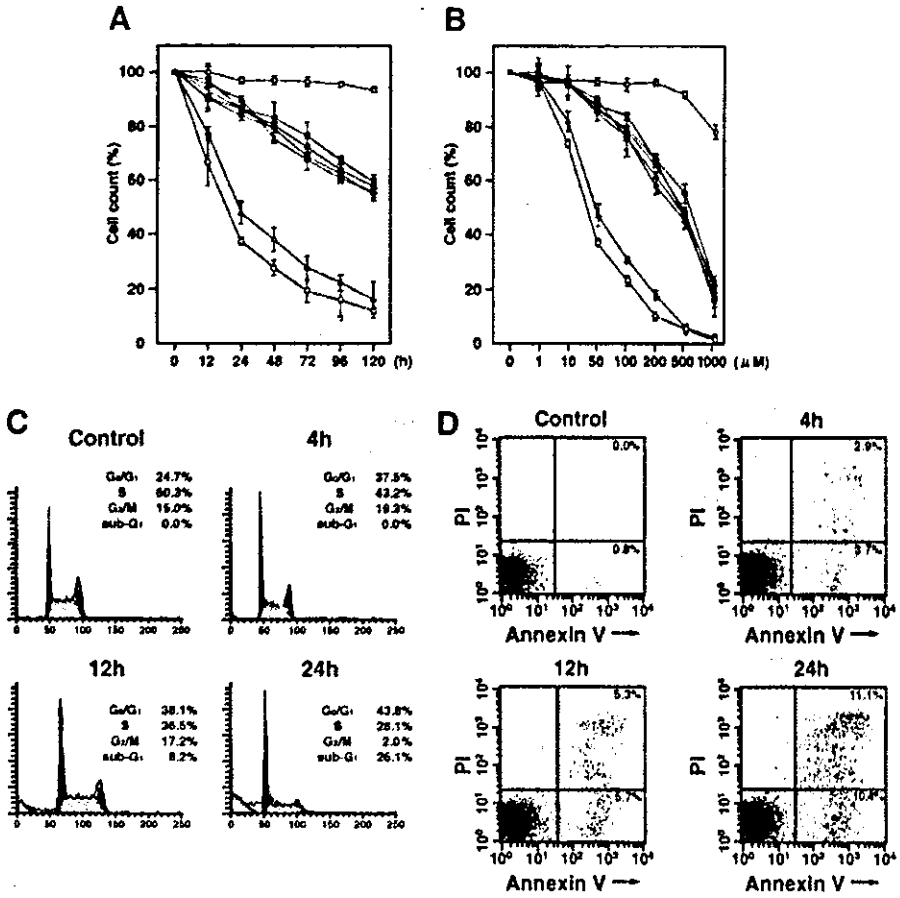

Fig. 1. Capsaicin inhibits growth of myeloid leukemic cells via G_0-G_1 phase cell cycle arrest followed by apoptosis. *A* and *B*, various myeloid leukemic cells [NB4 (□), Kasumi-1 (△), UF-1 (●), HL-60 (■), U937 (▲), KU812 (◆), K562 (▼)] and peripheral blood cells from a healthy donor (○) were treated with 50 μM capsaicin for various times (0–120 h; *A*) and concentrations (0–1000 μM) for 24 h (*B*). Cell viability was assessed by trypan blue dye exclusion. Results are expressed as the mean of three duplicate experiments, and the SD was within 5% of the mean. *C*, cell cycle analysis. Cells were cultured with 50 μM capsaicin for 0–24 h and stained with propidium iodide (PI) as described in "Materials and Methods." DNA content was analyzed by flow cytometry. G_0-G_1, G_2-M, and S indicate the cell phase, and sub-G_1 DNA content refers to the proportion of apoptotic cells. Each phase was calculated by using the cell ModiFIT program. Three duplicate experiments were performed with similar results. *D*, induction of apoptosis by the treatment of capsaicin in NB4 cells. Cells were cultured with 50 μM capsaicin for the indicated times, stained with Annexin V-FITC, and analyzed by flow cytometry. Three duplicate experiments were performed with similar results.

necrosis in addition to apoptosis in this fraction (Fig. 1*D*). These results showed that capsaicin-induced *in vitro* growth inhibition of leukemic cells was mediated by causing G_0-G_1 cell cycle arrest and apoptosis.

Capsaicin-Induced Death Signaling Is Mediated through the Mitochondrial Pathway. Treatment with capsaicin for 3 h significantly induced caspase-3 activity in NB4 cells (Fig. 3*A*). Capsaicin-induced apoptosis was completely blocked by the treatment with *N*-tert-butoxy-carbonyl-Val-Ala-Asp-fluoromethylketone (pan caspase inhibitor; Fig. 3*B*). After treatment with capsaicin for 3 h, low Rh123 staining in NB4 cells indicated an increase in the loss of mitochondrial $\Delta\psi m$ (Fig. 3*C*). Capsaicin induced a substantial release of cytochrome *c* from the mitochondria into the cytosol within 3 h. In addition, capsaicin induced a translocation of Bax from cytosol to mitochondria (Fig. 3*D*). These results indicate that capsaicin-induced apoptosis in early phase NB4 cells is mediated through the mitochondrial-dependent pathway.

ROS Generation Triggers Capsaicin-Induced Apoptosis. Previous investigation has reported that capsaicin induces inhibition of growth and the NADH oxidase activity in HeLa cells (43). Other examination has shown that capsaicin-induced apoptosis in tumor cells is associated with the generation of ROS (44). Therefore, we analyzed the production of intracellular ROS in NB4 cells. Treatment with capsaicin in NB4 cells showed within 0.5 h a dramatic increase in intracellular ROS compared with control cells (Fig. 4*A*). Treatment with a thiol antioxidant, NAC, completely blocked the generation of ROS and attenuated capsaicin-induced apoptosis in NB4 cells (Fig. 4*B*). The addition of 1 mM buthionine sulfoximine, a specific inhibitor of γ-glutamylcysteine synthetase, induced glutathione (GSH) depletion and synergistically enhanced capsaicin-induced apoptosis (data not shown). Superoxide is a major component of ROS in the mitochondria and is converted rapidly to H_2O_2 by superoxide dismutase. Most H_2O_2 is degraded further to H_2O by the enzymes catalase and glutathione peroxidase. We thus examined the effect of more specific antioxidants on capsaicin-induced cell death. Interestingly, apoptosis induced by capsaicin could be blocked completely by pretreatment with catalase in both NB4 (Fig. 4*C*) and Kasumi-1 (Fig. 4*D*) cells. However, superoxide dismutase partially inhibited capsaicin-induced cell death (Fig. 4, *C* and *D*).

Expression of Cell Cycle- and Apoptosis-Associated Proteins in NB4 Cells. To characterize the molecular mechanism of capsaicin-induced cell cycle arrest followed by apoptosis in NB4 cells, we examined the expression of cell cycle- and apoptosis-associated proteins during the treatment with capsaicin. Expression of $p21^{WAF1/CIP1}$ proteins was dramatically increased with reduction of cyclin D1 protein expression, dephosphorylation of Rb, and up-regulation of p53 and Bax at 3 h after treatment (Fig. 5). Interestingly, the Ser-15 residue of p53 became significantly phosphorylated after an hour of exposure to capsaicin (Fig. 5). In contrast, the phosphorylation levels of p53 at other residues did not change in response to treatment with capsaicin (data not shown).

Phosphorylation of p53 at the Ser-15 Residue in NB4 Cells Treated with Capsaicin. p53 protein was accumulated during the treatment with capsaicin in NB4 cells expressing wild-type p53 (Fig. 5). In addition, Western blot analysis using the antibody specific to the phosphorylated Ser-15 of p53 revealed that the Ser-15 residue of p53 became phosphorylated immediately after the treatment with capsaicin (Figs. 5 and 6*A*). Interestingly, the inhibition of ROS generation by pretreatment of NAC inhibited capsaicin-induced phosphorylation of p53 at the Ser-15 residue (Fig. 6*A*). In addition, pretreatment of cells with catalase caused complete inhibition of capsaicin-induced p53

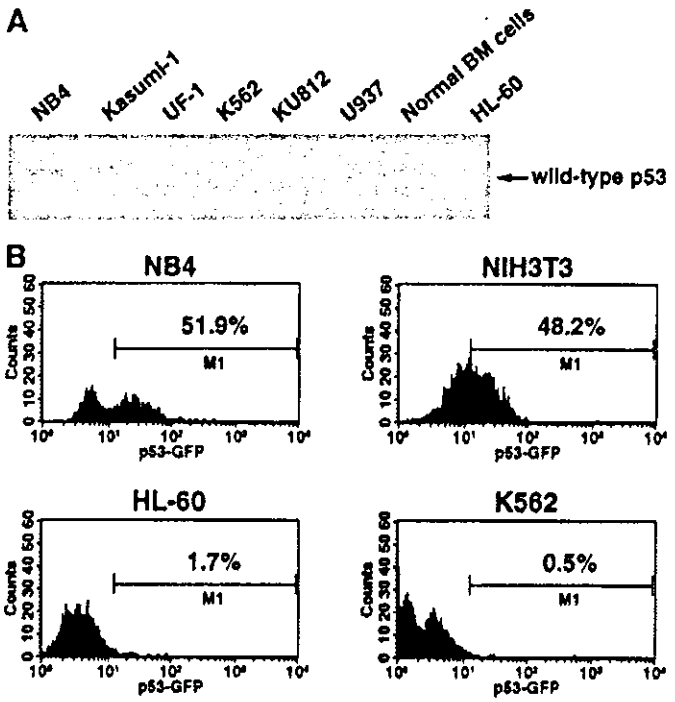

Fig. 2. Expression of wild-type p53 protein in various leukemic cells and normal bone marrow cells. *A*, wild-type p53 protein was immunoprecipitated with anti-p53 wild-type monoclonal antibody followed by blotting with the p53 polyclonal antibody. NB4 and Kasumi-1 cells are positive, but the other leukemic cells (HL-60, UF-1, K562, KU812, and U937) are negative for wild-type p53. Also, normal bone marrow mononuclear cells are positive. *B*, expression of wild-type p53 protein was analyzed by flow cytometry. NIH3T3 cells were used as a positive control. *GFP*, green fluorescent protein.

activation (Fig. 6*A*). These results suggest that H_2O_2 generation plays an essential role in p53 stabilization by phosphorylation at the Ser-15 residue (31, 32).

To examine whether inhibition of p53 expression can block capsaicin-induced cell cycle arrest and apoptosis in NB4 cells, we used antisense (AS) oligonucleotide for p53. Pretreatment with 1 μM p53 AS oligonucleotides prevented the capsaicin-induced increase in p53 protein levels in NB4 cells (Fig. 6*B*). In contrast, pretreatment with 1 μM scrambled and mismatch oligonucleotides did not significantly alter the expression of p53, and AS oligonucleotide did not modulate the expression of MDM2 and Bcl-2 proteins, indicating the specificity of the p53 AS oligonucleotide used in this study (Fig. 6*B*). Pretreatment with 1 μM p53 AS, but not scrambled or mismatched, oligonucleotides for 24 h significantly abrogated capsaicin-induced cell cycle arrest as well as apoptosis (Fig. 6*C*), suggesting that apoptotic cell death in capsaicin-treated NB4 cells is because of the ability of capsaicin to stimulate the accumulation of p53.

Effects of Capsaicin on Primary Cells from Patients with Leukemia. Among the leukemia cell lines, NB4 and Kasumi-1 cells expressing wild-type p53 were the most sensitive to capsaicin, whereas p53-defective cells including HL-60 and UF-1 cells were less sensitive to this agent. We therefore analyzed the association between the sensitivity to capsaicin and the status of p53 in freshly isolated cells from eight patients and found that the expression of wild-type p53 mRNA contributed to the sensitivity to capsaicin-induced apoptosis in the leukemic cells (Table 1). We also analyzed the levels of intracellular ROS generation during the treatment with capsaicin in capsaicin-sensitive and capsaicin-less sensitive cells and found that treatment with capsaicin caused a significant ROS generation with decreased intracellular GSH in capsaicin-sensitive cells (NB4 and leukemic cells from patient 1; Fig. 7, *A* and *B*). In contrast, in capsaicin-less sensitive cells (HL-60, UF-1, and leukemic cells from patient 6), intracellular ROS generation and GSH levels were less modulated by capsaicin (Fig. 7, *A* and *B*).

Capsaicin Induced Apoptosis *in Vivo*. Our *in vitro* data prompted us to examine whether the effects of capsaicin are equally demonstrable *in vivo*. Tumor weight significantly decreased in the mice that received an injection of capsaicin ($P < 0.001$, mean weight: 2.16 ± 0.53 g in the capsaicin-treated

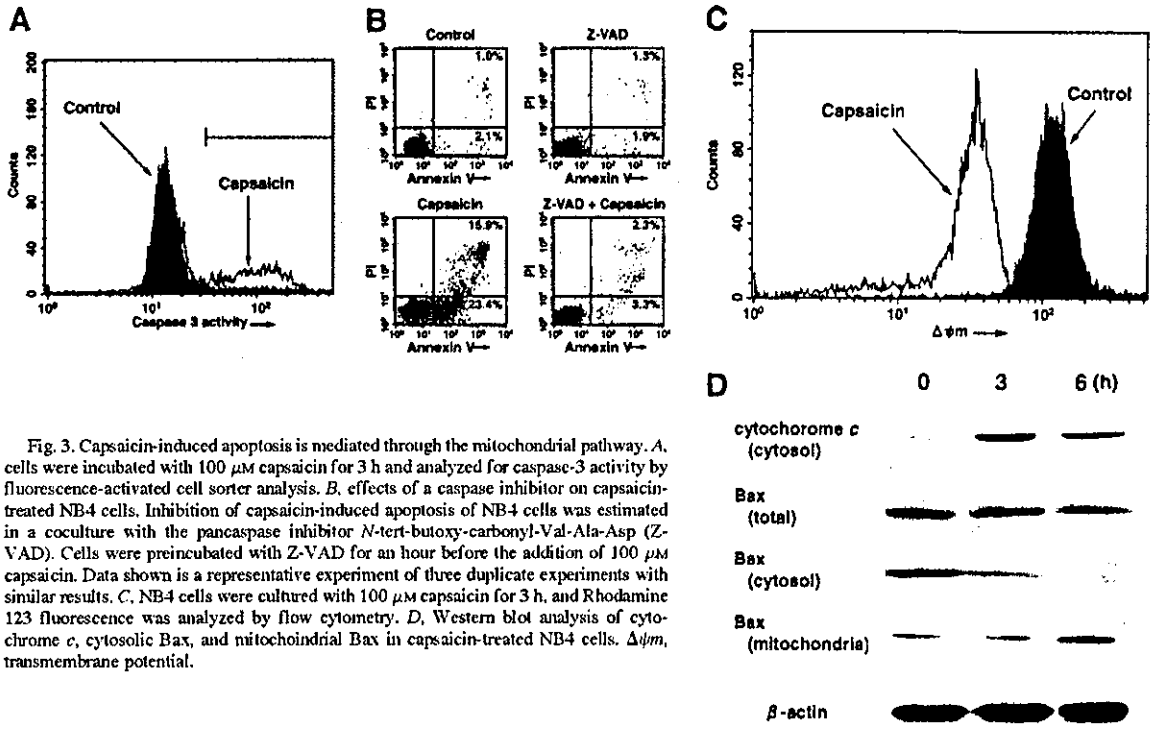

Fig. 3. Capsaicin-induced apoptosis is mediated through the mitochondrial pathway. *A*, cells were incubated with 100 μM capsaicin for 3 h and analyzed for caspase-3 activity by fluorescence-activated cell sorter analysis. *B*, effects of a caspase inhibitor on capsaicin-treated NB4 cells. Inhibition of capsaicin-induced apoptosis of NB4 cells was estimated in a coculture with the pancaspase inhibitor *N*-tert-butoxy-carbonyl-Val-Ala-Asp (Z-VAD). Cells were preincubated with Z-VAD for an hour before the addition of 100 μM capsaicin. Data shown is a representative experiment of three duplicate experiments with similar results. *C*, NB4 cells were cultured with 100 μM capsaicin for 3 h, and Rhodamine 123 fluorescence was analyzed by flow cytometry. *D*, Western blot analysis of cytochrome *c*, cytosolic Bax, and mitochoindrial Bax in capsaicin-treated NB4 cells. $\Delta\psi m$, transmembrane potential.

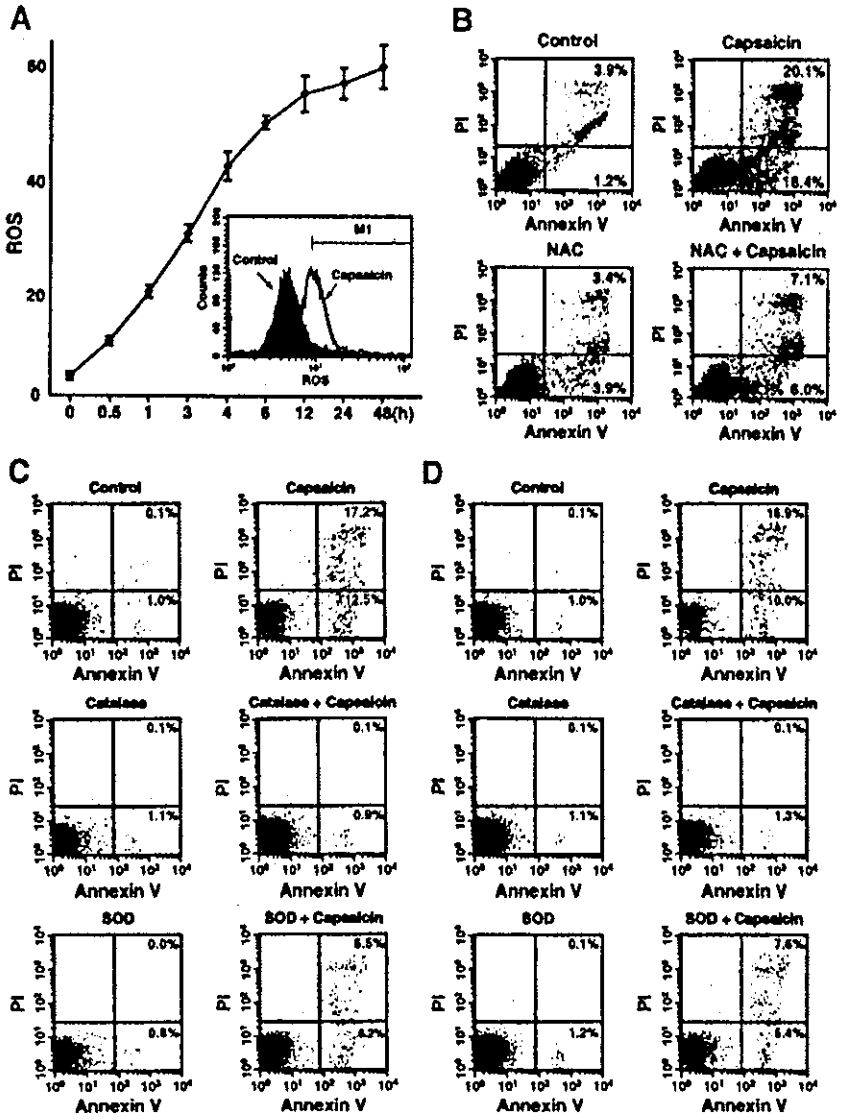

Fig. 4. Reactive oxygen species (ROS) generation by capsaicin in NB4 cells. *A*, to determine the intracellular concentration of ROS, we cultured NB4 cells treated with capsaicin for the indicated time with dihydroethidium (DHE), and the fluorescence was measured by flow cytometry. DHE-derived fluorescence in NB4 cells treated for 6 h with 100 μM of capsaicin is shown in the boxed panel. *B*, cells were pretreated with 100 μM *N*-acetyl-L-cystein (NAC) and then treated with 100 μM of capsaicin for 24 h, and induction of apoptosis was examined via Annexin V/propidium iodide (PI)-double staining. *C* and *D*, effect of specific antioxidants on capsaicin-induced apoptosis in NB4 (*C*) and Kasumi-1 (*D*) cells. Cells were treated with 400 units/ml catalase or 200 units ml superoxide dismutase and then treated with 50 μM capsaicin for 24 h. Induction of apoptosis was examined by Annexin V/PI-double staining. Representative data of three independent experiments were shown. *SOD*, superoxide dismutase.

group *versus* 4.71 ± 1.49 g in the control group; Fig. 8*A*). Pathological analysis at autopsy revealed no capsaicin-induced tissue changes in any of the organs. These results suggest that capsaicin had no toxic effects on mice during this treatment. When we evaluated tumor cell proliferation by counting the number of apoptotic cells by single-strand DNA staining, we observed a significant increase in the capsaicin-treated group ($P < 0.001$, approximately 8-fold increase; Fig. 8*B*).

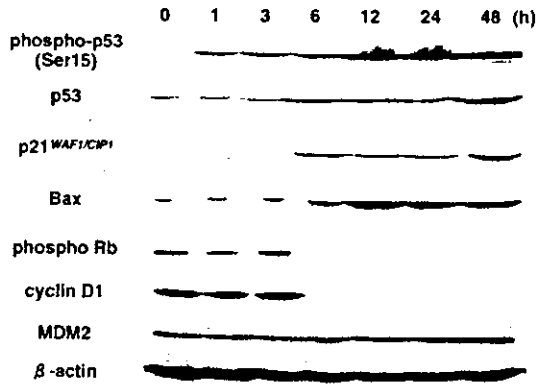

Fig. 5. Expression of the apoptosis- and cell cycle-associated proteins. NB4 cells were treated with 100 μM capsaicin for the indicated times. Cell lysates (15 μg/each lane) were fractionated on 12.5% SDS-polyacrylamide gels and analyzed by Western blotting with antibodies against apoptosis- and cell cycle-associated proteins. (pSer15-p53, p53, cyclin-dependent kinase inhibitors $p21^{WAF1/CIP1}$, Bax, pSer-Rb, cyclin D_1, and MDM2) Reblotting with β-actin staining demonstrated that equal amounts of protein were present in each lane.

DISCUSSION

To date, several studies have revealed the ability of capsaicin to inhibit events associated with the initiation, promotion, and progression of cancer (5, 12–14). In contrast, other studies have suggested the tumor-initiating potential of capsaicin (8, 9), and several epidemiological studies have shown that chilli pepper consumers were at greater risk for gastric cancer than were nonconsumers (2). In this study, we have showed that capsaicin inhibits cellular growth of leukemic cells via inducing apoptosis-modulating ROS production. Interestingly, capsaicin dramatically induced apoptosis of myeloid leukemic cells expressing wild-type p53.

In our study, capsaicin-sensitive cells expressed wild-type p53 among the leukemic cell lines (NB4 and Kasumi-1 cells) and fresh samples. There was a report that NB4 promyelocytic leukemia cells have point mutations at codon 273 and 248 of the p53 gene (45), but we could not detect any mutations of the p53 gene in NB4

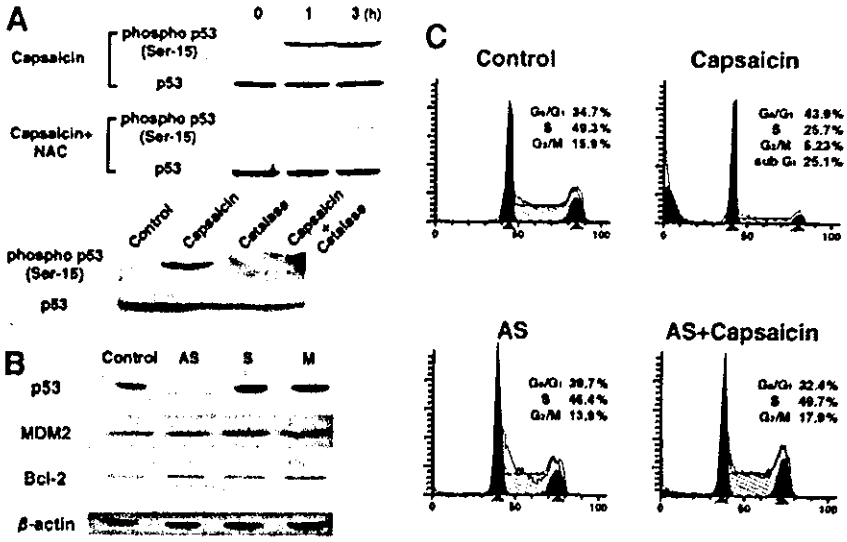

Fig. 6. Cell cycle arrest and apoptosis via a p53-dependent pathway in capsaicin-sensitive NB4 cells. *A*, phosphorylated Ser-15 residue of p53 was increased during capsaicin treatment of NB4 cells within an hour, and *N*-acetyl-L-cystein (NAC) inhibited phosphorylation of p53 by the treatment with capsaicin. In addition, specific antioxidant, catalase, completely blocked capsaicin-induced phosphorylation of the Ser-15 residue of p53. *B*, antisense (AS) oligonucleotide for p53 inhibits p53 expression in a sequence-specific manner. After 24 h of preincubation with 1 μM antisense, sense (S), or mismatch (M) oligonucleotides, NB4 cells were cultured in the presence of capsaicin and fresh oligonucleotides (1 μM) for 24 h. Cell lysates (30 μg/each lane) were fractionated on 12.5% SDS-polyacrylamide gels and analyzed by Western blotting with antibodies against p53, MDM2, and Bcl-2 proteins. Reblotting with β-actin staining demonstrated that equal amounts of protein were present in each lane. *C*, capsaicin-induced cell cycle arrest and apoptosis were abrogated by p53 AS oligonucleotide. Cells were preincubated with 1 μM oligonucleotide for 24 h before treatment with capsaicin. After preincubation, cells were treated with capsaicin for 24 h, and then cell cycle distribution was examined.

cells used in this investigation by sequencing analysis. In addition, we confirmed that our NB4 and Kasumi-1 cells expressed wild-type p53 protein using monoclonal antibody that recognizes conformational epitope of wild-type p53; therefore, we conclude that our NB4 and Kasumi-1 cells have wild-type p53. In contrast, we did not observe phosphorylation and accumulation of p53 during the treatment with capsaicin in less sensitive cells, which did not express wild-type p53. These cell lines have been reported to have mutated p53 gene as follows: HL-60 (major deletions), K562 (an early translational stop codon at 148), KU812 (a point mutation in codon 132 resulting in replacement of lysine with arginine), and U937 (46 base pairs deletion from codon 132) cells (39–42). Bax and $p21^{WAF1/CIP1}$ are well-known target genes of p53, and we demonstrated that capsaicin induced higher levels of these proteins in capsaicin-sensitive NB4 cells.

We showed that capsaicin induces phosphorylation of p53 at the Ser-15 residue after an hour of exposure, resulting in stabilization and protein accumulation. In addition, abrogation of p53 expression by the AS oligonucleotides could significantly inhibit induction of G_0-G_1 phase cell cycle arrest and apoptosis after treatment with capsaicin. However, normal bone marrow cells expressed wild-type p53, but capsaicin did not inhibit growth of the normal cells. Therefore, expression of wild-type p53 may be necessary but not sufficient for inducing apoptosis by capsaicin. Previous studies have reported that p53 gene mutations are infrequent and are found in only 5–10% of fresh acute myelogenous leukemia patients (46–48). In contrast, alterations of p53 gene are more frequent in myeloid leukemia cell lines, which might have the advantage in establishment of cell lines (42). Moreover, it has been reported that p53 mutations are associated with significantly poorer response to intensive chemotherapy and induce drug resistance by interfering with the normal apoptotic pathway in patients with acute myelogenous leukemia (49). Consistent with these reports, we showed that leukemic cells from seven of eight acute myelogenous leukemia patients express wild-type p53, and these cells are sensitive to capsaicin. Taken together, these results suggest that induction of p53 plays an essential role in G_0-G_1 cell cycle arrest and apoptosis in capsaicin-treated leukemic cells but not in normal cells.

Table 1 *Expression of wild-type p53 mRNA and sensitivity to capsaicin-induced apoptosis*[a]

Cells	Expression of wild-type p53 mRNA[d]	Annexin V single-positive cells		
		Control (%)	Capsaicin (%)	Fold increase[b]
Pt 1[e]	+	8.9 (12.6)[c]	63.4 (88.2)	7.1
Pt 2	−	4.5 (5.2)	5.2 (6.8)	1.2
Pt 3	+	3.7 (5.2)	40.3 (57.7)	10.9
Pt 4	+	6.8 (12.5)	50.9 (88.8)	7.5
Pt 5	+	6.2 (8.3)	64.3 (84.7)	10.4
Pt 6	+	4.2 (9.5)	53.4 (84.6)	12.7
Pt 7	+	3.8 (8.0)	43.5 (72.8)	11.4
Pt 8	+	3.3 (7.2)	46.2 (63.4)	14.0

[a] Cells were separated by Lymphoprep sedimentation procedure and subsequently cultured with 100 μM capsaicin for 24 h.

[b] Induction of apoptosis was measured by Annexin V single-positive cells and expressed as a fold increase of the percentage of control Annexin V single-positive cells.

[c] Percentage of Annexin V/P propidium iodide-double positive cells.

[d] Expression of wild-type p53 mRNA was examined by reverse transcription-PCR, and then PCR products were analyzed on 1% agarose gel. Result was expressed as positive (+) or negative (−).

[e] Pt 1, 2: acute lymphoblastic leukemia (ALL), Pt 3–8; acute myeloid leukemia (AML). *Pt.* patient.

Recent studies have demonstrated that mitochondria play an essential role in death signal transduction (50, 51). Bax in mitochondria is known to play an important role in the loss of $\Delta\psi m$ (50, 51), and distribution of $\Delta\psi m$ constitutes a critical step in a p53-dependent apoptotic pathway. In response to a capsaicin signal, Bax is induced and transported from the cytosol to mitochondria (data not shown), corresponding to a decline in $\Delta\psi m$ followed by cytochrome *c* release and caspase activation. These results suggest that capsaicin-induced death signaling is mediated through the mitochondrial-dependent pathway.

Several studies have demonstrated that ROS generation phosphorylates p53 at the Ser-15 residue in an ataxia telangiectasia mutated-dependent manner (31, 32). Consistent with previous studies (52–54), we detected that capsaicin-induced apoptosis in NB4 cells and in fresh leukemic cells from patients expressing wild-type p53 was associated with a significant increase in the levels of intracellular ROS, after GSH depletion. Capsaicin-less sensitive cells have defective p53; however, capsaicin could generate intracellular ROS in these cells. Interestingly, pretreatment with NAC, an excellent supplier of GSH, inhibited phosphorylation of p53 at the Ser-15 residue in the presence of capsaicin, indicating that ROS acts upstream of p53 phosphorylation by capsaicin. Moreover, reduction of H_2O_2 by catalase inhibited phosphorylation of p53 at the Ser-15 residue and apoptosis. In contrast, capsaicin-less sensitive cells were p53 defective, and capsaicin induced lower levels of ROS generation with less modulation of GSH. In addition, we failed to observe phosphorylation or induction of p53 during the treatment with capsaicin in these cells. We

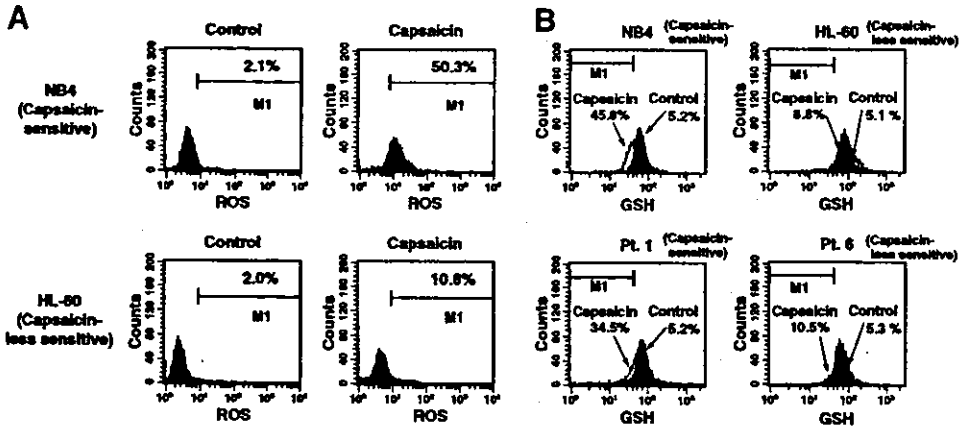

Fig. 7. Sensitivity to capsaicin in various leukemic cell lines and primary cells from patients. Intracellular levels of reactive oxygen species (ROS; *A*) and glutathione (GSH; *B*) were measured by flow cytometry in representative capsaicin-sensitive (NB4) and -less sensitive (HL-60) cell lines. *Pt.*, patient.

demonstrated that NAC also prevented capsaicin-less sensitive cells from apoptosis induced by a high dose of capsaicin (data not shown). Recent studies have reported that ROS is not only an upstream activator of the p53 pathway, but it is also a critical component of the downstream mediator of p53-dependent apoptosis, because overexpression of wild-type p53 produces ROS in association with apoptosis (55). The generation of ROS has been suggested to be a representative pathway of mitochondrial disruption in a p53-independent manner (56). It is likely that overgeneration of ROS plays some role in capsaicin-induced mitochondrial depolarization and apoptosis in p53-defective cells (57). Our data indicate that H_2O_2 can be a specific ROS species that plays mainly an essential role in capsaicin-induced p53 activation. Taken together, these data strongly indicate the existence of the following two downstream pathways that reduce $\Delta\psi m$ originating from H_2O_2 production by capsaicin: (*a*) one rapidly and with high sensitivity phosphorylatin p53 at the Ser-15 residue, leading to transportation of Bax to mitochondria, loss of $\Delta\psi m$, and early phase apoptosis in capsaicin-sensitive leukemic cells and (*b*) a second inducing a direct disruption of $\Delta\psi m$ by H_2O_2 generation independent of p53 in p53-defective cells but with less sensitivity to capsaicin. Additional studies are needed to clarify the exact mechanism of capsaicin-induced apoptosis in leukemic cells.

The therapeutic approach to acute leukemia is usually chemotherapy, but severe side effects and complications such as serious infection and bleeding because of anticancer drugs are major problems in the clinical setting. In particular, the side effects of drugs might be fatal in older patients or in immunocompromised patients, which highlights the urgent need for novel effective and less toxic therapeutic approaches. A component of the hot pepper Capsicum, capsaicin, is a natural compound and widely consumed as a food additive throughout the world, which indicates that it is less toxic to humans than current chemotherapeutic drugs. Because we could not observe any organ damage during *in vivo* experiments using a NOD/SCID mice leukemia model and because capsaicin did not affect cellular proliferation of normal bone marrow cells from healthy volunteers, we conclude that capsaicin might be developed as a new potent anticancer agent for the management of hematological malignancies.

In summary, we propose a model of cell cycle arrest and apoptosis induced by capsaicin through an oxidative stress in leukemic cells. Our data strongly indicate that capsaicin is particularly sensitive to leukemic cells expressing wild-type p53, which phosphorylated at Ser-15 residue by producing ROS during the treatment of capsaicin. Therefore, homovanillic acid derivative, capsaicin, has potential as a novel molecular targeted therapeutic agent against serine residue of the p53 gene for the treatment of leukemia, particularly in elderly and immunocompromised patients.

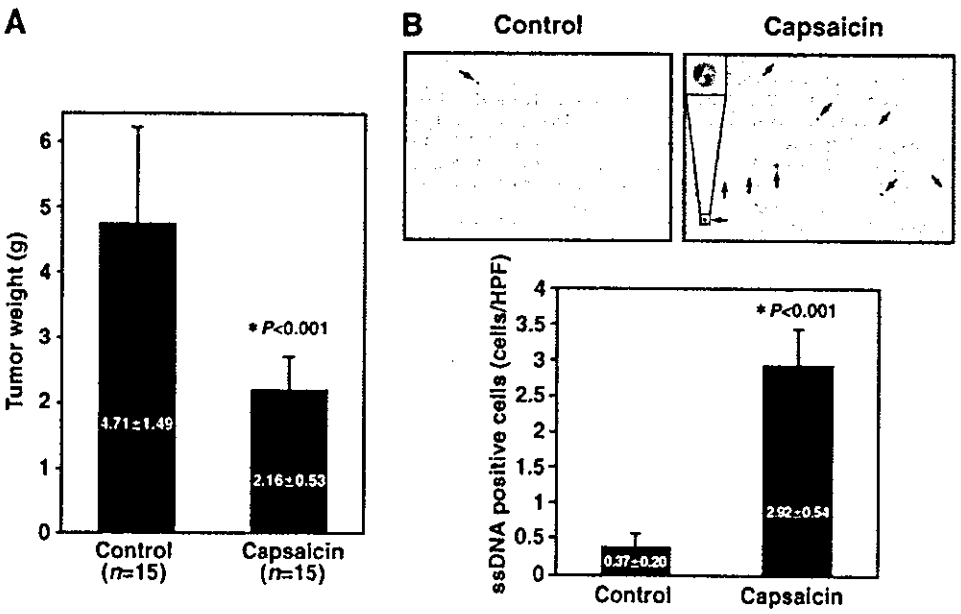

Fig. 8. Capsaicin-mediated apoptosis of leukemic cells *in vivo* using a NOD/SCID mice model. *A*, NB4 cells (1×10^7 cells) were inoculated s.c. into NOD/SCID mice. Fourteen days after transplantation, 5% ethanol (control; $n = 15$) or capsaicin (15 mg/kg; $n = 15$) was given daily for 6 days, after which mice were sacrificed and tumor weights were measured. *B*, the tumor sections were fixed and stained with anti-single-strand (ss)DNA antibody. We counted the apoptotic cells in the corresponding fields of control and capsaicin-treated tumor sections (10 sections/mouse, $n = 15$). *Arrows* indicate single-strand DNA (ssDNA)-positive cells. Original magnification, ×400. *HPF*, high power field.

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Editor-Communicated Paper

CD72 Stimulation Modulates Anti-IgM Induced Apoptotic Signaling through the Pathway of NF-κB, c-Myc and $p27^{Kip1}$

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Abstract: **Engagement of mIgM induces G1 arrest and apoptosis in immature B cells. The biochemical mechanism(s) regulating the cell death process are poorly understood. Cross-linking of CD72 (a B cell co-receptor) with anti-CD72 antibody was shown to protect B cells from apoptosis. We investigated the molecular mechanism involved in apoptosis preventing signaling mediated by CD72 ligation using a derivative (WEHIδ) of the WEHI231 cell line which is representative of immature B cells. Apoptotic WEHIδ cells following cross-linking of mIgM demonstrate a dramatic loss of c-Myc protein after transient up-regulation. In contrast, pre-ligation of CD72 was able to sustain c-Myc expression after transient up-regulation. Cross-linking of mIgM of WEHIδ cells causes accumulation of the Cdk inhibitor, $p27^{Kip1}$. CD72 pre-ligation was shown to inhibit the accumulation of $p27^{Kip1}$ protein. Moreover, NF-κB activity was not suppressed in WEHIδ cells after mIgM cross-linking when the cells were pre-treated with anti-CD72 antibody. These results strongly suggest that the apoptosis preventing signal evoked by CD72 ligation is delivered through the pathway of NF-κB, c-Myc, $p27^{Kip1}$ and cyclin.**

Key words: **c-Myc, $p27^{Kip1}$, NF-κB, CD72**

Apoptosis is a fundamental mechanism by which the immune system eliminates self-reactive, over-reactive, and/or un-reactive lymphocytes (5). Cross-linking (antigenic stimulation) of the B cell antigen receptor (BCR) is believed to induce apoptosis in immature B cells, eliminating self-reactive B cells before differentiation into mature B cells and antibody (Ab) secreting plasma cells (7, 22). Surface co-receptor molecules, however, modulate the BCR generated signals. One such modulator is CD72, a 45 kDa type II transmembrane protein, expressed predominantly in B lineage cells, except plasma cells (18). The molecule has an immunoreceptor tyrosine-based inhibition motif (ITIM) and an ITIM-like sequence in its cytoplasmic domain (3). SH2 containing protein tyrosine phosphatase-1 (SHP-1) binds to the ITIM sequence (ITIM1) located proximal to the N terminus, whereas Grb2 is recruited to the ITIM-like sequence (ITIM2) (1, 12, 41).

We have shown that the phosphorylation state of the CD72 ITIMs strongly correlates with BCR mediated signaling leading to apoptosis or cellular growth in WEHI231 cells and splenic B cells (12, 40, 41). The WEHI231 cell line expressing membrane immunoglobulin (mIg) M is representative of immature B cells. Engagement of mIgM of the B cell line evokes apoptosis (24, 32). CD72 of growing WEHI231 cells has the tyrosine residues of ITIMs de-phosphorylated by SHP-1 (41). Thus, CD72 is an *in vivo* substrate of SHP-1. In contrast, apoptotic WEHI231 cells carry phosphorylated CD72.

A large body of evidence demonstrates that transmembrane molecules carrying ITIMs are involved in negative regulation of signaling pathways of lymphocytes (4, 31). In contrast to this view, cross-linking of CD72 with anti-CD72 Ab seems to deliver a positive signal to splenic B cells resulting in increased expression of MHC

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Abbreviations: Ab, antibody; BCR, B cell antigen receptor; CDK, cyclin dependent kinase; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibition motif; mIgM, membrane immunoglobulin M; NF-κB, nuclear factor κB; PBS, phosphate buffered saline; SHP-1, src homology 2 containing tyrosine phosphatase.

class II molecules, enhanced cell proliferation and partial rescue from apoptosis induced by hyper-cross-linking of BCR (19, 28). Recent reports on CD72 deficient mice, however, described that B cells from the mice are hyper-responsive (20), suggesting that CD72 is involved in a negative modulation of BCR generated signaling. Thus, the function of CD72 in BCR mediated signaling is controversial.

Scott and his co-workers have reported that cross-linking of mIgM of WEHI231 cells leads to the blockade of the cell cycle in the G1 phase and subsequent apoptosis (8, 11). The G1 to S transition requires the activation of cyclin E/cyclin dependent kinase (Cdk)2 and cyclin A/Cdk2 resulting in phosphorylation of retinoblastoma protein (pRb). This kinase activity is down-regulated in WEHI231 cells treated with anti-IgM Ab.

Cdk inhibitors (CKI) or the Kip/Cip family in the case of Cdk2, provide one regulatory mechanism of Cdk activity (14). Apoptotic WEHI231 cells following mIgM cross-linking demonstrate accumulation of CKI, $p27^{Kip1}$ (8, 11). The decreased NF-κB activity leads to the down-regulation of c-Myc in anti-IgM Ab treated WEHI231 cells resulting in $p27^{Kip1}$ accumulation (8, 9, 27).

Very little is known about the molecular mechanism that rescues B cells from apoptosis by CD72 mediated signaling. One means to a better understanding of the CD72 mediated signaling pathway is to elucidate molecular events involved in the regulation of the G1 to S transition. In this paper, we find that CD72 mediated signaling plays a role in a pathway involved in the regulation of NF-κB, c-Myc and $p27^{Kip1}$.

Materials and Methods

Cell culture. WEHIδ murine B lymphoma cell line (IgD transfected WEHI231 cell) was maintained in DMEM supplemented with 10% fetal calf serum (FCS), 2-ME and MEM sodium pyruvate solution as previously described (12, 32, 41). Cells were incubated in 5% CO_2 at 37 C.

Western blot analysis. Western blotting was performed as already described (12, 41). Briefly, cells were treated with anti-mouse IgM Ab (Bet-2, American Tissue Type Collection) and incubated for 24 hr at a density of 5×10^5 cells/ml after pre-incubation with or without anti-CD72 Ab (K10, Cederlane Labs, Ontario Canada). 2.4G2 Ab (Pharmingen) was included to block FcγRIIB binding prior to the addition of Bet-2 Ab. Then, cells were solubilized in NP-40 lysis buffer supplemented with protease and phosphatase inhibitors. Lysates were separated by SDS-PAGE, transferred to PVDF membranes (Millipore), and detected by anti-p27 Ab (Santa Cruz) or anti-c-Myc Ab (Santa Cruz) using the ECL system (Amersham Biosciences).

RNA isolation and Northern blot analysis. Total cellular RNA was isolated from WEHIδ cells using ISOGEN (Nippon Gene) following the manufacturer's instruction. RNA (20 μg/lane) was fractionated on 1.1% formaldehyde/agarose gel, transferred to nylon transfer membrane, Hybond-N+ (Amersham Biosciences) and hybridized with a c-*myc* DNA probe. The DNA fragment (403 bp) was prepared by BamHI-XhoI digestion of exon 1 of a murine c-*myc* vector. The vector was obtained from D. Kitamura (Research Institute for Biological Sciences, Tokyo University of Science). The fragment was labeled with [^{32}P]dCTP using Prime It II Random Primer Labeling kit (Stratagene). Densitometry quantification was done using NIH image program.

Apoptosis analyses. Apoptosis analyses were carried out by propidium iodide (PI) staining and trypan blue exclusion assays. In PI staining assay, cells were washed with PBS and fixed with ice cold 70% ethanol on ice for 30 min. Then, cells were treated with 25 μg of RNase (Sigma) in PBS, re-suspended in 500 μl of PI solution containing 25 μg of PI and were analyzed using a FACScan flow cytometer (Becton Dickinson & Co.). In trypan blue exclusion assay, cells were incubated with 0.2% trypan blue (Gibco BRL) for 2 min, and the percentage of cells excluding dye or staining positive was determined.

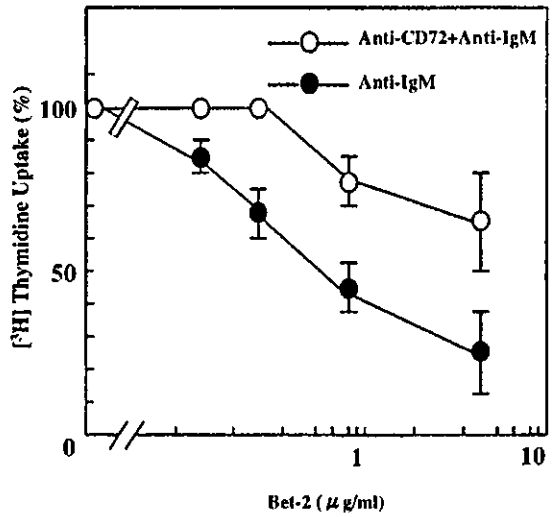

Fig. 1. Cross-linking of CD72 protects WEHIδ cells from anti-IgM induced growth arrest. WEHIδ cells (4×10^5) were pretreated with anti-CD72 Ab (K10) for 1 hr before the addition of varying amounts of anti-IgM Ab (Bet-2) as indicated. [^{3}H]thymidine incorporation was determined. Results are expressed as percentage when compared to results in the absence of Bet-2 Ab. Isotype-matched nonspecific antibody had no effect on the experiment (data not shown) (5).

Electrophoretic mobility shift assay. Cells were washed with cold PBS and were swelled in 500 µl of hypotonic reticulocyte standard buffer [10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4)] for 10 min followed by homogenization with Dounce homogenizer. Nuclei were precipitated by centrifugation and were suspended in 2 packed vols of extraction buffer [100 mM HEPES (pH 7.5), 350 mM NaCl, 5 mM EDTA] on ice with occasional tapping for 45 min. Samples were centrifuged at 10,000×*g* for 10 min. Nuclear protein concentration was determined by Coomassie Plus Protein assay kit (Pierce). Double stranded oligonucleotide containing κB site from the mouse Ig κ enhancer 5′-GATCGAGGGGACTTTCCGAGAGATC-3′ was end-labeled using T4 polynucleotide kinase and [γ-^{32}P]dATP. The labeled fragment was purified using quick spin column G-25 (Roche) and was used in the binding assay. Nuclear protein was mixed with poly(dI-dC) (1 mg/µl) and ^{32}P labeled probe in a binding buffer, and was incubated for 30 min at room temperature. Samples were then subjected to electrophoretic separation on a TAE/polyacrylamide gel. The gel was then dried, and labeled DNA was visualized by autoradiography.

Growth arrest assay. Growth arrest was determined by [^{3}H]thymidine incorporation assay as previously described (32, 41). Triplicates of 2×10^4 cells in a volume of 200 µl/well were incubated with anti-mouse IgM Ab (Bet-2) after pre-ligation with anti-CD72 Ab (K10) for 24 hr at 37 C. Cells were then pulsed with [^{3}H] thymidine (1 µCi/well) for 6 hr, before harvesting. The radioactivity was quantitated using Matrix 96 direct beta counter (Packard).

Results

Cross-Linking of CD72 with K10 Ab Protects WEHIδ Cells from Anti-IgM Induced Growth Arrest

In these experiments, we utilized WEHIδ cells (WEHI

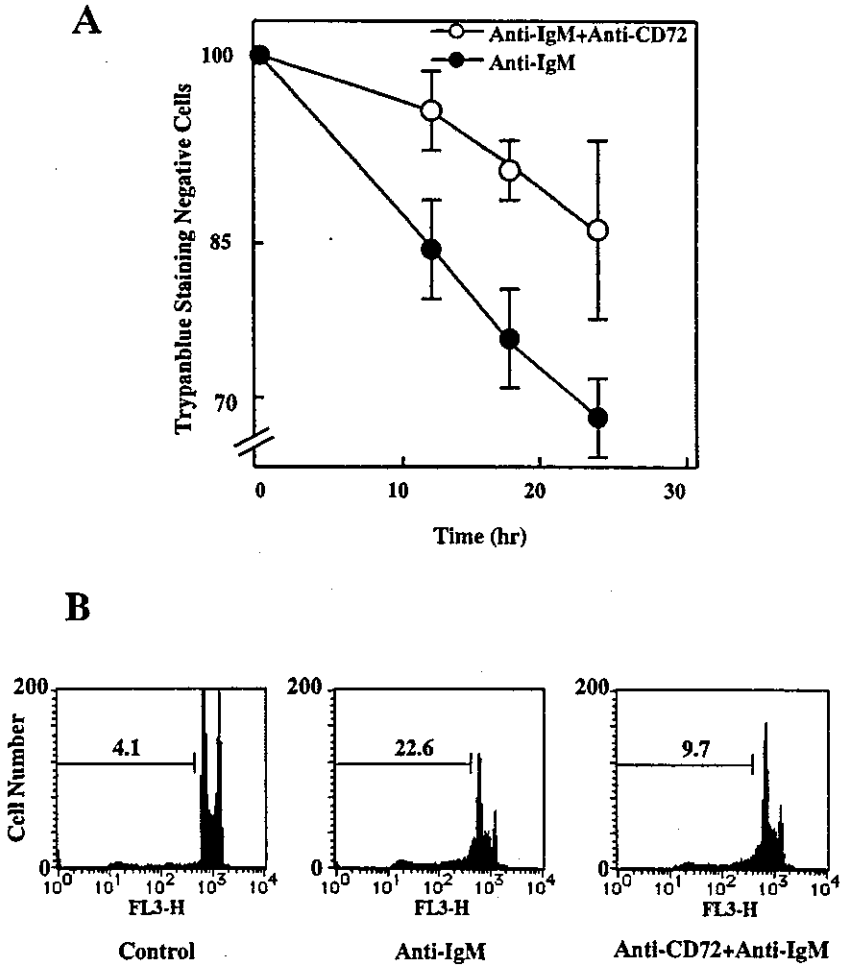

Fig. 2. Cross-linking of CD72 rescues WEHIδ cells from IgM ligation induced apoptosis. (A) Cells were cultured for indicated times with Bet-2 Ab alone or Bet-2 Ab+K10 Ab. Induction of cell death was determined by trypan blue staining. Percentages of survival populations are shown. (B) WEHIδ cells were cultured for 24 hr in the presence of the indicated Abs. Cells were stained with PI and analyzed on a FACScan flow cytometer. Percentages of apoptotic populations are shown.

231 cells transfected with the Igδ gene) expressing mIgM and mIgD. We have shown that cross-linking of mIgD, unlike mIgM stimulation, does not cause apoptosis in the transfectant (32). BCR stimulation to WEHI231 cells induces growth arrest before the induction of apoptosis. The effect of CD72 generated signaling was analyzed by a thymidine uptake experiment. WEHIδ cells were treated with anti-IgM Ab (Bet-2), following prior ligation of CD72 by K10 Ab. Prior ligation of CD72 partially protected the B cell line from growth arrest induced by mIgM engagement to some extent (Fig. 1). However, CD72 stimulation alone did not induce enhancement of thymidine uptake in the cell line (data not shown).

Cross-Linking of CD72 with K10 Ab Rescues WEHIδ Cells from Apoptosis

Ligation of CD72 by K10 Ab evokes anti-apoptotic response to splenic B cells (19). Next, we carried out experiments to test whether signaling through CD72 antagonizes apoptosis in WEHIδ cells. CD72 was pre-ligated before the addition of anti-IgM Ab and apoptotic response was assayed by the trypan blue exclusion test. WEHIδ cells (30%) became apoptotic at 25 hr. In contrast, 14% of cells pre-ligated with anti-CD72 Ab were committed to apoptosis at the same time point (Fig. 2A).

The cell cycle of WEHIδ cells was analyzed by PI staining. The flow cytometric data clearly demonstrated that percentages of apoptotic cells were much higher in mIgM engaged WEHIδ cells compared to control cells. Moreover, pre-ligation of CD72 rescues the cells from apoptosis (Fig. 2B). These results indicated that CD72 mediated signals promoted anti-apoptotic response in WEHIδ cells.

*Cross-Linking of CD72 with K10 Ab Sustains Expression Levels of c-*myc *mRNA and Protein*

We and others have shown that ligation of mIgM in WEHI231 and WEHIδ cells causes a temporal increase in the expression of the c-*myc* gene, followed by a rapid loss (8, 27, 32). Then, mRNA expression levels of the c-*myc* gene were analyzed by Northern blot experiments. To avoid the negative effect of FcγRIIB, we used Fc blocking 2.4G2 Ab prior to the addition of cross-linking Abs. Pre-ligation of CD72 resulted in a marked increase in c-*myc* mRNA expression compared to mIgM engagement alone (Fig. 3, A and B). Next, we investigated the changes in c-Myc protein expression. The expression level of c-Myc protein before mIgM cross-linking was high suggesting that c-Myc protein in WEHIδ cells without mIgM ligation was stable. The expression level of c-Myc protein was considerably decreased in WEHIδ

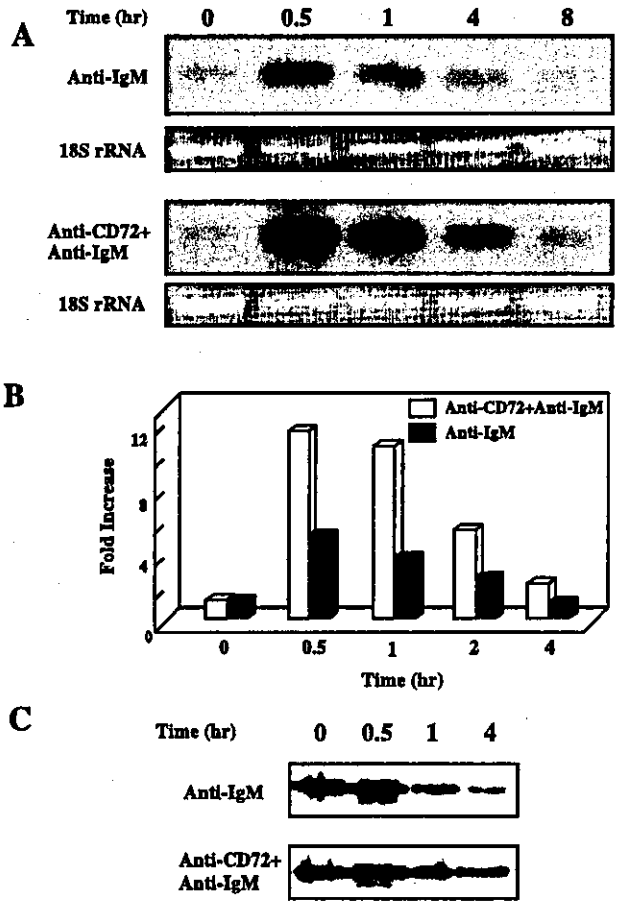

Fig. 3. Cross-linking of CD72 enhances c-*myc* gene and protein expression. (A) Cells were cultured and treated with Bet-2 Ab or Bet-2 Ab + K10 Ab for the indicated times. RNA was prepared from cells and samples were analyzed for c-*myc* gene expression by Northern blot. 18s rRNA is presented as a control for equal loading. (B) Densitometry data of Northern blot results are shown. Calculations are expressed as fold increase when compared to zero time results. (C) Cells (2×10^5/ml) were cultured for indicated time with Bet-2 Ab in either the presence or absence of K10 Ab and were lysed in 1% NP-40 lysis buffer. Equal volume proteins were loaded in each lane. Levels of c-Myc proteins were determined by Western blot analysis.

cells following cross-linking of mIgM after 4 hr, however the decrease was attenuated when CD72 was pre-ligated (Fig. 3C). We speculate that pre-ligation of CD72 is able to prevent apoptosis by sustaining c-Myc expression.

Cross-Linking of CD72 with K10 Ab Generates Anti-Apoptotic Signaling through a Pathway of NF-κB, c-Myc and $p27^{Kip1}$

Cyclin-dependent kinase (CDK)/cyclin complexes, Cdk2/cyclin A, Cdk2/cyclin E, Cdk4/cyclin D and Cdk6/cyclin D are responsible for the G1 to S transition (8). CDK enzymatic activity is regulated by cyclin expression and CDK inhibitors (CKIs) (9). Kip/Cip

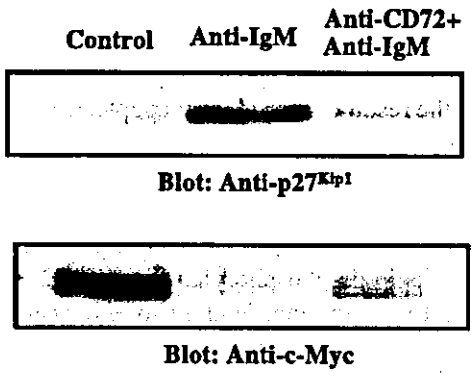

Fig. 4. CD72 pre-ligation prevents c-Myc down-regulation and $p27^{Kip1}$ accumulation in WEHIδ cells. Cells (2×10^5/ml) were cultured for 24 hr with Bet-2 Ab in either the presence or absence of K10 Ab and were lysed in 1% NP-40 lysis buffer. Equal volume proteins were loaded in each lane. Levels of c-Myc and $p27^{Kip1}$ proteins were determined by Western blot analysis using the indicated Abs.

group that includes $p27^{Kip1}$, $p57^{Kip2}$ and $p21^{WAF1/CIP1}$ is one of the CKI family. $p27^{Kip1}$ up-regulation leads to a decreased Cdk2 activity, retinoblastoma protein hypophosphorylation, G1 arrest and apoptosis (9). It was shown that $p27^{Kip1}$ is up-regulated in WEHI231 cells upon mIgM cross-linking (8). We examined an effect of CD72 pre-ligation on the expression level of $p27^{Kip1}$ (Fig. 4). No induction of $p27^{Kip1}$ was observed when CD72 was pre-ligated compared to a considerable accumulation of the same protein in mIgM stimulated WEHIδ cells without pre-ligation of CD72. The changes in $p27^{Kip1}$ expression vary inversely with the pattern of c-Myc expression. Notably, c-Myc protein expression was maintained in the cells with pre-ligation of CD72 after 24 hr. In contrast, mIgM cross-linking without CD72 pre-ligation resulted in a remarkable decrease in the accumulation of c-Myc protein (Fig. 4).

NF-κB is speculated to be involved in the modulation of anti-apoptotic signaling (10, 15, 25). Activity of NF-κB was investigated by gel shift assay (Fig. 5). Pre-ligation of CD72 sustained NF-κB activity considerably compared to mIgM engagement alone. These results suggest the anti-apoptotic signaling mediated via CD72 is delivered through the NF-κB, c-Myc and $p27^{Kip1}$ pathway.

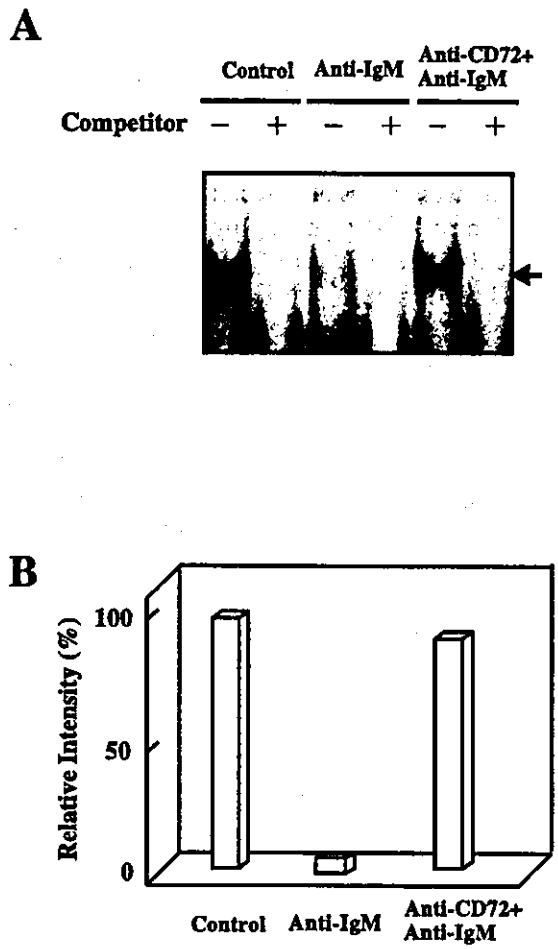

Fig. 5. IgM cross-linking inactivates NF-κB in WEHIδ cells, and CD72 pre-ligation prevents NF-κB inactivation. (A) Cells (2×10^6) were cultured with Bet-2 Ab in the presence or absence of K10 Ab for 30 min. Nuclear extracts were prepared, and equal volumes of nuclear proteins were loaded on each lane. NF-κB activity was determined by EMSA. The arrow indicates NF-κB. (B) Densitometry data of EMSA results are shown. The intensity of each band is expressed relative to that of non-stimulated sample (control) as 100%.

Discussion

BCR mediated signaling is modulated either positively or negatively by co-receptor molecules (6, 30, 34). In this report, we investigated the molecular mechanism involved in modulation of BCR stimulation induced apoptosis by the B cell co-receptor molecule, CD72. Components of antigen recognizing receptor complexes such as BCR and T cell receptor carry immunoreceptor tyrosine-based activation motif (ITAM) (29, 36). Negative regulators controlling antigen receptor-mediated signals have ITIMs in their cytoplasmic domains (34). Several reports including our own described how ITIM functions as a scaffold or a substrate of SHP-1 (2, 41). CD72 is of considerable interest from the point of a view of the regulation mechanisms, since the molecule may exert the dual regulatory functions (both positive and negative) (21).

The experiments with CD72 deficient mice have demonstrated that B cells from the mice are hyper-responsive to BCR stimulation (20). These results sug-

gest CD72 plays a negative regulatory role in modulation of BCR mediated signaling. In addition, expression of CD72 in a B lymphoma line deficient in CD72 molecule was reported to down-regulate BCR generated signaling (2).

Several investigators have reported a possible positive modulation of BCR signaling by CD72 (28, 35, 42). In particular, pre-ligation of CD72 partially rescues splenic B cells from apoptosis induced by hyper-cross-linking of BCR (19). We have reported a similar phenomenon following CD72 ligation in apoptotic WEHI231 cells (41). Venkataraman et al. reported that CD72 ligation activates the src-family kinases such as Lyn, Blk and Fyn, but not Syk (35). The proliferation response of B cells through CD72 was shown to be blocked by cycrosporin A and FK506, suggesting involvement of Ca^{2+} regulated activation pathways in CD72 mediated signaling (35).

BCR engagement with anti-IgM Ab causes growth arrest and apoptosis in WEHI231 cells (Figs. 1 and 2). The expression level of the c-*myc* gene is initially low and rapidly down-regulated after transient up-regulation by BCR stimulation (8, 27, 32). We have shown that ligation of CD72 sustains the expression levels of c-Myc protein and mRNA (Figs. 3 and 4). We have previously demonstrated that cross-linking of mIgD expressed in WEHIδ cells is not able to evoke apoptotic response (32). The expression level of the c-*myc* gene is increased temporally upon mIgD ligation, but the subsequent decline below the original expression level was not observed, unlike in mIgM stimulation. Furthermore, microinjection of an anti-sense oligonucleotide for the c-*myc* transcripts induced apoptosis in WEHI231 cells (39). It has been reported that the cell cycle is blocked at the G1 stage in apoptotic WEHI231 cells (40). Down-regulation of c-Myc protein expression was shown to lead to G1 growth arrest (27, 37). Therefore we speculate that resistance to apoptosis after ligation of CD72 is due to the sustained expression of c-Myc.

A major functional role of NF-κB is in the regulation of anti-apoptotic signaling (38). The NF-κB dependent gene products interfere with molecules involved in apoptotic cascades, such as Bcl-xL (4). Cross-linking of CD72 sustained NF-κB activity compared to anti-IgM cross-linking (Fig. 5). These results strongly suggest that CD72 generated anti-apoptotic signaling is mediated through the NF-κB pathway.

Increased c-Myc level promotes CDK activity leading to the cell cycle and cell proliferation. $p27^{Kip1}$ which is located down-stream of c-Myc functions as CKI. The protein level of the molecule is high in a variety of cells at the G1 to S transition (14). The results described in this paper suggest that CD72 generated signaling may rescue apoptotic WEHIδ cells from G1 arrest by declining the protein level of $p27^{Kip1}$ (Fig. 4). We are not able, however, to rule out the possibility that a decrease in $p27^{Kip1}$ is the result of rescue from apoptosis rather than the cause.

Recently, CD100 (a semaphorin family molecule) was identified as a ligand for CD72 (16, 26). The molecule is expressed on T cells. Binding of CD100 to CD72 results in de-phosphorylation of CD72 and prevents negative signaling, leading to enhanced B cell activation. The association of the ligand and the receptor is speculated to play a significant role in T cell-B cell interaction.

CD40 stimulation is able to prevent apoptosis induced by BCR stimulation in WEHI231 cells (13, 17, 23, 26, 33). As well, CD40 stimulation causes down-modulation of $p27^{Kip1}$ protein level (8, 13, 39). At present, we do not know where the apoptosis preventing signaling pathways of CD72 and CD40 merge. Currently, we are investigating the CD72 signaling pathway upstream of NF-κB. CD100 mediated CD72 stimulation to splenic B cells synergistically enhances CD40 induced B cell activation responses (16). The signaling mechanisms mediated via CD72 and CD40 may cross-talk to each other. It is quite interesting to elucidate and compare the signaling pathways mediated via CD72 and CD40.

In summary, pre-ligation of CD72 preventing apoptotic response in WEHIδ cells was demonstrated to exert an effect on the G1 to S transition influencing the signaling pathway of NF-κB, c-Myc, $p27^{Kip1}$ and cyclin for the first time. Further elucidation of the positive signaling pathway involved in apoptosis prevention will bring a new insight into a homeostasis mechanism in the immune system.

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Inducible costimulator-dependent IL-10 production by regulatory T cells specific for self-antigen

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In this study, we investigated the relationship between the expression levels of self-antigen and the function of self-reactive T cells in the periphery. To this end, we used two rat insulin promoter-ovalbumin (RIP-OVA) transgenic mice (RIP-OVA^{high}, RIP-OVA^{low}) in which was produced only in pancreatic β-islet cells. The OVA-producing transgenic mice were crossed to DO.11.10 (DO) mice expressing a T cell antigen receptor specific for $OVA_{323-339}$. The responsiveness of peripheral CD4$^+$ T cells in the double transgenic mice was examined. We demonstrated that hyporesponsive but highly IL-10-producing T cells were developed in DO × OVA^{high} mice only, not in DO × OVA^{low} mice. These IL-10-producing T cells exhibited regulatory activity both in *in vitro* and *in vivo* experiments. Moreover, these IL-10-producing regulatory T (Tr) cells expressed high levels of inducible costimulator (ICOS) before *in vitro* stimulation. Blockade of ICOS-signaling inhibited the production of IL-10 and abrogated the inhibitory function of these Tr cells. Thus, these results suggested that the development of IL-10-producing Tr cells depends on the expression levels of self-antigen *in vivo* and that ICOS signal plays a critical role in immune regulation by IL-10-producing Tr cells in self-tolerance.

Self-tolerance is mediated by central and peripheral mechanisms. Clonal deletion in the thymus and the induction of unresponsiveness (anergy) are well characterized mechanisms for the establishment and maintenance of tolerance (1). However, it is now clear that these processes are imperfect. In addition to these mechanisms, active suppression by regulatory T (Tr) cells has been proposed for tolerance to both self and foreign antigens. Various subsets of Tr cells have been described, and much effort has been focused on understanding their ontogeny, function, and mechanisms of action. Within the CD4$^+$ T cell subsets, at least three different types of cells with suppressive function may exist: CD4$^+$ CD25$^+$ T cells (2–4), T helper type 3 cells (5), and type 1 Tr (Tr1) cells (6, 7). These T cell subsets appear to be distinguishable based on their cytokine production profiles and their ability to suppress immune responses through direct cell-to-cell interaction. CD4$^+$ CD25$^+$ T cells are well characterized Tr cells among these subsets. Depletion of CD4$^+$ CD25$^+$ T cells results in the development of severe autoimmunity, which can be prevented by the injection of CD4$^+$ CD25$^+$ T cells (2, 8, 9). In addition to CD25 expression, they express cytotoxic T lymphocyte-associated antigen 4 and glucocorticoid-induced tumor necrosis factor receptor at higher levels (10, 11), and antibody against glucocorticoid-induced tumor necrosis factor receptor abolishes the suppressive activity (12). Moreover, the transcription factor Foxp3 is highly expressed, and this is associated with the suppressive ability and phenotype of these cells (13–15). T helper type 3 cells were identified in studies of oral tolerance. These cells secrete transforming growth factor type β (TGF-β), and their suppressive ability is mediated through a TGF-β-dependent mechanism (16, 17). Tr1 cells were initially defined in studies of CD4$^+$ T cells, which were activated in the presence of IL-10 and rendered anergic (6). The Tr1 cells produce high levels of IL-10 and TGF-β, moderate amounts of IFN-γ and IL-5, but little or no IL-2 or IL-4. Importantly, Tr1 cells were shown to be involved in the down-regulation of immune responses *in vitro* and *in vivo* through the production of the immunosuppressive cytokines IL-10 and TGF-β (6). Similarly, IL-10-producing Tr cells were also induced *in vitro* by culturing T cells with immature dendritic cells (18, 19) by using immunosuppressive drugs (20) or by stimulation with CD2 (21). The importance of suppression by IL-10-producing Tr cells is noteworthy; however, specific cell markers or instances of specific gene expression have not been clarified.

The importance of costimulatory molecules, such as CD28, cytotoxic T lymphocyte-associated antigen 4, or PD-1 (programmed death 1), for the activation of T cells is well known (22). A new member of the CD28 family, inducible costimulator (ICOS) is a T cell-specific cell-surface molecule structurally related to CD28 and cytotoxic T lymphocyte-associated antigen 4 (23, 24). Both CD28 and ICOS molecules are able to amplify the secretion of several cytokines, but only CD28 induces substantial amounts of IL-2, whereas ICOS shows a certain preference for the induction of IL-10 (23). Recently, Tr cells with the ability to produce IL-10 were reported in the respiratory tolerance system (25). In that report, IL-10-producing Tr cells developed by means of the ICOS signaling pathway. More recently, Lohning *et al.* (26) suggested a correlation between stable ICOS expression and T cell effector capacity; they showed that the expression of ICOS *in vivo* was strongly biased to CD4$^+$ T cells for IL-10 production but did not provide any direct information on the regulatory function of these cells.

Another important problem to be solved is the relationship between expression levels of self-antigens and the mechanisms of self-tolerance. It was reported that the distribution and amount of self-antigen influenced the induction and mechanisms of tolerance in self-antigen-specific CD4$^+$ T cells (27). Depending on the pattern of self-antigen expression, deletion of double-positive thymocytes ranged from minimal to complete, and peripheral CD4$^+$ T cells exhibited graded reduction in T cell antigen receptor (TCR) expression and *in vitro* proliferation. However, the relationship between the amount of self-antigen and the function of these CD4$^+$ T cells in the periphery remains to be clarified.

In this report, we investigated the relationship between the expression levels of self-antigens and the function of self-reactive CD4$^+$ T cells in the periphery by using two rat insulin promoter-ovalbumin (RIP-OVA) mouse lines in which OVA is produced only in pancreatic β-islet cells as a self-antigen (28). We demonstrate that the development of IL-10-secreting cells depends

Abbreviations: ICOS, inducible costimulator; RIP, rat insulin promoter; OVA, ovalbumin; Tg, transgenic; Tr, regulatory T; DO, DO.11.10; TGF-β, transforming growth factor type β; TCR, T cell antigen receptor; OVAp, $OVA_{323-339}$; CFSE, carboxyfluorescein diacetate succinimidyl ester.

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on the expression levels of self-antigen and that the ICOS molecule is the critical factor for IL-10 production from self-antigen-specific Tr cells.

Materials and Methods

Mice. BALB/c mice were purchased from CLEA Japan (Tokyo). RIP-OVA^{high} (hereafter referred to as OVA^{high}) and RIP-OVA^{low} (hereafter referred to as OVA^{low}) transgenic (Tg) mice on a C57BL/6 background were donated by W. R. Heath (The Walter and Eliza Hall Institute of Medical Research, Melbourne) (28). Both OVA Tg mice on a BALB/c background were produced by crossing OVA Tg C57BL/6 mice to BALB/c mice for more than six generations. DO.11.10 (DO) Tg mice carrying a TCR specific for $OVA_{323-339}$ (OVAp) were kindly provided by M. Kubo (Research Institute for Biological Sciences, Tokyo University of Science). DO × OVA double-Tg mice were bred in our animal facility.

Preparation of CD4$^+$ T Cells. CD4$^+$ T cells were purified with CD4 microbeads (Miltenyi Biotec, Bergish Gladbach, Germany) as described (29). The purity of CD4$^+$ T cells was routinely estimated to be ≈94–98%.

Flow Cytometry Analysis for Cell-Surface Molecules. Biotin-conjugated anti-ICOS mAb (15F9) was purchased from eBioscience (San Diego). Phycoerythrin- or FITC-labeled anti-CD4 mAbs, biotin-conjugated-CD45RB (RA3–6B2), CD44 (IM7), CD25 (7D4), and CD69 (H1.2F3) mAbs were purchased from BD Pharmingen. Clonotype-specific KJ1.26 mAb was purified from ascites and conjugated with FITC in our laboratory. Anti-B7h mAb (HK5.3, rat IgG2a) was prepared as described (30).

T Cell Proliferation Assay. The T cell proliferation assay was performed in 96-well flat-bottom plates. CD4$^+$ T cells (5×10^4 per well) in a total volume of 200 μl were stimulated with ≈0–5 μM OVAp in the presence of irradiated syngeneic spleen cells (2×10^5 per well). The cells were cultured for 54 h. Proliferation was assessed by measuring the incorporation of [^{3}H]thymidine (1 μCi per well) added for the final 18 h of culture.

Cytokine ELISA. CD4$^+$ T cells (1×10^6) in a total volume of 1 ml were cultured with 0.5 μM OVAp in the presence of antigen-presenting cells (2×10^6) in 24-well plates. The cultured supernatants were recovered 24 h later for measurement of IL-2 or 72 h later for measurement of IFN-γ, IL-10, and IL-4. The cytokine content was determined by means of a two-site ELISA. All Abs used in ELISA were purchased from BD Pharmingen.

***In Vitro* Assay of Suppressive Activity.** Target CD4$^+$ T cells (5×10^4 cells per well) from DO mice and effector CD4$^+$ T cells (1×10^5 cells per well) from DO × OVA^{high} were cultured with irradiated syngeneic spleen cells (3×10^5 cells per well) in the presence of 5 μM OVAp for 54 h with or without anti-B7h mAb. Proliferation was assessed as described above.

***In Vivo* Assay of Suppressive Activity.** CD4$^+$ T cells from DO mice were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) at a final concentration of 0.1 μM. A total of 4×10^6 CFSE-labeled CD4$^+$ T cells with 4×10^6 CD4$^+$ KJ1.26$^+$ T cells from DO × OVA^{high} mice were transferred into the tail vein of BALB/c mice. Some mice received 0.5 mg of anti-B7h mAb or isotype control IgG. Two days after the transfer, mice received one i.p treatment of OVA in complete Freund's adjuvant. Division of CFSE-labeled CD4$^+$ T cells in the spleen was monitored 2 d later.

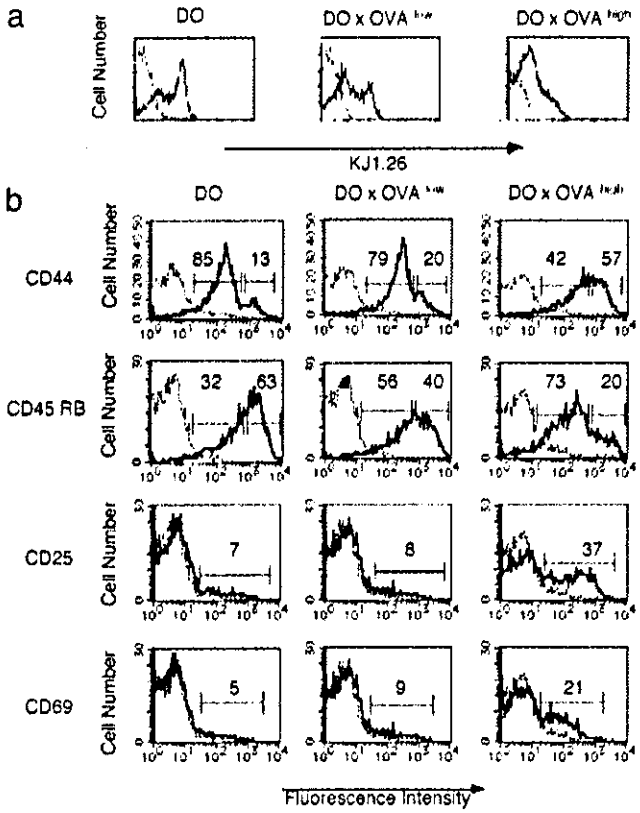

Fig. 1. Changes in cell-surface molecules on CD4$^+$ KJ1.26$^+$ T cells of DO × OVA mice. (*a*) Decreased expression of transgenic KJ1.26$^+$ TCR on spleen CD4$^+$ T cells from DO × OVA double-Tg mice. (*b*) Expression of CD44, CD45RB, CD25, and CD69 on spleen CD4 and KJ1.26 double-positive T cells. One representative data set of five is shown.

Results

Altered Numbers and Phenotypes of Peripheral CD4$^+$ T Cells in Tolerant Mice. Previously, for the study of reactivity of self-antigen specific CD8$^+$ T cells, two RIP-OVA Tg mouse lines (OVA^{high} mice and OVA^{low} mice) that expressed different amounts of OVA under the control of RIP were generated (28). OVA^{high} mice expressed 1.0 ± 0.4 ng of OVA per μg of protein in the pancreatic β-islet cells, and OVA^{low} mice expressed a lower level of antigen that was <0.03 ng of OVA per μg of protein (28). Both CD8$^+$ T cells (28) and CD4$^+$ T cells (Fig. 6, which is published as supporting information on the PNAS web site) of these RIP-OVA mice were tolerant to OVA. To compare the fate of self-reactive CD4$^+$ T cells recognizing OVA expressed in the pancreas, OVA^{high} mice and OVA^{low} mice were crossed to DO mice. Numbers and phenotypes of peripheral CD4$^+$ T cells were changed in DO × OVA double-Tg mice. In DO × OVA^{high} mice, the number of spleen CD4$^+$ T cells was reduced 3- to 5-fold compared with DO mice. However, in DO × OVA^{low} mice, the change was minimal, and the number of CD4$^+$ T cells in the spleen was only reduced by 1.5- to 2-fold. Both DO × OVA double-Tg mice carried CD4 subsets which expressed low levels of KJ1.26 (Fig. 1). CD4$^+$ T cells from DO × OVA double-Tg mice expressed increased levels of CD44 and decreased levels of CD45RB compared with DO mice, and the change was more dramatic in DO × OVA^{high} mice (Fig. 1). These results indicated that peripheral CD4$^+$ T cells from DO × OVA double-Tg mice displayed a phenotype of previously activated cells in proportion to the amount of self-antigen expression. Moreover, in DO × OVA^{high} mice, some CD4$^+$ T

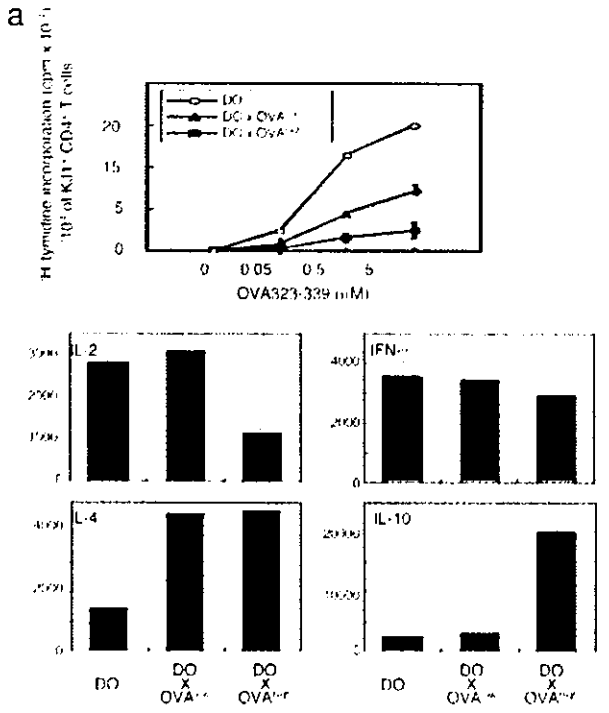

Fig. 2. Proliferation and cytokine production of CD4+ T cells from DO, DO × OVA^{low}, and DO × OVA^{high} mice after antigenic stimulation *in vitro*. CD4+ T cells were purified from splenocytes of DO, DO × OVA^{low}, or DO × OVA^{high} mice. (*a*) CD4+ T cells were stimulated with irradiated splenocytes in the presence of the indicated amounts of OVAp. Total cpm values were divided by the number of KJ1.26+ cells as determined by flow cytometry. (*b*) CD4+ T cells were stimulated with irradiated splenocytes in presence of 0.5 μM OVAp. Cytokines in the culture supernatant were measured by ELISA. The amount of cytokines was divided by the number of KJ1.26+ cells as determined by flow cytometry. Data are representative of five separate experiments with similar results.

cells also expressed the early activation markers CD69 and CD25 (Fig. 1).

CD4+ T Cells from DO × OVA^{high} Mice Produce High Levels of IL-10. To compare the functional ability of peripheral CD4+ T cells from DO × OVA double-Tg mice, the proliferation ability and cytokine production in response to OVAp were examined. Corresponding to the different degrees of TCR expression described above (Fig. 1), CD4+ T cells from the different DO × OVA double-Tg mice displayed reduced proliferative ability when stimulated with OVAp (Fig. 2*a*). To assess whether the observed hyporesponsiveness could be reversed by the addition of IL-2, as in the case with anergic T cells *in vitro* (31), recombinant IL-2 was added to the culture when stimulated with OVAp. However, proliferative capacity of CD4+ T cells from these DO × OVA double-Tg mice was not recovered (data not shown). To further characterize the functional difference of CD4+ T cells, purified CD4+ T cells were examined for cytokine production. To this end, CD4+ T cells from either DO or DO × OVA double-Tg mice were purified from spleen cells and stimulated with OVAp (Fig. 2*b*). IL-2, IFN-γ, IL-4, and IL-10 were detected in all mice, whereas TGF-β1 was not (data not shown). IL-4 production was increased in both of the DO × OVA double-Tg mice compared with DO mice. The most significant difference was observed in IL-2 and IL-10 production. CD4+ T cells from DO × OVA^{high} mice produced low levels of IL-2 but very high levels of IL-10 (Fig. 2*b*). Thus, CD4+ T cells in DO × OVA^{high} mice are hyporesponsive and are able to produce high amounts of IL-10.

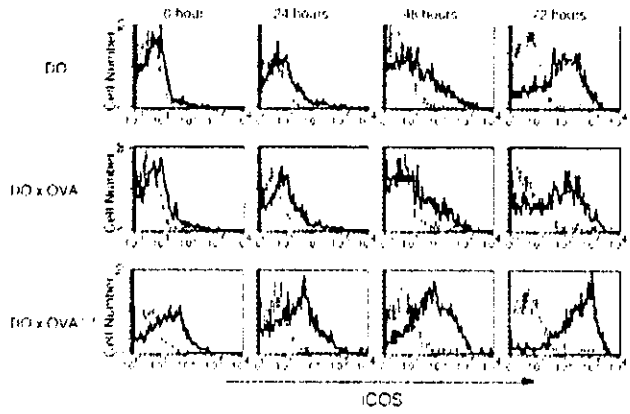

Fig. 3. CD4+ KJ1.26+ T cells from DO × OVA^{high} mice highly express ICOS. CD4+ T cells from the spleen of DO, DO × OVA^{low}, and DO × OVA^{high} mice were stimulated with 0.5 μM OVAp, and the expression levels of ICOS were examined at the indicated times after stimulation *in vitro*. CD4+ T cells were stained with mAb against CD4, KJ1.26, and ICOS. ICOS expression was analyzed by gating on CD4 and KJ1.26 double-positive T cells. One representative experiment of three is shown

ICOS Expression Is Highly Correlated with IL-10 Production in CD4+ T Cells from DO × OVA^{high} Mice. It has been suggested that stimulation of ICOS on CD4+ T cells preferentially promotes IL-10 production (23, 32, 33). Moreover, the importance of the ICOS molecule in IL-10-producing Tr cells was reported in respiratory tolerance (25). To clarify the relationship between IL-10 production and ICOS expression in our self-tolerance system, we analyzed ICOS expression in CD4+ T cells. CD4+ T cells isolated from DO mice and DO × OVA^{low} mice expressed low levels of ICOS, but from DO × OVA^{high} mouse expressed a high level of ICOS (Fig. 3). When these cells were stimulated with OVAp *in vitro*, the expression of ICOS was enhanced in all mice after 24–72 h. Interestingly, at any time point after stimulation, induced ICOS expression was significantly higher in DO × OVA^{high} mice compared with other Tg mice.

To determine the contribution of the ICOS signaling pathway to IL-10 production in our system, we examined whether blocking ICOS signaling reversed IL-10 production in CD4+ T cells from DO × OVA^{high} mice. As shown in Fig. 4, the blocking of ICOS signaling by the mAb to B7h, a ligand of ICOS, reduced IL-10 production of CD4+ T cells from DO × OVA^{high} mice but had no effect on CD4+ T cells from DO mice. In addition, IFN-γ production was decreased and IL-4 production was increased, but the effect was not as prominent. The blocking of the ICOS signaling pathway had no effect on proliferation (data not shown). These results suggest that the ICOS signaling pathway is specifically involved in IL-10 production of CD4+ T cells from DO × OVA^{high} mice.

IL-10-Producing CD4+ T Cells from DO × OVA^{high} Mice Inhibit T Cell Proliferation. We investigated whether IL-10-producing T cells in our double-Tg mouse system had the ability to suppress the activation of T cells *in vitro*. CD4+ T cells from DO × OVA ^{high} mice and DO mice were cocultured with irradiated syngeneic spleen cells in the presence of OVAp. CD4+ T cells from DO × OVA ^{high} mice inhibited the proliferation of CD4+ T cell from DO mice (Fig. 5*a*). The inhibitory ability of CD4+ T cells from DO × OVA^{high} mice depended on IL-10 production, because the addition of anti-IL-10 mAb reversed the inhibitory effect. Moreover, the blockade of the ICOS signaling pathway abrogated the *in vitro* suppressive function. Next, we examined the *in vivo* suppressive ability. CFSE-labeled CD4+ T cells from DO mice were transferred into BALB/c mice with CD4+ T cells from

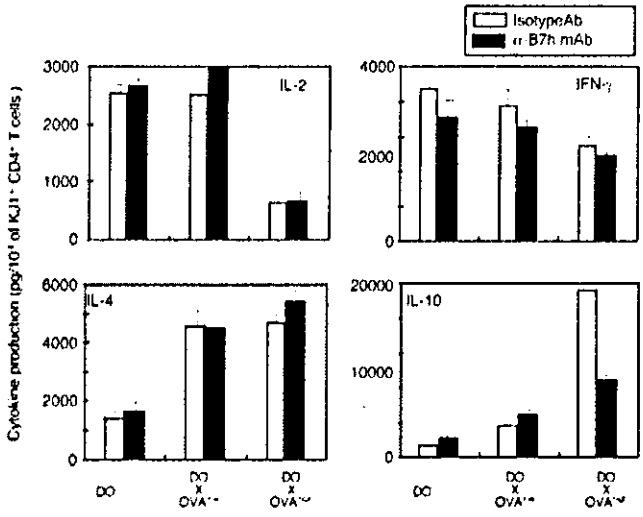

Fig. 4. Blocking the ICOS signaling pathway inhibits IL-10 production of CD4$^+$ T cells from DO × OVA^{high} mice after antigenic stimulation *in vitro*. CD4$^+$ T cells were purified from splenocytes of DO, DO × OVA^{low}, or DO × OVA^{high} mice. CD4$^+$ T cells were stimulated with irradiated splenocytes in the presence of 0.5 μM OVAp with or without α-B7h mAb. Cytokines in the culture supernatant were measured by ELISA. The amount of cytokines was divided by the number of KJ1.26$^+$ cells as determined by flow cytometry. Data are representative of three separate experiments with similar results.

DO × OVA ^{high} mice or DO mice by i.v. injection. Two days after cell transfer, the recipient mice were immunized with OVA/complete Freund's adjuvant. The splenic cells from these recipient mice were harvested 4 d after transfer. Fluorescence-activated cell sorter analysis of these populations was performed gaiting on the CD4, KJ1.26, and CFSE triple-positive cells. As shown in Fig. 5*b*, CFSE-labeled cells cotransferred with CD4$^+$ T cells from DO mice divided readily in response to OVA, with only 38% remaining undivided. The same cells cotransferred with DO × OVA ^{high} mice also divided; however, a greater proportion of these cells (≈80%) did not undergo cell division. To investigate the role of ICOS in mediating this *in vivo* suppression, recipient mice transferred with CD4$^+$ T cells from DO × OVA ^{high} mice were treated with anti-B7h mAb 1 h before antigen challenge. The suppression of proliferation was significantly reduced when the ICOS signal was blocked (Fig. 5*c*). These results suggested that CD4$^+$ T cells from the DO × OVA ^{high} mice contained the Tr cells and that their suppressive activity on proliferation was mediated by ICOS-induced IL-10.

Discussion

Here we demonstrate that the expression levels of self-antigen determine the function of self-reactive CD4$^+$ T cells in the periphery and that IL-10-producing Tr cells developed in mice expressing high levels of self-antigen. Furthermore we show that IL-10 production and the inhibitory activity of these Tr cells are mediated by ICOS stimulation. Although IL-10-producing Tr cells have been reported in another self-tolerance system (34), we demonstrated that ICOS is crucial for IL-10 production by self-antigen specific Tr cells.

Previously, the effect of distribution and expression levels of self-antigen to tolerance induction of self-reactive CD4$^+$ T cells has been examined by using several types of hen egg lysozyme (HEL) expressing Tg mouse lines: e.g., expression on the thyroid epithelium, the pancreatic B-islet cell, or systemically (27). These HEL Tg mice were crossed to TCR Tg mice specific for HEL. The analysis of the double-Tg mice has revealed that the deletion of double-positive thymocytes, reduction of TCR expression, and proliferative response of peripheral CD4$^+$ T cells were

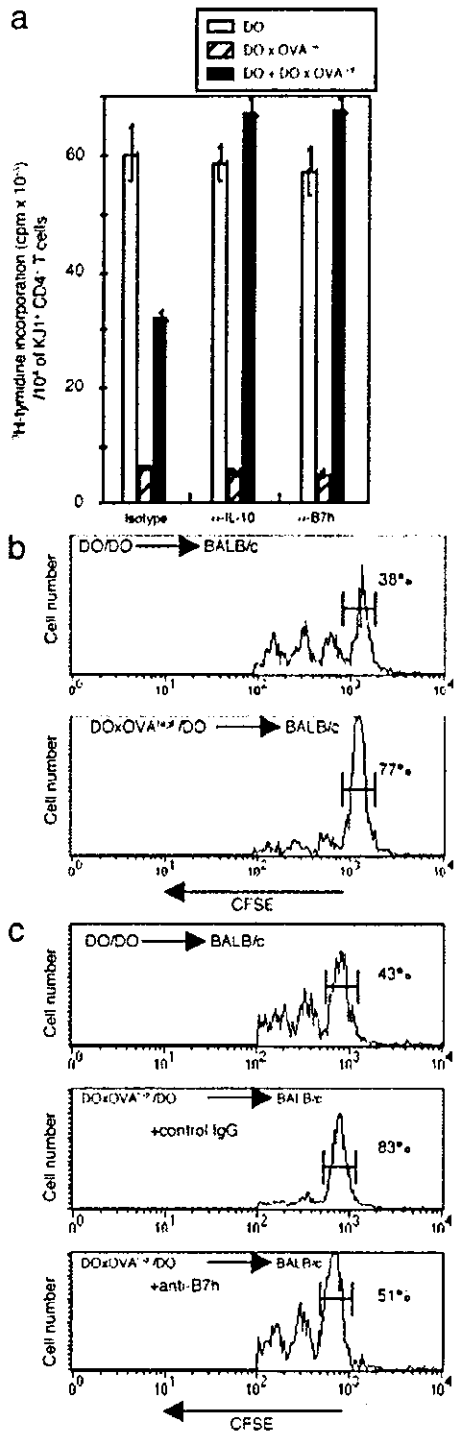

Fig. 5. CD4$^+$ T cells from DO × OVA^{high} mice suppress the proliferation of CD4$^+$ T cells from DO mice. (*a*) CD4$^+$ T cells from DO × OVA^{high} mice and DO mice were cultured with irradiated syngeneic spleen cells in the presence of 0.5 μM OVAp with or without both anti-IL-10 or anti-B7h mAb. One representative experiment of three is shown. (*b* and *c*) CD4$^+$ T cells from DO were labeled with CFSE and transferred with CD4$^+$ T cells from DO × OVA^{high} mice or from DO into BALB/c mice. Some mice received anti-B7h mAb or control IgG before OVA injection. Two days after the transfer, the recipient mice were injected with OVA in complete Freund's adjuvant. Two days after immunization, spleen cells were collected from the recipients and stained with anti-CD4 and anti-KJ1.26 mAb. Fluorescence-activated cell sorter analysis was performed, gating on the CD4, KJ1.26, and CFSE triple-positive cells. The results are representative examples of experimental groups containing three mice. The experiments have been repeated three times with similar results.

influenced by the distribution and amount of the self-antigen. However, the cytokine production profile and regulatory function of the T cells involved in tolerance were not examined. In this study, we clearly demonstrated that the proliferative response of T cells from both DO × OVA^{high} and DO × OVA^{low} mice was suppressed in proportion to the expression levels of the self-antigen (Fig. 2*a*), which is in accordance with a previous report (27). These hypoproliferative T cells from the double-Tg mice produce high amounts of IL-4 in response to *in vitro* antigenic stimulation. However, enhanced IL-10 production was detected only in CD4$^+$ T cells from DO × OVA^{high} mice, which show a greater suppression of proliferation. These results led us to hypothesize that self-specific T cells in the periphery become hypoproliferative irrespective of the expression level of self-antigen, but they gain the ability to produce the immunosuppressive cytokine (IL-10) and regulatory function when the self-antigen is expressed at a level beyond a certain threshold, and, as a result, chronic antigen stimulation occurs. This hypothesis is in agreement with Buer's work (34) showing that deeply anergic, but IL-10-producing, T cells were generated in the double-Tg tolerance mouse model in which a specific antigen was expressed in hematopoietic cells. Furthermore, IL-10 production by anergic T cells has also been induced by the repeated administration of superantigen (35, 36) or repeated intranasal administration of a high-affinity MHC-binding analog (37).

Our results clearly demonstrate the specific role of the ICOS signal in IL-10 production and immune regulation by Tr cells. Blocking of the ICOS signal with anti-B7h mAb inhibited production of IL-10 but not of IL-4 and IFN-γ from Tr cells in DO × OVA^{high} mice (Fig. 4). Furthermore, the treatment abrogated the inhibition of proliferation by Tr cells in both *in vitro* and *in vivo* experiments (Fig. 5). These data are in agreement with recent studies showing that the regulatory function of IL-10-producing T cells in intranasal tolerance completely depends on ICOS (25). Thus, the ICOS signal seems to be critical for Tr cell-mediated self and nonself tolerance. ICOS knockout mice showed enhanced susceptibility to experimental autoimmune encephalomyelitis, suggesting that ICOS signaling is involved in protection from autoimmune disease (38). CD4$^+$ T cells from the ICOS knockout mice, however, have produced comparable levels of IL-10 to those from WT mice. The role of ICOS in IL-10 production may depend on the nature of the T cells: i.e., T helper or Tr, and this point should be clarified in future experiments.

A recent study has also demonstrated a clear correlation between the ability of T cells to produce IL-10 and the levels of their ICOS expression. Lohning *et al.* (26) have shown that ≈1% of splenic CD4$^+$ T cells from nonimmunized mice express ICOS. These T cells show an activated/memory phenotype and that a proportion of their ICOS expression is linked to their IL-10 production ability; i.e., IL-10 is the dominant cytokine produced by T cells with high levels of ICOS. These findings are consistent with our present study in that CD4$^+$ T cells in DO × OVA^{high} mice express high levels of ICOS before *in vitro* stimulation (Fig. 3). Thus, ICOS expression could be one of the molecular markers of T cells with IL-10-producing ability. It remains unclear why IL-10-producing T cells express high levels of ICOS and how these high levels are maintained. Taking into account that ICOS is one of the activation markers of T cells, continuous stimulation of self-antigens *in vivo* may sustain ICOS expression.

IL-10-producing Tr cells have been generated by culturing T cells in the presence of large quantities of exogenous IL-10 (6), culturing T cells with immature IL-10-producing dendritic cells (19), or by using immunosuppressive drugs (20). These studies suggested that the presence of IL-10 was critical for the development of IL-10-producing cells. From these observations, the differentiation of IL-10-producing Tr cells in our double-Tg mouse system might be also induced by IL-10-producing dendritic cells presenting OVA as the self-antigen. Induced IL-10 production from T cells lends credence to the finding that, after exposure to intranasally applied OVA pulmonary, dendritic cells produced IL-10 transiently (25). On the other hand, this study has also demonstrated the involvement of ICOS itself in the development of IL-10-producing T cells. In future studies, it will be important to clarify whether a signal by means of ICOS is also required for the development of self-specific Tr cells with IL-10-producing ability.

Usually, IL-10 production from normal mouse T cells can be observed 48–72 h after antigenic stimulation, but CD4$^+$ T cells producing IL-10 in the DO × OVA^{high} mouse were detected immediately, and significant levels of IL-10 were observed only 24 h after stimulation (Fig. 7, which is published as supporting information on the PNAS web site). The characteristics of rapid IL-10 production and the dependency of suppressive ability on IL-10 are reminiscent of Tr 1 cells (39). However CD4$^+$ T cells in the DO × OVA^{high} mouse differed from conventional Tr1 cells in other cytokine production profiles. Tr1 cells produced high levels of IL-10, TGF-β1, and moderate amounts of IFN-γ, but little or no IL-2 or IL-4 (6). On the other hand, Tr cells in DO × OVA^{high} mice produce high levels of IL-4 in addition to IL-10 (Fig. 3*b*). It may be possible that Tr cells from DO × OVA^{high} mice consist of several types of cells, such as Tr cells producing IL-10 alone, IL-10 plus IL-4-producing T helper type 2 cells, and T cells producing IL-4 alone. Nonetheless, it is important that the regulatory activity of the Tr cells depends on IL-10 production. This point should be clarified by future experiments.

Sakaguchi *et al.* (2) reported that CD4$^+$CD25$^+$ T cells exhibit suppressive activity by cell-to-cell contact. These cells highly express Foxp3, and the introduction of the Foxp3 gene is able to convert naïve T cells toward Tr cells (13). Although the inhibitory activity of Tr cells in our double-Tg mice is mediated by IL-10 (Fig. 5), we found higher levels of Foxp3 mRNA expression in CD4$^+$ T cells of DO × OVA^{high} mice than those of DO mice (data not shown). Papiernik *et al.* (40) reported that CD4$^+$CD25$^+$ Tr cells secreted higher levels of IL-10 than did CD4$^-$CD25$^+$ T cells, and CD4$^+$CD25$^+$ Tr cells suppressed inflammatory bowel disease through an IL-10- and TGF-β-dependent mechanism (10, 41). From our findings and these previous reports, we are not able to rule out the possibility that at least a population of Tr cells in our system consist of so-called CD4$^+$CD25$^+$ Tr cells.

In conclusion, this study demonstrates that the development of self-specific IL-10-producing Tr cells depends on the amount of self-antigen expressed and the critical role of ICOS for the active function of these cells. The ICOS signaling pathway may be a novel target for the treatment of autoimmune disease, because ICOS costimulation seems to be specifically linked to IL-10 production of Tr cells.

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