IL-12 Provides Proliferation and Survival Signals to Murine CD4^T Cells Through Phosphatidylinositol 3-Kinase/Akt Signaling Pathway

Jae Kwang Yoo, Jae Ho Cho, Seung Woo Lee and Young Chul Sung

*J Immunol* 2002; 169:3637-3643; doi: 10.4049/jimmunol.169.7.3637

http://www.jimmunol.org/content/169/7/3637

---

**References**  This article cites 36 articles, 25 of which you can access for free at:
http://www.jimmunol.org/content/169/7/3637.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
IL-12 Provides Proliferation and Survival Signals to Murine CD4⁺ T Cells Through Phosphatidylinositol 3-Kinase/Akt Signaling Pathway

Jae Kwang Yoo, Jae Ho Cho, Seung Woo Lee, and Young Chul Sung

IL-12 is a pleiotropic cytokine that plays an important role in innate and adaptive immunity. IL-12 induces T cell proliferation and IFN-γ secretion from activated T cells. It was also reported that IL-12 prevents apoptosis of CD4⁺ T cells. However, the signaling mechanism that regulates these IL-12-induced responses is poorly understood yet. In this study, we demonstrated that IL-12 activates phosphatidylinositol 3-kinase (PI3K)/Akt pathway in murine CD4⁺ T cells, and that this signaling pathway is required for IL-12-induced T cell proliferation and antiapoptotic function, but not for IFN-γ induction. Through PI3K/Akt pathway, IL-12 up-regulates the expression of cell cycle-related molecule such as cyclin D3, and antiapoptotic molecules such as Bcl-2 and cellular inhibitors of apoptosis proteins-2, followed by down-regulation of active caspase-3. These results suggest that PI3K/Akt pathway is critical for mediating IL-12-induced CD4⁺ T cell responses such as T cell proliferation and survival. The Journal of Immunology, 2002, 169: 3637–3643.

Interleukin-12 (IL-12) is a pleiotropic cytokine that is secreted by activated professional APCs (1–3). IL-12 can induce Th1-type cellular immune responses, T cell proliferation, and IFN-γ secretion from activated T and NK cells (1–5). In addition, it has been reported that IL-12 prevents apoptotic cell death of the activated human and mouse peripheral T cells (6–9).

IL-12R is composed of two subunits, designated β1 and β2. IL-12 activates the Janus family of protein tyrosine kinases (Jaks) such as Jak2 and Tyk2 through IL-12R (10). These activated Jaks phosphorylate tyrosine residues on IL-12Rβ2, providing docking sites for the Src homology 2 domain of STAT-4, and then STAT-4 is phosphorylated and dimerized to bind to the IL-12-responsive genes (11). STAT-4 appears to be an important mediator in IL-12R signaling, because IL-12-induced IFN-γ secretion and T cell proliferation are impaired in STAT-4⁻/⁻ mice (5). Besides the Jak-STAT pathway, IL-12 was also shown to activate mitogen-activated protein kinase (MAPK) kinase (MKK) 6/3 MAPK pathway, but not extracellular signal-regulated kinases (Erk) 1/2 or e-Jun N-terminal kinase pathway (12, 13). In particular, MKK 6/3 MAPK pathway is required to promote IL-12-induced IFN-γ secretion and STAT-4 serine phosphorylation on serine 721, but not T cell proliferation (12–15). These results suggest that IL-12 might activate multiple signaling pathways for a variety of IL-12-induced T cell responses.

Many cytokines and growth factors activate phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway in T cells (16). In mammalian cells, multiple isoforms of PI3K were found and subdivided into three classes. Among them, class I PI3Ks are generally coupled to extracellular stimuli and transmit signals to induce protein synthesis, cytoskeletal changes, cell cycle regulation, and apoptosis regulation (17). In the case of IL-2R signaling, it was reported that PI3K/Akt pathway activated by IL-2 regulates cell cycle-related molecules such as cyclin D3 and p27kip1 to induce T cell proliferation (18). Recently, it was also shown that PI3K/Akt pathway activated by IL-2 family cytokines such as IL-2, IL-4, and IL-15 promotes T cell survival through the modulation of an antiapoptotic molecule such as Bcl-2 (19). However, it is not clear yet whether IL-12 activates the PI3K/Akt pathway, leading to the modulation of IL-12-induced T cell responses. In this study, we clearly demonstrated that IL-12 stimulates the PI3K/Akt signaling pathway in activated CD4⁺ T cells, and that this signaling pathway is critical for IL-12-induced T cell proliferation and inhibition of apoptosis. In particular, IL-12 modulates the expression of cell cycle-related molecule such as cyclin D3, and antiapoptotic molecules such as Bcl-2 and cellular inhibitors of apoptosis proteins (cIAP)-2 through PI3K/Akt pathway.

Materials and Methods

Mice

Five- to 10-wk-old C57BL/6 female mice were purchased from Japan SLC (Shizuoka, Japan) and used for the isolation of lymph nodes (LN). The mice were maintained in specific pathogen-free conditions and fed autoclaved food and water.

Reagents and Abs

For MACS, anti-mouse CD11b, MHC class II, CD8, and B220 microbeads were obtained from Miltenyi Biotec (Auburn, CA). Purified hamster anti-mouse CD3e and CD20 N/A/LE Abs were purchased from Southern Biotechnology Associates (Birmingham, AL). For the flow cytometric analysis and cytokine ELISA, anti-mouse CD4 FITC, active caspase 3 FITC, annexin V FITC, and anti-mouse IFN-γ capture and detection Abs were purchased from BD PharMingen (San Diego, CA). Recombinant mouse
IL-2, IL-4, and IL-12 were all obtained from R&D Systems (Minneapolis, MN) and reconstituted as described in technological notes. General reagents used were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated. SB203580 and LY294002 were purchased from Calbiochem (La Jolla, CA). RPMI 1640 and FBS were purchased from Life Technologies (Grand Island, NY) and HyClone Laboratories (Logan, UT), respectively. For immunoblot assay, rabbit Abs against p38 MAPK, Erks, Akt, Thr<sup>17/20</sup>/Tyr<sup>18/22</sup>-phosphorylated p38 MAPK, Thr<sup>18/20</sup>/Tyr<sup>202/204</sup>-phosphorylated Erks, and Ser<sup>17/18/20</sup>/Thr<sup>202/204</sup>-phosphorylated Akt were all purchased from New England Biolabs (Beverly, MA). Rabbit Abs against STAT-4 and cIAP-1/2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse Abs against p27<sup>kip1</sup>, Bcl-2, and cyclin D3 were also obtained from Santa Cruz Biotechnology. For IFN-γ response region (GRR) affinity purification, streptavidin-agarose beads were obtained from Sigma-Aldrich.

**Preparation of murine primary CD4<sup>+</sup> T lymphocytes**

Mouse naïve CD4<sup>+</sup> T cells were purified from the LNs of C57BL/6 female mice. CD4<sup>+</sup> T cells were negative selected through V8<sup>+</sup> columns using the high gradient MACS (Miltenyi Biotec), as described previously (20). Briefly, harvested LN cells were treated with ammonium chloride lysing buffer to clear RBC. Then remaining cells were treated with anti-CD11b, anti-MHC class II, anti-B220, and anti-CD8 microbeads, and purified by negative selection using the MACS Separation System. Purified cell populations were 92% CD4<sup>+</sup> T cells. The purity was assessed by flow cytometric analysis on a FACScan flow cytometer (BD Biosciences, Heidelberg, Germany) after staining with anti-CD4 FITC Abs.

**In vitro activation and culture of CD4<sup>+</sup> T cells**

Purified CD4<sup>+</sup> T cells were in vitro activated for 72 h with plate-bound anti-CD3 (300 ng/well) and anti-CD28 (100 ng/well) hamster Abs in 96-well plate. T cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS (HyClone Laboratories), 2 mM l-glutamine, 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 1 mM PMSF, 1 mM DTT, protease inhibitors, and 0.1% Triton X-100. The oligonucleotide sequence used was GTATTCTCCA GAAAGGGAC. Cell lysates were incubated with GRR-bound agarose beads, which were then washed and subjected to SDS-PAGE. Oligo-precipitated proteins were electrophoretically transferred to nitrocellulose transfer membrane and immunoblotted with anti-STAT-4 Abs (Santa Cruz Biotechnology).

**Preparation of whole cell lysates and immunoblot analysis**

The Ab-activated T cells (5 × 10<sup>6</sup> cells/ml) were pretreated for 20 min with medium alone, 5 μM LY294002, or 10 μM SB203580 (Calbiochem). After pretreatment, T cells were incubated with medium, 20 ng/ml IL-2, 100 ng/ml IL-4, or 10 ng/ml IL-12p70 (R&D Systems) for the indicated times. Then cells were harvested and whole cell lysates were prepared, as described previously (10). To quantify the phosphorylation of Akt, p38 MAPK, and Erks, equal amounts of whole cell lysates were resolved by 10% SDS-PAGE. Then Western blots were immunoaanalyzed using specific Abs against the phosphorylated form of each protein. To confirm that the same amount of proteins was loaded in each lane, primary Ab/secondary Abs. Data are representative of two independent experiments.

**Cytokine ELISA and intracellular staining**

Murine IFN-γ ELISA was performed as described in the recommended protocol (BD Pharmingen). Ab-activated T cells were harvested and treated with indicated inhibitors and cytokines. At 36 h after treatment, cells were collected and washed twice with PBS. Cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD Pharmingen) for 20 min at 4°C, and then washed with Perm/Wash buffer (BD Pharmingen). Permeabilized cells were resuspended with the wash buffer containing FITC-conjugated rabbit anti-mouse Abs. Data are representative of two independent experiments.
Results
IL-12 directly activates the PI3K/Akt signaling pathway
To examine which signaling pathways are activated by IL-12, we investigated the activation of STAT-4 molecule and several kinases such as PI3K/Akt, p38 MAPK, and Erk1/2 by immunoblot analysis. As expected, IL-12 activated STAT-4 and induced their binding to the GRR DNA (Fig. 1A). When STAT-4 is activated by IL-12, it is translocated to the nucleus and regulates the transcription of the IL-12-responsive genes through the binding of conserved DNA sequences, GRR (23, 24). In addition, the treatment of IL-12 induced the phosphorylation of p38 MAPK, which was significantly inhibited by 10 μM p38 MAPK inhibitor, SB203580. However, Erk1/2 were not activated by the treatment of IL-12. These results agree well with the previous reports that IL-12 stimulates MKK6/p38 MAPK, but not Erk1/2 signaling pathway in activated CD4⁺ T cells (12, 13). Particularly, IL-12 induced Akt phosphorylation, which was significantly blocked by 3.5 IC₅₀ (5 μM) of PI3K-specific inhibitor, LY294002 (25) (Fig. 1B). As reported previously (16, 19), IL-2 family cytokines such as IL-2 and IL-4 also induced Akt phosphorylations, which appeared to be less than those by IL-12 (Fig. 1C). Therefore, we demonstrated that, in addition to STAT-4 and p38 MAPK, IL-12 directly activates the PI3K/Akt signaling pathway in murine CD4⁺ T cells.

FIGURE 2. IL-12 regulates the expression of cyclin D3 to induce T cell proliferation through PI3K/Akt signaling pathway. A, Seventy-two hours after Ab stimulation, cells (1 × 10⁵ cells/sample) were harvested and stimulated with medium alone or IL-12 (10 ng/ml) for the indicated times. PI3K-specific inhibitor LY294002 (5 μM) or p38 MAPK-specific inhibitor SB203580 (10 μM) was added and incubated for 20 min before IL-12 treatment. T cell proliferation was measured through the counting of total cell numbers at the indicated times. Data are representative of three independent experiments. B, Ab-activated T cells were incubated with LY294002 (5 μM) or SB203580 (10 μM) for 20 min, and then stimulated with medium alone or IL-12 (10 ng/ml) for 20 h. Total cell lysates were resolved by SDS-PAGE, followed by immunoblot with each indicated Ab. Data are representative of two independent results.

IL-12 induces CD4⁺ T cell proliferation via PI3K/Akt pathway-dependent modulation of cyclin D3 molecules
IL-12 has been known as a cytokine that induces the proliferation of activated CD4⁺ T cells (3, 5). In addition, it has also been reported that the PI3K/Akt pathway is an important cellular signaling pathway that regulates multiple cellular responses, one of which is to induce cellular proliferation through the modulation of cell cycle-related molecules such as cyclin D3 and cyclin-dependent kinase (cdk) inhibitor, p27kip1 (17, 18). As shown in Fig. 2A, the proliferation of CD4⁺ T cells was enhanced by IL-12 treatment compared with the cells treated with medium alone. This IL-12 effect was completely inhibited by LY294002, but not by SB203580, suggesting that PI3K/Akt, but not p38 MAPK pathway is critical for IL-12-induced CD4⁺ T cell proliferation.

It has been well known that both up-regulation of cyclin D molecules and down-regulation of cdk inhibitors such as p27kip1 or p21WAF1/Cip1 are needed and sufficient to induce the cell cycle progression through the G1/S phase boundary in many cell types (26). Particularly, it was reported that PI3K/Akt pathway is involved in IL-2-induced T cell proliferation through the regulation of both cyclin D3 and p27kip1 expression (18). Interestingly, the treatment of IL-12 up-regulated the expression of cyclin D3, but down-regulated p27kip1, compared with medium alone (Fig. 2B). In addition, IL-12-induced up-regulation of cyclin D3 was significantly inhibited by the treatment of LY294002, but not by SB203580. However, the modulation of p27kip1 was not affected by both LY294002 and SB203580, which suggests that another signaling pathway except both PI3K/Akt and p38 MAPK pathways might be involved in IL-12-induced down-regulation of p27kip1. Taken together, these results demonstrated that IL-12 induces CD4⁺ T cell proliferation through the modulation of cell cycle-related molecules such as cyclin D3 and p27kip1, and that PI3K/Akt pathway activated by IL-12 is associated with the regulation of cyclin D3, but not with that of p27kip1.

FIGURE 3. The PI3K/Akt signaling pathway is not involved in IL-12-induced IFN-γ secretion from CD4⁺ T cells. Ab-stimulated T cells were preincubated with each indicated inhibitor, as described in Fig. 2A, and then treated with medium alone or IL-12 (10 ng/ml) for 24 h. Cell culture supernatants were harvested and tested for murine IFN-γ production by cytokine ELISA. Data are shown as the mean ± SD of triplicate cultures and representative of two independent experiments.
The PI3K/Akt pathway via IL-12 is not required for IL-12-induced IFN-γ production

One of the important properties of IL-12 is its ability to induce the production of IFN-γ from activated T cells (3). As expected, IL-12 induced large amounts of IFN-γ production from activated CD4⁺ T cells, which was inhibited by the treatment of SB203580 (Fig. 3). This result is consistent with previous reports that p38 MAPK pathway is required for IL-12-induced IFN-γ production from activated T cells (14, 15). In contrast, the treatment of LY294002 did not significantly inhibit the IL-12-induced IFN-γ production, indicating that PI3K/Akt pathway is not required for IL-12-induced IFN-γ production from activated CD4⁺ T cells.

IL-12 suppresses the apoptosis of activated CD4⁺ T cells by up-regulation of antiapoptotic molecules through PI3K/Akt pathway

Because IL-12 has been shown to prevent the apoptosis of activated CD4⁺ T cells in various conditions (3, 6–9), we tested...
whether PI3K/Akt pathway is involved in antiapoptotic effects of IL-12 in passive cell death.

Apoptotic T cell death was measured through flow cytometric analysis stained with both annexin V and propidium iodide (PI). Preactivated CD4⁺ T cells showed spontaneous apoptosis over time, but the addition of IL-12 significantly reduced apoptosis in a dose-dependent manner (Fig. 4A), which is consistent with previous reports (7, 27). As shown in Fig. 4, B and C, LY294002 inhibited the antiapoptotic effect of IL-12 in a dose-dependent manner, but SB203580 did not, suggesting that IL-12 induces CD4⁺ T cell survival through PI3K/Akt pathway, but not through p38 MAPK pathway.

The PI3K/Akt pathway gives survival signals to many cell types by regulating the expression of antiapoptotic molecules such as Bcl-2 and cIAP-1/2, or by controlling the activity of proapoptotic molecules (16). Particularly, it has been reported that PI3K/Akt pathway activated by cytokines inhibits the T cell apoptosis through induction of Bcl-2 expression (19, 28). As expected, IL-12 enhanced the expression of Bcl-2 and cIAP-2 molecules, which was inhibited by the treatment of LY294002, but not by SB203580 (Fig. 5A). However, the expression of cIAP-1 appeared not to be affected by the treatment of IL-12. These results suggest that IL-12 up-regulates the expression of Bcl-2 and cIAP-2 molecules through PI3K/Akt pathway in activated CD4⁺ T cells.

Bcl-2 and cIAP-2 are known as antiapoptotic molecules that promote cell survival through inhibition of the processing and activity of caspase molecules (16, 29). As expected, IL-12 decreased the level of active caspase-3 from 70 to 58% (Fig. 5B). This result correlates with a recent report that IL-12 prevents apoptosis of human CD4⁺ T cells costimulated with ICAM-1 through inhibition of the processing of caspase-3/9 (9). Moreover, the inhibitory effect of IL-12 was completely blocked by the treatment of LY294002, but not by SB203580, suggesting that IL-12 induces CD4⁺ T cell survival in passive cell death through up-regulation of Bcl-2 and cIAP-2, followed by the down-regulation of active caspase-3 via PI3K/Akt pathway.

**Discussion**

It has been known that multiple cytokines and growth factors such as IL-2, IL-4, and insulin-like growth factors activate PI3K/Akt signaling pathway (16). In this study, we demonstrated that like IL-2 family cytokines, IL-12 activates the PI3K/Akt signaling
pathway in murine CD4+ T cells, which is essential for IL-12-induced T cell proliferation and survival. On the contrary, Veronica et al. (24) recently reported that IL-12 could not activate PI3K/Akt pathway in human PBL T cells. This discrepancy might be due to the difference of origin of cells that were harvested, or the difference of experimental conditions in which T cells were prestimulated.

The PI3K/Akt signaling pathway is associated with cellular proliferation in T cells. It was reported that IL-2 induces T cell proliferation by modulation of both cyclin D3 and p27kip1 through PI3K/Akt pathway (18). Similarly, IL-12 appeared to regulate these two molecules to induce CD4+ T cell proliferation. However, the expression of cyclin D3, but not that of p27kip1, appeared to be regulated via PI3K/Akt signaling pathway. Previously, it was reported that the regulation of p27kip1 by IL-12 is mediated through STAT-4-dependent pathway (30). In addition to p27kip1, p21WAF1/cip1 has been known as a cdK inhibitor molecule that inhibits cellular proliferation (26). In contrast to p27kip1 regulation, the expression of p21WAF1/cip1 was up-regulated by IL-12 (data not shown). This result was consistent with previous reports that IL-12 suppresses apoptotic cell death of activated T cells, but the molecular mechanisms are not clear yet (3, 6–9, 33). In this study, we demonstrated that IL-12 provides survival signals to CD4+ T cells through the up-regulation of Bcl-2 and cIAP-2, and the down-regulation of active caspase-3 via PI3K/Akt pathway. Our results are similar to previous reports that PI3K/Akt signaling pathway via IL-2 family cytokines promotes T cell survival through the modulation of antiapoptotic molecule, Bcl-2 (19, 28). In addition, the activation of PI3K/Akt pathway was reported to prevent the apoptosis of T cells via NF-kB activation, which was known to induce the expression of cIAP molecules (34–36). Bcl-2 eventually regulates caspase-3 activation through modifying the mitochondrial membrane potential, and cIAP-2 is potent inhibitor of caspases (29). Therefore, it is likely that IL-12 inhibits caspase-3 activation by up-regulation of both Bcl-2 and cIAP-2 molecules through PI3K/Akt pathway. Estaquier et al. (6) showed that IL-12 prevents apoptosis of CD4+ T cells induced by anti-Fas or anti-CD3. cIAP-2 has also been reported to inhibit death receptor-mediated apoptosis through suppression of caspase-8 activation (35, 36). Therefore, it is possible that IL-12 may prevent the death receptor-mediated apoptosis by up-regulation of antiapoptotic molecules such as cIAP-2 through PI3K/Akt pathway.

Even if p38 MAPK pathway is not involved in IL-12-induced T cell proliferation and antiapoptotic function, this pathway is required for IL-12-induced IFN-γ production from activated CD4+ T cells (14, 15). It has also been reported that MKK6/p38 MAPK pathway is involved in IL-12-induced STAT-4 serine phosphorylation that is important for the trans-activation activity of STAT-4 (12, 13). Although we have demonstrated that the PI3K/Akt pathway is not critical for IL-12-induced IFN-γ induction, it should be further studied whether this pathway is associated with STAT-4 serine phosphorylation. Consequently, these results suggest that IL-12 differently employs signaling pathways such as PI3K/Akt, MKK6/p38 MAPK, and Jak/STAT to perform diverse functions of activated CD4+ T cells.

Acknowledgments

We thank Sang Chon Lee for devoted animal care and Chang Guen Lee and Kwan Hyuck Baek for technical assistance.

References


