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Epigallocatechin-3-Gallate, a Histone Acetyltransferase Inhibitor, Inhibits EBV-Induced B Lymphocyte Transformation via Suppression of RelA Acetylation

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Abstract

Because the p300/CBP-mediated hyperacetylation of RelA (p65) is critical for nuclear factor-κB (NF-κB) activation, the attenuation of p65 acetylation is a potential molecular target for the prevention of chronic inflammation. During our ongoing screening study to identify natural compounds with histone acetyltransferase inhibitor (HATi) activity, we identified epigallocatechin-3-gallate (EGCG) as a novel HATi with global specificity for the majority of HAT enzymes but with no activity toward epigenetic enzymes including HDAC, SIRT1, and HMTase. At a dose of 100 μmol/L, EGCG abrogates p300-induced p65 acetylation *in vitro* and *in vivo*, increases the level of cytosolic IκBα, and suppresses tumor necrosis factor α (TNFα)–induced NF-κB activation. We also showed that EGCG prevents TNFα-induced p65 translocation to the nucleus, confirming that hyperacetylation is critical for NF-κB translocation as well as activity. Furthermore, EGCG treatment inhibited the acetylation of p65 and the expression of NF-κB target genes in response to diverse stimuli. Finally, EGCG reduced the binding of p300 to the promoter region of *interleukin-6* gene with an increased recruitment of HDAC3, which highlights the importance of the balance between HATs and histone deacetylases in the NF-κB–mediated inflammatory signaling pathway. Importantly, EGCG at 50 μmol/L dose completely blocks EBV infection-induced cytokine expression and subsequently the EBV-induced B lymphocyte transformation. These results show the crucial role of acetylation in the development of inflammatory-related diseases. [Cancer Res 2009;69(2):583–92]

Introduction

Protein acetylation influences a broad set of cellular processes, including diverse aspects of transcriptional regulation, through the recruitment of enzymes: the histone deacetylases (HDAC) and the histone acetyltransferases (HAT; ref. 1). The packaging of eukaryotic DNA into chromatin plays an active role in transcriptional regulation by interfering with the accessibility to the transcription factors (2). Acetylation of specific lysine residues within the NH2-terminal tails of nucleosomal histones is generally linked to chromatin disruption and transcriptional activation of genes (3). Consistent with their role in altering chromatin structure, many transcriptional coactivators, including hGCN5, p300/CBP, PCAF, and SRC-1, possess intrinsic acetyltransferase activity that is critical for their function (4, 5). Similarly, corepressor complexes include proteins that have deacetylase activity (6). Until now, more than 40 transcription factors and 30 other proteins are acetylated on lysine residues, and their function is thereby regulated (7–13). Depending on the functional domain that is modified, acetylation can regulate different functions of these nonhistone proteins, such as DNA recognition, protein stability, protein-protein interaction, and subcellular localization (14). Thus, dysregulation of the balance between protein acetylation and deacetylation is often associated with the initiation of tumorigenesis and other diseases (15).

Nuclear factor-κB (NF-κB) is a ubiquitously expressed family of transcription factors controlling the expression of numerous genes involved in inflammatory and immune responses and cellular proliferation (16). In mammals, the most abundant form of NF-κB is a heterodimer of p50 and p65 (17). The RelA (p65) subunit of NF-κB is also known to be activated in an acetylation-dependent manner in response to cytokine stimulation (18, 19). Deacetylation of p65 promotes its effective binding to IκBα and leads to IκBα-dependent nuclear export of the NF-κB complex by a CRM-1–dependent pathway (18). Reversible acetylation of p65 thus functions as a molecular switch that both controls the duration of the NF-κB transcriptional response (20). Given the ubiquitous expression of NF-κB and the important number of inducing stimuli as well as target genes, it is not surprising that NF-κB is involved in numerous and diverse diseases, such as the maintenance of chronic inflammation and asthma, the survival of cancer cells, and the resistance of cancer cells to treatment (17, 21). Therefore, NF-κB is an interesting target for pharmaceutical interference with the establishment and the progression of these pathologic states.

Among the small molecules that are capable of modulating epigenetic status, HDAC inhibitors have been extensively studied and several are currently in clinical trials (22). One example is vorinostat, which has been approved by the Food and Drug Administration for the treatment of cutaneous T-cell lymphoma (23). On the other hand, there is little information available on inhibitors of HATs (HATi). As suggested in recent studies, the

development of HATis from dietary compounds, such as garcinol, curcumin, and anacardic acid, is the next therapeutic goal (24, 25). Garcinol has been found to inhibit p300 and PCAF *in vitro* and *in vivo*, anacardic acid to inhibit TIP60 as well as p300 and PCAF, and curcumin to inhibit p300 and PCAF (24). These dietary compounds are associated with the prevention of cancer and other diseases.

Epigallocatechin-3-gallate (EGCG), the major polyphenol found in green tea, is reported to have an anti-NF-κB transactivation activity in a broad range of human malignancies, such as colon cancer, lung cancer, breast cancer, and in chronic inflammation (26, 27). Recently, EGCG has been shown to inhibit the production of nitric oxide synthase (NOS2) by blocking the NF-κB signal transduction pathway (28). Although EGCG is also known to suppress NF-κB activation and phosphorylation of p38 mitogen-activated protein kinase and c-Jun NH$_2$-terminal kinase (29), it is still unclear how EGCG regulates NF-κB–dependent activation whether via inhibiting the stabilities or activities of proteins. In this study, we first report that EGCG possesses potent anti-HAT activity and shows global specificity for the majority of HAT enzymes. Our studies show that EGCG generally induces hypoacetylation of p65 by directly inhibiting the activity of HAT enzymes. This hypoacetylation of p65 led to the down-regulation of NF-κB function by diverse inflammatory signals. In addition, EGCG totally blocks EBV-induced B-cell transformation by suppressing viral protein-induced acetylation of p65. In summation, this study shows that selective modulation of NF-κB acetylation by HATi is a potential mechanism for a new class of anti-inflammatory and chemopreventive drugs.

Materials and Methods

Cell culture and reagents. All cell lines were obtained from the American Type Culture Collection. HEK293 cells were cultured in DMEM and THP-1 cells were cultured in RPMI 1640 (Hyclone) and supplemented with 10% fetal bovine serum (FBS; Hyclone), 1% antibiotics, and antimycotics (Hyclone). All cell lines were grown at 37°C in 5% CO$_2$. The Effectene transfection reagent was purchased from Qiagen.

Mouse experiments. We maintained male BALB/c mice (6 wk old) approximately 20 to 25 g in body weight in accordance with the guidelines and under approval of the Animal Care Committee of Yonsei University (Seoul, South Korea). Mouse peritoneal macrophages were isolated according to the method described previously (30). Peritoneal cells were washed in PBS and cultured at 37°C under 5% CO$_2$ in DMEM supplemented with L-glutamine, antibiotics, and 10% FBS. After incubation for 2 d, cells treated with EGCG were cultured for 2 h by tumor necrosis factor α (TNFα; 20 ng/mL) treatment and then harvested.

HAT and HDAC activity assay. HeLa cell nuclear extract (NE) was prepared as previously described (31). HAT activity and HDAC activity assays were determined using a commercial available kit (Biovision Biotechnology) according to the manufacturer's instruction. SIRT1 deacetylase activity was assayed with SIRT1/Sir2 Deacetylase Fluorometric Assay kit (CycLex).

RNA extraction, reverse transcription-PCR, and chromatin immunoprecipitation analysis. RNA extraction, reverse transcription-PCR (RT-PCR), and chromatin immunoprecipitation (ChIP) were performed as described (31). The antibodies against p65, acetyl-p65, HDAC3, and p300 were purchased from Upstate Biotechnology. Primer sequences for the amplification of human interleukin (IL)-6, murine IL-6, human cyclo-oxygenase-2 (COX-2), murine COX-2, human NOS2, murine NOS2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are reported in Supplementary Table S1.

Proliferation assays. The EBV virus was obtained by treating B95.8 cells (gift from Richard F. Ambinder, Oncology Center, Johns Hopkins University, Baltimore, MD) with 30 ng/mL 12-*O*-tetradecanoylphorbol-13-acetate

(Sigma). Virus supernatant was concentrated by using Centricon Plus-80 filters (Millipore). Peripheral blood mononuclear cells (PBMC; Yonsei University Health System Blood Bank) were infected with concentrated virus for 3 h and cultured in RPMI 1640 plus 10% FCS for 3 wk. Medium, plus or minus EGCG, was replaced weekly. Assays were performed in five-well replicates. Colony outgrowth was monitored by using phase-contrast microscopy.

ELISA and cytokine antibody array. IL-6 was measured with a human IL-6 TiterZyme Enzyme Immunometric Assay kit (Assay Designs) according to the manufacturer's instructions. Values were calculated based on a standard curve constructed for each assay. For analyzing cytokine expression profile, TransSignal Human Cytokine Antibody Array 3.0 membranes (Panomics) were incubated with conditioned medium from EBV-positive B cells untreated or treated with EGCG. Images were visualized using LAS 3000 image analyzer (Fuji).

Results

EGCG is a specific inhibitor of HAT activity. During our ongoing screening study to identify natural compounds with anti-HAT activity, we found that green tea extract had potent anti-HAT activity. Green tea extract contains polyphenols, which are composed of catechin derivatives; thus, we first decided to examine whether catechin derivatives possess anti-HAT activity. Most catechin derivatives, including catechin, epicatechin, and epigallocatechin, exhibited low levels of anti-HAT activity, whereas EGCG inhibited 90% of HAT activity in a dose-dependent manner (Fig. 1*A* and *B*). To examine the enzyme specificity, we first sought to assess the effect of EGCG on HDAC and histone methyltransferase (HMT) activities. When HeLa NE was used as a source of histone deacetylase enzymes, deacetylation activity was not affected by the presence of EGCG, but trichostatin A (TSA) efficiently blocked nuclear HDAC activity (Fig. 1*C*). This suggests that EGCG did not possess specificity for HDAC. We next investigated the effect of EGCG on HMT activity. HeLa core histones were methylated with [^{3}H]*S*-adenosylmethionine by recombinant lysine methyltransferase SET7/9, which specifically methylates lysine residues 9 and 27 of histone H3. As shown by Fig. 1*D*, histone methylation by SET7/9 remains the same in the presence or absence of EGCG. The same result was also observed with NE as an enzyme source (data not shown). Similar to the results of the HDAC and HMT assays, the SIRT deacetylase assay also showed no difference either in the presence or absence of EGCG (Supplementary Fig. S1*A*). We next determined whether EGCG was a general HATi or was a specific HATi. The HAT activity was measured using recombinant HAT p300 and CBP as enzyme sources. EGCG was found to be a highly efficient inhibitor of p300 and CBP acetyltransferase activities with an IC$_{50}$ of ∼30 μmol/L and ∼50 μmol/L, respectively (Fig. 2*A*). Under similar conditions, EGCG also inhibited immunoprecipitated PCAF and TIP60 acetyltransferase activities with an IC$_{50}$ of ∼60 μmol/L and ∼70 μmol/L, respectively, although to a lesser extent, suggesting that EGCG generally inhibited most HAT enzyme activities (Fig. 2*B*). To understand the mechanism of EGCG-mediated p300/CBP HAT activity, we investigated the enzyme kinetics by changing the histone concentration while keeping all other concentrations constant at once. The K_m and V_{max} were decreased by EGCG treatment in both cases, showing that EGCG does not bind to the active sites of histone but to some other site on the enzyme. These data show that EGCG uncompetitively inhibits p300/CBP HAT activity (Supplementary Fig. S1*B*).

To assess the EGCG-mediated inhibition of HAT activity toward the synthetic H4 tail peptide, we analyzed radiolabeled H4 tail

peptide by SDS-PAGE and fluorography. The results were similar to the observation made for the HAT activity colorimetric assays. Compared with other catechin derivatives, EGCG strongly quenched HAT activity (Fig. 2C). After establishing EGCG as a strong inhibitor of HATs *in vitro*, we further investigated whether it could also affect the acetylation of histone tails *in vivo*. For this purpose, we examined the histone acetylation status of chromatin by ChIP assay. It has been reported that T3 hormone induces histone acetylation in the promoter region of the thyroid hormone receptor-regulated gene, *deiodinase 1* (*D1*; refs. 6, 31). Treatment of EGCG reversed the T3-stimulated acetylation of histone H3 and H4 by p300/CBP in the D1 promoter but not the GAPDH promoter negative control (Fig. 2D). These results therefore established that EGCG is a specific and global inhibitor of HAT activity but not other enzymes for which histones are the substrate.

EGCG-induced hypoacetylation of p65 resulted in exchange between HDACs and HATs in the promoter region of a NF-κB–regulated gene. Based on recent studies showing that EGCG inhibits inflammatory signaling in a broad range of human cancer cells and bronchial epithelial cells, we next sought to investigate whether EGCG inhibits the acetylation of p65 both *in vitro* and *in vivo*. As p300 acetyltransferase has been already known to play a major role in the acetylation of p65, we generated the active p300 (HAT) protein and the p300 (ΔHAT) HAT activity-defective mutant to confirm whether p300 directly acetylates p65. As shown in

Supplementary Fig. S1C, histone H4 tail peptide *in vitro* was acetylated efficiently by GST-p300 (HAT) but not by GST-p300 (ΔHAT). We next performed *in vitro* acetylation assays with recombinant p300 domain proteins in the presence or absence of EGCG using GST-p65 protein as a substrate. As shown in Fig. 3A, the acetylation of GST-p65 was detected in the presence of active GST-p300 but not in the presence of GST-p300 (ΔHAT). On EGCG treatment, the p300-induced acetylation was abrogated, confirming that EGCG prevented the hyperacetylation of p65 *in vitro*. To further substantiate the prevention of p65 acetylation by EGCG, we next determined the effect of EGCG treatment on TNFα-induced acetylation of p65 in HEK293 cells. TNFα-treated HEK293 cells were cultured either in the presence or absence of EGCG. The cells were collected, and subsequent Western blot analysis was performed using antibody against acetylated p65 (lysine 310). As shown in Fig. 3B, EGCG treatment reversed TNFα-enhanced acetylation of p65 in HEK293 cells. Taken together, we conclude that EGCG prevents the hyperacetylation of p65 by inhibiting the HAT activity of p300/CBP.

NF-κB has also been shown to interact with HDAC3, which seems to be crucial for the deacetylation of p65 (20). It has been reported that the reversible acetylation causes exchange between the HDACs and HATs in the promoter region of a T3R-regulated gene (31). Therefore, we hypothesized that the balance between HDAC and HAT activity determines the level of p65 acetylation and
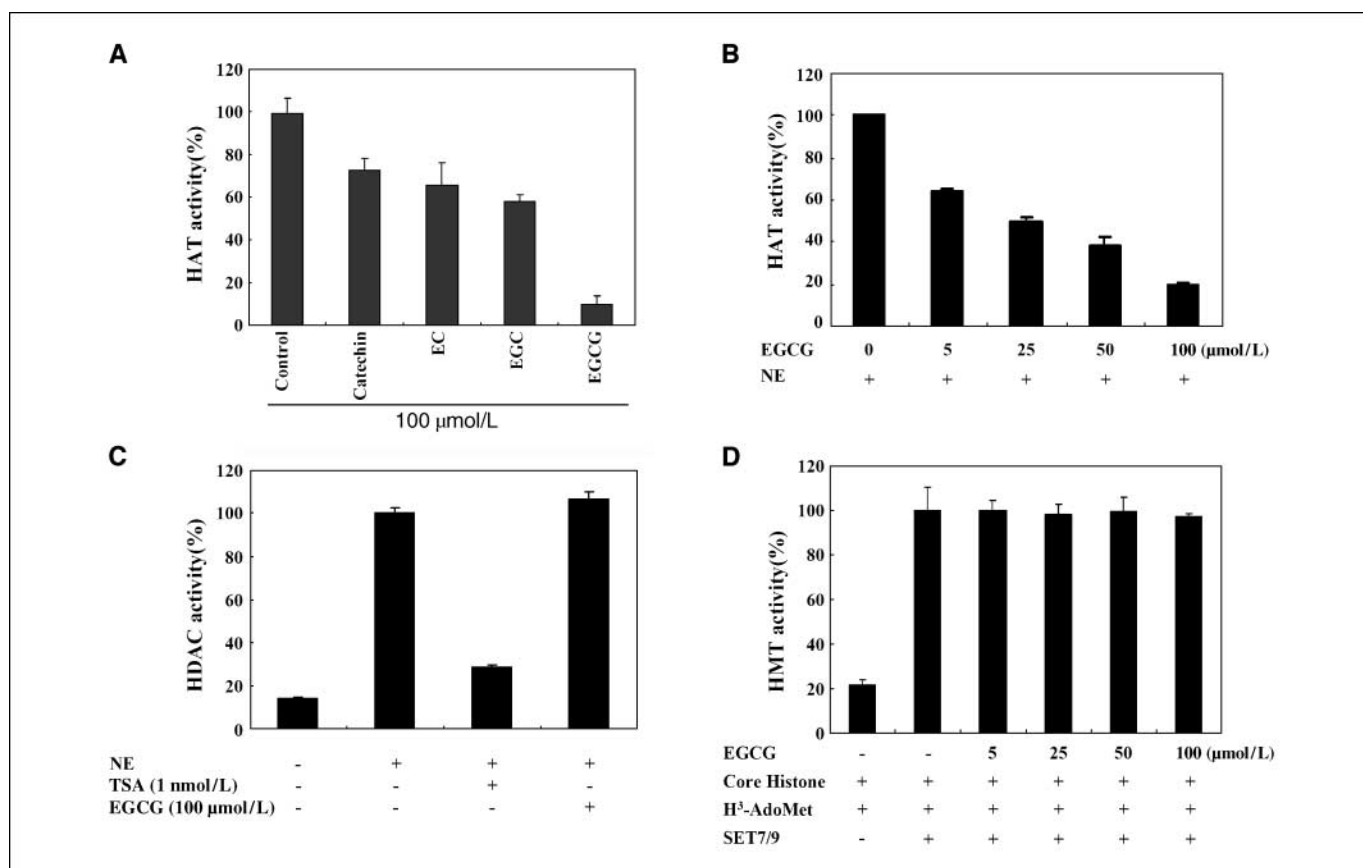

Figure 1. EGCG has specific anti-HAT activity. *A*, EGCG has potent anti-HAT activity toward catechin derivatives. HAT activity was assayed with a HAT activity colorimetric assay kit and HeLa NE as a source of HAT enzymes. *Columns*, average of three independent experiments; *bars*, SD. *EC*, epicatechin; *EGC*, epigallocatechin. *B*, EGCG dose dependently inhibits HAT activity. HAT activity colorimetric assays were performed with indicated concentrations of EGCG. *C*, EGCG treatment had no effect on the HDAC activity. HDAC activity was assayed with an HDAC activity colorimetric assay kit. TSA was used as a control inhibitor for HDAC activity. *Columns*, average of three independent experiments; *bars*, SD. *D*, HMT assays were performed in 30 μL reaction in the presence or absence of EGCG using recombinant SET7/9 as the enzyme sources and then processed for filter binding assay.
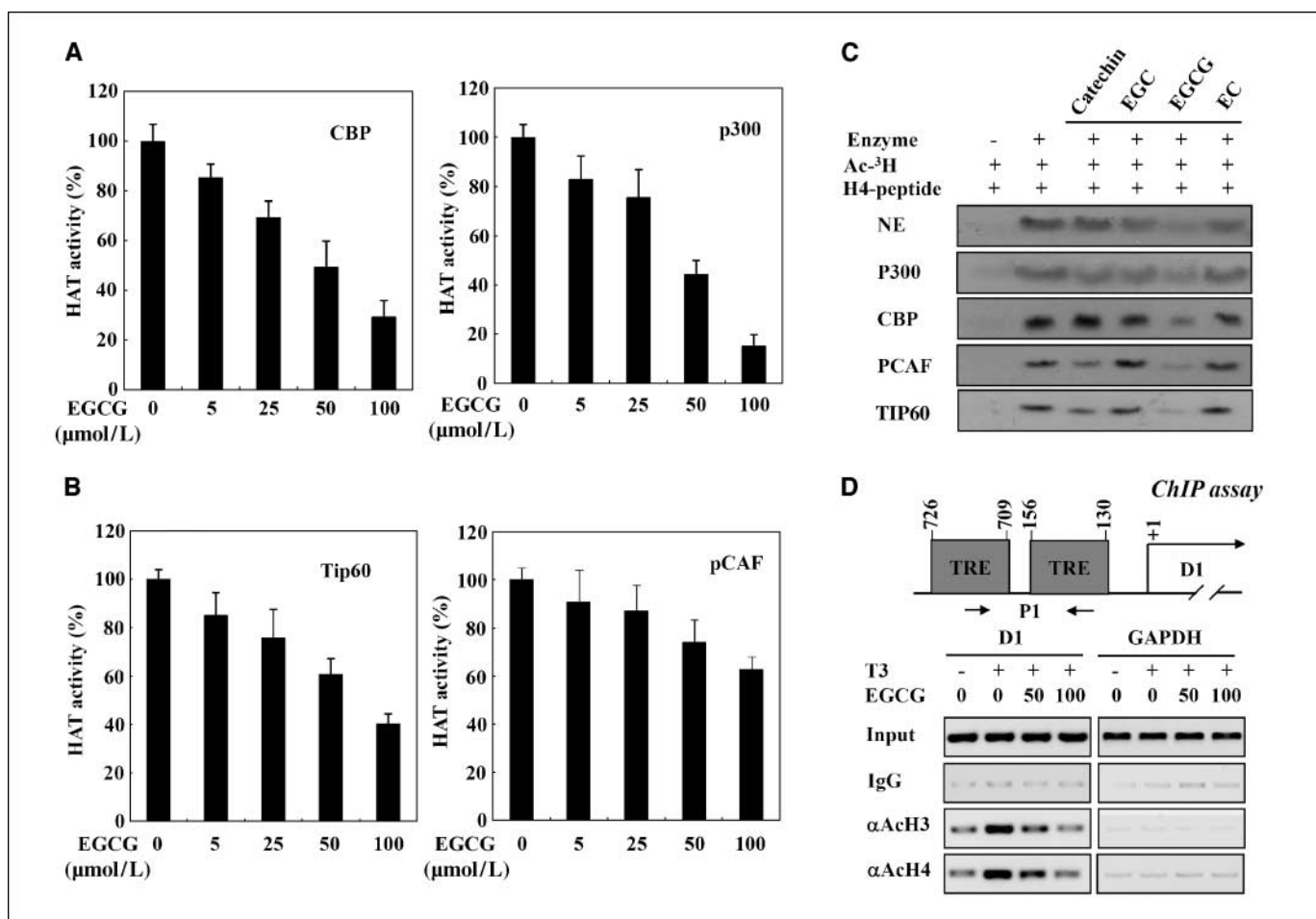
Figure 2. EGCG exhibits a broad range of substrate specificity. HAT activity colorimetric assays were performed with an increased amount of GST-CBP (*A, left*), GST-p300 (*A, right*), immunoprecipitated TIP60 (*B, left*), and PCAF (*B, right*). *C, in vitro* HAT activity assays were performed with HAT enzymes in the presence or absence of catechin derivatives (100 μmol/L) using a synthetic H4 tail peptide and subsequently processed for fluorography. *D,* EGCG inhibits hypoacetylation of histone tails in chromatin. A diagram of the D1 promoter showing the positions of TREs and primers used for PCR amplification in ChIP assays. The P1 pair primers amplify a DNA fragment ~2.5 kb downstream of the transcriptional start site. A ChIP assay was performed with HeLa α2 treated with EGCG in the presence of T3 (1 h). The histone modification of the *D1* gene was then assessed by ChIP assays using the indicated antibodies.

subsequently the expression levels of NF-κB–regulated genes. We tested this hypothesis by assessing the effect of EGCG on the recruitment of HDAC3 or p300 to the promoter region of the *IL-6* gene. The *IL-6* gene promoter contains a well-characterized NF-κB–binding site located at −75 to −63 relative to the transcriptional start site (Fig. 3*C*). We used ChIP assays to determine whether HDAC3 or p300 associated with the IL-6 promoter in THP-1 cells treated with or without TNFα. As shown in Fig. 3*C*, TNFα treatment led to a significant increase in binding of p300 to the NF-κB–binding site of the *IL-6* gene. However, addition of EGCG resulted in the enhanced recruitment of HDAC3 to the NF-κB–binding site of the *IL-6* gene. The recruitment of p300 was abolished by treatment with EGCG, confirming the importance of the balance between HATs and HDACs to the NF-κB–mediated inflammatory signal.

Hypoacetylation of p65 by EGCG treatment led to loss of NF-κB function. RelA acetylation plays a critical role in the nuclear function of NF-κB (18, 19, 32). Therefore, we first examined the effect of EGCG on TNFα-induced DNA-binding activity of NF-κB by performing electrophoretic mobility shift assays (EMSA). When TNFα-treated HEK293 NE was incubated with radiolabeled

NF-κB probe, a protein-DNA complex specific for the NF-κB probe was observed in the gel shift assay (Fig. 4*A, right, lanes 1* and *2*). However, EGCG treatment reduced the enhanced NF-κB DNA-binding activity induced by TSA treatment, suggesting that the level of acetylation determines the DNA-binding activity of NF-κB (Fig. 4*A, left, lanes 3–5*). Supershift analysis indicated that the protein/DNA complex observed actually contained p65 (Fig. 4*A, left*). To substantiate this result, we carried out ChIP assays over the promoter region of the *IL-6* gene in HEK293 and THP-1 cells. Cells were then collected and processed for ChIP assay using antibodies to p65 or acetylated p65. To more precisely measure the recruitment of p65 and acetylated p65, we used real-time PCR analysis. A representative result is presented in Fig. 4*B* and shows that TNFα treatment increased the recruitment of acetylated p65 to the promoter of the *IL-6* gene but not the *β-actin* gene. The enhanced recruitment of acetylated p65 was abolished by treatment with EGCG, which agrees with the results of the EMSA.

Because the dissociation of IκBα and nuclear translocation of NF-κB are critical for NF-κB activation (18), we determined whether EGCG-mediated hypoacetylation of p65 enhances the association of IκBα with p65. As shown in Fig. 4*C*, EGCG treatment

led to the restoration of the association of IκBα with p65, which was abrogated by TNFα treatment. In support of this result, EGCG increased the level of cytosolic IκBαeven in the presence of TNFα. These data indicated that deacetylation events lead to a cytoplasmic accumulation of IκBαand subsequent cytosolic sequestration of NF-κB. We next determined whether hypoacetylation by EGCG inhibits the nuclear translocation of p65 by performing immunohistochemistry analysis. As shown in Fig. 4D, EGCG reversed TNFα-induced nuclear translocation of p65 and enhanced nuclear export of hypoacetylated p65. It is noteworthy that the treatment with LMB, a specific inhibitor of nuclear protein export, abrogated nuclear export of hypoacetylated p65, suggesting that the level of acetylation is critical for NF-κB translocation. These results therefore established that hypoacetylation of p65 by EGCG induced the loss of NF-κB nuclear function by enhancing nuclear export of p65, increasing the subsequent association with the inhibitory protein IκBα, and impairing DNA-binding activity.

EGCG down-regulates the TNFα-induced NF-κB–dependent inflammatory response. To determine whether EGCG regulates TNFα-induced NF-κB activation and the inflammatory response, we measured NF-κB–dependent promoter activity. EGCG dose dependently inhibited TNFα-induced NF-κB activation (Fig. 5A).

Due to the important role of NF-κB in the regulation of a variety of key inflammatory mediators, we next determined whether EGCG inhibited TNFα-induced transcription of NF-κB–regulated genes by performing real-time PCR analysis. As shown in Fig. 5B, EGCG greatly reduced TNFα-induced expression of *IL-6, COX-2,* and *NOS2* in both HEK293 and THP-1 cells. Similar results were also observed in primary peritoneal macrophages. Consistent with these results, EGCG dramatically reduced protein levels of these target genes (Supplementary Fig. S2A). In addition, we also assessed the effect of EGCG on the release of inflammatory markers from THP-1 cell cultures. EGCG treatment of cells resulted in a significant inhibition of the TNFα-induced increase in the expression of *IL-6,* depicting EGCG as a potent anti-inflammatory molecule (Fig. 5C). Next, we investigated whether EGCG could also inhibit the production of TNFα-induced inflammatory molecules *in vivo*. We pretreated 6-week-old male mice with 50 mg EGCG/ kg body weight by oral administration for 4 weeks, and then we i.p. injected 0.5 mg TNFα/kg body weight. After 2 hours, serum IL-6 levels were measured. As shown in Fig. 5D, EGCG dramatically decreased TNFα-induced serum levels of IL-6 compared with levels in the control group. Consistent with this, we also observed reduced levels of p65 acetylation in primary peritoneal
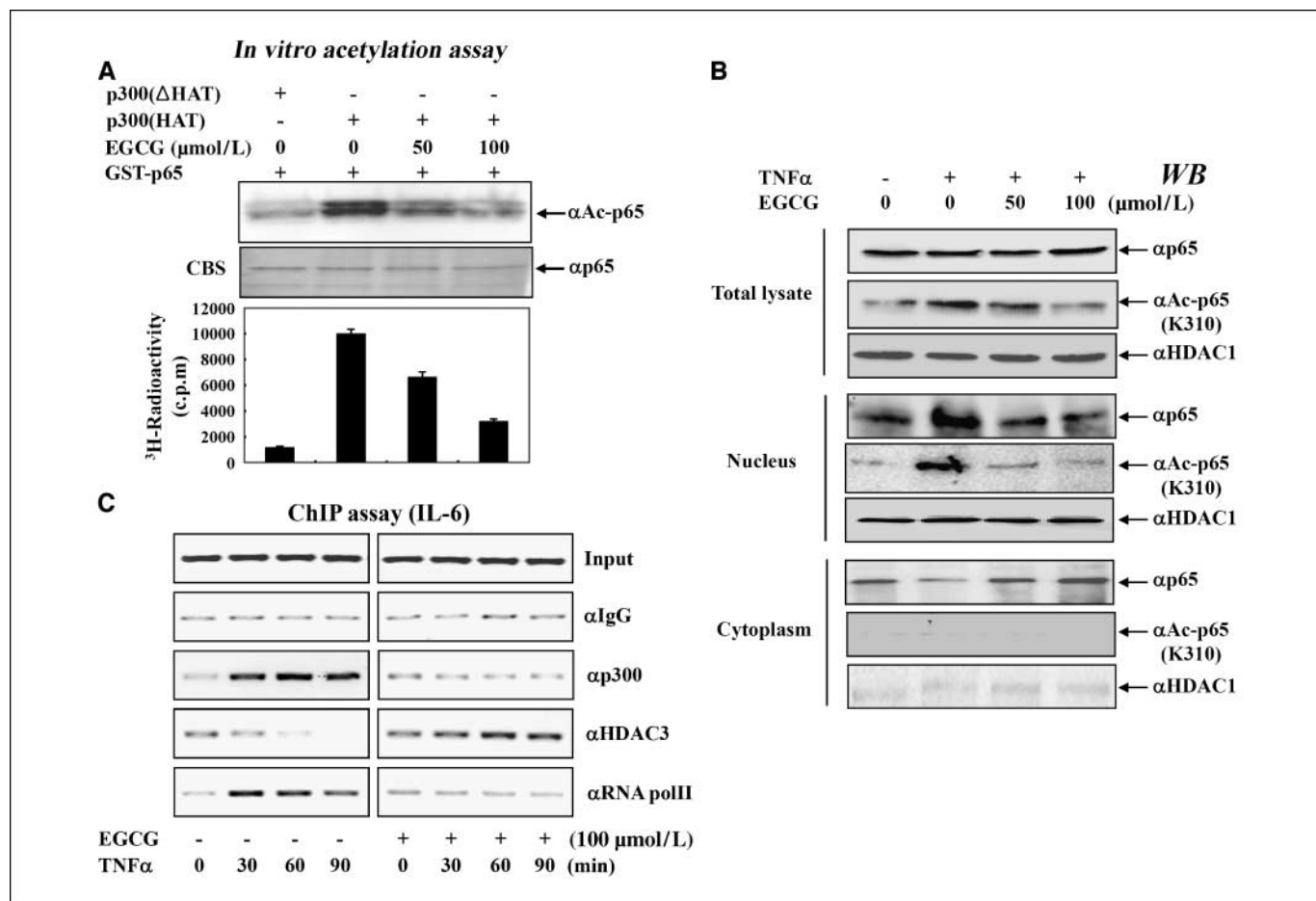

Figure 3. EGCG-induced hypoacetylation of p65 leads to an exchange between p300 and HDAC3 in the promoter region of the NF-κB–regulated gene *IL-6. A,* EGCG prevents p300-mediated p65 acetylation *in vitro*. Either recombinant GST-p300 or GST-p300 (Δ) was incubated with GST-p65 in the presence or absence of EGCG and subsequently processed for Western blot analysis using antibody against acetylated lysine (310) p65. *CBS,* Coomassie blue staining. *B,* EGCG treatment prevented TNFα-induced p65 acetylation *in vivo*. HEK293 cells were treated with TNFα and/or 100 μmol/L EGCG. The level of p65 acetylation was assayed by Western blot analysis using antibody against acetylated lysine (310) p65. *C,* EGCG-induced p65 hypoacetylation resulted in the association of HDA3 and the dissociation of p300 with the IL-6 promoter region. HEK293 cells were treated with TNFα and/or EGCG for various times as indicated. ChIP assay was then carried out to determine the binding of HDAC3 and p300.
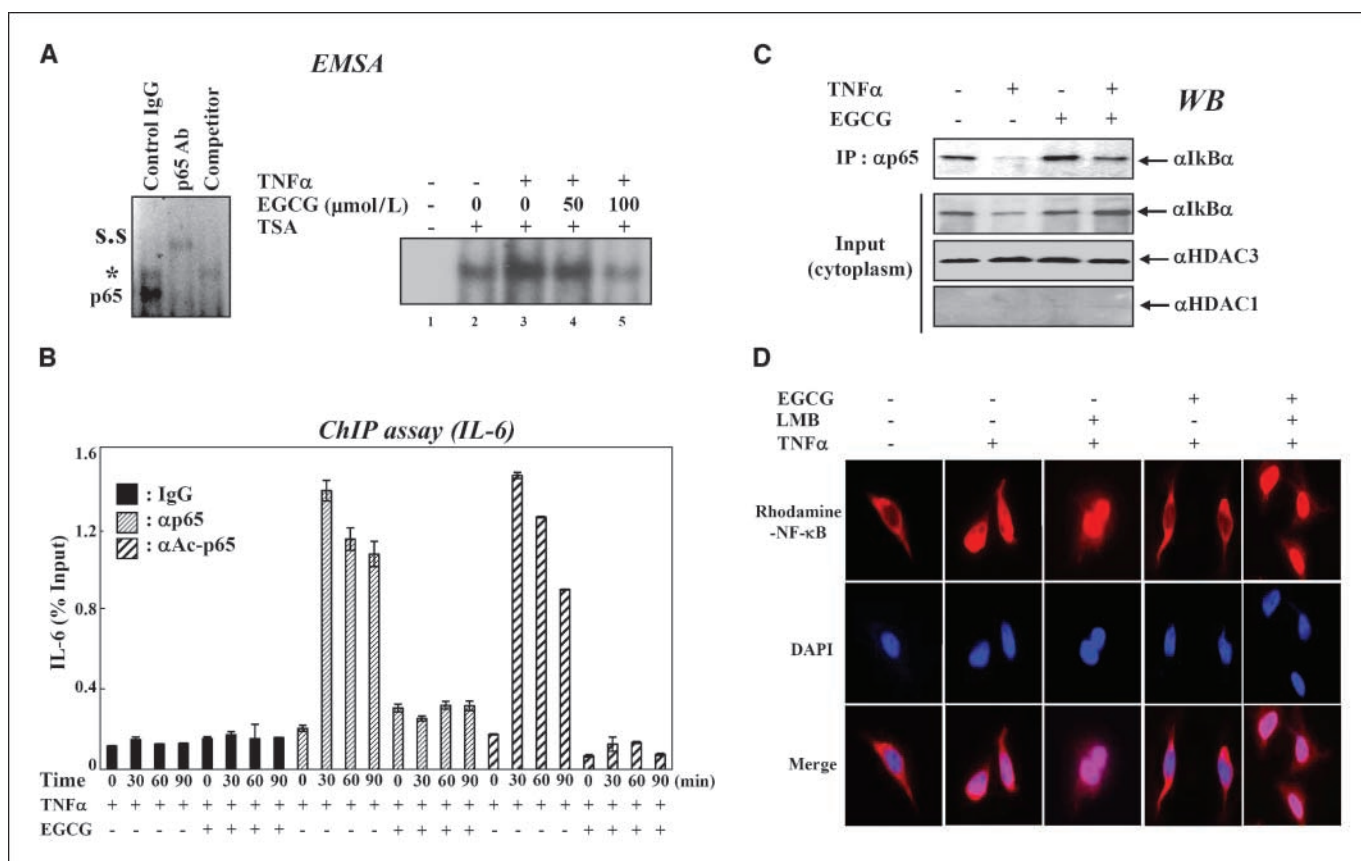
Figure 4. Hypoacetylation of p65 by EGCG treatment led to loss of NF-κB function. *A,* an electromobility assay was performed in the presence of TNFα and/or EGCG as indicated. EGCG treatment resulted in a dose-dependent reduction of DNA binding of NF-κB in cells treated with TNFα. Note that EGCG treatment reversed the effect of TSA on the DNA-binding activity of p65. The nonspecific band is indicated by *. *S.S,* supershift. *B,* the increased association of p65 and acetylated p65 with the *IL-6* gene was abolished on EGCG treatment. HEK293 cells were treated with TNFα and/or EGCG for various times as indicated. ChIP assay was then carried out to determine the binding of HDAC3 and p300. The results were analyzed by real-time PCR and shown as the percentage of input. *Columns,* mean of three independent experiments; *bars,* SD. *C,* EGCG treatment led to a cytoplasmic accumulation of IκBα. HEK293 cells were treated with TNFα and/or EGCG. Cytoplasmic extracts were prepared and an immunoprecipitation assay was processed for Western blot analysis. *D,* EGCG-induced hypoacetylation of p65 enhanced the nuclear export of p65 as assessed by immunostaining after treating with EGCG and LMB, a specific inhibitor of nuclear export.

macrophages following EGCG treatment (Supplementary Fig. S2*B*). Taken together, these data show that EGCG inhibits the TNFα-induced p65-dependent inflammatory response *in vitro* and *in vivo*.

Because multiple stimuli-mediated inflammatory responses have been reported, we next examined whether EGCG generally inhibits inflammatory responses by other stimuli, including lipopolysaccharide (LPS), IFN-γ, and IL-1β. EGCG efficiently blocked acetylation of p65 stimulated by LPS, IFN-γ, and IL-1β. Consistently, EGCG also inhibited the release of IL-6 by HEK293 cell cultures exposed to various inflammatory stimuli, suggesting that EGCG generally inhibits inflammatory responses (Supplementary Fig. S2*C*).

The acetylation of RelA (p65) is a potent therapeutic target for EBV-associated B lymphocyte transformation. We next tested the therapeutic potential of EGCG for the treatment of chronic inflammation–associated diseases. Chronic inflammation is linked to carcinogenesis in several organ systems and infection-related tumorigenesis, such as EBV-associated B-cell lymphoma and human papillomavirus–related cervical cancer (33, 34). Importantly, the continuous low-grade activation of NF-κB is required for EBV-induced B-cell transformation and its inhibition rapidly results in cell death (35). Therefore, we sought to test whether EGCG down-regulates the NF-κB signal induced by EBV

infection of B cells. As shown in Fig. 6*A*, EGCG treatment abrogated EBV-enhanced acetylation of p65 in the B cells. Consistent with this result, EGCG increased the level of cytosolic IκBα even in EBV-infected B cells. The activation of NF-κB by viral proteins is also related to the induction of proinflammatory cytokines, including IL-6 and IL-12, and these cytokines are elevated in EBV-associated cancer patients (36). Thus, we examined the effect of EGCG on the expression of proinflammatory cytokine genes on EBV infection. EBV infection dramatically increased the mRNA levels of *IL-6* and *IL-12,* whereas EGCG treatment reduced both mRNA and protein levels of these target genes (Fig. 6*B*). Moreover, Human Cytokine Antibody Array 3.0 analysis showed that levels of IL-4, IL-6, IL-12, transforming growth factor β, IFN-γ, and IL-1β were all increased from EBV-positive cells compared with controls. Conversely, treatment of EBV-positive cells with EGCG reversed the elevated cytokine expression profile in the EBV-positive B cells (Fig. 6*C*). Together, these results suggest that EGCG suppressed EBV infection-mediated induction of proinflammatory cytokines.

We next evaluated the ability of EGCG to prevent EBV-induced initiation of B-cell proliferation by using human PBMCs. Human PBMCs were incubated in the presence or absence of EBV virus obtained from induced B95.8 cells. After 3 weeks of incubation, the

wells were examined for outgrowth of clumps of proliferating B cells. EGCG was added just before virus infection and replenished weekly. Cells in uninfected controls and EBV-infected EGCG-pretreated wells were dying and no microscopic colonies were visible, whereas wells that were infected with EBV without EGCG pretreatment contained large numbers of macroscopic colonies (Fig. 6D). Thus, the EGCG inhibited the EBV-mediated B-cell transformation by blocking acetylation of p65. These data raise the possibility that HATis can be developed for chemoprevention at the tumor-promoting stage in lymphoma high-risk groups and for treatment of diverse inflammatory diseases.

Discussion

Dysregulation in acetylation of histones and other proteins has been linked to malignant transformation and other diseases (15).

NF-κB is also known to be activated in an acetylation-dependent manner on treatment with cytokines (18). Thus, acetylation of p65 increases its DNA-binding affinity, transcriptional activation, and IκBα disassembly. Coactivators, especially p300 and CBP, contain intrinsic HAT activity toward p65 and therefore can regulate inflammatory signals (19). Interestingly, the deacetylase inhibitor TSA was shown to potentiate the acetyltransferase activity of p300/CBP and thereby enhance NF-κB–dependent activation (20). This phenomenon created an opportunity for the identification of selective acetylation inhibitors for the attenuation of hyper-inflammatory responses.

During our ongoing screening study to identify natural compounds with anti-HAT activity, we first found potent anti-HAT activity in a green tea extract that contained polyphenol compounds such as EGCG. EGCG possessed a global specificity for the majority of HAT enzymes but not other epigenetic enzymes,
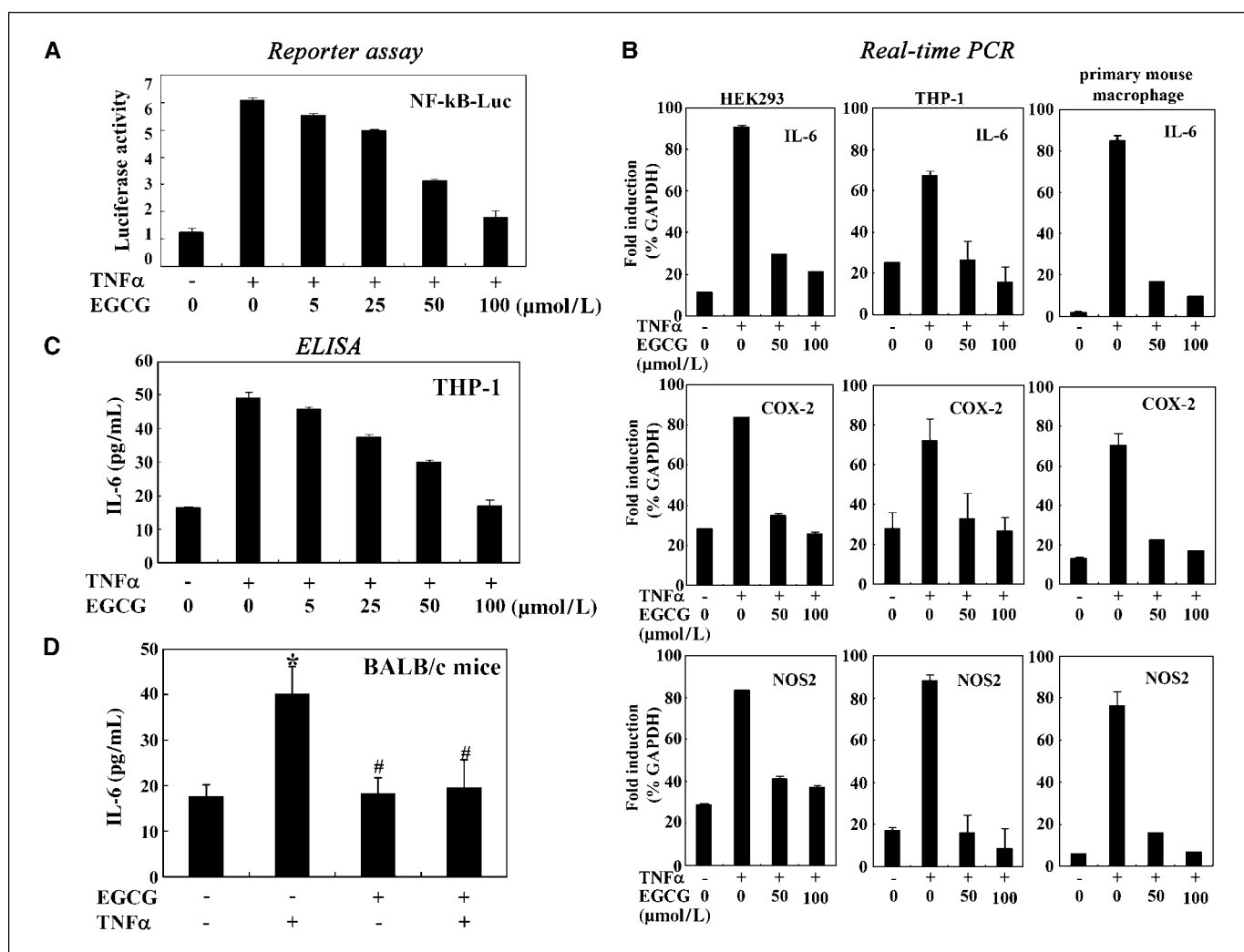

Figure 5. EGCG suppressed the TNFα-induced NF-κB–dependent inflammatory response. *A,* EGCG dose dependently inhibited TNFα-induced NF-κB–dependent promoter activity in HEK293 cells. HEK293 cells were transfected with a NF-κB–binding site-driven luciferase reporter plasmid and treated with TNF and/or various concentrations of EGCG. Whole-cell extracts were used in luciferase assay. Results are presented as the means of two independent experiments performed in triplicate. *B,* EGCG reduced mRNA levels of these target genes. Total RNA was prepared from each sample and used for quantitative RT-PCR to measure the expression of the NF-κB target genes. As controls, the level of GAPDH mRNA was also measured by quantitative RT-PCR using the same batch of RNA samples. *C,* EGCG treatment of cells resulted in a significant inhibition of the TNFα-induced IL-6 production. The release of IL-6 into the HEK293 cell culture medium was analyzed with an ELISA kit. *D,* EGCG inhibits the production of TNFα-induced inflammatory molecules *in vivo*. Male BALB/c mice were pretreated with the indicated doses of EGCG by oral administration for 4 wk, and then all were treated with TNFα as indicated. After 2 h, mice were killed, and serum was collected for serum IL-6 levels. A one-way ANOVA test was used to determine the effects of the treatment. *Columns,* mean (*n* = 8); *bars,* SD. *, $P < 0.05$ versus control; #, $P < 0.05$ versus TNFα treated.
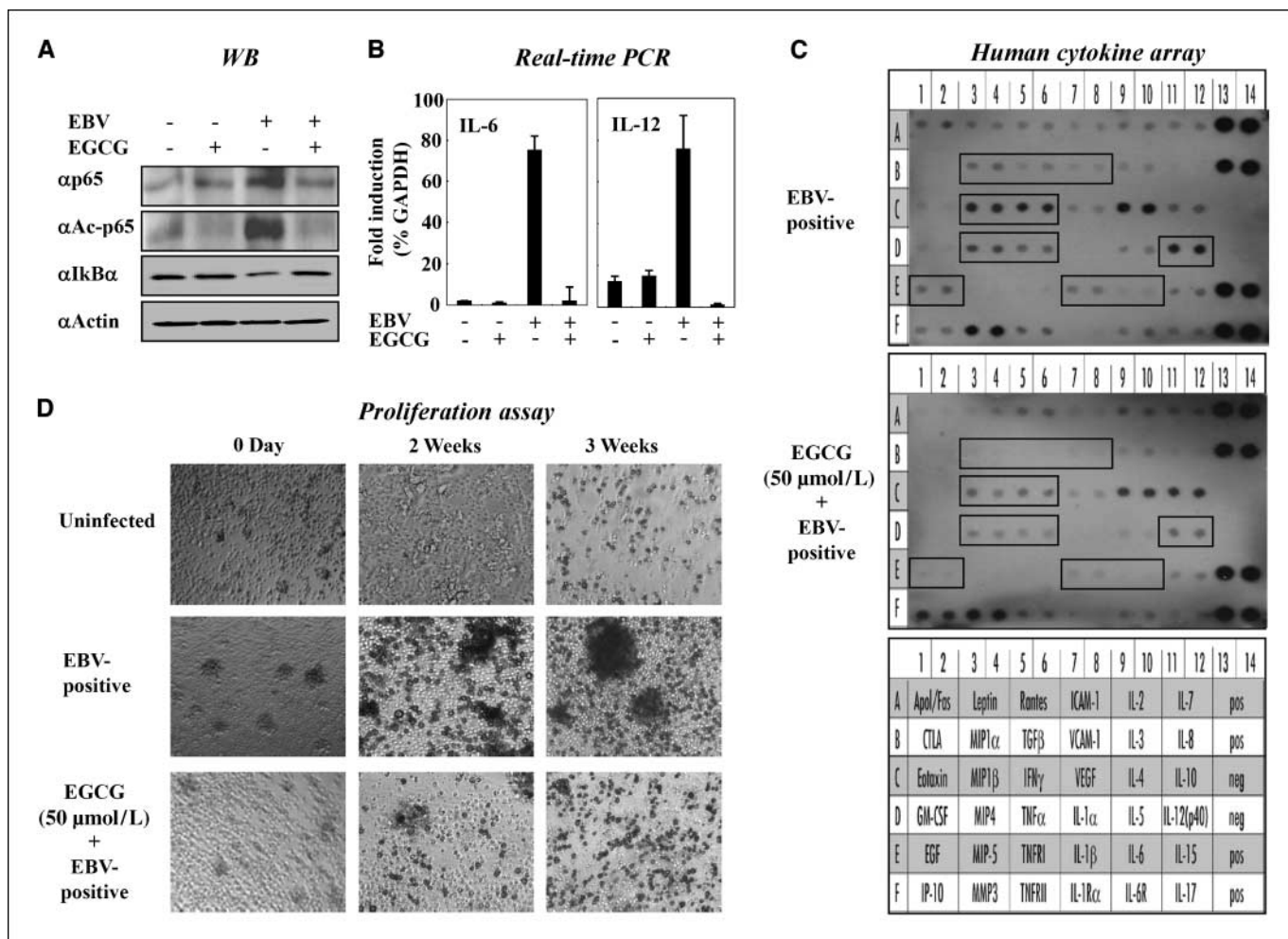
Figure 6. EGCG suppresses EBV-induced B lymphocyte transformation via inhibition of p65 acetylation. *A,* EGCG inhibited the EBV-mediated B-cell transformation by blocking acetylation of p65. The level of p65 acetylation was measured by Western blot analysis of the EBV-associated Burkitt's lymphoma cell lines in the presence or absence of EGCG. *B,* the effect of EGCG on the production of the proinflammatory cytokines IL-6 and IL-12 in EBV-positive cells was assessed by real-time PCR analysis. *C,* EGCG reversed the elevated cytokine expression profile in the EBV-positive B cells. Human Cytokine Antibody Array 3.0 membranes were incubated with conditioned medium from EBV-positive B cells untreated or treated with EGCG. Images were visualized using LAS 3000 image analyzer. *D,* EGCG inhibited the EBV-mediated B-cell transformation by blocking acetylation of p65. The outgrowth of proliferating B cells was examined by proliferation assay. Human PBMCs were incubated in the presence or absence of EBV virus obtained from induced B95.8 cells. After 3 wk of incubation, the wells were examined for the outgrowth of proliferating B cells by using phase-contrast microscopy.

including HDAC, SIRT, and HMTase. EGCG also reversed the acetylation of histone H3 and H4 by p300/CBP on the chromatin, showing that EGCG is a novel anti-HATi. The p65 acetylation has been shown to play a critical role in NF-κB–dependent activation. Although EGCG has been shown to suppress the inflammatory response through inhibition of NF-κB activation, there has been no evidence whether EGCG directly suppresses p300/CBP-mediated p65 acetylation and subsequent NF-κB activation. *In vitro* and *in vivo* acetylation assays with p65 as a substrate clearly showed that EGCG abrogated the p300-induced p65 acetylation. In addition, EGCG increased the level of cytosolic IκBα even in the presence of TNFα. Thus, our study provides firm evidence that EGCG suppresses p300/CBP-mediated p65 acetylation.

Another important result of this study is that EGCG reduced the binding of p300 to the promoter region of *IL-6* gene with an increased recruitment of HDAC3, which highlights the importance of the balance between HATs and HDACs in the NF-κB–mediated inflammatory signaling pathway. NF-κB has also been shown to

interact with HDAC3, which is crucial for the deacetylation of p65. We thus investigated our hypothesis that the balance between HDAC and HAT activity determines the level of p65 acetylation and subsequent expression of NF-κB–regulated genes. ChIP analysis showed that addition of EGCG resulted in increased association of HDAC3 with and dissociation of p300 from the NF-κB–binding site within the IL-6 gene promoter, which shows the importance of the HAT/HDAC balance in NF-κB–mediated inflammatory signaling. The importance of exchange between acetylation and deacetylation has been emphasized in the functional control of several proteins. The acetylation of the androgen receptor (AR) is also a key posttranslational modification regulating growth control in human prostate cancer cells. Like p65, AR is also acetylated by p300 and PCAF/TIP60, and acetylation of AR regulates the recruitment of coregulators to the basal transcriptional machinery of AR target genes (37). The balance between corepressors and coactivators determines the levels of histone and AR acetylation and subsequent transcription (31). Thus, our study provides firm evidence to

support the hypothesis that HAT coactivators compete with HDAC corepressors for binding to promoter regions and/or protein substrates and determine the level of transcription.

Chronic inflammation is linked to carcinogenesis in several organ systems (33, 34). Our findings in this study imply that EGCG can mediate a host defense system against environmental pathogens. EGCG inhibited p65 acetylation and overexpression of NF-κB target genes induced by EBV viral proteins implicated in B-cell transformation and the development of EBV-associated malignancies. EBV is a lymphotropic virus associated with a variety of human malignancies, including Burkitt's lymphoma, nasopharyngeal carcinoma, and acquired immunodeficiency syndrome–associated lymphomas (38). EBV reverse genetic analyses in the context of primary B lymphocyte transformation indicate that the latent viral proteins EBNA2, EBNA3A, EBNA3C, and LMP1 have effects on cell growth regulation and are required for EBV latent infection and B-cell transformation (39, 40). In particular, LMP1 constitutively activates NF-κB signaling pathways, and NF-κB activation up-regulates antiapoptotic genes, such as *A20, Bfl-1*, and *BCL-2*, which contribute to B-cell transformation (41, 42). As NF-κB activation is one of the critical events during EBV-induced B-cell transformation, the inhibition of NF-κB activation could be a potential target for the prevention of EBV-associated malignancies. Importantly, we found that EBV-induced B-cell outgrowth was dramatically inhibited by treatment with EGCG, supporting the important role of acetylation in cancer initiation and progression.

EGCG is known to have anti-NF-κB activation activity in sepsis and autoimmune encephalomyelitis as well as in specific cell types, such as vascular smooth muscle cells, and various cancer cell types (43–45). However, it remains unclear how EGCG suppresses NF-κB–dependent signaling. EGCG was recently shown to inhibit cigarette smoke condensate–induced p65 phosphorylation in normal human bronchial epithelial cells (46). EGCG also seems to indirectly inhibit IκB kinase activity in epithelial cells (47). Thus, these results raise the possibility that EGCG directly or indirectly inhibits the activities of protein kinases involved in p65 phosphorylation. However, EGCG already has been reported to be a nonspecific inhibitor of various kinases involved in the phosphorylation of p65, with the exception of two protein kinases: tyrosine-phosphorylated and regulated kinase 1A and p38-regulated/activated kinase (48). Consistent with this result, we also

failed to detect the inhibitory effect of EGCG on protein kinases involved in p65 phosphorylation, including protein kinase A, protein kinase C, CK2, and glycogen synthase kinase 3β (data not shown). Based on these results, the mechanism of anti-NF-κB activation by EGCG is closely related to the inhibition of p65 acetylation rather than the direct inhibition of p65 phosphorylation.

In conclusion, we have provided evidence that EGCG, a HATi, inhibits p65 acetylation-dependent NF-κB activation and suppresses the tumor-promoting stage in lymphoma. Similar to the use of HDAC inhibitors, the disruption of histones and nonhistone protein acetylation can be exploited to develop new anticancer drugs. It seems contradictory to find both HAT and HDAC inhibitors because these enzymes have opposing catalytic actions. However, the molecular basis of HATs and HDACs is complex and still being elucidated, and the molecular mechanisms of reversible acetylation in cancer development are not likely to be simple. Until now, dysfunction of HATs has been found to be associated with several diseases, such as cardiac hypertrophy, asthma, and cancer. In all of these diseases, the cellular histone and nonhistone proteins are hyperacetylated. Therefore, the work described here has shown that selective and signaling-dependent interferences of protein acetylation by HATi, like that of EGCG, will be useful pharmacologic tools to enhance our understanding of acetylation events in cellular function and may lead to a new class of therapeutic or chemopreventive drugs.

Disclosure of Potential Conflicts of Interest

Acknowledgments

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