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Different Pattern of $p27^{kip1}$ and $p21^{cip1}$ Expression Following *Ex Vivo* Activation of $CD8^+$ T Lymphocytes

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Abstract – T cell proliferation is a pivotal to an effective immune response. Cyclin-dependent kinase (cdk) inhibitor, $p27^{kip1}$ is degraded to initiate T cell expansion. In this study, we show that although the expression of $p27^{kip1}$ protein was down-regulated, that of $p21^{cip1}$, another cdk inhibitor, was up-regulated in $CD8^+$ T cells following *in vitro* stimulation. *Ex vivo* gB antigen-stimulation following HSV immunization increased $p21^{cip1}$ positive cells that co-expressed IFN-γ. Moreover, $p21^{cip1}$ was co-expressed with IFN-γ in E7 antigen-stimulated $CD8^+$ T cells, whereas $p27^{kip1}$ was not. Our findings imply a role of $p21^{cip1}$ proteins in antigen-induced effector $CD8^+$ T cells differentiation *in vivo*.

Key words □ $p27^{kip1}$, $p21^{cip1}$, effector $CD8^+$ T cells, differentiation, virus antigens

INTRODUCTION

The T cell expansion and differentiation have a critical role in T cell-mediated antigen clearance (Mueller *et al*., 1989). Antigen-evoked T cell expansion occurs by signaling via antigen-specific TCR, co-stimulatory receptors, and cytokine receptors. During the expansion, naïve T cells become differentiated into effector cells that are responsible for clearing primary Ag infection. Following antigen-specific T cell expansion, some T cells further differentiate into long-lived memory cells that respond to secondary Ag infection.

Molecular mechanisms how antigen presenting on MHC of antigen presenting cells (APC) drives T cell proliferation has been extensively studied (Mueller *et al.*, 1989; Vella *et al*., 1997; Vinay *et al.*, 1998). To adapt such antigen-specific proliferation, T cells require two signals: ligation of the T cell receptor (TCR) with the MHC/peptide complex on the antigen presenting cell (APC), and cross-linking of co-stimulatory receptors on the T cell with the corresponding ligands on the APC. Occupancy of TCR with antigen on APC gives rise to TCR-mediated signal transduction pathways evoking antigen-specific T cell expansion. Co-stimulatory signals in T cells are necessary to TCR-mediated activation of T cells. If co-stimulatory signals provide insufficiently for T cells, T cells could become anergy (antigen-specific inactivation). Several molecules including cyclin-dependent kinase (cdk) inhibitor $p27^{kip1}$ that play key roles in regulating the cell cycle in T cells have been identified (Botz *et al*., 1996; Leone *et al*., 1997; Sherr *et al.*, 1999). Studies of cyclin/cyclin-dependent kinase (cdk) holoenzymes and cdk inhibitors such as $p27^{kip1}$ and $p21^{cip1}$ have resulted in important advances in understanding the cell cycle of T cells (Toyoshima *et al*., 1994; Poltak *et al*., 1994). CD28 co-stimulation enhances the clonal expansion of T cells via PI3K/Akt-pathway-mediated down-regulation of $p27^{kip1}$ (Appleman *et al.*, 2002). Mitogenic ligands initiate up-regulation of cyclin D protein and inactivate the cyclin D/cdk4 or 6 complex, which then sequesters $p27^{kip1}$ from the inactive cyclin E/cdk2/$p27^{kip1}$ complex and generates an active cyclin D/cdk4 or 6/$p27^{kip1}$ complex (Sherr *et al*., 1999). This active form phosphorylates and inhibits the activity of the retinoblastoma tumor suppressor gene product (Rb) that binds and blocks the activity of transcription factor E2F. Active E2F increases transcription of genes such as cyclin E required for S phase entry (Matakeyama *et al*., 1994; Resnitzky *et al.*, 1995). Sequestration of $p27^{kip1}$ from inactive cyclin E/cdk2/ $p27^{kip1}$ onto cyclin D/cdk4 or 6 also results in increased levels of active cyclin E/cdk2, which further phosphorylates Rb and activates E2F. As the levels of cyclin E and cyclinE/cdk2 rise, $p27^{kip1}$

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protein is phosphorylated and degraded via the ubiquitin-proteasome pathway (Montagnoli *et al.*, 1999; Tsvetkov *et al.*, 1999). Degradation of $p27^{kip1}$ further increases the level of active cyclin E/cdk2 complexes. As a general rule, mitogenic stimuli promote the G_1/S phase transition by means of this positive auto-regulatory feedback loop. We also showed that stimulation of $CD8^+$ T cell expansion occurs by a combinatorial signaling pathway. MEK/ERK and PI3K/Akt/mTOR pathways activated by TCR, 4-1BB and IL-2R increased transcription and translation of cyclin D and cyclin E, and concomitantly down-regulated $p27^{kip1}$, which initiate S phase entry and cell cycle progression (Lee, *et al*., 2003). Although molecular mechanisms underlying T cell expansion have been delineated, the mechanism by which T cells differentiate to effector or memory cells has remained elusive. We here show a potential role of $p21^{cip1}$ in 4-1BB-mediated differentiation of $CD8^+$ T cell.

MATERIALS AND METHODS

Mice, reagent, virus, peptide and Abs

Six-to eight-week-old C57BL/6 mice were purchased from the Halran Laboratories (Indianapolis, IN). Male BALB/c mice were obtained from Harlan (Indianapolis, Indiana). Animals were maintained under specific pathogen-free conditions. 4-$1BB^{-/-}$ ($H\text{-}2K^b$) mice were bred in our specific pathogen-free facility. The HSV-1 KOS stain was grown on CV-1 cells in Minimal Essential Media (MEM)-2% FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml). Virus was tittered on CV-1 cells by plaque assay. Virus was aliquoted in 1-ml vials and stored at -70ºC. The $gB_{498\text{-}505}$ peptide (SSIEFARL) was synthesized from Peptron (Dae-jeon, korea). Anti-CD3 mAb (145-2C11 clone), biotin & PE-labeled anti-CD8 mAb, and isotype control antibody were purchased from BD Pharmingen (San Diego, CA). Agonistic anti-4-1BB mAbs (3H3 and 3E1) were kindly provided by Dr. Robert S. Mittler (Emory University, GA). Streptavidin-conjugated microbeads and LS columns were purchased from Miltenyi Biotech (Auburn, CA). All antibodies for Western blotting were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). E7 peptide (RAHYNIVTF) was synthesized from peptron (Dae-jeon, korea). Anti-m4-1BB mAb producing hybridoma cells (3H3) were a kind gift from Dr. Robert Mittler at Emory University. The antibodies were produced from ascites and were purified by protein G-column (Sigma, St. Louis, MO) and then tested on anti-CD3 mAb stimulated T cells. Purified rat IgG was purchased from Sigma and served as a control antibody.

$CD8^+$ T cell purification

Cell suspensions were prepared from the spleens and lymph nodes of BALB/c mice. Cells were incubated at 37ºC for 1 h in flasks to eliminate adherent cells before purification. $CD8^+$ T cells were purified using the MACS magnetic separation system according to the manufacturer's instructions (Miltenyi Biotech). In brief, cells were resuspended at a concentration of 10^8 cells/ml in PBS containing 5% FBS, incubated with anti-CD8-mAb conjugated with biotin, and collected by incubating with streptavidin-microbeads at 4ºC for 15min. LS columns (Miltenyi Biotech) were used for the selection of $CD8^+$ T cells. $CD8^+$ T cell purity was assessed, and routinely showed >95% by flow cytometric analysis.

T cell stimulation

The purified $CD8^+$ T cells were plated at 10^6 cells/well in 96 well round microplates with 0.5 μg/ml anti-CD3-mAb (BD Pharmingen) for 16 h. After incubation, cells were stained with anti-4-1BB-FITC (3E1-FITC), and >70% of the cells routinely showed 4-1BB expression on their surface by flow cytometric analysis. After verifying 4-1BB expression on the purified $CD8^+$ T cells, we pre-incubated cells with or without various pharmacological inhibitors for 1 h, and then added 5 μg/ml agonistic anti-4-1BB mAb (3H3) or rat IgG_{2a} as an isotype control for the indicated periods.

HSV infection and antibody treatment

Mice were anesthetized with ketamine hydrochloride (1 mg/kg, Vetamine; Phoenix Scientific Inc., St. Joseph, MO), and xylazine (0.5 mg/kg, Ben Venue Laboratories, Bedford, OH). Mice were injected in each hind footpad with 5×10^5 pfu HSV-1 in 50 μl of PBS. Anti-4-1BB mAb(3H3, 200mg) or rat IgG i.p. was injected into HSV-1 infected mice on day 0 and 2. T cells were separated from lymph nodes and were re-stimulated with gB peptide for 6 h.

E7 injection and antibody treatment

Mice were anesthetized with ketamine hydrochloride (1 mg/kg, Vetamine; Phoenix Scientific Inc., St. Joseph, MO), and xylazine (0.5 mg/kg, Ben Venue Laboratories, Bedford, OH). Human papillomavirus type 18 E7 peptide was emulsified 1:1 with complete Freund's adjuvant (Sigma, St. Louis) and injected to mice. At 5 days later, draining lymph node was isolated and separated T cells for seeding in 7×10^6 cell/well and

was re-stimulated for 2 days with E7 and 3H3.

Intracellular staining

The cells were washed with PBS, fixed in 4% paraformaldehyde for 5 min at room temperature, permeabilized with 0.05% Triton X-100 for 30 min at room temperature, and stained with a 1:100 dilution of primary antibody for 30 min. After three washes with PBS, the cells were stained with the appropriate secondary antibody for 30 min, and washed three times with PBS. A cover slip was applied with GVA mounting solution (Zymed, San Franscisco, CA), and the cells were visualized with an Olympus FV500 confocal laser-scanning microscope (Olympus, Tokyo, Japan). To minimize cross talk of the dual colored samples between channels, we used a sequential scanning technique exciting one dye at a time. Cells were incubated with FITC-conjugated anti-$p27^{kip1}$ or anti-$p21^{cip1}$ plus PE-conjugated anti-IFN-γ mAb with the Cytofix/cytoperm kit (BD Pharmingen), according to the manufacture's instructions

Western blotting analysis

The purified $CD8^+$ T cells were stimulated as described earlier, and proteins were extracted with lysis buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM EDTA, 30 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate, 1% Triton X-100, 0.5% NP-40, 1mM PMSF, and protease inhibitor cocktail). Equal amount of proteins from each sample was diluted with 4x SDS sample buffer, applied onto SDS-PAGE gels, separated and transferred to nitrocellulose membranes (Millipore, Bedford, MA). Each protein of interest was detected with primary Abs and secondary Ab-HRP. Bound Abs were detected by ECL chemiluminescence (Amersham Phrmacia Biotech, Little Chalfont, England).

RESULTS

Up-regulation of $p21^{cip1}$ expression upon T cell activation

We previously showed that cross-linking 4-1BB on $CD8^+$ T cells up-regulated cylin D and E and down-regulates cyclin-dependent kinase (cdk) inhibitor $p27^{kip1}$, which were responsible for 4-1BB-mediated $CD8^+$ T cell expansion (Lee, *et al*., 2003). Unlike $p27^{kip1}$, the expression of $p21^{cip1}$, another cdk inhibitor, was increased following cross-linking of 4-1BB (Fig. 1). Since 4-1BB ligation has known to enhance cell cycle progression of $CD8^+$ T cells, it was unexpected that cell cycle inhibitor $p21^{cip1}$ up-regulated following the treatment cells with agonist anti-4-1BB. 4-1BB is an important co-stimulatory molecule for T cells to be committed to differentiate to effector or/and memory cells (Shuford *et al.*, 1997). Zezula J. *et al*. reported that $p21^{cip1}$ had a critical role in the differentiation of oligodendrocytes (Zezla, *et al.*, 2001). These results suggest that 4-1BB-mediated $CD8^+$ T cell differentiation may be due to the up-regulation of $p21^{cip1}$ expression.

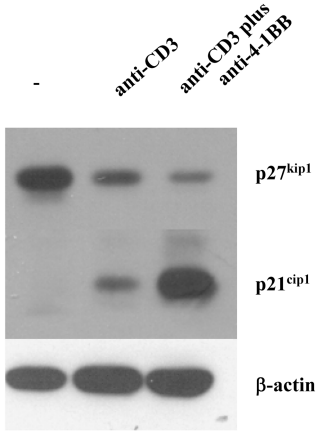

Fig. 1. Reverse expression of $p27^{kip1}$ and $p21^{cip1}$ during activation of $CD8^+$ T cell. Purified $CD8^+$ T cell were incubated with rat IgG (-), anti-CD3 mAb plus IgG or anti-CD3 Ab plus anti-4-1BB Ab for 36 h. Equal amount of proteins extracted from $CD8^+$ T cell were separated by SDS-PAGE and transferred into nitrocellulose membrane. $p27^{kip1}$ was detected by Western blotting analysis with anti-$p27^{kip1}$ Ab used as the primary Ab. After the membrane was stripped, $p21^{cip1}$ was detected by re-probing with andti-$p21^{cip1}$ Ab. Similar results were obtained from three independent experiments.

Expression of $p21^{cip1}$ protein in HSV-immunized T cell

To test whether $p21^{cip1}$ has a potential role in differentiating $CD8^+$ T cell to effector cells, we infected mice with HSV in combination with anti-4-1BB in vivo, isolated T cells and re-stimulated with or without HSV epitope peptide gB. Our Previous study showed that in vivo treatment with HSV- 1 plus anti-4-1BB followed by in vitro pulsing T cells with gB enhances $CD8^+$ T cell activation and differentiation to effector cells (Seo, *et al*., 2003). Fig. 2 showed that effector T cells, as indicated by positively being stained with anti-IFN-γ Ab, were generated by in vivo stimulation in combination with in vitro re-challenging with HSV epitope gB. Effector T cells produced by HSV-1 challenge co-expressed $p21^{cip1}$, suggesting that $p21^{cip1}$ protein may be involved in processes of antigen-specific differentiation to effector cells.

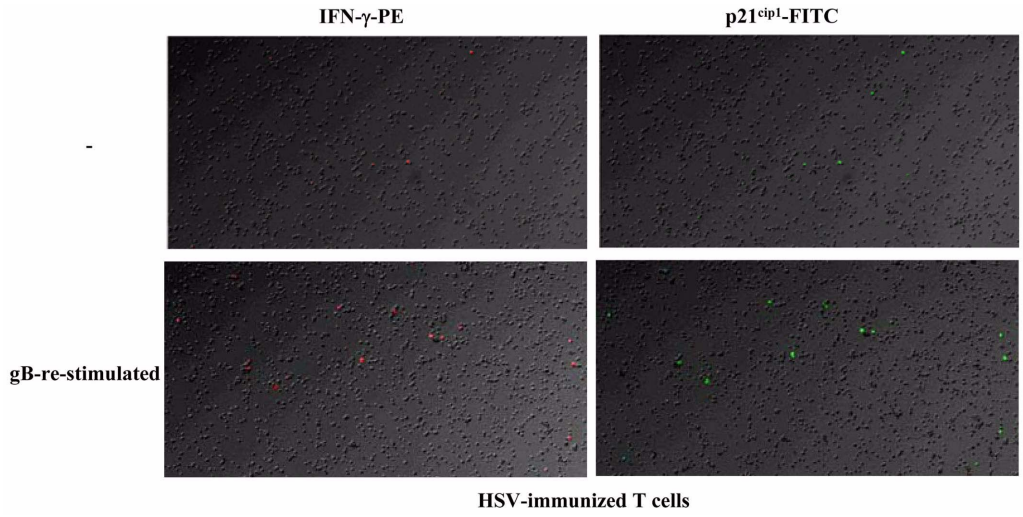

Fig. 2. Mice were anesthetized with ketamine hydrochloride, and zylazin. Mice were injected in each hind footpad with 5×10^5 pfu HSV-1 in PBS. Anti-4-1BB (3H3) or rat IgG was injected into HSV-1 infected mice on day 0 and 2. T cells were separated from lymph nodes and were re-stimulated with gB peptide for 6 h. Cells were double-stained with PE-conjugated anti-IFN-γ (red) and FITC-conjugated anti-$p21^{cip1}$ (green). This is a representative of three independent experiments.

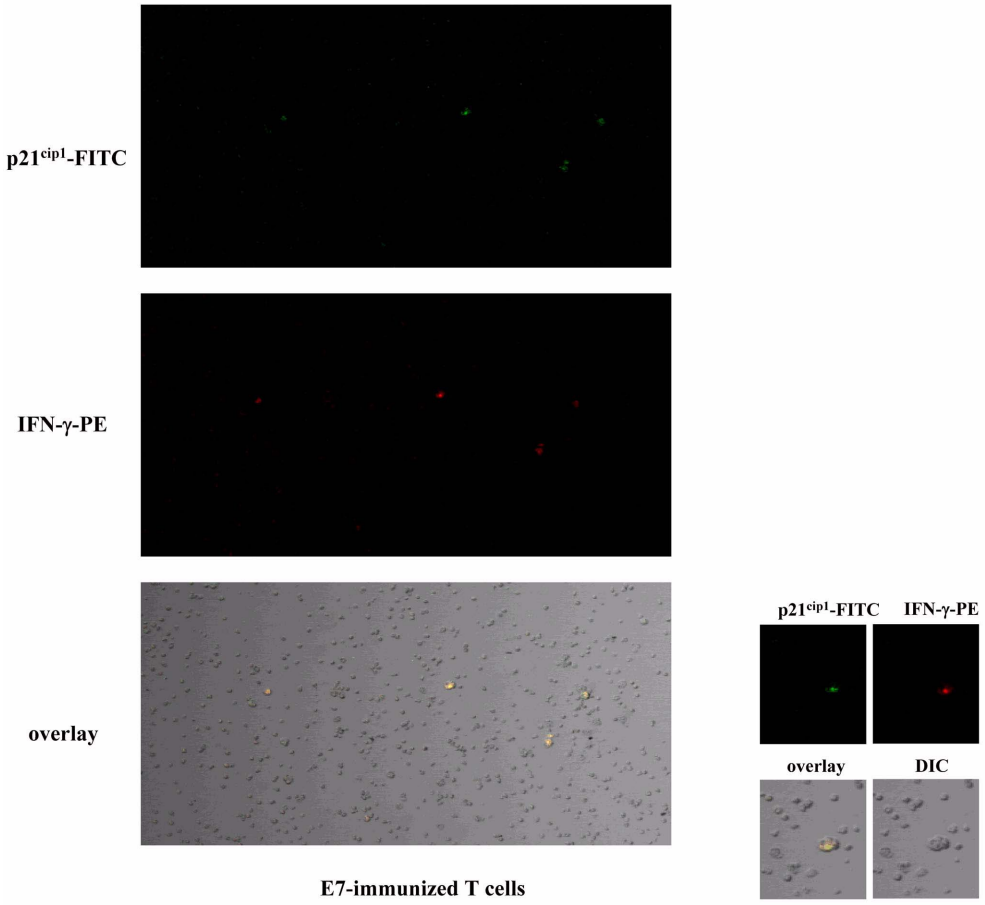

Fig. 3. C57/B6 mice were immunized with human palillomavirus type 18 antigen E7 for 5 days. $CD8^+$ T cells from draining lymph nodes were purified by MACS. $CD8^+$ T cells were re-stimulated with E7 peptide plus anti-4-1BB for 2 days. The cells were fixed, permeabilized and stained with $p21^{cip1}$-FITC (green) and IFN-γ-PE (red). Stained cells were observed by confocal microscopy. Similar results were obtained from three independent experiments.

Expression of $p21^{cip1}$ and $p27^{kip1}$ protein in E7-immunized T cell

To examine whether effector T cells produced by treating mice with human papillomavirus type 18 E7 peptide express $p21^{cip1}$ protein, we injected mice with E7 peptide for 5 days, purified $CD8^+$ T cells from draining lymph nodes using MACS and re-stimulated with E7 peptide plus anti-4-1BB for 2 days. Fig. 3 showed that IFN-γ-positive effector cells co-expressed $p21^{cip1}$ protein. However, $p27^{kip1}$ protein was expressed in IFN-γ-negative cells (Fig. 4). These data suggest that $p21^{cip1}$, not $p27^{kip1}$, may have a role in E7-evoked differentiation of T cell to effector cells.

DISCUSSION

It has been shown previously that signaling through the 4-1BB transmits a potent costimulatory signal to T cells in terms of their survival, differentiation, and cytokine expression (Vinay *et al.*, 1998). Recent *in vivo* studies reported that 4-1BB is a critical costimulatory molecule enhancing long-term survival and expansion of $CD8^+$ T lymphocytes (Shuford *et al*., 1997; Jang, *et al*., 1998; Maus, *et al.,* 2002). Here, we present several new findings in terms of a role of $p21^{cip1}$ in antigen/4-1BB-mediated differentiation of $CD8^+$ T cell. First, the expression of $p21^{cip1}$, contrast to that of $p27^{kip1}$, was up-regulated following cross-linking 4-1BB, which is known to enhance the expansion *in vitro* or *in vivo* and the differentiation of $CD8^+$ T cells *in vivo*. Second, $p21^{cip}$ was expressed in effector T cells generated by injecting with virus antigens such as HSV-1 gB peptide or human papillomavirus type 18 E7 peptide plus agonistic anti-4-1BB mAb. Third, $p27^{kip1}$ was not expressed in effector $CD8^+$ T cells, suggesting that $p27^{kip1}$ protein is degraded for T cells to be proliferated and differentiated.

Both $p21^{cip1}$ and $p27^{kip1}$ are cdk inhibitor that block mitogen-evoked cell cycle progression (Botz *et al*., 1996; Leone *et al.*, 1997). Antigen/costimlation-induced T cell proliferation is attributable to changes in the expression of various proteins precisely coordinating cell cycle progression. One of these

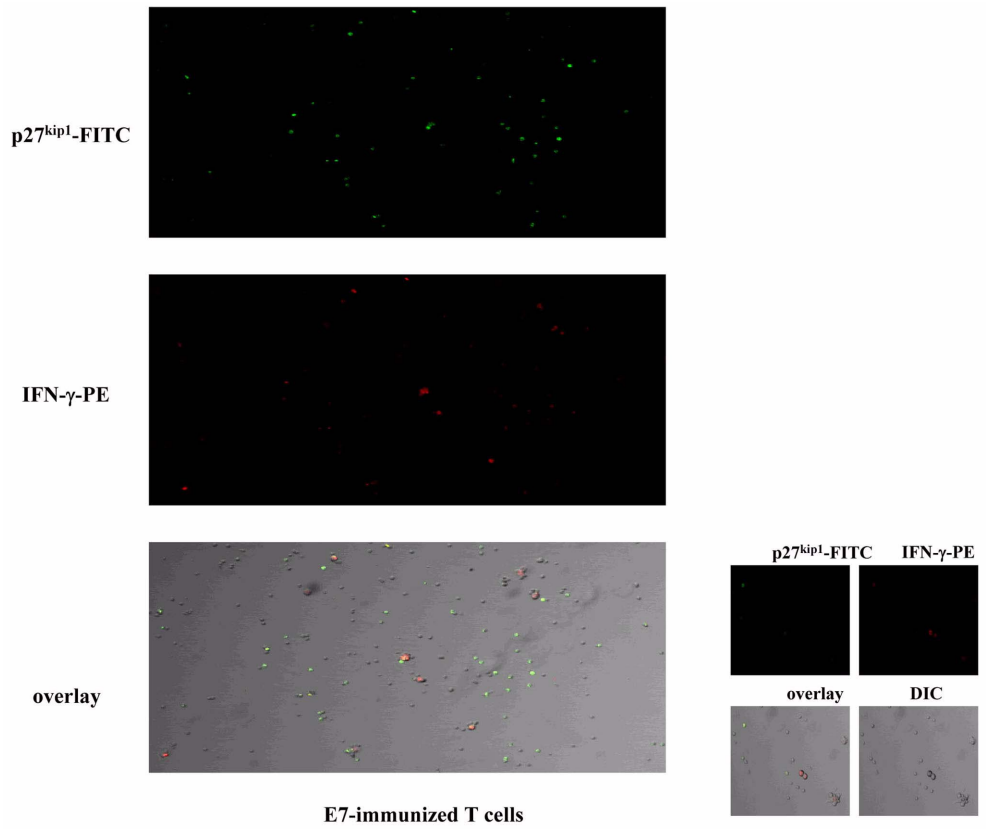

Fig. 4. C57/B6 mice were immunized with human palillomavirus type 18 antigen E7 for 5 days. $CD8^+$ T cells from draining lymph nodes were purified by MACS. $CD8^+$ T cells were re-stimulated with E7 peptide plus anti-4-1BB for 2 days. The cells were fixed, permeabilized and stained with $p27^{kip1}$-FITC (green) and IFN-γ-PE (red). Stained cells were observed by confocal microscopy. Similar results were obtained from three independent experiments.

changes is the down-regulation of expression of cdk inhibitors. This is first study showing that the expression of $p21^{cip1}$ is up-regulated whereas that of $p27^{kip1}$ is down-regulated following ex vivo antigen challenge. Molecular mechanisms how T cells are proliferated upon TCR ligation with antigen have been widely studied. However, the mechanism for T cell differentiation still needs to be scrutinized. Origins of effector or memory T cells are controversial. It is still uncertain whether differentiated T cells are raised concurred with the proliferation of naïve T cells or independent of the proliferation. Even if there are a few studies performed in non T cell systems, they suggested a potential role of $p21^{cip1}$ in the cell differentiation (Wang *et al*., 2000; Zezla *et al.*, 2001).

The present results clearly show that antigen/4-1BB-mediated up-regulation of $p21^{cip1}$ expression is responsible for the differentiation of $CD8^+$ T cells although precise mechanisms by which $p21^{cip1}$ causes T cells to be differentiated remains uncovered.

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