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# Interleukin-11 induces proliferation of human T-cells and its activity is associated with downregulation of $p27^{kip1}$

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*Background and Objectives*. We have recently shown that interleukin (IL-)11 induces polarization of human T-cells by inhibiting macrophage production of IL-12 and by exerting a direct effect on $CD4^+$ T-cells. In this study, we investigated the effects of IL-11 on the kinetic activation and apoptosis of T-cell subsets stimulated with anti-CD3/CD28 antibodies, anti-CD3 and IL-2 or dendritic cells.

*Design and Methods*. Apoptosis and cell cycle analysis of T-cells were assesed by double staining with propidium iodide and intracellular Ki-67 and by acridine orange staining. The expression of the negative regulator of the cell cycle $p27^{Kip1}$ (p27) was also determined by flow cytometry.

*Results*. Our results show that 18 hours of incubation with IL-11 resulted in a significantly higher number of cycling $CD4^+$ cells, $CD4^+CD45RA^+$ naive T-cells and $CD4^+CD45RO^+$ memory T-cells, but not of $CD8^+$ cells. The kinetic activity of IL-11 was observed up to 72 hours, when the peak value of S-phase cells occurred. IL-11 also significantly enhanced $CD4^+$ and $CD4^+CD45RA^+$ cell proliferation when T-cells were co-incubated with allogeneic dendritic cells. Conversely, IL-11 did not protect any of the T-cell subsets from apoptosis. At the functional level, a type-2 cytokine pattern of cultured T-lymphocytes was observed after 5 days of incubation with IL-11. Proliferation and functional activation of T-cells were preceeded by downregulation of p27, which occurred as early as 12 hours after incubation with IL-11.

*Interpretation and Conclusions*. IL-11 induces Th-2 polarization and cell-cycle entry of human $CD4^+$, $CD4^+CD45RA^+$ and $CD4^+CD45RO^+$cells and their activation is associated with the downregulation of p27.


Key words: interleukin-11, cell cycle, T-cell proliferation, p27, CKI

Interleukin (IL)-11 is a multifunctional cytokine which stimulates hematopoietic progenitor cells and exerts a series of important immunomodulatory effects.[1] The strong anti-inflammatory activity of IL-11 *in vitro* and *in vivo* is mediated by inhibition of macrophage production of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-$\alpha$, IL-1$\beta$, IL-6 and IL-12.[2-4] When administered in multiple animal models of inflammatory disorders, IL-11 reduces signs of disease.[5-7] Moreover, in a well-characterized murine model of graft-versus-host disease (GVHD), treatment with IL-11 strongly inhibited GVHD and enhanced recipient survival without affecting the cytolytic function and graft-versus-leukemia (GVL) effect of $CD8^+$ T-cells.[8] The significant reduction of CD4-dependent GVHD toxicity was in part due to suppression of TNF-$\alpha$ and to polarization of donor T-cells to a Th2 response with decreased interferon (IFN)-$\gamma$ and augmented IL-4 production.

We have recently shown that IL-11 induces polarization of human T-cells by inhibiting macrophage production of IL-12 and by exerting a direct effect on $CD4^+$ naive T-cells that express IL-11 receptor on their surface.[9] On the other hand, we did not observe any activity of IL-11 on the development and functional activation of dendritic cells

(DC). In the present study, we investigated whether incubation with IL-11 would, as well as promoting T-cell survival, also trigger kinetic activation of quiescent T-cells, as determined by entry into cell cycle and progression to S-phase. Moreover, as recent studies have demonstrated the pivotal role of the cyclin-dependent kinase inhibitor (CKI) $p27^{Kip1}$ (p27) in the development and function of murine T-cells,[10,11] we investigated the role of p27 in IL-11-mediated proliferation and functional activation of human T-cells. Our results indicate that, along with polarization of $CD4^+$ Th0 cells to Th2 phenotype, IL-11 induces cycling of $G_0$-phase $CD4^+$ cells, $CD4^+$ $CD45RA^+$ naive T-cells and $CD4^+CD45RO^+$ memory T-cells. This kinetic activity is due to early down-regulation of p27.

## Design and Methods

### *Cell sample collection and processing*

Peripheral blood (PB) mononuclear cells (MNC) were obtained by gradient centrifugation (Lymphoprep; 1.077 g/mL; Nycomed Pharma, Oslo, Norway). Light-density cells were washed twice in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA; Sigma Chemical Co. St. Louis, MO, USA) and $CD4^+$, $CD4^+CD45RA^+$, $CD4^+CD45RO^+$, $CD8^+$ or $CD14^+$ cells were highly purified from the MNC fraction by MiniMacs high-gradient magnetic separation column (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions.[9] Flow cytometric re-analysis of purified cell fractions was performed on a gated population set on scatter properties using FACScan equipment (Becton Dickinson, Mountain View, CA, USA), as earlier described.[9,12] A minimum of 10,000 events was collected in list mode on FACScan software. The purity of enriched populations was always greater than 90% of the total cell yield.

### *Generation of DC in liquid culture*

DC were generated from PB $CD14^+$ cells (Mo-DC) as earlier reported.[13] Briefly, $1\times10^6$ purified $CD14^+$ cells were cultured for 5-7 days in 1 mL of RPMI 1640 supplemented with 10% fetal calf serum (FCS; Sera Lab, Crawley Down, Sussex, UK), antibiotics, L-glutamine, 50 ng/mL of granulocyte macrophage colony-stimulating factor (GM-CSF) (Novartis, Basel, Switzerland) and 800 U/mL of IL-4 (Schering Plough, Kenilworth, NJ, USA). Twenty-five ng/mL of TNF-$\alpha$ (Innogenetics, Zwijndrecht, Belgium) were then added to the culture for a further 24-36 hours to induce terminal maturation of DC. Cultures were maintained at 37°C in 5 % $CO_2$ by replacing culture medium and cytokines at day +3. At the end of the culture, the generation of functionally active DC was assessed by phase-contrast microscopy, immunophenotyping, evaluation of cytokine secretion and mixed lymphocyte reactions (MLR) as previously described.[13,14]

### *In vitro priming of $CD4^+$ T-cells*

Highly purified T-cells were resuspended in RPMI 1640 containing 5% human AB serum and stimulated with 1 $\mu$g/mL of plate-coated anti-CD3 MoAb (Immunotech, Marseille, France) and 1 $\mu$g/mL of soluble anti-CD28 MoAb (Pharmingen, San Diego, CA, USA) in the presence or absence of recombinant human IL-11 (100 U/mL; Endogen, Woburn, MA, USA). The concentration of IL-11 was chosen based on our previous experience[9] and a dose-response curve (0.1-1000 U/mL) to determine the optimal proliferative effects on T-cells (*data not shown*). In selected experiments, 100 U/mL of recombinant human IL-2 (Proleukin, Chiron; Emervylle, CA, USA) replaced soluble anti-CD28 MoAb. After 5 days of culture, T-cells were transferred to uncoated plates in medium containing 30 U/mL of IL-2. After 7 to 8 days of expansion, T-cells were treated for intracellular cytokine staining.[9] Briefly, T-cells were counted and reactivated with IS Cyto Activation Kit, containing PMA, ionomycin and monensin (Immune Source, Los Altos, California, USA). After 6 hours of incubation at 37°C, cells were washed, fixed in Fixation Buffer (Immune Source) and permeabilized with permeabilization/wash buffer (Immune Source), following the manufacturer's instructions. T-cells were then stained with anti-IL-4-phycoerythrin (PE) and anti-IFN$\gamma$-FITC antibodies (Immune Source) and finally analyzed with a flow cytometer.

### *Allogeneic MLR*

In order to assess the allogeneic MLR, $5 \times 10^4$ highly purified $CD4^+$ and $CD4^+CD45RA^+$ T-cells were stimulated with increasing numbers of irradiated (30 Gy) DC in the presence or absence of 100 U/mL of IL-11. Cells were resuspended in RPMI-1640, 25 mM HEPES, antibiotics and 5% AB human serum that had been inactivated at 56° C for 30 min, in round-bottomed 96-well plates for 6 days at 37°C in a 5% $CO_2$ humidified atmosphere. Cells were pulsed with 1 $\mu$Ci/well $^3$H-thymidine for 18 h before harvest on day 6. The stimulation index (SI) was calculated for each individual experiment as follows: SI= counts per minute (c.p.m.) (T-cell responders+ stimulators)/c.p.m. (T-cell responders) as earlier reported.[13,14]

*Assessment of apoptosis and cell cycle analysis*

Purified T-cell subsets (i.e. $CD4^+$, $CD4^+CD45RA^+$, $CD8^+$ and $CD4^+CD45RO^+$ T-lymphocytes) were grown in the presence of anti-CD3 MoAb and anti-CD28 MoAb or IL-2 (see above) with or without IL-11 (100U/mL). Cell-cycle analysis and assessment of apoptosis were performed at baseline and daily, after priming, up to 5 days as earlier reported.[15] Briefly, cells were fixed and permeabilized in 70% ethanol. RNAse was then added at 5 μg/mL and the cells were incubated at 37°C for 15 minutes before staining with 50 μg/mL propidium iodide (PI) for 60 minutes at 4°C. The fractions of viable cells in the $G_0/G_1$-, S- and $G_2$/M-phases of cell cycle were determined by flow cytometric analysis (FacScan; Becton Dickinson). In some experiments, T-cells were fixed in 70% ethanol, permeabilized as described above and then stained for intracellular Ki-67 by using MIB-1 Moab (Immunotech). Early recruitment of T-cells in cell cycle was evaluated by an acridine orange flow cytometric technique.[12,16] The percentage of cells in $G_0$, $G_1$ and the mean RNA content of $G_0/G_1$ cells were determined by measuring simultaneously the DNA and RNA total cellular content. RNA content of $G_0/G_1$ cells was expressed as an RNA-index (RNA-I $G_0/G_1$), calculated as the ratio of the mean RNA content of $G_0/G_1$ cells of the samples multiplied by 10 and divided by the median RNA content of control PB lymphocytes. $G_0$ cells were considered the cells with an RNA content equal to or lower than that of control PB lymphocytes.[12,16] Apoptosis was evaluated by flow cytometry measuring annexin V binding simultaneously with the PI exclusion test.[15]

*Flow cytometry analysis of p27 expression*

Expression of p27 CKI by T-cells was evaluated using a flow cytometric technique. Briefly, 0.5 $\times 10^6$/mL cells were fixed and permeabilized using the Fix & Perm kit (Caltag Laboratories, Burlingame, CA, USA) and then incubated for 30 min in the dark at room temperature with the mouse monoclonal antibodies, anti-human p27 (clone 57) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a concentration of 1 μg/mL. Irrelevant mouse antibody of the appropriate subclass was used as the negative control to determine background fluorescence. After two washes, cells were incubated with FITC-conjugated goat $F(ab')_2$ fragment mouse IgG (H-L) for 30 min in the dark at room temperature. After washing, cells were immediately measured at flow cytometry. Results were expressed as the mean fluorescence index (MFI), corresponding to the mean fluorescence of the sample stained with p27 MoAb divided by the mean fluorescence of the sample stained with the irrelevant MoAb.

*Statistical analysis*

The results are expressed as the mean ± the standard deviation (SD). Where indicated, statistical analysis was performed by the non-parametric paired Wilcoxon rank-sum test.

## Results

*Kinetic activity of IL-11 on T-cells: recruitment into cell cycle and progression to S-phase*

In the first set of experiments we investigated whether IL-11-induced functional activation of $CD4^+$ cells is associated with kinetic activation and expansion of T-cells. To this end, $CD4^+$ cells were stimulated with anti-CD3/anti-CD28 antibodies and exposed to IL-11 or mock medium. Cell-cycle analysis and assessment of apoptosis were performed at baseline and for up to 5 days after priming; the overall results are shown in Figure 1A. Incubation with IL-11 resulted in significantly higher percentages of cycling ($Ki\text{-}67^+$) and S-phase $CD4^+$ cells at day +3 (63±8% vs. 28±4% and 23±6% vs. 14±4%, respectively; both $p < 0.04$). This kinetic difference disappeared at day +5, when the type-2 cytokine pattern of cultured $CD4^+$ cells became evident ($IFN\text{-}\gamma^+$ cells decreased from 69±8% to 36±7% while $IL\text{-}4^+$ cells augmented from 35±6% to 54±7%; both $p < 0.04$). The same proliferation pattern was found when anti-CD28 antibodies were replaced by IL-2 (*data not shown*). An example of the striking T-cell kinetic activation induced by IL-11 is shown in Figure 1B.

We then asked whether the proliferative effects of IL-11 are exerted on both naive and memory $CD4^+$-cell subsets. To this end, in the next set of experiments we evaluated whether IL-11 induces the early exit of quiescent $CD4^+$, $CD4^+CD45RA^+$ and $CD4^+CD45RO^+$ T-cells from $G_0$-phase (Figure 2). At baseline, >97% and >99% of $CD4^+$ and $CD4^+$ $CD45RA^+$ naive T-cells, respectively, were in $G_0$-phase. After 18 hours of incubation with IL-11, the percentage of $G_0$ $CD4^+$ cells was 19.8% as compared with 48.9% in control cultures. Concomitantly, the percentage of $CD4^+$ cells in $G_1$-phase reached as high as 80.2% compared to the 51.1% in control cultures. Similarly, 18 hours of incubation in the presence of IL-11 reduced the percentage of $CD4^+CD45RA^+$ naive T-cells in $G_0$-phase to 10.7% ($G_1$ = 89.3%), as compared with 24.8% in control samples ($G_1$= 75.2%). Furthermore, when we determined the cell cycle state of $CD4^+CD45RO^+$

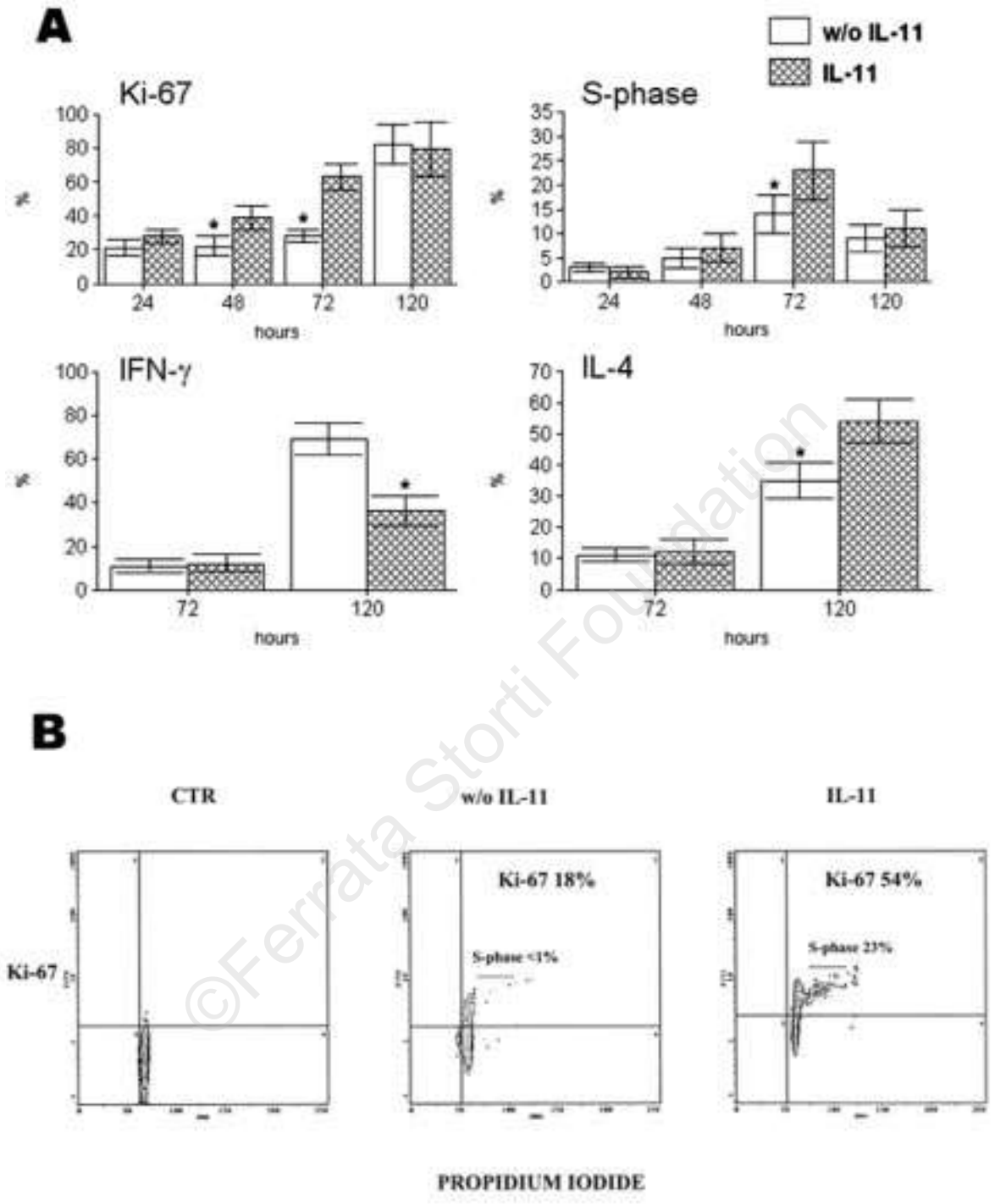




**Figure 1. IL-11 recruits CD4⁺ T-cells into the cell cycle: it promotes their entry into S-phase and polarization to Th2 cells. Purified CD4⁺ T-cells were grown in the presence of anti-CD3 and anti-CD28 MoAbs with or without (w/o) IL-11. Cell-cycle analysis and assessment of cytokine secretion were performed at baseline and daily, after priming, for up to 5 days as reported in the *Design and Methods* section. Addition of IL-11 induced a significantly higher number of cycling and S-phase CD4⁺ cells after 3 days of incubation. This kinetic activation was followed by a type-2 cytokine pattern as determined by intracellular cytokine staining. In A, the overall results of three experiments performed in triplicate are reported. In B, an example of T-cell kinetic activation induced by IL-11 is shown. *indicates $p < 0.05$.**

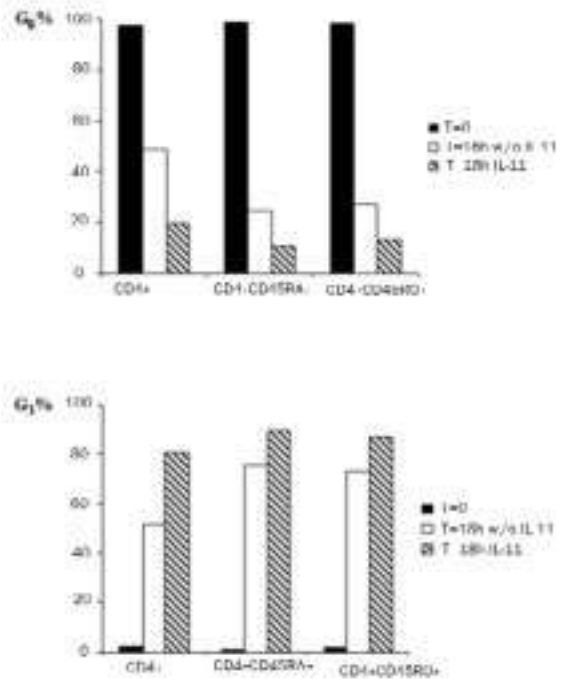

**Figure 2. IL-11 induces early exit of quiescent T-cells from $G_0$-phase. $CD4^+$ cells, $CD4^+CD45RA^+$ naive T-cells and $CD4^+CD45RO^+$ memory T-cells were incubated with anti-CD3 / IL-2 with or without (w / o) IL-11, and the percentages of $G_0$-, $G_1$-phase cells were determined by the AO flow cytometric technique. IL-11 rapidly recruited T-cells into $G_1$-phase. The results of one representative experiment out of 3 are shown.**

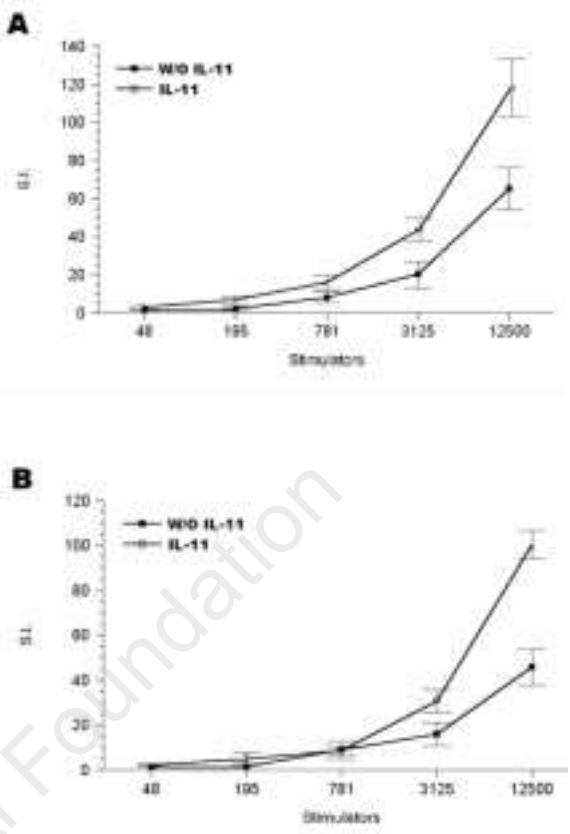

**Figure 3. IL-11 enhances $CD4^+$ and $CD4^+CD45RA^+$ T-cell proliferation upon co-colture with DC. Fifty thousand ($5\times10^4$) purified $CD4^+$ (A) or $CD4^+CD45RA^+$ (B) cells were incubated with increasing numbers of allogeneic DC with or without (w / o) IL-11. The negative control was autologous MNC. In both series of experiments (n=5), the stimulation index (S.I.) was significantly higher ($p < 0.03$) when IL-11 was added to MLR.**

memory T-cells, we found that 18 and 36 hours of incubation with IL-11 decreased the percentage of $G_0$ cells from 27% to 13% and from 24.7% to 6.6%, respectively (Figure 2). By contrast, we did not observe a significant effect of IL-11 on cell-cycle recruitment of cytotoxic $CD8^+$ cells (*data not shown*).

The proliferative activity of IL-11 on purified $CD4^+$ and $CD4^+CD45RA^+$ T-cells was also tested when T-cells were co-incubated with mature Mo-DC in a conventional allogeneic MLR. As shown in Figure 3, the presence of IL-11 significantly enhanced proliferation of both T-cell subsets ($p < 0.03$).

Apoptosis of T-cells was evaluated during up to 5 days of incubation with and without IL-11 by the AO technique and (for early events) by simultaneous staining with PI/annexin V. Neither assay revealed any effect of IL-11 on protection from apoptosis in any of the T-cell subsets. In particular, the percentages of apoptotic $CD4^+$, $CD4^+CD45RA^+$ and $CD4^+CD45RO^+$ cells after 72 hours of culture (at the time of the peak activity of IL-11) were 2.4%, 3.4% and 3.8%, without IL-11, vs 2%, 2.7% and 2.2% in presence of IL-11, respectively.

### *IL-11 inhibits p27 expression*

We then investigated whether the proliferative effect of IL-11 is associated with p27 inhibition, which has been shown to be important for the development and function of normal and leukemic T-cells.[10,11,17] Flow cytometric analysis of p27 demonstrated that as little as 12 hours of incubation with IL-11 significantly reduced ($p< 0.05$) the expression of this CKI on $CD4^+CD45RA^+$ cells (Figure 4). p27 remained downregulated at 18 hours

($p$= 0.06) before returning to the baseline level after 24 hours. Taken together, these results indicate that the exit from kinetic quiescence, progression into cell cycle and Th2 polarization of T-cells are preceeded by transient inhibition of p27 induced by IL-11.

## Discussion

IL-11 belongs to the IL-6 family of cytokines, all of whose members share the gp 130 molecule in their receptor complexes.[1] Like IL-6, IL-11 exerts anti-inflammatory activity by inhibiting pro-inflammatory cytokine gene expression in macrophages[3,4,18] and by inducing T-cell polarization to Th2-type cells with reduced production of IFN-γ and increased IL-4.[8] However, whereas IL-6 causes differentiation of Th2 cells by increasing T-cell production of IL-4 and by inhibiting DC development,[19,20] IL-11 inhibits the production of IL-12 secreted by activated macrophages and exerts a direct effect on CD4+ naive T-cells bearing the IL-11 receptor.[9,21] The finding that IL-11 is a potent anti-inflammatory and Th2-polarizing cytokine has prompted its therapeutic use in Th1-predominant inflammatory diseases, such as autoimmune disorders, and for the prevention of GVHD.[8,22]

CKIs are negative regulators of the cell cycle that inhibit the kinase activity of the cyclin-CDK complex. p27 belongs to the Kip family of CKI, which also includes p21 and p57.[23] In normal cells, p27 is expressed during $G_0$-phase, but it is readily downregulated when cells are recruited into the cell cycle by cytokine stimulation.[24] In the T-lineage, p27 is highly expressed in resting PB T-cells,[25] while it is downregulated during maturation of thymocytes.[26] Recent studies in mice have shown that forced expression of p27 arrested the development of T-cells and impaired T-cell-dependent immunity.[11] On the other hand, mice deficient in p27 but not in p21 showed increased proliferative response to multiple cytokines including IL-2, IL-4 and IL-12.[10] Moreover, upregulation of p27 inhibits IL-2 transcription and clonal expansion of alloreactive T-cells inducing antigen-specific anergy.[27] p27 also appears to play a critical role in tumor progression, as p27 levels correlate with prognosis[28,29] and its downregulation may be the mechanism by which certain cytokines promote leukemia cell expansion.[17] Thus, p27 seems to play a crucial role in the proliferation and development of both normal and leukemic T-cells.

In the present study, we investigated whether the polarization of T-cells to a Th2 phenotype induced by IL-11 correlates with effects on T-cell proliferation and apoptosis and whether functional activation of T-cells is regulated by the modulation of p27 expression. Our results show that incubation with IL-11 results in early recruitment of quiescent CD4+ cells, CD4+CD45RA+ naive T-cells and CD4+ CD45RO+ memory T-cells into the cell cycle and their fast progression to S-phase, but that it has no effect on apoptosis. IL-11 significantly enhanced proliferation when T-cells were stimulated by anti-CD3/CD28 antibodies, anti-CD3/IL-2 or allogeneic DC (the most potent stimulants of T-cell activation). Proliferation and expansion of antigen-specific T-cells and of naive T-cells are essential requirements for efficient memory and primary immune responses, respectively. Thus, the finding that IL-11 increases proliferation of both CD4+ CD45RA+ and CD4+CD45RO+ cells underscores the relevance of this cytokine in the regulation of T-cell response, especially when a secondary challenge occurs. Interestingly, we did not observe any kinetic effect on CD8+ cytotoxic lymphocytes, despite their reported IL-11 receptor expression.[9] Further experiments are in progress to investigate the activity of IL-11 on CD8+ cells.

When we evaluated p27 expression, we found that the activity of IL-11 on the proliferation and polarization of CD4+CD45RA+ T-cells was preceded by downregulation of this CKI. The relation between p27 expression, cell cycle status and T-cell activation, suggests that p27 is downregulated during proliferation and returns to baseline levels at the post-mitotic stage. Thus, whereas IL-6 exerts a

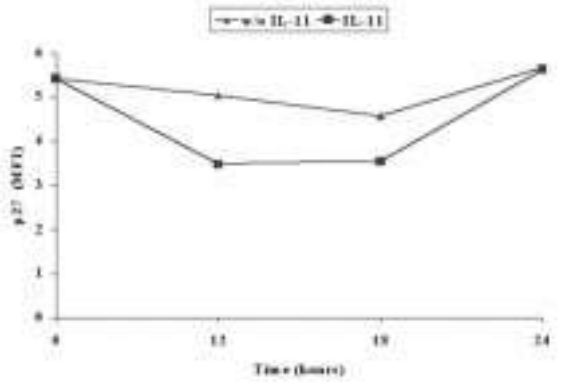


**Figure 4. IL-11 downregulates p27 expression in CD4+ CD45RA+ T-cells. Expression of p27 was evaluated by flow cytometry on highly purified T-cells stimulated with anti-CD3/IL-2 with or without (w/o) IL-11. A significant decrease of p27 was observed on CD4+CD45RA+ cells after 12 and 18 hours of incubation with IL-11. Results (expressed as MFI as described in *Design and Methods* section) of one representative experiment out of 3 are shown.**

mainly antiapoptotic effect on T-cells mediated by Stat 3 activation,[30] IL-11 modulates proteins responsible for cell-cycle regulation without affecting apoptosis. In this regard, it is noteworthy that leukemic T-cells stimulated by IL-7 showed enhanced proliferation and modulation of Bcl-2 and both effects were mediated by the downregulation of p27.[17] However, p27-mediated protection from apoptosis in leukemic (as opposed to normal) T-cells, may be a consequence of neoplastic transformation itself.

In conclusion, by showing that IL-11 increases proliferation of both $CD4^+CD45RA^+$ and $CD4^+CD45RO^+$ cells, our results further underscore the importance of this cytokine in T-cell activation. In addition, we demonstrate that inhibition of p27 may mediate the proliferation and immune competence of human T-cells stimulated by IL-11. Since downregulation of p27 seems to be a general mechanism to activate T-cell function, strategies aimed at preventing p27 degradation could be developed to induce antigen-specific T-cell anergy and leukemic cell expansion. For instance, rapamycin, which prevents downregulation of p27 and induces T-cell anergy even in the presence of co-stimulation,[31] may be the preferred immunosuppressive drug in the setting of allogeneic stem cell transplantation.

*Contributions and Acknowledgments*

*AC and RML designed the experiments; MRR, PT, MTP, MR, EF performed the experiments and helped in the analysis and interpretation of the data; AT and RML drafted the manuscript and revised it critically; MB and ST gave final approval of the version to be submitted.*

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*Disclosures*

*Conflict of interest: none.*

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## Peer Review Outcomes

### *Manuscript processing*

This manuscript was peer-reviewed by two external referees and by Dr. Andrea Velardi, who acted as as Associate Editor. The final decision to accept this paper for publication was taken jointly by Dr. Velardi and the Editors. Manuscript received December 10, 2001; accepted February 15, 2002.

### *What is already known on this topic*

IL-11 induces polarization of human T cells by inhibiting macrophage production of IL-12 and by exerting a direct effect on $CD4^+$ T cells.

### *What this study adds*

IL-11 induces Th-2 polarization and cell-cycle entry of human $CD4^+$, $CD4^+CD45RA^+$ and $CD4^+CD45RO^+$ cells, and their activation is associated with the downregulation of the negative regulator of the cell cycle p27.

### *Potential implications for clinical practice*

Studies such as this one might allow the development of new strategies for preventing graft-versus-host disease while preserving the graft-versus-leukemia effect in allogeneic hematopoietic transplantation.

*Andrea Velardi, Associate Editor*