $CD4^+$ cells from either genotype do not secrete significant amounts of IL-4. Thus, the IL-4-secreting cells in $p27^{Kip1}$-deficient spleens do not have a naive phenotype.

Increased $\gamma\delta$ T cells in $p27^{Kip1}$-deficient mice do not secrete significant amounts of IL-4

We next wanted to determine whether the IL-4-producing T cells in $p2^{7kip1}$-deficient mice have an $\alpha\beta$ or $\gamma\delta$ TCR, because $\gamma\delta$ T cells are also known to be a source of IL-4 in vivo (19). Fig. 3*A* demonstrates that the $\gamma\delta$ T cell population is expanded in $p27^{Kip1}$-deficient mice about 2-fold above levels seen in wild-type mice. However, when wild-type and $p27^{Kip1}$-deficient spleen cells were stimulated with anti-TCRβ or anti-TCR$\gamma\delta$, only stimulation of the TCRβ-positive cells yielded IL-4 production comparable with anti-CD3 stimulation (Fig. 3*B*). To confirm the lack of involvement of $\gamma\delta$ T cells in this phenotype, we sorted $\gamma\delta$ T cells by flow cytometry and stimulated them with anti-CD3. Both wild-type and $p27^{Kip1}$-deficient $\gamma\delta$ T cells produced low and similar levels of IL-4 (Fig. 3*C*). Thus, while numbers of $\gamma\delta$ T cells are increased in $p27^{Kip1}$-deficient mice, they are not responsible for the increased levels of IL-4 following anti-CD3 stimulation.

IL-4-producing cells have a $CD4^+DX5^+$ phenotype

We next wanted to examine the NKT cell populations in wild-type and $p27^{Kip1}$-deficient spleen. Unfortunately, the $p27^{Kip1}$ gene is located amid the NK complex located on mouse chromosome 6. So while the $p27^{Kip1}$-deficient mice have been backcrossed six generations to the C57BL/6 genetic background, the NK1 gene is of

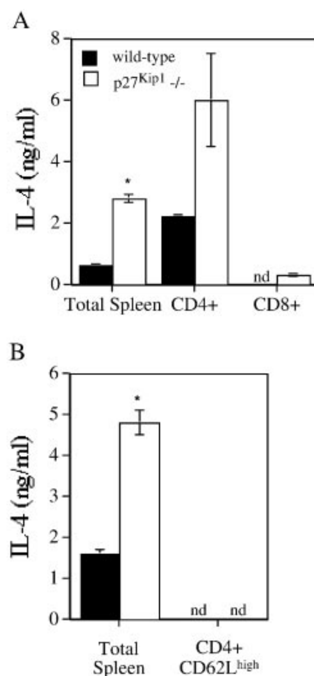

FIGURE 2. Stimulated $CD4^+$ T cells secrete increased amounts of IL-4. *A*, Spleen cells from wild-type or $p27^{kip1-/-}$ mice were sorted into $CD4^+$ and $CD8^+$ subpopulations and stimulated with anti-CD3. Culture supernatants (48 h) were assayed for IL-4 production. *B*, Naive T cells ($CD4^+CD62L^{high}$) were specifically sorted and stimulated by anti-CD3. Culture supernatants (72 h) were tested for IL-4 production. *, Significantly different from wild type ($p < 0.05$).

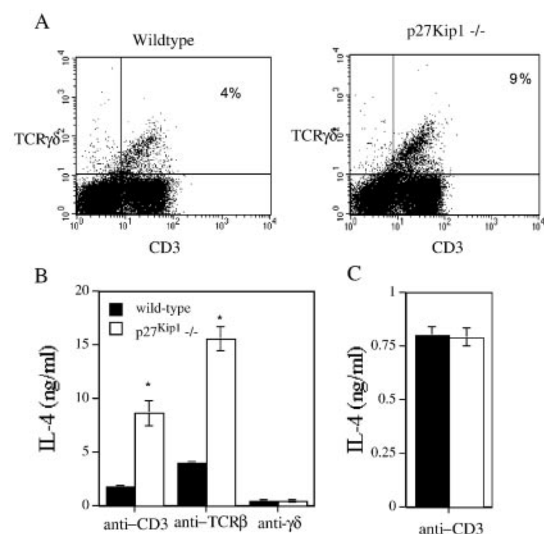

FIGURE 3. $p27^{kip1-/-}$ mice have increased percentages of $\gamma\delta$ T cells. *A*, FACS analysis of spleen cells stained with TCR$\gamma\delta$ and CD3. *B*, Whole spleen cells were stimulated by plate-bound anti-CD3, anti-TCRβ, or anti-TCR$\gamma\delta$ (all at 2 μg/ml). After 72 h, supernatants were harvested and levels of IL-4 were determined. *C*, TCR$\gamma\delta^+$ T cells were sorted and stimulated with anti-CD3. ELISA of culture supernatants (72 h) were used to detect IL-4, as above. *, Significantly different from wild type ($p < 0.05$).

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129 origin because it lies adjacent to the p27^{Kip1} gene targeted in 129 stem cells. Thus, despite the C57BL/6 genetic background, p27^{Kip1}-deficient mice remain NK1.1 negative. This necessitated the use of the DX5 mAb, which recognizes CD49b (20), as a marker for the NKT population. Wild-type and p27^{Kip1}-deficient spleen had similar levels of DX5$^+$TCRβ^+ cells present (Fig. 4*A*). To determine the DX5 status of IL-4-secreting cells, wild-type and p27^{Kip1}-deficient CD4$^+$ cells were sorted into DX5$^-$ and DX5$^+$ populations by flow cytometry. These cells were then stimulated with anti-CD3 and supernatants were analyzed for IL-4 levels. The majority of IL-4 produced by p27^{Kip1}-deficient cells is secreted by the CD4$^+$DX5$^+$ population (Fig. 4*B*). To confirm that this population was indeed responsible for the IL-4 production in total spleen, spleen cells from wild-type and p27^{Kip1}-deficient mice were depleted using DX5 and complement before stimulation with anti-CD3. This treatment decreased dramatically the amount of IL-4 secreted by p27^{Kip1}-deficient cells and minimized the differences between the wild-type and p27^{Kip1}-deficient spleen cells (Fig. 4*C*). To directly demonstrate that DX5$^+$ cells were responsible for the differences in Th2 differentiation between wild-type and p27^{Kip1}-deficient T cells, CD4$^+$ T cells purified from spleen of both genotypes of mice were differentiated in culture with or without DX5 depletion. Fig. 4*D* demonstrates that DX5 depletion of CD4$^+$ T cells before culture minimizes the differences in IL-4 and IL-5 production between wild-type and p27^{Kip1}-deficient Th2 cultures. Interestingly, depletion of DX5$^+$ cells from the wild-type population actually increased IL-4 production, suggesting that a DX5$^+$ population may also be negatively regulating Th2 differentiation. Importantly, however, the levels of IL-4 production from wild-type and p27^{Kip1}-deficient cultures are not significantly different following DX5 depletion.

These results were consistent with our hypothesis that increased endogenous IL-4 secretion from p27^{Kip1}-deficient CD4$^+$DX5$^+$ T cells was increasing the generation of Th2 cells. It is also possible that the CD4$^+$DX5$^+$ T cells are contributing to the IL-4 production by the differentiated cultures. To distinguish these possibilities, we examined IL-4 production by Th2 cultures with intracellular staining while counterstaining with DX5. We observed that the majority of IL-4 production in both wild-type and p27^{Kip1}-deficient Th2 cultures was from CD4$^+$DX5$^-$ T cells (data not shown). Thus, p27^{Kip1}-deficient CD4$^+$DX5$^+$ primary T cells increase Th2 generation in our in vitro culture conditions but do not significantly contribute to the increased IL-4 production compared with wild-type Th2 cultures.

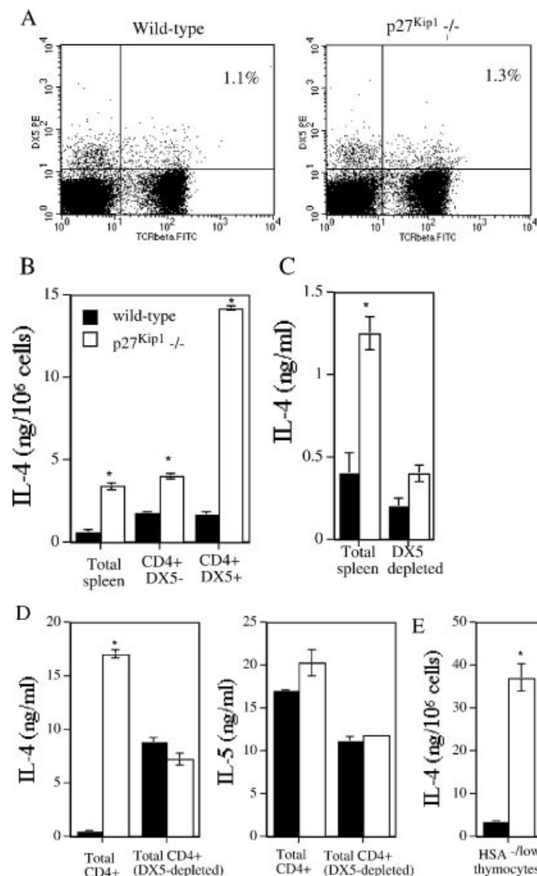

FIGURE 4. DX5$^+$ cells in p27$^{kip1-/-}$ mice secrete elevated IL-4 levels. *A*, FACS analysis of spleen cells stained with TCRβ and DX5. *B*, CD4$^+$DX5$^+$ and CD4$^+$ T cells (CD4$^+$DX5$^-$) were specifically sorted and stimulated with anti-CD3. ELISA of culture supernatants (72 h) were tested for levels of IL-4. *C*, Wild-type and p27^{Kip1}-deficient spleen were stimulated with anti-CD3 with or without depletion of DX5$^+$ cells by Ab plus complement. *D*, MiniMacs-purified CD4$^+$ cells (untreated or depleted of DX5$^+$ cells with Ab and complement) were differentiated under unskewed conditions and analyzed as in Fig. 1. *E*, Thymocytes enriched by HSA (J11d)-mediated complement depletion were stimulated by anti-CD3, and 72-h supernatants were analyzed for IL-4 levels. *, Significantly different from wild type ($p < 0.05$).

Increased IL-4-secreting cells in p27^{Kip1}-deficient thymus

NKT cells also reside in the thymus and are responsible for a large portion of the IL-4 secreted by HSA$^{-/low}$ thymocytes (21). To determine whether thymic NKT cells are also secreting more IL-4, we isolated thymocytes from wild-type and p27^{Kip1}-deficient mice and complement-depleted HSA$^+$ cells. Remaining cells were stimulated with anti-CD3 and IL-4 levels were determined by ELISA. Fig. 4*E* shows that greatly increased levels of IL-4 were secreted by p27^{Kip1}-deficient HSA$^{-/low}$ thymocytes compared with wild-type controls.

Enhanced proliferation but not differentiation of T cells doubly deficient in STAT4 or STAT6 and p27^{Kip1}

We have previously correlated a defect in p27^{Kip1} regulation with the inability of STAT4- and STAT6-deficient lymphocytes to proliferate in response to IL-12 and IL-4, respectively (11). We have also determined that p27^{Kip1}-deficient T cells are hyperproliferative to cytokine stimulus (8). Moreover, T cells from mice that are doubly deficient in p27^{Kip1} and STAT6 significantly recover proliferative responses to IL-4, though not completely (8). To further characterize the proliferative responses in wild-type, STAT6-deficient, and STAT6/p27^{Kip1}-deficient T cells, CD4$^+$ cells from these mice were activated with anti-CD3 for 72 h. Cells were then labeled with CFSE, a dye that allows enumeration of cell division. Importantly, because the cells labeled are preactivated, the CFSE patterns differ from the patterns of division seen in anti-CD3- or Ag-stimulated resting cells. However, division in the population is still reflected as lower CFSE staining (22). Fig. 5*A*, *left panels*, demonstrates that T cells from mice of all three genotypes have similar responses to IL-2 at 24, 48, and 72 h following IL-2 stimulation. In contrast, proliferative responses to IL-4 are altered in T cells lacking STAT6 expression. As shown in previous work, the proliferative response of STAT6-deficient cells is diminished compared with wild-type cells. This is reflected in Fig. 5*A* as higher CFSE staining of the STAT6-deficient cells, indicating that the population has gone through fewer divisions than similarly treated wild-type cells. It also demonstrates that STAT6-deficient T cells respond to IL-4 as a single population and there is not only a subpopulation of cells dividing. We previously observed that double deficiency in STAT6 and p27^{Kip1} recovers IL-4-stimulated

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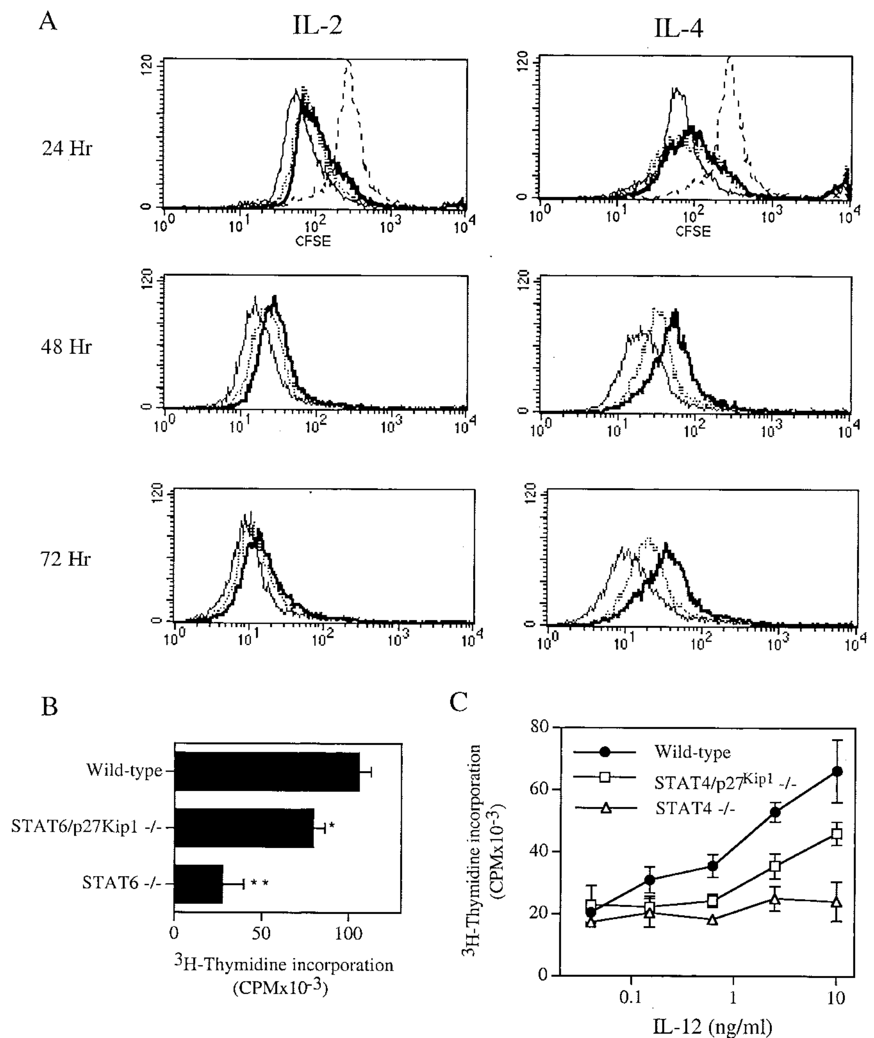

FIGURE 5. Activated T lymphocytes from STAT6/p27$^{kip1-/-}$ or STAT4/p27$^{kip1-/-}$ mice recover proliferative ability. *A*, Proliferation after IL-2 (20 U/ml, *left panels*) or IL-4 (10 ng/ml, *right panels*) treatment of wild-type (thin line), STAT6$^{-/-}$ (thick line), and STAT6/p27$^{kip1-/-}$ (dotted line) 72-h activated T cells was monitored by CFSE and FACS analysis after 24, 48, and 72 h poststimulation. Dashed line represents 0-h CFSE staining. *B*, Proliferation of wild-type, STAT6-deficient, and STAT6/p27^{Kip1}-deficient 72-h activated T cells stimulated with 1 ng/ml IL-4 was determined by [^{3}H]thymidine incorporation. *, Significantly different from wild-type and STAT6-deficient cells ($p < 0.05$); **, significantly different from wild-type and double-deficient cells ($p < 0.05$). *C*, Proliferation from wild-type, STAT4-deficient, or STAT4/p27^{Kip1}-deficient 72-h activated T cells was determined by [^{3}H]thymidine incorporation.

proliferation, compared with STAT6-deficient cells, but is still slightly less than wild-type, particularly at lower doses of IL-4 (Fig. 5*B*) (8). This observation is supported by CFSE staining that demonstrates STAT6/p27^{Kip1}-deficient T cells only obtain an intermediate level of division (Fig. 5*A*, *right panels*) compared with wild-type cell proliferation.

We next tested whether double deficiency of STAT4 and p27^{Kip1} would similarly restore some of the proliferative capacity of T cells in response to IL-12. To test this we activated CD4$^+$ cells from wild-type, STAT4-deficient, and STAT4/p27^{Kip1}-deficient mice with anti-CD3 for 72 h. Activated T cells were then plated with increasing concentrations of IL-12. While STAT4-deficient mice lack a proliferative response to IL-12 (14, 23), STAT4/p27^{Kip1}-deficient mice display an IL-12-stimulated proliferative response (Fig. 5*C*). This response is intermediate, between the STAT4-deficient response and wild-type response, and suggests that, like STAT6, STAT4 may affect expression of other factors, aside from p27^{Kip1}, that are required for IL-12-stimulated proliferation. These results suggest that p27^{Kip1} deficiency can correct for some but not all of the proliferative defects in STAT4- and STAT6-deficient T cells.

Proliferation of differentiating Th cells has recently been proposed to be essential for the acquisition of restricted cytokine expression patterns (16–18). In addition to their lack of ability to respond by proliferation to IL-4 stimulation, STAT6-deficient T cells also have dramatically reduced differentiation of Th2 cells (13, 24–26). The STAT6/p27^{Kip1}-deficient mice provided a unique opportunity to assess the relative importance of STAT6-dependent proliferation vs the requirement for a STAT6 instructive step in Th2 differentiation. To determine whether the increased proliferative response of double-deficient lymphocytes could increase their ability to become Th2 cells, we stimulated wild-type, STAT6-, p27^{Kip1}-, and STAT6/p27^{Kip1}-deficient CD4$^+$ T cells with anti-CD3 supplemented with 10 ng/ml IL-4 and 10 μg/ml anti-IFN-γ. After 6 days in culture, cells were washed and restimulated with anti-CD3 alone. Supernatants were harvested after 24 h and tested for IL-4 levels by ELISA. As previously shown, STAT6-deficient lymphocytes secreted negligible levels of IL-4 compared with wild-type cells (Fig. 6*A*). p27^{Kip1}-deficient cells were consistently better at differentiating into Th2 than wild-type cells, as indicated in Fig. 1. Importantly, STAT6/p27^{Kip1}-deficient cells did not have any increased capacity to differentiate into Th2 cells (Fig. 6*A*), supporting our conclusion that increased p27^{Kip1}-deficient Th2 generation is due to increased IL-4 production and not to an intrinsic ability of p27^{Kip1}-deficient T cells to become Th2 cells. To determine whether increased IL-12-stimulated proliferation was sufficient to increase the capacity of STAT4/p27^{Kip1}-deficient T cells to become Th1 cells, wild-type, STAT4-, p27^{Kip1}-, and STAT4/p27^{Kip1}-deficient CD4$^+$ T cells were differentiated to the Th1 phenotype by culture with 1 ng/ml IL-12 and 10 μg/ml anti-IL-4. Following restimulation with anti-CD3, supernatants were collected for ELISA analysis. Wild-type and

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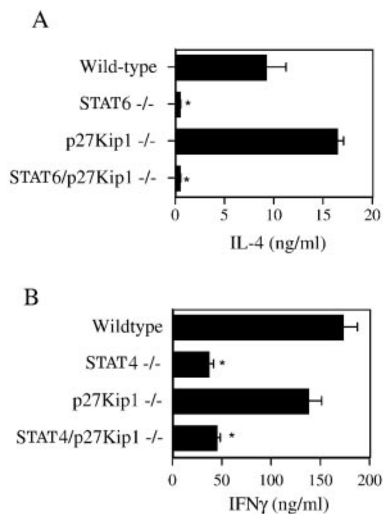

FIGURE 6. Enhanced proliferation does not compensate for STAT-dependent instruction of Th cell differentiation. *A*, $CD4^+$ cells from the indicated genotypes were incubated under Th2 culture conditions as indicated in Fig. 1. After 6 days in culture, cells were washed and restimulated with anti-CD3. Supernatants (24 h) were tested for IL-4 levels. *B*, $CD4^+$ cells from the indicated genotypes were incubated under Th1 culture conditions (anti-CD3, anti-CD28, 1 ng/ml IL-12, and 10 μg/ml anti-IL-4). After 6 days in culture, cells were washed and restimulated with anti-CD3. Supernatants (24 h) were tested for IFN-γ levels. *, Significantly different from wild type ($p < 0.05$).

$p27^{Kip1}$-deficient cultures secreted high levels of IFN-γ that were indistinguishable over repeated assays. However, the reduced Th1 differentiation in STAT4-deficient cultures was not corrected by the additional deficiency in $p27^{Kip1}$ (Fig. 6*B*). Thus, while elimination of $p27^{Kip1}$ is sufficient to increase STAT4- and STAT6-mediated proliferation, the subsequent increase in proliferation is not sufficient to alter the differentiative capacity of STAT4- and STAT6-deficient T cells.

Discussion

We have previously demonstrated that T cells deficient in $p27^{Kip1}$ have increased proliferative responses to cytokine stimulation. In this report, we demonstrate that $p27^{Kip1}$-deficient lymphocytes have an enhanced differentiation to Th2 cells in vitro, apparently resulting from an increase in IL-4-secreting T cells that express CD4 and CD49b. In our previous report (8) and in this report, we demonstrate that $p27^{Kip1}$ deficiency is able, at least partially, to correct the proliferative defects in STAT4- and STAT6-deficient T cells. However, despite increased cytokine-stimulated proliferation and enhanced Th2 differentiation in $p27^{Kip1}$-deficient cells, T cells from STAT4/$p27^{Kip1}$-deficient or STAT6/$p27^{Kip1}$-deficient mice have no more ability to differentiate to Th1 or Th2 cells, respectively, than either of the single STAT4- or STAT6-deficient T cells. Together, these data demonstrate the important role of $p27^{Kip1}$ in regulating T cell expansion but not differentiation.

The potential role of proliferation in Th cell differentiation has received considerable attention since several studies suggested that cell division was required for the secretion of particular cytokines (16–18). A subsequent report demonstrated that cytokines could be secreted without division, although levels of secretion were higher following division (27). However, these reports still did not distinguish whether the expansion of cytokine-secreting cells was selective or instructive following the cytokine stimulus. Farrar et al. (28) used retroviral tagging to demonstrate that IL-4-secreting cells are not selectively expanded in Th1 or Th2 populations and supported the idea that GATA-3 delivers an instructive signal to generate Th2 cells. Cytokine secretion at near wild-type levels are initially seen in STAT4- and STAT6-deficient T cells, although T-bet and GATA-3 expression are not maintained, respectively, in the STAT-deficient T cells (29). This also suggests that STAT4 and STAT6 have instructive roles in sustaining expression of transcription factors involved in the differentiated phenotype. To further address this issue, we mated STAT4- and STAT6-deficient mice to $p27^{Kip1}$-deficient mice and demonstrated that cytokine-induced proliferation is, at least in part, recovered in T cells deficient in STAT4 or STAT6 and $p27^{Kip1}$. If a simple selective hypothesis were correct, the generation of Th1 cells in STAT4/$p27^{Kip1}$-deficient or Th2 cells in STAT6/$p27^{Kip1}$-deficient T cell cultures would be expected to increase. However, our studies demonstrate that even when cytokine-driven proliferation (selection) is increased, and protection from apoptosis should be normal (11, 12, 30, 31), differentiation is not affected (Fig. 6). Indeed, using CFSE analysis, STAT6/$p27^{Kip1}$-deficient T cells cultured under Th2 conditions go through as many cell divisions as wild-type cells yet still acquire a Th1 cytokine-secreting phenotype (data not shown). Thus, while cell division may be required for successful Th differentiation, it is not sufficient in the absence of STAT-regulated differentiation programs.

STAT proteins may control growth and proliferation at several levels through distinct mechanisms. For example, STAT3 is important for protection from apoptosis in lymphocytes (32, 33). STAT3 may also regulate cell cycle transition in lymphoid cells (34). In nonlymphoid cells, STAT3 is a cofactor for transformation of cells by Src and can function as an oncogene by itself (35–37). STAT5a-deficient cells display impaired proliferative responses to IL-2 and GM-CSF (38, 39). The proliferative response of STAT5a-deficient T cells to IL-2 can be overcome by high doses of IL-2 to compensate for the low level of CD25 expression (39). The phenotype in STAT5b-deficient mice was more profound in that T cells had reduced expression of both CD25 and CD122 and proliferation to IL-2 could not be recovered with high doses of IL-2 (40). STAT5a/STAT5b double deficient T cells are completely refractory to anti-CD3 stimulation (41). This correlated with a lack of anti-CD3-induced expression of cyclin D2, cyclin D3, and cyclin-dependent kinase 6 (41). Importantly, resting T cell stimulation is strictly dependent on STAT5 because STAT6 activation cannot compensate for the lack of STAT5 signals. In contrast, the cytokine-stimulated proliferative defects in STAT4- and STAT6-deficient T cells are restricted to the cognate activating cytokines, IL-12 and IL-4, respectively (13, 14, 23–25). This phenotype correlated with a dysregulation of $p27^{Kip1}$ levels following cytokine stimulation (11). Furthermore, mice deficient in $p27^{Kip1}$ and either STAT4 or STAT6 recover proliferative responses to the requisite cytokines (Ref. 8 and this report). The result that $p27^{Kip1}$ deficiency does not completely recover STAT4- or STAT6-dependent proliferation only highlights the multiple targets of STAT proteins in these processes. Other potentially important targets include the receptors for IL-4 and IL-12, which we have shown are regulated by STAT6 and STAT4, respectively (13, 42). Additional targets are also currently being pursued.

An increase in thymocytes and splenic T cells in $p27^{Kip1}$-deficient mice has been previously described (5–7). Thymus size is increased, although the proportions of subsets therein appear normal. Splenic T cell percentages (both $CD4^+$ and $CD8^+$) are also increased in $p27^{Kip1}$-deficient mice. In our previous report, we observed that $CD4^+$ memory ($CD62L^{-/low}$) cells are expanded in $p27^{Kip1}$-deficient spleens (8). In this report, we further observe an increase in $\gamma\delta$ T cells similar to increases in $CD4^+$ and $CD8^+$ cells (Fig. 3). We also note that, functionally, there is an increase in

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IL-4-secreting cells in the spleen and these cells have a $CD4^+TCR\alpha\beta^+CD62L^{-/low}DX5^+$ phenotype (Figs. 2–4). However, we did not see an increase in the percentage of $TCR\alpha\beta^+$ $DX5^+$ or $CD4^+DX5^+$ T cells (Fig. 4 and data not shown). It is possible and likely that IL-4-secreting cells comprise only a small proportion of the $CD4^+DX5^+$ cells and that the expansion cannot be detected using only these surface markers. Overall, p27^{Kip1} appears to play a critical role in regulating the expansion and homeostasis of various T cell subsets.

The exact identity of the IL-4-secreting population is unclear. These cells have many of the characteristics of NKT cells that, it is becoming clear, are a very heterogeneous population of cells. Classical NKT cells express both NK1.1 and TCRs, recognize glycolipids in the context of CD1 (43, 44), and are dependent on CD1 for their development (21, 45–47). Some studies suggest that non-CD1-restricted T cells can express NK1 or CD49b and thus still might be classified, in a general sense, as NKT cells (48, 49). Importantly, a subset of $CD4^+DX5^+$ (and $NK1.1^-$) cells has recently been identified as regulatory cells in a model of diabetes (50). In the future, p27^{Kip1}-deficient mice may be a useful system to examine these functionally expanded cells.

In summary, we have demonstrated that p27^{Kip1} deficiency can partially compensate for STAT4- and STAT6-dependent proliferative defects in response to IL-12 and IL-4, respectively. Despite this, double deficient T cells do not have any increased capacity to respond to these cytokines and differentiate into Th1 and Th2 cells, respectively. Mice deficient in p27^{Kip1} have increased populations of $\gamma\delta$ T cells and $CD4^+DX5^+$ T cells that secrete high levels of IL-4. Thus, p27^{Kip1} is an important regulator of T cell expansion but is not a regulator of the Th cell differentiative process.

Acknowledgments

We thank Michael Grusby and Jim Roberts for supplying mice, Shangming Zhang for technical assistance, Hua-Chen Chang for statistical analysis, and Heather Bruns, Randy Brutkiewicz, and Cheong-Hee Chang for critical review of this manuscript.

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