factor-α (TNF-α) and interleukin (IL)-1β in several types of cells and NF-κB activates multiple genes, including IL-8.[7,8] Therefore, agents that suppress the expression of inflammatory mediators and the activation of NF-κB have the potential to treat endometriosis.

Sirtuin 1 (SIRT1), a mammalian homolog of yeast silent information-regulator 2 (Sir2), is an NAD^+-dependent class III histone deacetylase. Its initially investigated activity was as a mediator of increased lifespan that follows calorie restriction in yeast.[9] SIRT1 has been shown to be involved in various pathophysiological processes including inflammation, carcinogenesis and modulation of metabolism by deacetylating target proteins.[10–12] SIRT1 is known to exhibit suppressive effects on a variety of biological functions, especially inflammation, in various cell types. For example, activated SIRT1 inhibits TNF-α-induced inflammation in NIH/3T3 fibroblasts.[13] In turn, siRNA-mediated knockdown of SIRT1 increases the expression of inflammatory cytokines in 3T3-L1 adipocytes treated with TNF-α.[14] In liver-specific SIRT1 knockout mice, high-fat diets induce hepatic steatosis and hepatic inflammation.[15,16] Furthermore, SIRT1 has inhibitory effects on chronic inflammatory diseases such as chronic obstructive pulmonary disease and colitis.[17,18] SIRT1 is reported to suppress the production of pro-inflammatory cytokines by deacetylating the RelA/p65 subunit of NF-κB at lysine 310 and inhibiting NF-κB activity.[19]

Resveratrol, a phytoalexin polyphenol produced naturally by several plants that have been invaded by bacterial and fungal pathogens, is a potent activator of SIRT1.[20] Previous reports indicate that resveratrol exerts anti-inflammatory effects via blockade of NF-κB transcription activities.[13,21–24] Resveratrol decreases the expression of the NF-κB subunit RelA/p65 and attenuates translocation of p65 from the cytosol to the nucleus, thereby stabilizing inhibitory I-κB.

A previous study of the effects of resveratrol on experimental endometriosis have focused on changes in cell proliferation and invasion upon resveratrol exposure.[25] The authors demonstrated that endometriotic cell growth and infiltration were suppressed by treatment with resveratrol. However, there have been no prior reports describing either the expression of SIRT1 in endometriotic lesions or the involvement of SIRT1 in endometriotic cell functions. We previously reported the expression of SIRT1 in human ovarian granulosa cells and resveratrol enhancement of granulosa cell steroidogenesis.[26]

Viewing the relationship between resveratrol and SIRT1 in gynecological tissues, we hypothesized that resveratrol would have suppressive effects on endometriotic lesions through the activation of SIRT1-mediated anti-inflammatory pathways. In the present study, we investigated the expression of endogenous SIRT1 in endometriomas. We addressed TNF-α-induced IL-8 production in primary endometriotic stromal cells (ESC) cultured in the presence of an activator (resveratrol) or an inhibitor (sirtinol) of SIRT1.

Methods

Patients and samples

Endometriotic tissues were obtained from patients with ovarian endometriomas undergoing laparoscopy.[27] Diagnosis was confirmed by histopathological examination. Normal endometrial tissues were obtained from patients undergoing hysterectomy for benign gynecological conditions. All patients had regular menstrual cycles and none had received hormonal treatment for at least 6 months before surgery. The tissues were collected under sterile conditions and processed for primary cell culture. All experimental procedures were approved by the institutional review board at The University of Tokyo and signed informed consent for use of these tissues was obtained from each participant.

Isolation and culture of endometriotic stromal cells (ESC) and normal endometrial stromal cells (NES)

Isolation and culture of stromal cells from ovarian endometriotic tissues and normal endometrial tissues were processed as described previously.[27,28] Fresh endometriotic biopsy specimens and endometrium collected in sterile medium were rinsed to remove blood cells. The tissues were minced into small pieces and incubated in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Gibco, Grand Island, NY, USA) containing type I collagenase (0.25%; Sigma-Aldrich, St Louis, MO, USA) and deoxynuclease I (15 U/mL; TaKaRa, Tokyo, Japan) for 120 min at 37°C. The resultant dispersed cells were separated by filtration through nylon cell strainers (70 μm; BD, Franklin Lakes, NJ, USA). Stromal cells passing through the strainer and into the filtrate were collected for analysis.

Filtrates were collected, centrifuged and the stromal cells in the resulting pellets were resuspended in phenol-red free DMEM/F12 containing 5% fetal

bovine serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.25 mg/L amphotericin B. Stromal cells were plated into 12-well culture plates at a density of 2×10^5 cells/mL. The cells reached confluence in 2 or 3 days and were then used for experiments. Recombinant human TNF-α, resveratrol and sirtinol were purchased from Sigma-Aldrich. Resveratrol at 10, 20 or 40 μM, and sirtinol at 20 μM were added 1 h before TNF-α (5 ng/mL) stimulation.

RNA extraction and quantitative real-time polymerase chain reaction (qRT–PCR)

Total RNA of ESC and NES were extracted using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). Extracted RNA was reverse transcribed using an RT–PCR kit (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. To assess mRNA expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), IL-8 and SIRT1, qRT–PCR was performed using a Light Cycler 480 (Roche Diagnostics, Mannheim, Germany). Expression of IL-8 was normalized using GAPDH mRNA as an internal standard. Primer pairs for IL-8, SIRT1 and GAPDH are shown in Table 1. The PCR conditions for GAPDH, IL-8 and SIRT1 were as follows: for GAPDH, 35 cycles at 95°C for 10 s, 64°C for 10 s and 72°C for 18 s; for IL-8, 40 cycles at 95°C for 10 s, 66°C for 10 s and 72°C for 11 s; for SIRT1, 40 cycles at 95°C for 10 s, 66°C for 10 s and 72°C for 9 s. All PCR reactions were followed using melting curve analysis.

Measurement of IL-8 protein

The concentration of IL-8 in the conditioned media was measured using a standard IL-8 enzyme-linked immunoassay (ELISA) kit (Duoset; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

Cell proliferation assay

Stromal cells were plated into 96-well culture plates at a concentration of 1×10^5 cells/mL and incubated for 24 h. Cells were treated with resveratrol (0, 20 and 40 μM) for 1 h followed by the TNF-α (5 ng/mL) stimulation for 24 h. Cell proliferation was assessed by Cell Counting Kit-8 (CCK08) assay (DOJINDO, Osaka, Japan) according to the manufacturer's instruction; absorbance at 490 nm was measured using a microplate reader (BioTek, Seattle, WA, USA) and the mean ratio of the absorbance was calculated.

Immunohistochemistry

The endometriotic tissues and endometrial tissues used in this study were obtained from five discrete female patients. Immunohistochemistry was performed as described previously.[26] Paraffin sections (4 μm) were dewaxed in xylene and rehydrated through graded ethanol to water. Antigens were retrieved by boiling in 10 mmol citrate buffer (pH 6.0) for 30 min. The cooled sections were incubated in DAKO REAL Peroxidase-Blocking solution (DAKO, Carpentaria, CA, USA) for 10 min to quench endogenous peroxidase. To block non-specific binding, sections were incubated in DAKO Protein Blocking solution (DAKO) for 10 min at room temperature. Sections were then incubated with the rabbit polyclonal antibody to SIRT1 (sc-15404, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in DAKO REAL Antibody Diluent (DAKO) overnight at 4°C. Negative controls were incubated with pre-immune serum immunoglobulin G fractions. A ChemMate EnVision Detection system (DAKO) was used to visualize signals. The sections were developed with 3,3′-diaminobenzidine tetrahydrochloride substrate (DAKO), lightly counterstained with Mayer's hematoxylin (Wako Chemical, Osaka, Japan), dehydrated through ethanol and xylene and mounted.

Statistical analysis

Data are presented as means ± standard error of the mean. Statistical analyses were carried out using

Table 1 Primer sequences

Genes	Primers	Sequences (5′–3′)	Expected size (bp)
GAPDH	Sense	ACCACAGTCCATGCCATCAC	452
	Antisense	TCCACCACCCTGTTGCTGTA	
IL-8	Sense	ACTTCCAAGCTGGCCGTGGCTCTCTTGGCA	295
	Antisense	TGAATTCTCAGCCCTCTTCAAAAACTTCTC	
SIRT1	Sense	GAGATAACCTTCTGTTCGGTGATGAA	194
	Antisense	CGGCAATAAATCTTTAAGAATTGTTCG	

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; SIRT1, sirtuin 1.

Student's *t*-test or Dunnett's analysis using JMP software. A value of $P < 0.05$ was considered significant. Asterisks indicate those comparisons with statistical significance ($P < 0.05$).

Results

SIRT1 is expressed in the nuclei of human ESC and NES

There have been no reports on the expression of SIRT1 in endometriosis lesions or endometria, so here we characterized the expression of SIRT1 protein in the endometriosis lesions and normal endometria using immunohistochemistry and the expression of SIRT1 mRNA in cultured ESC and NES by RT–PCR. In both endometrial lesions and normal endometria, the nuclei of both epithelial cells and stromal cells were immunoreactive for SIRT1 (Fig. 1a–d). To evaluate the expression of SIRT1 mRNA in these cells, confluent ESC and NES were cultured in DMEM/F12 for 5 h. Total RNA was collected from these cells and SIRT1 mRNA was measured by RT–PCR. RT-PCR analysis demonstrated the expression of SIRT1 mRNA in ESC and NES in culture (Fig. 1e). There was no significant difference in the expression level of SIRT1 mRNA between ESC and NES. No signal was detected in negative controls (water alone).

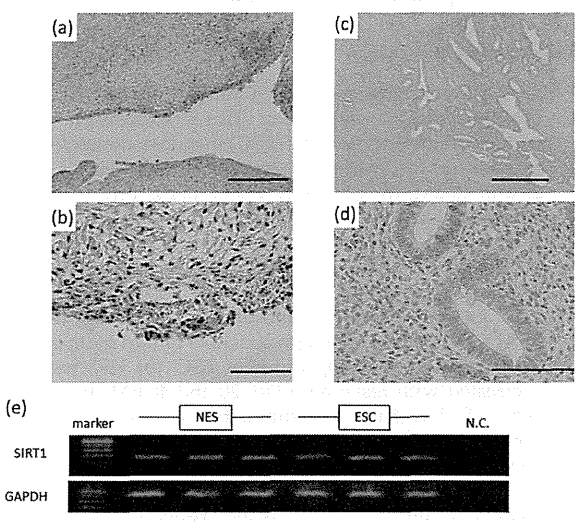

Figure 1 Expression of sirtuin 1 (SIRT1) in endometriosis and endometrium. (a–d) Representative data of immunohistochemical detection of SIRT1 in endometriotic lesions (a,b) and endometria (c,d) are shown. Bars indicate 200 μm in low-power fields (a,c), 50 μm in high-power fields (b,d). (e) Expression of SIRT1 mRNA in endometriotic stromal cells (ESC) and normal endometrial stromal cells (NES). SIRT1 mRNA was detected by reverse transcription polymerase chain reaction. Total RNA was extracted from cultured ESC and NES of three patients. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; N.C., negative control.

Resveratrol suppresses IL-8 expression in ESC

Peritoneal macrophages from patients with endometriosis secrete large amounts of TNF-α.[1,6] TNF-α activates a variety of classical and non-classical immune cells and induces inflammatory cytokine production. ESC exposed to TNF-α are also known to release IL-8.[7] Therefore, to address the involvement of SIRT1 in endometriosis, we evaluated IL-8 expression in ESC in the presence and absence of TNF-α. Because resveratrol is a well-known activator of SIRT1, we utilized resveratrol to better define SIRT1-related anti-inflammatory activities. First, we examined whether cell proliferation of ESC was altered upon exposure to resveratrol (20 and 40 μM) and/or TNF-α (5 ng/mL) (Fig. 2). ESC exposed to TNF-α for 24 h showed a tendency to proliferate. However, exposure to resveratrol alone did not alter cell proliferation of ESC (Fig. 2). Then, ESC were cultured in the presence or absence of 20 μM of resveratrol for 1 h followed by incubation

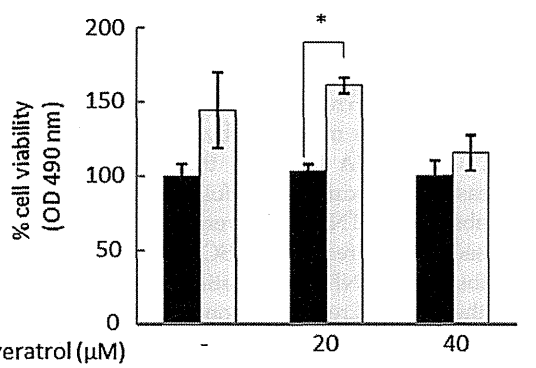

Figure 2 Effects of resveratrol and tumor necrosis factor (TNF)-α on the proliferation of endometriotic stromal cells (ESC). ESC were cultured in the presence or absence of resveratrol (20 and 40 μM) for 1 h followed by incubation with 5 ng/mL of TNF-α for 24 h. Cell proliferation of ESC was assessed by cell proliferation assay. Absorbance (490 nm) of the ESC with no treatment was compared with that of non-treated ESC. Data are representative of two independent experiments using different ESC. The data were analyzed using Student's *t*-test. ■, TNF-α (–); □, TNF-α (+).

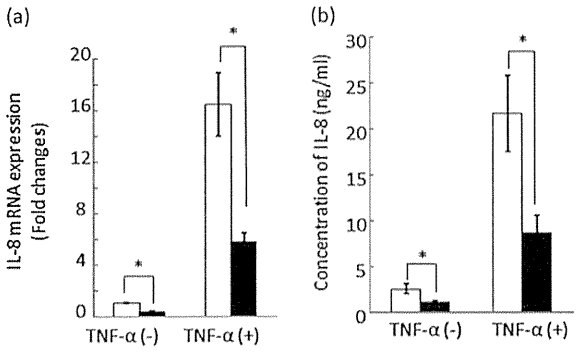

Figure 3 Resveratrol suppressed the expression of interleukin (IL)-8 in endometriotic stromal cells (ESC). (a) ESC were cultured in the presence or absence of 20 μM of resveratrol for 1 h followed by incubation with or without 5 ng/mL of tumor necrosis factor (TNF)-α for 4 h. Total RNA from ESC was reverse transcribed and IL-8 mRNA levels were measured by quantitative reverse transcription polymerase chain reaction. Expression levels of IL-8 were corrected for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are the mean ± standard error of the mean (SEM) of three independent experiments using different ESC. The data were analyzed using Student's *t*-test. (b) ESC were cultured with or without 20 μM of resveratrol for 1 h followed by incubation with or without 5 ng/mL of TNF-α for 24 h. Conditioned media were collected and concentrations of IL-8 were measured by enzyme-linked immunoassay. Data are the mean ± SEM of three independent experiments using different ESC. The data were analyzed using Student's *t*-test. □, resveratrol (–); ■, resveratrol (+).

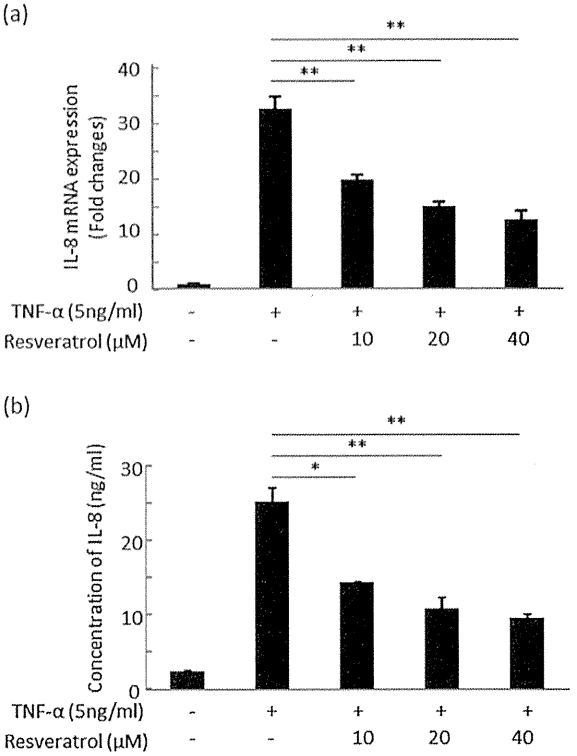

Figure 4 Resveratrol suppressed tumor necrosis factor (TNF)-α-induced interleukin (IL)-8 expression in endometriotic stromal cells (ESC) in a dose-dependent manner. (a) ESC were pretreated with resveratrol (10, 20 and 40 μM) for 1 h before TNF-α (5 ng/mL) stimulation for 4 h. Total RNA from ESC was reverse transcribed and IL-8 mRNA levels were measured by quantitative reverse transcription polymerase chain reaction. Expression levels of IL-8 were corrected for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are the representative of two independent experiments using different ESC. (b) ESC were pretreated with resveratrol (10, 20 and 40 μM) for 1 h before TNF-α (5 ng/mL) stimulation for 24 h. Conditioned media were collected and concentrations of IL-8 were measured by enzyme-linked immunoassay. Data are representative of two independent experiments using ESC derived from distinct patients. The data were analyzed by Dunnett's analysis using JMP software. $^{*}P < 0.05$, $^{**}P < 0.01$.

with or without 5 ng/mL of TNF-α for 4 h. IL-8 mRNA levels were then measured by qRT–PCR (Fig. 3a). In ESC, IL-8 mRNA levels in the presence of TNF-α increased approximately 20-fold higher than those in the absence of TNF-α. Resveratrol significantly suppressed IL-8 expression in ESC in the presence and in the absence of TNF-α stimulation (Fig. 3a). To confirm that the effects of resveratrol on ESC IL-8 mRNA levels translated into similar effects on protein production, IL-8 levels in ESC conditioned media were measured by ELISA (Fig. 3b). ESC were pretreated with 20 μM of resveratrol for 1 h and cultured in the presence or absence of 5 ng/mL of TNF-α for 24 h. IL-8 secretion was significantly suppressed by resveratrol in the presence or absence of TNF-α, recapitulating our mRNA expression patterns (Fig. 3b). Both results indicate that resveratrol suppresses IL-8 expression in ESC.

Furthermore, when ESC were treated with resveratrol (10, 20 and 40 μM) for 1 h before TNF-α (5 ng/mL) stimulation, resveratrol significantly decreased the expression of IL-8 in a dose-dependent manner (Fig. 4).

Sirtinol promotes IL-8 expression in ESC

Sirtinol is an inhibitor of sirtuin enzymatic activities, which includes SIRT1 activity, and this effect is

independent of class I or class II histone deacetylases (HDAC).[29,30] We therefore hypothesized that, in contrast to SIRT1, sirtinol may provoke IL-8 release from ESC. To test this hypothesis, ESC were cultured in the presence or absence of 20 μM of sirtinol for 1 h followed by incubation with or without 5 ng/mL of TNF-α for 4 h (Fig. 5a). IL-8 mRNA levels were measured as a surrogate for inflammatory cytokine release. Upon TNF-α stimulation, ESC IL-8 mRNA levels increased but sirtinol had no effect on IL-8 expression. In contrast, IL-8 expression in ESC was dramatically increased upon sirtinol exposure in the absence of TNF-α stimulation. To confirm these effects at the protein level, secretion of IL-8 from ESC into the conditioned media were measured by specific ELISA. ESC were pretreated with 20 μM of sirtinol for 1 h and cultured in the presence or absence of 5 ng/mL of TNF-α for 24 h. Like its effects on IL-8 mRNA, sirtinol enhanced IL-8 secretion from ESC only in the absence of TNF-α (Fig. 5b).

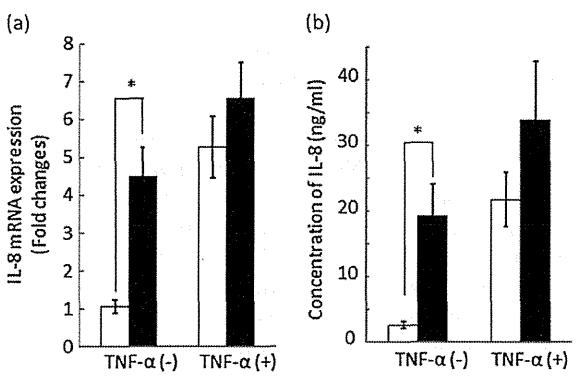

Figure 5 Sirtinol enhanced the expression of interleukin (IL)-8 in endometriotic stromal cells (ESC) in the absence of tumor necrosis factor (TNF)-α. (a) ESC were cultured in the presence or absence of 20 μM of sirtinol for 1 h followed by incubation with or without 5 ng/mL of TNF-α for 4 h. IL-8 mRNA levels were assessed using quantitative reverse transcription polymerase chain reaction. Data are the mean ± standard error of the mean (SEM) of three independent experiments using different ESC. The data were analyzed using Student's *t*-test. (b) ESC were cultured with or without 20 μM of sirtinol for 1 h followed by incubation with or without 5 ng/mL of TNF-α for 24 h. Conditioned media were collected and concentrations of IL-8 were measured by ELISA. Data are the mean ± SEM of three independent experiments using different ESC. The data were analyzed using Student's *t*-test. □, sirtinol (–); ■, sirtinol (+).

Effects of resveratrol and sirtinol on NES

In order to investigate whether resveratrol and sirtinol had different effects on NES when compared to those seen using ESC, NES were cultured in the presence or absence of 20 μM of resveratrol or sirtinol for 1 h followed by incubation with or without 5 ng/mL of TNF-α for 4 h (Fig. 6). IL-8 mRNA levels were measures as a surrogate for inflammatory cytokine release. Although resveratrol also tended to suppress the expression of IL-8 in NES, it did not do so to the same extent as that seen using ESC. In contrast, like ESC, IL-8 expression in NES was dramatically increased upon sirtinol exposure in the absence of TNF-α stimulation.

Discussion

Here we have described the expression of SIRT1 in endometriomas using immunohistochemical analysis. SIRT1 is involved in a number of cellular processes including gene silencing at telomere and mating loci, DNA repair and recombination, and aging.[10,31,32] Recent studies have established that SIRT1 plays an important role in the regulation of cell fate and stress responses in mammalian cells; it promotes cell survival by inhibiting apoptosis or cellular senescence induced by stressors such as DNA damage.[32] Both overexpression of SIRT1 and increased activity of SIRT1 significantly

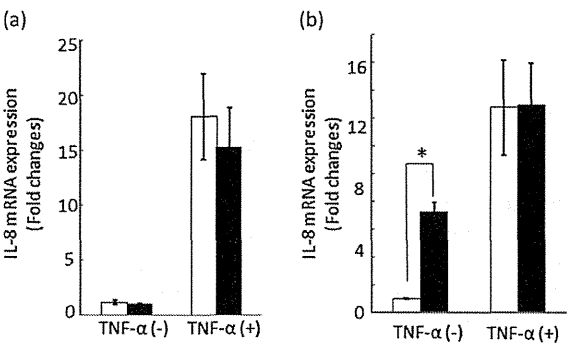

Figure 6 Effects of resveratrol and sirtinol on normal endometrial stromal cells (NES). NES were cultured in the presence or absence of 20 μM of resveratrol (a) or sirtinol (b) for 1 h followed by incubation with or without 5 ng/mL of tumor necrosis factor (TNF)-α for 4 h. IL-8 mRNA levels were assessed using quantitative reverse transcription polymerase chain reaction. Data are the mean ± standard error of the mean of three independent experiments using different NES. The data were analyzed by Student's *t*-test. □, resveratrol (–); ■, resveratrol (+); □, sirtinol (–); ■, sirtinol (–).

suppress the production of inflammatory cytokines in animal models,[18,26] whereas depletion of SIRT1 stimulates inflammatory responses.[33] The acetylation of lysine residues in NF-κB is essential to its normal activity.[34] SIRT1 suppresses NF-κB-related pro-inflammatory cytokine production through deacetylation of the RelA/p65 subunit of NF-κB at lysine 310.[19] Resveratrol has also been shown to possess anti-oxidant and anti-inflammatory activities[21,35] and is implicated in the activation of SIRT1.[10]

Endometriosis is an estrogen-dependent disease that significantly worsens the lives of reproductive aged women.[36] To date, medical therapies for endometriosis are mainly based on hormone-suppressing drugs, however, these therapies are accompanied by severe side-effects. Recently, many investigators have demonstrated that pro-inflammatory mediators are also involved in the progression of endometriosis[2-4] and that inhibiting inflammation is important in the control of the disease. In this study, activation of SIRT1 by resveratrol significantly suppressed IL-8 release from ESC even under TNF-α stimulation. In turn, suppression of SIRT1 by sirtinol promoted IL-8 secretion from ESC. These data suggest a central role for SIRT1 in regulating inflammation in endometriosis.

We have provided insight into the possibility that the SIRT1 activator, resveratrol, may represent a novel treatment modality for endometriosis that would both suppress the invasion of stromal cells[25] and promote the development of a local anti-inflammatory environment. This approach is made even more attractive by the relative paucity of side-effects reported with resveratrol administration.[37,38]

Peritoneal immune cells, particularly macrophages, also play a crucial role in the pathogenesis of endometriosis.[3,39] Several studies have reported that NF-κB is activated in macrophages from endometriosis patients and the genes upregulated in association with this activation[39] include those involved in cell proliferation, cell adhesion and neovascularization. A number of studies have reported that resveratrol inhibits inflammatory responses in macrophages.[40-42] Resveratrol appears to inhibit inflammatory responses not only of ESC but also of peritoneal immune cells, suggesting it may represent a promising and novel therapeutic anti-inflammatory agent for endometriosis.

It is intriguing that the anti-inflammatory effects of resveratrol are more prominent in ESC than in NES in our studies. Human endometrial stromal cells express NF-κB,[8,43] and NF-κB is activated by the elevated expression of TNF-α and IL-1β in cultured ESC.[7,44,45] A constitutive activation of NF-κB has been demonstrated in endometriotic lesions from affected women[44] as well as in endometriotic lesions induced in nude mice.[46] Therefore, we hypothesize that the exaggerated response to resveratrol in ESC might be attributed to the extent of activation of the NF-κB pathways. In order to investigate the inhibitory effects of resveratrol on NF-κB pathways, we have also performed NF-κB luciferase assays using ESC and 293T cells (data not shown). Although NF-κB transcriptional activities were markedly suppressed by resveratrol in 293T cells, the variability in NF-κB transcriptional activities in ESC was too great to allow detection of significant differences. However, a number of reports have previously shown that resveratrol exerts its anti-inflammatory effects by inhibiting NF-κB activities,[13,22,23] strongly suggesting that the anti-inflammatory effects of resveratrol are due to inhibition of NF-κB activation in ESC. Further studies are required to fully detail the mechanisms that underlie the inhibition of inflammation by resveratrol.

Sirtuin 1 is also a HDAC. In contrast to the sirtuins, other HDAC, such as HDAC1 and HDAC2, are reported to promote cancer progression[47,48] and to exacerbate inflammatory diseases like arthritis.[49] Therefore, HDAC inhibitors have emerged as a new family of anticancer and anti-inflammatory therapeutics.[49-52] In endometriosis, Kawano *et al.* reported that acetylated histones were more significantly decreased in ESC when compared to NES, and that HDAC inhibitors repressed the proliferation of ESC.[53] In our results, although there was no significant difference between ESC and NES in the levels of SIRT1 mRNA, the effects of resveratrol were more potent in ESC. The differences in resveratrol sensitivity between ESC and NES may reflect differences in the level of basal acetylation of NF-κB in the two cell types.

In conclusion, our data indicate that, in endometriosis, activation of SIRT1 plays an important role in regulating the expression of inflammatory cytokines and that resveratrol has the potential to be a novel therapy for endometriosis by suppressing inflammatory reactions mediated through endogenous SIRT1 activation.

Acknowledgments

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High-risk human papillomavirus correlates with recurrence after laser ablation for treatment of patients with cervical intraepithelial neoplasia 3: A long-term follow-up retrospective study

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Abstract

Aim: The purpose of our study was to evaluate the efficacy of laser ablation as a conservative treatment for cervical intraepithelial neoplasia 3 (CIN3) and assess whether the human papillomavirus (HPV) test is useful to predict recurrence after treatment.

Materials and Methods: A total of 134 patients who received laser ablation for treatment of CIN3 were enrolled in this study. During the follow-up period, patients were followed with cytological and colposcopic evaluations. Recurrence of CIN3 was regarded as the primary end-point. HPV genotype was tested before and after treatment. Post-treatment cumulative recurrence rates were estimated and comparisons by both patient age and HPV genotype were performed.

Results: Overall cumulative recurrence rate of CIN3 in the first year after treatment was 22.6% for all patients. No significant correlation was shown between patient age and recurrence. Patients infected by specific genotypes (16, 18, 31, 33, 52, and 58) frequently failed to clear the infection after treatment. The 1-year recurrence-free survival in those positive after treatment for eight high-risk genotypes (16, 18, 31, 33, 35, 45, 52, and 58) was significantly lower (66.7%), compared to that in those positive for other high-risk types (78.6%). The recurrence-free survival of those who remained HPV-positive after treatment was significantly lower than those who turned negative.

Conclusion: Laser ablation should be performed prudently with appropriate patient counseling about recurrence rate. Considering its minimal invasiveness, laser ablation is effective, especially for young patients who are negative for eight high-risk genotypes. With regard to HPV testing, although genotyping has significant value for predicting recurrence, screening for all genotypes warrants further evaluation.

Key words: cervical intraepithelial neoplasia 3, human papillomavirus testing, laser ablation, recurrence, treatment efficacy.

Introduction

The spread of systematic screening programs has detected more cervical intraepithelial neoplasia (CIN) and has succeeded in producing marked declines in cervical carcinoma incidence and mortality in the developed countries where screening programs and treatment for pre-invasive lesions are

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widespread. Many women who receive treatment for high-grade CIN are of reproductive age with a mean age of approximately 30 years old.[1] Therefore, the treatment should not only be effective but also have minimum adverse effects on future fertility and obstetrical outcomes. Cold-knife conization, laser conization, loop electrosurgical excisional procedure (LEEP), cryotherapy, and laser ablation are all conservative methods used to treat high-grade CIN by removing or destroying the transformation zone containing abnormal epithelial cells and thereby preserving cervical function. According to data from the Japan Society of Obstetrics and Gynecology (JSOG), conservative conization methods were chosen for only 33% of women with carcinoma *in situ* (CIS) in 1990, for which hysterectomy had been the treatment standard, rising as high as 79.3% in 2009. These data robustly represent the increasing demand for conservative CIN treatments at the present time.

Many studies have demonstrated that these methods show similarly low morbidity and are equally successful at preventing invasive cervical cancer.[2–5] Of these conservative methods, characteristics of laser ablation have been well reported due to its fertility-sparing advantage. Laser ablation is usually performed under local anesthesia as an outpatient procedure whereas conization procedures need general anesthesia and inpatient care. Regarding pregnancy outcomes, excisional treatment procedures, including cold-knife conization, laser conization, and LEEP, are associated with increased risk of adverse obstetric morbidity. In contrast, ablative procedures, including cryotherapy and laser ablation, are free of any of these untoward outcomes.[5,6] However, resected specimens from excisional procedures allow for precise histological diagnosis, including presence of unexpected microinvasive diseases, while ablative methods, by destroying cervical tissue, preclude this investigation and require additional pre-treatment biopsy. This ability to combine diagnosis with treatment in a single procedure remains an advantage of excisional treatments.

Women with high-grade CIN frequently undergo excisional treatments because, while more invasive, they are more definitive than ablative therapies. As such, there are few reports available regarding the efficacy of ablative treatments, such as laser ablation for high-grade CIN. Moreover, most studies comparing efficacy between treatments show both the rate of recurrence and residual disease, which is the failure rate; since these study populations often include a variety of CIN1 to CIN3 patients, and definitions for recurrence/residual disease depend on treatments, failure rates also vary markedly between studies. Both randomized and non-randomized studies demonstrate a failure rate of 5–30% for laser ablation and 5–16% for LEEP in a 6-month follow-up period;[7,8] however, in 2002, Dey *et al.* demonstrated that the cumulative risk of cytological abnormality reported as moderate dysplasia or worse is higher after laser ablation than LEEP.[9] A recent long-term follow-up study found cryotherapy was associated with the highest rate of recurrence compared with conization, LEEP, and laser ablation.[10] In total, the treatment efficacy against CIN has been still inconsistent comparing laser ablation against other excisional methods, such as conization and LEEP, and there is little information for the safety and efficacy of laser ablation for high-grade lesion.

In this study, we propose that laser ablation is a useful modality for the treatment of CIN in terms of obstetrics outcomes, even for high-grade lesions, if satisfactory colposcopy and consecutive cytology after treatment are available. In addition, we aimed to perform a descriptive investigation of the recurrence of high-grade CIN after laser ablation. Furthermore, the International Agency for Research on Cancer (IARC) announced in 2003 that among the over 100 HPV genotypes, 13 types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82) should be considered carcinogenic, thus defined as 'high-risk types'.[11] Since then, HPV testing has been proposed as part of the follow-up of patients treated for high-grade CIN due to its very high sensitivity and negative predictive value for detecting residual/recurrent disease,[12] suggesting that it may be a good indicator of disease clearance. Indeed, a growing body of evidence has demonstrated that HPV testing together with cytology is useful in monitoring women treated for high-grade CIN.[12,13] To further clarify this in the setting of ablative therapy, we focused on the correlation between high-grade CIN recurrence rates and HPV genotype before and after laser ablation.

Methods

Patients

Following Japanese standard treatment protocols, in our study, those patients whose cervical biopsy demonstrated CIN3 (excluding CIS) and whose histology, cytology, and colposcopy were in concordance, were

treated with laser ablation between 2004 and 2010 at the Tokyo University Hospital. Patients were followed up with cytology and colposcopy 3 months after laser ablation, and those who showed residual disease were excluded from this study. Patients with negative cytology and normal colposcopic findings 3 months after treatment were included in this retrospective study. A total of 144 patients (mean age, 36.9 years; range, 25–71 years) met these criteria and were included in this retrospective study. Patients were followed up for at least 5 years with cytological and colposcopic evaluations conducted at intervals of 3–4 months. No residual lesion was confirmed by satisfactory colposcopic findings with negative cytology on the ecto-endocervix together. The recurrence of CIN3 was regarded as the primary end-point. Referring to the previous publications,[9,10] date of recurrence was defined as the mid-point between the date of the examination when abnormal cytology or histology (such as moderate-severe dysplasia, atypical squamous cells that cannot exclude HSIL [ASC-H], or high-grade squamous intraepithelial lesion [HSIL]) was first detected with satisfactory colposcopy, and the most recent preceding examination in which the colposcopic evaluations and smear (ecto-endocervix) were normal.

In total, 83 patients (median age, 36 years) were further examined for the efficacy of laser ablation by HPV genotypes, identified by polymerase chain reaction (PCR)-based HPV DNA testing before and after ablation, comparing the post-treatment persistent infection and recurrence-free survival (RFS) rates. HPV genotyping was performed in each patient. Regarding the natural history of CIN in Japan, a recent prospective cohort study by Matsumoto *et al.*[14] showed that the cumulative progression rate for CIN3 within 5 years was 20.5% for eight types of high-risk HPV (16, 18, 31, 33, 35, 45, 52, and 58), which was significantly higher than the 6.0% observed for five other high-risk types (39, 51, 56, 59, and 68), demonstrating that differences in progression exists even in the 13 HPV types defined by IARC as high-risk. In our study, therefore, we classified the study population according to this report and focused on the eight 'higher-risk' types. Informed consent was obtained in all cases. The median follow-up period was 17 months, with a minimum of at least 6 months. Recurrence was defined as emergence of CIN3 in complete responders.

PCR-based HPV DNA testing

DNA was extracted from cervical smear samples by using the QIAGEN DNeasy Blood & Tissue Kits. PCR-based HPV DNA testing was performed using the PGMY-CHUV assay. Briefly, standard PCR was conducted using the PGMY09/11 L1 consensus primer sets and HLA-dQ primer sets. Reverse blotting hybridization was subsequently performed as described previously.[15]

Laser ablation

Outpatient carbon dioxide laser procedures were carried out under colposcopic guidance, taking about 10 min, without anesthesia or premedication. Water in the tissue absorbs the laser energy, which destroys tissue by vaporization. To be effective, the lesion is typically ablated to a depth of 5 mm on the ectocervix and 8–9 mm around the endocervix. After ablation, the epithelium regenerates in 2–3 weeks. All cases were performed by gynecologic oncologists using CO2 laser, MEDILASER-30S (Model mel-30S, Mochida) with a power density of 8–12W in continuous mode.

Statistics

Date of recurrence was defined as the mid-point between the date of the examination when abnormal cytology was first detected and the most recent preceding examination in which the smear was normal. The log–rank test was used to assess differences in cumulative risk between study groups; tests of significance were carried out at the 5% two-sided level.

Results

We initially identified 144 patients with CIN3 who received laser ablation at Tokyo University Hospital between 2004 and 2010, and showed both negative cytology and normal colposcopic findings 3 months after treatment. Ten patients were excluded because of incomplete data. A total of 134 cases of CIN3 (median age, 37 years; range, 27–71 years, excluding CIS) were monitored every 3–4 months during the follow-up period (6–95 months; median, 38 months). Seven (5.2%) were censored at 1 year, 19 (14.2%) at 2 years, and 105 (78.4%) at 5 years after treatment. The recurrence of CIN3 was regarded as the primary end-point. All the patients were evaluated with satisfactory colposcopy and histological examination of transitional zone.

First, we investigated the efficacy of laser ablation against all CIN cases. During the follow-up period, recurrence was identified in 57 (42.5%) of the 134 patients, and the overall cumulative CIN3 recurrence rate in the first 12 months after treatment was 22.6%

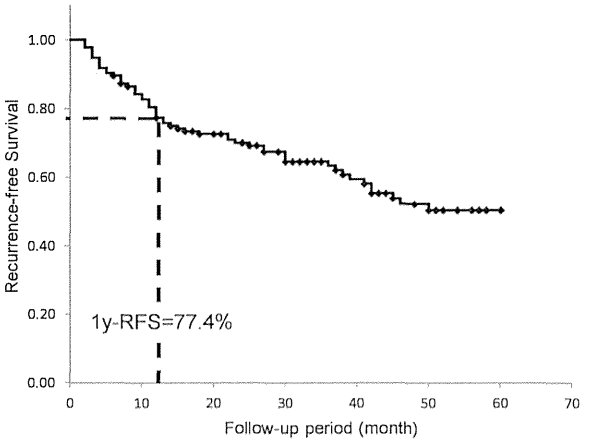

Figure 1 Overall cumulative recurrence rate after treatment. The overall cumulative recurrence rate of cervical intraepithelial neoplasia 3 in the first 12 months after treatment was 22.6%. RFS, recurrence-free survival.

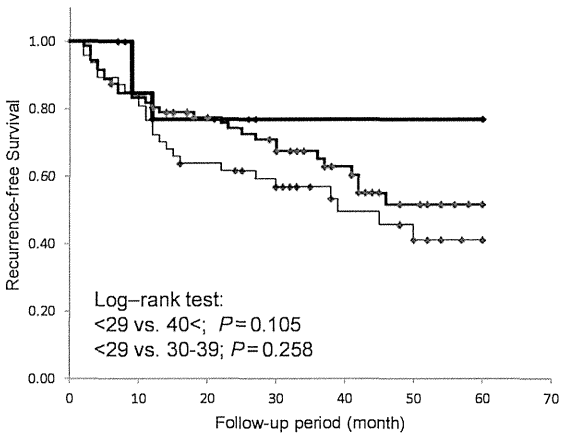

Figure 2 Recurrence rates compared by age. No significant elevated risk of recurrence was seen between younger age (<29) and older age (<29 vs >40; $P = 0.105$, <29 vs 30–39; $P = 0.258$, by log–rank test). ——, <29; ——, 30–39; ——, 40<.

(Fig. 1). Figure 2 shows recurrence rates compared by age. It is worth noting that no significant elevated risk of recurrence was seen between younger age (<29) and older age (<29 vs >40, $P = 0.105$; <29 vs 30–39, $P = 0.258$, by log–rank test). Among recurrence cases, 27 patients underwent second laser ablation, nine patients underwent cervical conization, and one patient underwent total hysterectomy. Other patients were followed up with intensive cytological and colposcopic evaluations

Table 1 Distribution of HPV type before treatment

HPV type before treatment	Rate (%)
16	34
18	2
31	2
33	5
52	20
58	13
Others	24

The HPV genotypes of 83 patients were identified by polymerase chain reaction-based HPV DNA testing both before and after ablation. Seventy-six women were positive on HPV-DNA testing (91.6%). The distribution of HPV genotypes was classified according to the Matsumoto criteria of 8 'higher-risk' types. Two of the higher-risk types (35, 45) were not detected in this study. All cervical intraepithelial neoplasia 3 cases in this study had monoinfection. HPV, human papillomavirus.

with informed consent. The re-recurrence rate of CIN3 after second ablation was 11.1%.

Next, we evaluated the association between HPV genotype and efficacy of laser ablation, assessing whether HPV genotyping might predict failure after laser ablation. Of the 134 patients, we examined the HPV genotype of 83 patients, identified by PCR-based HPV DNA testing both before and after ablation. Single infection was observed in all CIN3 cases in this study, although there is a possibility that some patients might have multiple infections, especially in the CIN1-2 population. The median follow-up period was 17 months, with a minimum follow-up of 6 months. The median age of these 83 patients was 36 years; 76 women were positive on HPV-DNA testing (91.6%). The distribution of HPV genotypes, classified according to the Matsumoto criteria of eight 'higher-risk' types, before laser ablation is shown in Table 1.

Table 2 shows detection rates – that is, whether the same HPV type was detected before and after laser ablation, which can be interpreted as a good indicator of disease clearance. HPV was persistently detected after treatment more often in the higher-risk types, especially in type 16 and 18, than in other HPV types (16/18 vs 31/33/52/58 vs others; 66.6% vs 52.4% vs 25.0%; $P < 0.0001$ by Cochran–Armitage test).

In addition, we compared post-treatment RFS by HPV genotypes. As shown in Figure 3, we first compared RFS by pre-treatment HPV genotypes, that is, between those who were positive for the 8 higher-risk types and those positive for other HPV types before ablation; no statistically significant difference was seen between the two ($P = 0.77$ by log–rank test). We then

Table 2 Detection rate of HPV after treatment for each HPV type before treatment

HPV type before treatment	Detection of HPV after treatment†	
	Positive (%)	Negative (%)
16/18	66.6	33.3
31/33/52/58	52.4	47.6
Others	25.0	75.0

†Cochran–Armitage test $P < 0.0001$. Detection rates – i.e. whether the same HPV type was detected before and after laser ablation, which can be interpreted as a good indicator of disease clearance. HPV was persistently detected after treatment more often in the higher-risk types, especially in types 16 and 18, than in other HPV types (16/18 vs 31/33/52/58 vs others; 66.6% vs 52.4% vs 25.0%; $P < 0.0001$ by Cochran–Armitage test). HPV, human papillomavirus.

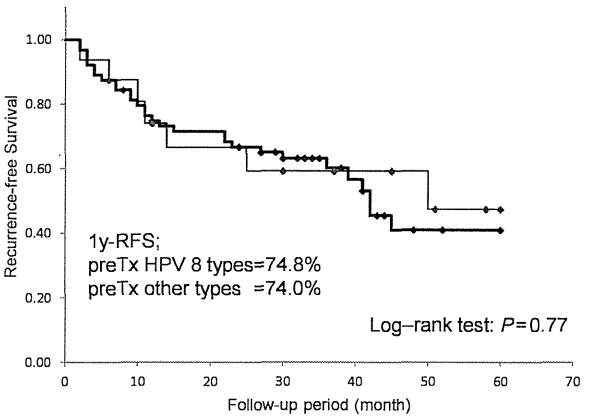

Figure 3 Recurrence-free survival (RFS) by pre-treatment human papillomavirus (HPV) genotype. We compared RFS between those who were positive for the 8 higher-risk types versus those positive for other HPV types prior to ablation; no significant difference between the two groups was identified. ——, 8 types; ——, Others.

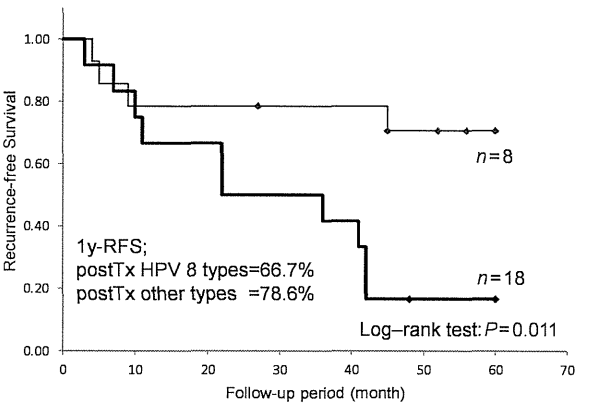

Figure 4 Recurrence-free survival (RFS) by post-treatment human papillomavirus (HPV) genotype. After ablation, 1-year RFS was 66.7% for those who were positive for the 8 higher-risk HPV types, which was significantly lower than the 78.6% observed in those positive for other HPV types. ——, 8 types; ——, Others.

compared RFS by post-treatment HPV genotypes. As shown in Figure 4, 1-year RFS was 66.7% for those who were positive for the 8 higher-risk types after ablation, which was significantly lower than the 78.6% observed in those positive for other HPV types ($P = 0.011$ by log–rank test). Furthermore, Figure 5 shows that the 1-year RFS for those positive for all high-risk 13 types of HPV after treatment was 61.1%, versus 100% in HPV-negative subjects ($P = 0.0013$ by log–rank test).

All these data suggest that the persistence of high-risk HPV genotypes, especially of the eight types, might play a key role in the development of recurrence.

Discussion

In 2011, a large Dutch multi-cohort study, one of the representative prospective studies on long-term efficacy of CIN treatment, reported on recurrence risk after treatment for CIN2/3.[16] According to that study, the recurrence risk of CIN was quite low after highly successful treatment followed by normal cytology or negative HPV testing. However, all participants were treated by excisional methods, such as cold-knife conization or LEEP, so the data did not present any information on the prognostic outcomes of conservative treatments, including laser ablation.

As mentioned, many studies have demonstrated that conservative methods, including cold-knife conization, LEEP, cryotherapy, and laser ablation, show similarly low morbidity and are equally successful at preventing invasive cervical cancer.[4,17,18] However, in 2009, Melnikow *et al.*[10] compared the long-term follow-up of 37 142 patients with CIN by treatment modality (cold-knife conization, LEEP, laser [conization and ablation

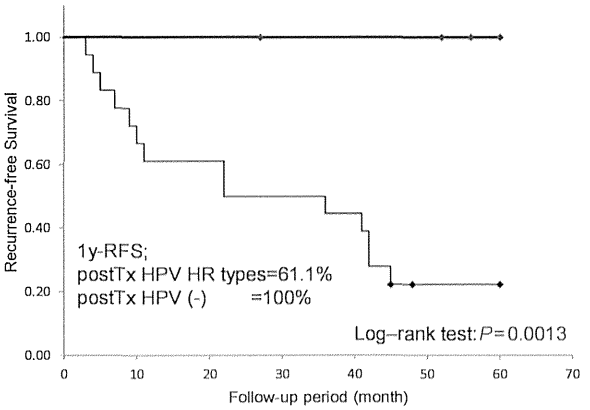

Figure 5 Recurrence-free survival (RFS) between post-treatment human papillomavirus (HPV) genotypes. Post-treatment 1-year RFS for those positive for 13 high-risk HPV was 61.1%, versus 100% for those who were HPV-negative. It is unsurprising that no recurrence was seen in any HPV-negative cases after laser ablation. —, negative; —, high-risk types.

combined], and cryotherapy), reporting that overall cumulative rate of CIN2/3 recurrence in the first 6 years after treatment of 14.0% for women originally treated for CIN3. Estimated rates of CIN2/3 after treatment were lowest for cold-knife conization, followed by LEEP and laser, and highest for cryotherapy. This result corroborates the invasiveness of the respective procedure.

Further evidences are warranted for the treatments of CIN3 though; considering many women with high-grade CIN are of reproductive age, treatment should not only be effective but also have minimum adverse effects on future fertility and obstetric outcomes. Several previous studies have clearly demonstrated that excisional treatments result in long-term adverse obstetric outcomes, including preterm delivery, low birthweight, or premature rupture of membranes; thus, an increasing number of patients are likely to choose less invasive treatment like laser ablation.[5]

The data in our study suggest that laser ablation is an optional conservative treatment for CIN3 with minimal invasiveness. Regarding obstetric outcomes, it is difficult to follow patients after CIN treatment in terms of pregnancy because of the premalignant nature of the lesion, so no data were available in this study. Nevertheless, as laser ablation has thus far been shown to be free of any adverse effect on obstetric outcomes, it should be taken into consideration especially for young patients of child-bearing age. Even for lesions, provisional use of laser ablation may provide a 'grace period' for young women with desire to bear children before more invasive interventions are performed. Therefore, many women can postpone, or even entirely avoid, the unnecessary adverse outcomes associated with invasive intervention.

With regard to follow-up surveillance after treatment of high-grade CIN, the role of HPV-DNA testing has been an area of interest due to its high sensitivity and negative predictive value (NPV) for detecting recurrent disease. In a meta-analysis of 11 studies by Zielinski *et al.*,[12] sensitivity, NPV, and specificity of high-risk HPV testing for recurrence were 91% (95% CI = 86–95), 98% (97–99), and 79% (76–82), respectively. That study demonstrated that combining cytology with high-risk HPV testing increases rates even further: sensitivity was 96% (89–99), NPV was 99% (98–100), and specificity was 81% (77–84). Specificity is indeed relatively low, but considering that this is not a screening test but rather a follow-up for a potentially lethal disease, NPV and sensitivity should be valued higher than specificity.

We demonstrated in this study that HPV infection is not likely to disappear, but persists after laser ablation in women positive for eight 'higher-risk' HPV. Considering that cervical cancer is caused by persistent infection with a subset of carcinogenic HPV, this failure of viral clearance should lead to the increase in cervical cancer in future. Indeed, we confirmed the significant difference in RFS between those who were positive for the eight higher-risk types of post-treatment versus those who were positive for other types or altogether negative. This result supports the efficacy of HPV testing and is consistent with several previous studies.

However, several critical problems with HPV-PCR testing remain. First, while PCR testing can distinguish individual genotype, detection sensitivities differ greatly among HPV genotypes. Furthermore, routine PCR-based HPV DNA testing is not suitable for daily clinical use due to its high cost and complex technique using specific primers. For the purposes of this study context, therefore, we recommend commercially available HPV testing using the hybrid capture method, Hybrid Capture II (HC2), which targets a group of 13 high-risk HPV genotypes but does not distinguish which genotypes are present. This intensive test also represents well the eight higher-risk genotypes; identification of the existence of high-risk HPV by HC2 may sufficiently inform the likelihood of recurrence. In Japan, HC2 has been covered by insurance since 2010, but the target of this test is only limited for patients

with atypical squamous cells of undetermined significance (ASC-US) detected by screening cytology test; we recommend that HC2 should also be used widely for the surveillance of patients after treatment for CIN.

In summary, laser ablation is a useful conservative treatment for CIN3, especially for young women of reproductive age. It will continue to be necessary for gynecologists to tailor treatment strategies for young patients according to their health needs, balancing minimal adverse obstetric outcomes with minimal attenuation of efficacy. In addition, it is well established that the rate of developing high-grade disease, including invasive cervical cancer, remains elevated in these post-treatment patients, even after a very long time subsequent to treatment. Indeed, a study in 2006 warns that the rate of invasive disease remained at 56 per 100 000 woman-years, 2.8 times greater than expected, for at least 20 years after treatment.[19] Because of this substantial long-term risk, close monitoring remains critical, and combined cytological evaluation together with HPV testing should increase the safety of the surveillance. For more rigorous and detailed follow-up algorithms, including surveillance intervals, obstetric outcomes, and cost-effectiveness, larger and longer-term studies are warranted in the future.

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Disclosure

The authors have no conflicts of interest related to this article.

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MAJOR ARTICLE

Epigenetic Repression of Interleukin 2 Expression in Senescent CD4$^+$ T Cells During Chronic HIV Type 1 Infection

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The molecular mechanisms for *IL2* gene–specific dysregulation during chronic human immunodeficiency virus type 1 (HIV-1) infection are unknown. Here, we investigated the role of DNA methylation in suppressing interleukin 2 (IL-2) expression in memory CD4$^+$ T cells during chronic HIV-1 infection. We observed that CpG sites in the *IL2* promoter of CD4$^+$ T cells were fully methylated in naive CD4$^+$ T cells and significantly demethylated in the memory populations. Interestingly, we found that the memory cells that had a terminally differentiated phenotype and expressed CD57 had increased *IL2* promoter methylation relative to less differentiated memory cells in healthy individuals. Importantly, early effector memory subsets from HIV-1–infected subjects expressed high levels of CD57 and were highly methylated at the *IL2* locus. Furthermore, the increased CD57 expression on memory CD4$^+$ T cells was inversely correlated with IL-2 production. These data suggest that DNA methylation at the *IL2* locus in CD4$^+$ T cells is coupled to immunosenescence and plays a critical role in the broad dysfunction that occurs in polyclonal T cells during HIV-1 infection.

Keywords. HIV-1; CD4$^+$ T-cell dysfunction; repression of IL-2 expression; DNA methylation; T-cell differentiation; immunosenescence.

During chronic human immunodeficiency virus type 1 (HIV-1) infection, CD4$^+$ T cells undergo cell-intrinsic phenotypic and functional impairments that are coupled to increased pathogenesis. These phenotypic changes include elevated levels of expression of activation, exhaustion, and senescent markers [1–5]. We have previously reported reduction in the expression of specific cytokines by T cells in HIV-1 noncontrollers, which is associated with activation/exhaustion status in both CD4$^+$ and CD8$^+$ memory T cells [6]. It has also been observed that both HIV-1-specific CD4$^+$ and CD8$^+$ T cells are less functional, with a particular impairment in interleukin 2 (IL-2) transcriptional expression, in individuals with disease progression, compared with long-term nonprogressors [7–10]. These data suggest that loss of function in T cells is, in part, due to gene-specific transcriptional dysregulation that is acquired during chronic HIV-1 infection. However, molecular mechanisms underlying the gene-specific reduction in expression have not been elucidated.

Epigenetic mechanisms of transcriptional regulation are critical for tissue and gene-specific transcript expression. Recently, it has become clear that epigenetic marks, such as DNA methylation and chromatin modification, modulate helper T-cell lineage commitment and function [11–14]. Furthermore, detailed analysis of epigenetic modification at the PD-1 locus in both mouse and human T cells indicates that the DNA methylation program modulates memory T-cell quality during persistent viral infection [15, 16]. Additionally, DNA demethylation at the promoter/enhancer region near the transcriptional start site of several genes coding

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for cytokines, including *IL2*, occurs after T-cell activation [17–19]. However, the role of epigenetic reprogramming of the *IL2* promoter in dysfunctional T cells during chronic HIV-1 infection has not been examined.

In the present study, we evaluated DNA methylation at the promoter/enhancer region of *IL2* in $CD4^+$ and $CD8^+$ T cells from HIV-1–infected noncontrollers with suppressed IL-2 expression. We also measured *IL2* promoter DNA methylation in T-cell subsets from HIV-1–uninfected donors to gain further insight into the link between changes in DNA methylation and downregulation of IL-2 expression. Finally, we investigated the relationship between *IL2* promoter DNA methylation and CD57, a marker of replicative senescence on T cells and a characteristic feature of T cells in HIV-1–infected individuals.

MATERIALS AND METHODS

Study Population

Peripheral blood mononuclear cells (PBMCs) were obtained from 16 viremic controllers (median HIV-1 RNA level, 410 copies/mL; interquartile range, 105–613 copies/mL) and 19 noncontrollers (median HIV-1 RNA level, 71 000 copies/mL; interquartile range, 60 500–86 000 copies/mL). Untreated chronic and viremic controller donors with $CD4^+$ T-cell counts of approximately 400 cells/μL were identified and used for this study, to ensure that differences in downstream assays were not due to reduction in $CD4^+$ T-cell quantity. Seven individuals in the acute stage of HIV-1 infection (defined as ≤3 months after diagnosis) were recruited. Individuals showing symptoms of acute HIV-1 infection had a dramatic decline in viral load, and 8 individuals received combination antiretroviral therapy (cART) for prolonged period and were also included in this study (Table 1). Eleven HIV-1–seronegative individuals were enrolled in this study. All participants gave written informed consent. The study was approved by the institutional review boards of the Institute of Medical Science at the University of Tokyo (20-47-210521).

Cell Culture and T-Cell Stimulation

The frozen PBMCs were thawed and rested overnight before use in Roswell Park Memorial Institute 1640 medium supplemented with 10% heat-inactivated fetal calf serum. The PBMCs were stimulated with 2 μg/mL phytohemagglutinin-L (Roche) for 2.5 or 5 hours. Total messenger RNA (mRNA) was extracted 2.5 hours after stimulation for quantification of cytokine gene expression. Culture supernatants were harvested after 5 hours of stimulation for measurement of cytokine production. The human cytokine 25-plex antibody kit (Invitrogen) was used for measurement of the concentrations of multiple cytokines in the supernatant [6].

Quantification of mRNA by Real-Time Polymerase Chain Reaction (PCR)

Total RNA was extracted from the stimulated PBMCs, using an RNeasy Mini Kit (Qiagen), and was reverse transcribed with SuperScript III reverse transcriptase (Invitrogen), according to the manufacturer's protocol, with oligo dT primers. Quantitative reverse-transcription PCR was performed with the LightCycler TaqMan Master Kit and the LightCycler 2.0 capillary-based system, using the Universal ProbeLibrary (Roche). All samples were run in duplicate. The gene encoding succinate dehydrogenase complex subunit A (*SDHA*) was used as a reference gene [20].

$CD4^+$ and $CD8^+$ T-Cell Isolation

$CD4^+$ and $CD8^+$ T cells were isolated from PBMCs by magnetic cell separation–positive selection, using anti CD4 and CD8 antibody-conjugated beads (Miltenyi Biotic), according to the

Table 1. Characteristics of Study Subjects, by Group

	Controller (n = 16)	Noncontroller (n = 19)	Acutely Infected (n = 7)	cART Recipient (n = 8)	HIV-1-uninfected (n = 11)	*P*[a]
Viral load, HIV-1 RNA copies/mL	410 (105–613)	71 000 (60 500–86 000)	230 000 (156 000–715 000)	Undetectable[b]	. . .	<.0001
T-cell count, cells/μL						
$CD4^+$	401 (377–468)	417 (329–442)	418 (300–492)	742 (606–795)	. . .	NS
$CD8^+$	893 (695–1103)	1302 (837–1760)	1262 (810–1668)	981 (809–1173)	. . .	.025
Age, y	34 (27–47)	39 (35–46)	41 (36–46)	44 (40–50)	38 (34–42)	NS
Duration after diagnosis, mo	50 (20–78)	29 (18–39)	≤3	82 (63–93)	. . .	NS
Treatment duration, mo	NA	NA	NA	60 (38–70)	. . .	. . .

Data are median (interquartile range).

Abbreviations: cART, combination antiretroviral therapy; HIV-1, human immunodeficiency virus type 1; NA, not applicable; NS, not significant.

[a] Data denote results of comparisons between controllers vs noncontrollers.

[b] Lower limit of detection (50 copies/mL), except a patient with 80 copies/mL.

manufacturer's instructions. The purity of each cell fraction was >95%, as determined by flow cytometry.

DNA Methylation Analysis

The analysis of DNA methylation was determined by bisulfate sequencing as previously described [21]. Briefly, genomic DNA from the purified $CD4^+$ T cells and $CD8^+$ T cells were treated with bisulfate, using the EpiTect Bisulfite Kit (Qiagen). Converted DNA (50 ng) was amplified by PCR with AccuPrime Taq DNA polymerase. The PCR was performed with the following locus-specific primers: 5′-GAGATAGGATTTTTT-TAAGTGTTTTTAGGT-3′ and 5′-CATTAACCCACACTT AAATAATAACTCTAA-3′ for the *IL2* gene, 5′-GTTAAGAGG-GAGAGAAGTAATTATAGATTT-3′ and 5′-AAATCTAT AATTACTTCTCTCCCTCTTAAC-3′ for the tumor necrosis factor gene (*TNF*), and 5′-TGGAAAGAGGAGAGTGACA-GAA-3′ and 5′-TTGGATGCTCTGGTCATCTTTA-3′ for the interferon γ gene (*IFNG*). The PCR products were cloned into the pGEM-T Easy vector system (Promega), and sequencing analysis was performed with at least 10 individual clones from each sample. All independent experiments were duplicated to avoid PCR amplification bias.

Antibodies

The following antibodies were used for flow cytometric analysis and cell sorting: CD57–fluorescein isothiocyanate (FITC), PD1-FITC, IL2-FITC, CD28–allophycocyanin (APC), CD45RA-APC, CCR7-phycoerythrin (PE)-Cy7, and CD3–Pacific blue (BD Pharmingen); CD27-FITC, CD8-PE, and CD3-peridinin chlorophyll protein-Cy5.5 (BD Biosciences); CD57-PE, CD45RA-APC-Cy7, CD4–Pacific blue, and CD8–Pacific blue (BioLegend); and CD4-APC-eFluor780 (eBioscience). Dead or dying cells were detected by staining with propidium iodide (Sigma).

Flow Cytometric Analysis and Cell Sorting of $CD4^+$ T-Cell Subsets

Multiparameter flow cytometry and cell sorting were performed with an Aria fluorescence-activated cell sorter (BD). Intracellular cytokine staining and surface staining were preformed as previously described [6]. For cell sorting, the sort logic was set by gating lymphocytes by forward scatter and side scatter and then gating on $CD3^+CD4^+$ cells. For methylation analysis of $CD4^+$ T-cell subsets, $CD4^+$ T cells were classified into 5 subsets based on expression of CD45RA, CCR7, CD27, and CD28: $CD45RA^+CCR7^+CD27^+CD28^+$ (naive), $CD45RA^-CCR7^+CD27^+CD28^+$ (central memory), $CD45RA^{+/-}CCR7^-CD27^+CD28^+$ (early effector memory), $CD45RA^{+/-}CCR7^-CD27^-CD28^+$ (intermediate effector memory), and $CD45RA^{+/-}CCR7^-CD27^-CD28^-$ (late effector memory). Memory $CD4^+$ T cells ($CD3^+CD4^+$ $CD45RA^-$) were classified into further subsets based on expression of CD57. The purity of the sorted cell populations was >99%.

Statistical Analysis

GraphPad Prism5 software (GraphPad Software) was used for all statistical analysis. The Mann–Whitney *U* test and the Wilcoxon matched paired test were used for unpaired and paired comparisons, respectively. Correlation analysis was performed using Spearman rank correlation. Correction for multiple comparisons was assessed by calculating *q* values, the *P* value analogue of the false-discovery rate [22]. The level of significance for all analyses was set at a *P* value of <.05 and a *q* value of <0.2.

RESULTS

IL2 Gene Expression Is Impaired in HIV-1–Infected Noncontrollers During Chronic Infection

To elucidate a qualitative difference between T cells from viremic controllers and those from noncontrollers during chronic HIV-1 infection, we broadly assayed cytokine expression profiles from phytohemagglutinin-L (PHA)-stimulated PBMCs isolated from individuals with chronic HIV-1 infection. We found that IL-2, tumor necrosis factor α, interleukin 6, and interleukin 7 expression was significantly impaired in noncontrollers (Figure 1*A*). Because *IL2*, *TNF*, and *IL6* are early response genes that trigger sequential multiple immune responses and share some signaling pathways, we further examined mRNA expression of these genes at an earlier time point after PHA stimulation, to determine the hierarchy of reexpression. Importantly, the level of *IL2* mRNA expression was almost 4-fold higher in controllers relative to noncontrollers, whereas *TNF* and *IL6* mRNA expression levels were similar (Figure 1*B*). Furthermore, impaired *IL2* mRNA expression in noncontroller PBMCs was also observed after anti-CD3/CD28 stimulation (Supplementary Figure 1*A*). These data suggest that $CD4^+$ T cells in noncontrollers were qualitatively distinct in *IL2* gene regulation.

The *IL2* Promoter/Enhancer Region Is Hypermethylated in HIV-1–Infected Noncontrollers

Epigenetic modifications are a critical mechanism for stable gene expression. Specifically, acquired DNA methylation–mediated gene silencing can be maintained in a dividing population of cells [23–26]. The human *IL2* promoter contains 6 CpG sites within 1 kb immediately upstream of the transcriptional start site (Figure 2*A*) [18]. To investigate the role of DNA methylation in the restricted *IL2* expression in noncontrollers, we performed bisulfate sequencing to assess the level of DNA methylation at the individual CpG sites of the *IL2* promoter in $CD4^+$ and $CD8^+$ T cells isolated from HIV-1–infected individuals. The methylation frequency at all CpG sites in $CD4^+$ T cells was significantly higher in noncontrollers relative to that for $CD4^+$ T cells from controllers and HIV-1-uninfected individuals (Figure 2*B* and 2*C*). Notably, the difference was prominent in CpG site 1. In contrast, there was no significant

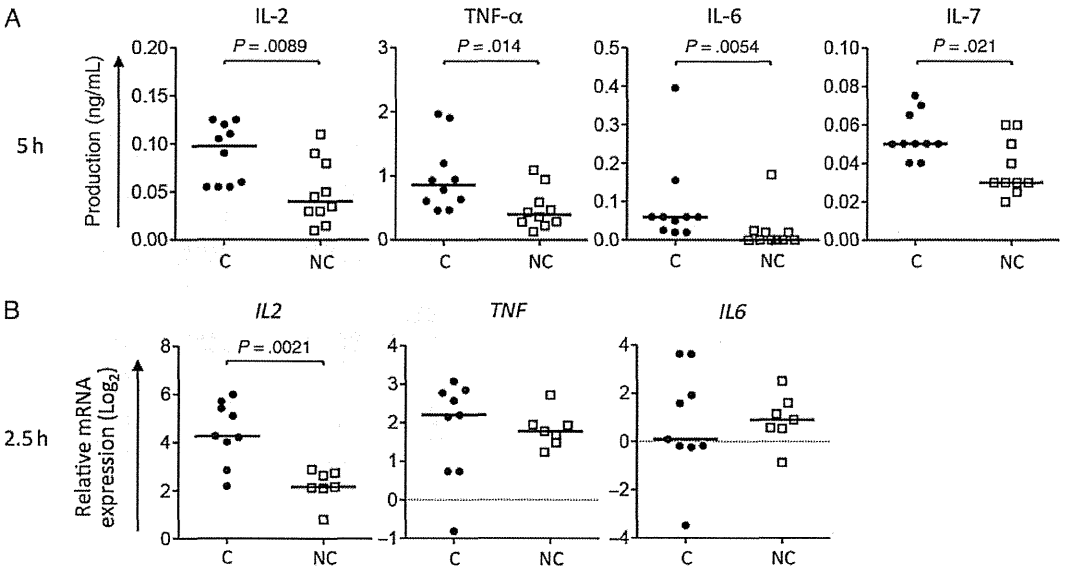

Figure 1. Cytokine transcription and protein expression profiling of phytohemagglutinin L (PHA)–stimulated peripheral blood mononuclear cells from individuals with chronic human immunodeficiency virus type 1 infection. Cytokine production (*A*) and messenger RNA (mRNA) expression of cytokines (*B*) after PHA stimulation. Data are normalized to the reference gene *SDHA* and are presented as fold change relative to unstimulated conditions. The horizontal bars indicate the median value. The Mann–Whitney *U* test was used for statistical analysis. Abbreviations: C, viremic controller; NC, noncontroller; IL-2, interleukin 2; IL-6, interleukin 6; IL-7, interleukin 7; TNF-α, tumor necrosis factor α.

difference in the methylation status of the *IL2* promoter in $CD8^+$ T cells between the groups (Figure 2*B* and Supplementary Figure 2*A*). It has previously been reported that *IFNG* and *TNF* promoters undergo methylation in aged people [27, 28]. Therefore, we sought to determine whether these promoters also acquired methylate promoters during chronic HIV-1 infection. Our bisulfite sequencing data indicate that there is not a significant difference in DNA methylation of the both *IFNG* and *TNF* promoter in $CD4^+$ and $CD8^+$ T cells between HIV-1 controllers and noncontrollers (Supplementary Figure 2*B* and 2*C*). To further determine whether the increase in methylation of the *IL2* promoter was coupled to a reduction in expression, we measured IL-2 expression in PHA-stimulated PBMCs and compared it to the *IL2* promoter methylation status in $CD4^+$ T cells. The DNA methylation status at CpG site 1 in $CD4^+$ T cells was inversely correlated to *IL2* mRNA expression and IL-2 production (Figure 2*D*). The inverse correlation was also observed when anti-CD3/CD28 was used as a stimulus (Supplementary Figure 1*B*). These data indicate that DNA hypermethylation at CpG site 1 in $CD4^+$ T cells is coupled to low IL-2 expression in noncontrollers during chronic HIV-1 infection.

$CD4^+$ T Cells From cART Recipients Have a Hypomethylated *IL2* Promoter

We next examined the DNA methylation status of the *IL2* promoter in $CD4^+$ T cells at different clinical stages of HIV-1 infection. We measured the methylation status of the *IL2* promoter in $CD4^+$ T cells from HIV-1–infected individuals with a high viral load in the acute stage of infection (Figure 2*E*). Surprisingly, the level of methylation of the *IL2* promoter in $CD4^+$ T cells from HIV-1–infected individuals in the acute stage of infection was similar to $CD4^+$ T cells from controllers and those from HIV-1-uninfected individuals. These data suggest that a high viral load itself does not affect the methylation status of the *IL2* locus.

We next sought to determine whether reduction of the HIV level in chronically infected individuals would result in demethylation of the *IL2* promoter. We performed a longitudinal analysis of *IL2* promoter methylation in $CD4^+$ T cells isolated from individuals before they received cART and after they achieved prolonged cART-mediated virus suppression. The subjects with prolonged viral suppression had recovery in $CD4^+$ T-cell counts, and, importantly, hypermethylation at CpG site 1 in the *IL2* locus before starting cART declined to normal levels after prolonged virus suppression in all individuals (Figure 2*F*). Indeed, the level of *IL2* promoter methylation in treated individuals was similar to the promoter methylation levels in HIV-1 controllers and uninfected individuals (Figure 2*E*). These data suggest that *IL2* promoter hypermethylation results from persistent high viral loads but is reversible after cART-mediated virus suppression.

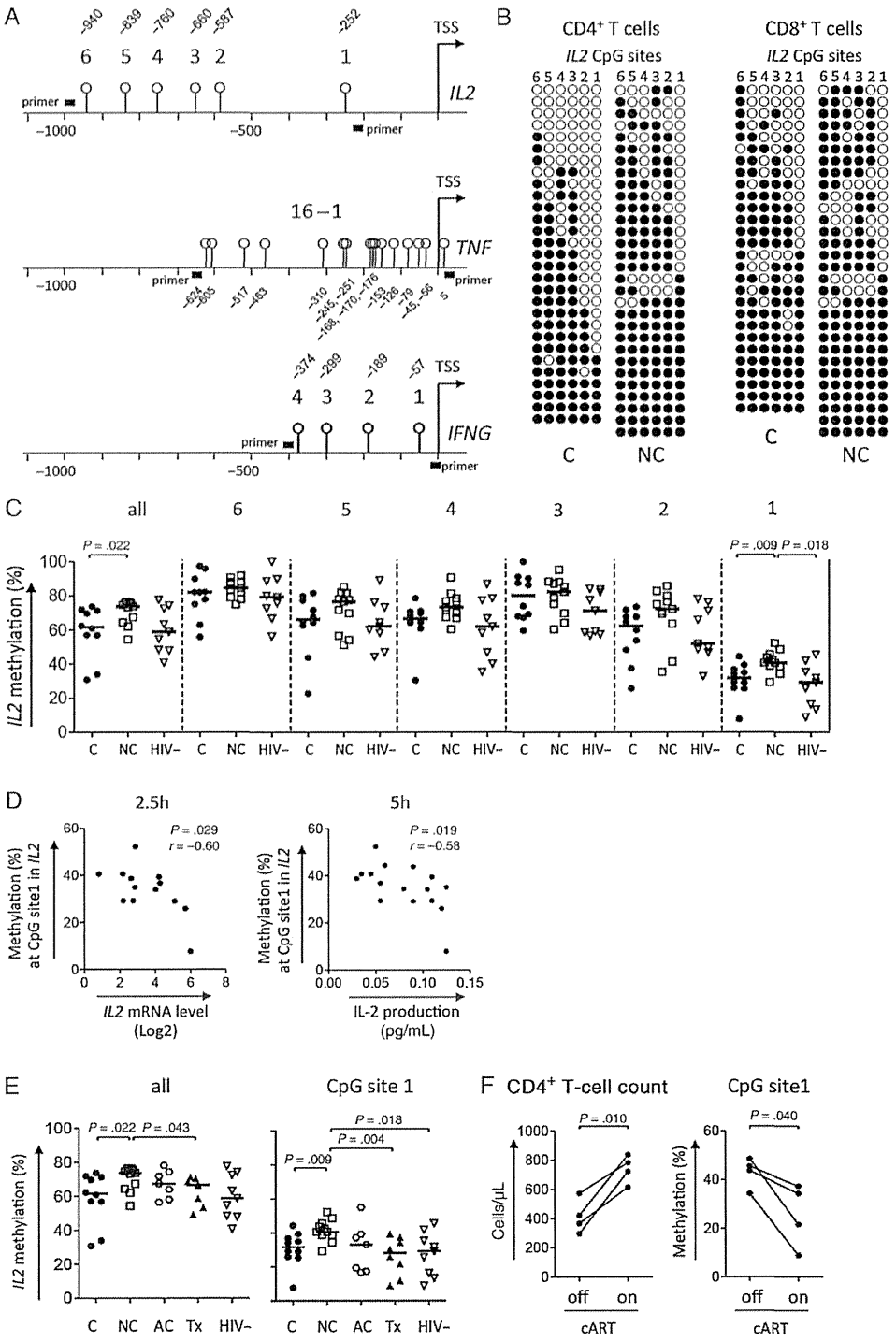

Figure 2. DNA methylation analysis of the *IL2* locus in human immunodeficiency virus type 1 (HIV-1)–infected individuals. *A*, Diagram of CpG position relative to the transcriptional start site of the *IL2, TNF,* and *IFNG* loci. *B*, Representative bisulfite sequencing DNA methylation analysis of the *IL2* locus in $CD4^+$ and $CD8^+$ T cells from viremic controllers and noncontrollers. Each line represents an individual clone picked for sequencing (filled circles, methylated cytosine; open circles, unmethylated cytosine). *C*, DNA methylation status of the *IL2* locus at CpG sites 1–6 in $CD4^+$ T cells from healthy and HIV-1–infected individuals. *D*, Correlation plot between methylation at CpG site 1 in $CD4^+$ T cells and interleukin 2 (IL-2) expression in peripheral blood mononuclear cells. *E*, DNA methylation status in all and at CpG site 1 in $CD4^+$ T cells at different clinical status in HIV-1 infection. *F*, Longitudinal change of methylation status at CpG site 1 and $CD4^+$ T-cell count in 4 subjects before and after combination antiretroviral therapy initiation. The Mann–Whitney *U* test (*C* and *E*) and the Wilcoxon matched paired test (*F*) were used for statistical analysis. Correlation coefficient and *P* values determined by the Spearman rank correlation test are shown in panel *D*. Abbreviations: AC, acute HIV-1 infection; C; controller, NC; noncontroller; Tx, treated with combination antiretroviral therapy; HIV-, HIV-1-uninfected.