Histone acetylation regulates the cell type specific CIITA promoters, MHC class II expression and antigen presentation in tumor cells

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Abstract

The regulation of MHC class II expression by the class II transactivator (CIITA) is complex and differs in various cell types depending on the relative activity of three CIITA promoters. Here we show that, in plasma cell tumors, the deacetylase inhibitor trichostatin A (TSA) elicits PIII-CIITA but does not activate the IFN-γ-inducible PIV-CIITA promoter. In trophoblast cells, all CIITA promoter types are constitutively silent and not induced by IFN-γ or TSA treatment. TSA induction of PI-CIITA was restricted to macrophage and dendritic cell lines. In the Colon 26 tumor IFN-γ induced endogenous PIV-CIITA but not PII-CIITA while TSA activated class II in the apparent absence of CIITA. Reporter assays in Colon 26 showed that TSA induced PII-CIITA but not PIV-CIITA. Transfection of a dominant negative CIITA plasmid in Colon 26 inhibited induction of class II by IFN-γ but not TSA. Thus, the potential for both CIITA-dependent and -independent pathways of MHC induction exists within a single cell. Further evidence of CIITA-independent class II expression elicited by TSA was obtained using knockout mice with defects in CIITA, STAT-1α and IRF-1 expression. TSA treatment can also activate class II expression in mutant cell lines with deficiencies in signaling molecules, transcription factors and the BRG-1 cofactor that are required for IFN-γ-induced CIITA expression. Importantly, after epigenetic activation by the deacetylase inhibitor, MHC class II is transported and displayed on the cell surface of a plasma cell tumor and it is converted to an efficient antigen presenting cell for protein and class II-peptide presentation.

Introduction

Covalent modifications of chromatin structure are well-established regulators of gene expression and growing evidence suggests that this includes many of the genes involved in immunity. An array of covalent modifications (acetylation, phosphorylation, methylation, ubiquitination and sumoylation) targets histones. These modifications appear to be inter-dependent and represent an evolutionarily conserved ‘histone code’ that alters the interactions of histones with DNA as well as marking binding sites on histones for effector proteins that regulate gene expression (1). Activation of promoters is accompanied by an ordered recruitment of transcription and chromatin-remodeling factors in which acetylation of histones often, but not always, precedes and is required for stable binding of remodeling complexes such as SWI/SNF (2). Acetylation of chromatin by agents that inhibit histone deacetylases (HDAC), such as trichostatin A (TSA), can directly or indirectly control other epigenetic modifications of histones, such as lysine methylation or ubiquitination and serine/threonine phosphorylation as well as DNA methylation (1, 3). Histone H3K9 methylation requires prior deacetylation and provides a binding site for the heterochromatin protein-1 (HP-1) that mediates heterochromatin silencing. Treatment with TSA can disrupt higher-order chromatin structure and inhibit heterochromatin pericentric staining with anti-HP-1 antibody (4). Certain genes, including MHC class II, have an upstream locus control region (LCR) which adds an additional layer of transcriptional regulation (5). The MHC class II LCR binds class II transactivator (CIITA) and the RFX-box-binding
proteins and induces long-range histone acetylation of downstream lysines. Deacetylase inhibitors, such as TSA, induce both targeted and also global acetylation and the transcriptional alteration accompanying deacetylase inhibitor treatments results from both the overall charge effects on histones as well as local residue-specific effects (6). Although many genes are transcriptionally activated by acetylation of histones, in some genes, HDACs are essential for activation and, in these cases, TSA may elicit repression (7, 8). In addition, acetylation can occur on non-histone proteins, such as transcription factors and cofactors (9), and alter their nuclear transport, DNA binding, interaction with other proteins and activity. Several previous studies of mouse and human tumor cells have shown that HDACs can repress MHC class II expression and that class II is activated by treatment with HDAC inhibitors (10, 11).

A subset of normal cells, that includes plasma cells and trophoblasts, as well as tumors arising from them, are MHC class II negative and expression is not inducible by IFN-γ. When either plasma cells or trophoblasts are fused to a class II-expressing B cell, the resulting hybridoma is class II negative, suggesting that both cell types may carry an inhibitor of MHC class II expression (12–14). The demonstration that high levels of the zinc finger DNA-binding protein Blimp-1 in plasma cells repress CIITA (15) suggests that it may recruit HDACs to the CIITA promoter. However, a recent report demonstrates that CIITA repression by Blimp-1 does not require deacetylase activity (16). Trophoblast cells may also produce an inhibitor of CIITA (17, 18), but since there is little, if any, Blimp-1 in trophoblast cells (S. Chou, unpublished results), the mechanism of silencing of CIITA in trophoblasts may be different from that of plasma cells.

CIITA is a ‘master’ regulator of MHC class II, and quantitative levels of class II in normal tissues parallel CIITA expression (19). CIITA knockout mice are markedly deficient in class II compared with wild-type mice [100-fold by quantitative measurement on dendritic cells (DCs)] (20). However, CIITA knockouts contain, by immunohistology, many more class II-expressing cells than do MHC class II knockouts which are essentially devoid of MHC staining (21, 22). This suggests the existence of additional mechanisms other than CIITA for activating class II expression. It is also unclear whether various class II negative tumor cells, including those derived from MHC class II-negative cells, utilize common mechanisms of class II silencing and whether this is based on chromatin structure. In this study, we use TSA as a probe to determine whether distinct patterns of chromatin repression may exist in tumors. Moreover, if chromatin repression is reversible, a tumor cell might be induced to express class II and, with the appropriate co-stimulatory molecules, be converted to an effective antigen presenting cell (APC). This could potentially contribute to tumor immunity in a fashion similar to that described for tumor vaccines generated with genetically modified tumor cells transfected for class II and co-stimulatory molecule expression (23). A recent study with HDAC inhibitor-altered tumor cells suggests their potential as an epigenetic tumor vaccine (24).

Our previously reported studies showed that the deacetylase inhibitor TSA activates MHC class II and CD40 in the murine J558 plasmacytoma cell and the Colon 26 tumor (10). Here, we extend this work and show distinct patterns of chromatin regulation of the cell-type-specific CIITAs in different tumor types. In plasma cells and several other cells of B lineage, TSA induces PIII-CIITA and class II expression while TSA induction of class II in Colon 26 and trophoblast cells is independent of CIITA. Using dominant negative CIITA transfection, we showed that the Colon 26 tumor line possessed both an IFN-γ-activated CIITA-dependent and a TSA-induced CIITA-independent pathway of class II expression. Further evidence that class II expression does not require CIITA was obtained in three types of knockout mice having defects in CIITA itself and/or components of its signaling pathway. TSA treatment also enhanced class II expression in mutant cell lines that lack signaling molecules or transcription factors and cofactors shown to be involved in the IFN-γ-induced class II expression pathway. MHC class II induction was found following TSA treatment in a cell defective in the BRG-1 and Brm chromatin-remodeling factors. That functional MHC class II protein can be induced by TSA and transported to the surface of tumor cells was demonstrated in a murine plasmacytoma by flow cytometry and the ability of deacetylase inhibitor-treated tumor cells to present antigen and class II-restricted peptides. This work suggests that chromatin can inhibit antigen presentation in certain tumor cells and that repression is potentially reversible by treatments that alter cellular acetylation patterns.

**Methods**

**Mice and cell culture**

Mouse plasmacytomas J558 and MPC-11, B cell line A20, macrophage cell line IC-21, DC-like cell JAWSII, embryonal carcinoma cell lines P19, trophoblast cell line SM9-1, human epithelial cell line HeLa, trophoblast line JAR and human small cell carcinoma SW-13 were cultured as described by American Type Tissue Collection. Mouse adenocarcinoma Colon 26 was provided by E. A. Repasky, the mouse trophoblast cell line SM9-1 by J. Hunt, mouse pro-B cell line L1210 by H. Fuji and the CIITA-deficient human B cell line RJ2.2.5 by C. H. Chang. For short-term primary kidney cultures, aseptically dissected kidneys were minced, disrupted by passage through nylon mesh and incubated for 6 h with collagenase (100 U ml⁻¹) (Worthington Biochemical Corporation, Freehold, NJ, USA) and 300 µM CaCl₂. Intact cells were recovered after density centrifugation with Ficoll-Paque™ PLUS (Amersham Biosciences, Piscataway, NJ, USA). The interface was collected, washed with RPMI 1640 and re-suspended in Opti-MEM supplemented with 4% fetal bovine serum, 50 µM β-mercaptoethanol, 4 µg ml⁻¹ insulin, 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin (Invitrogen, Grand Island, NY, USA). After culture for 4–7 days, cells were treated with TSA (Wako, Richmond, VA, USA) or recombinant IFN-γ (R&D Systems, Minneapolis, MN, USA) at concentrations and time periods indicated subsequently. C57BL/6 and BALB/c mice were provided by the Department of Laboratory Animal Resources at Roswell Park Cancer Institute. CIITA knockout mice, B6.129S2-CIita<sup>tm1Cumo</sup> (CIITA<sup>−/−</sup>), were provided by C. H. Chang (21). STAT-1α knockout mice, 129S6/SvEv-STAT<sup>tm1</sup> (STAT-1α<sup>−/−</sup>), were purchased...
from Taconic (Germantown, NY, USA) and IRF-1 knockout mice, B6.129s2-Ifn1<sup>tm1Mak</sup> (IRF-1<sup>−/−</sup>), from the Jackson Laboratory (Bar Harbor, ME, USA). All animal use complied with all relevant federal guidelines and institutional policies.

**Transient transfections and plasmid constructs**

Plasmid EdCIITA [green fluorescent protein (GFP)-dominant negative CIITA] was kindly provided by M. Peterlin. The EdCIITA construct contains a truncated human CIITA (residue 302–1130) fused to the GFP (25). The BRG-1 expression vector pREP7-BRG-1 was provided by K. Zhao (26). The pGL3-III contains the −1 to 322 region of the human PIIICIIA promoter without the upstream IFN-γ-responsive element. The pGL3-PIV reporter construct contains the −1 to −354 region of the human PIV-CIIA promoter region. Transfection was carried out with the Effectene kit (Qiagen, Valencia, CA, USA) using 2 μg of plasmid for most transfections and 4 μg for transfection of the prREP7-BRG1 plasmid; IFN-γ and TSA treatments began 4 h after transfection.

**RT-PCR and real-time quantitative RT-PCR**

Preliminary titration with IFN-γ (10–500 U ml<sup>−1</sup>) and TSA (1−250 nM) for 6−72 h was performed to determine expression levels. Each cell type varies in the optimal concentrations of IFN-γ and TSA giving maximal expression. RNA samples were harvested from cell cultures using the RNeasy kit (Qiagen). Reverse transcription (RT) reactions were performed with 2 μg of RNA and Superscript II<sup>TM</sup> RNase H<sup>−</sup> reverse transcriptase (Invitrogen). Hot start PCR was performed with 35 cycles of 30 s at 94°C and 1 min at 60°C [23 cycles for glyceraldehyde-3-phosphate dehydrogenase (GAPDH)], and was within the linear range of amplification. Real-time PCR was performed as previously described (10). RT-PCR primer sets for mouse CIITA, human CIITA, IAα and mouse GAPDH were as previously described (10). Primer and probe sets used in real-time quantitative RT-PCR for the analysis of mouse CIITA, IAα and GAPDH and human CIITA can be found at http://groups.yahoo.com/group/apcrlistserver/files/. Primers and probes for the human and mouse promoter-specific CIITA are listed in Supplementary Data (available at International Immunology Online). For real-time RT-PCR data analysis, the ΔΔCt method was used to calculate fold change from the untreated sample (10). GAPDH expression was shown to be unaffected under our treatment conditions and was used as reference gene for normalization of Cts (ΔCt). Samples that did not show amplification by 40 cycles were considered not detectable. The limits of linear detection for each gene determined by validation experiments were used as Ct-values for the undetectable control samples for the calculation of fold change. In these cases, the fold change indicated is a minimum fold change and is likely to underestimate the actual change. Each experiment included triplicate samples from each treatment and error bars represent the range of fold change calculated from the triplicates. A20 or Raji RNA was included in every assay as a positive control for CIITA and class II and to monitor inter-assay reliability. Experiments were repeated several times and representative data are displayed in figures and tables.

**Reporter assays**

Transfection of the reporter constructs was performed as described above. A β-galactosidase expression plasmid (0.2 μg) was co-transfected with the reporter constructs as a control for transfection efficiency. Cell extracts were prepared in passive lysis buffer (Promega) and incubated on ice for 15 min followed by centrifugation. Protein concentration was determined by Micro BCA protein assay (Pierce, Rockford, IL, USA). β-Galactosidase assays were performed for normalization based on transfection efficiency on each sample. The amount of protein extract used for the luciferase assays was based on normalization to the β-galactosidase activity. Both β-galactosidase and luciferase assays were performed according to standard procedures provided by the manufacturer (Promega). The luciferase assay data are presented as relative fold change by comparing the raw luminescence units (RLUs) of each sample with the RLU of the untreated sample.

**Western blotting**

Cell extracts were prepared in RIPA buffer (0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40 in 1× PBS) and incubated on ice for 30 min followed by passage through a 21G needle. Cell lysates were clarified by centrifugation. For western blot analysis, 40 μg of lysate samples were re-suspended in SDS sample buffer plus 0.13 M dithiothreitol, separated on 12% SDS-PAGE gels and transferred to Immobilon-P membrane (Millipore, Bedford, MA, USA). Primary polyclonal antibodies used were rabbit anti-IRF-1 polyclonal IgG, anti-phosphorylated STAT-1α (Tyr 701) and anti-STAT-1 (Santa Cruz, Santa Cruz, CA, USA). Goat anti-rabbit HRP (Promega) was used as the secondary antibody. Blots were developed with the western blotting luminol reagent kit (Santa Cruz). Cell extracts were harvested at different time points for assessment of optimal target protein expression.

**Antigen presentation and T cell proliferation assays**

Ppolitic lymph node T cells were isolated from BALB/c mice 7 days after priming by footpad injection of ovalbumin (ova) (Sigma, St Louis, MO, USA) in CFA (DIFCO Laboratories, Detroit, MI, USA) and purified over nylon wool (Polyscience, Warrington, PA, USA). Peritoneal exudate cells elicited by thioglycolate (3%) were isolated from BALB/c mice 3 days after intra-peritoneal injection and plated in plastic tissue culture plates. After 2 h of culture at 37°C in 5% CO<sub>2</sub>, the adherent population was recovered and these peritoneal macrophage (p<sup>mac</sup>) cells were used as positive controls. Irradiated p<sup>mac</sup>, TSA-treated J558 (25 nM for 24 h) or untreated irradiated J558 (40 Gy) were used as stimulators and incubated in triplicate in 96-well flat-bottom plates for 5 days with equal numbers (1 × 10<sup>5</sup>) of T cells as responders. The responder:stimulator ratio and incubation period used in these assays were chosen after multiple titration experiments with various cell numbers (2.5 × 10<sup>5</sup> to 5 × 10<sup>5</sup>) and different periods of incubation (3–5 days). Antigen additions were 10 μM ova, ova-peptide<sub>322–338</sub> or alanine-substituted (E333A) ova-peptide<sub>322–338</sub> in RPMI 1640 with 1.5% syngeneic mouse serum, 10 mM HEPEs and 50 μM β-mercaptoethanol. The optimal antigen concentration (10 μM) was chosen after
multiple antigen presentation experiments with different concentrations of ova (5–100 μM) and ova-peptide$_{322-338}$(2.5–50 μM). The ova-peptide$_{322-338}$ was specifically presented by APCs in the context of H-2d MHC class II to stimulate CD4$^+$ T cell proliferation. The alanine-substituted (E333A) ova-peptide$_{322-338}$, used as a negative control, was defective in binding to the TCR (27). T cell proliferation was measured by $[^{3}H]$thymidine (1 μCi per well) incorporation for the final 18 h of culture. Results are expressed as net counts per minute (c.p.m.) [average c.p.m. from mixed cultures of T cells with J558 cells – (average c.p.m. from parallel cultures without T cells + c.p.m. from cultures with T cells only)]. Stimulation index (SI) was calculated as (average c.p.m. from parallel cultures without T cells + c.p.m. from cultures with T cells only).

Results

MHC class II expression patterns in different tumor types
Previous studies have shown that histone acetylation status may be important in determining expression of MHC genes in certain tumors (10). To further clarify the role of histone acetylation in the silencing of MHC class II and the cell-type-specific co-activator (CIITA) genes in specific tumor types, we treated various tumor types with TSA in vitro. Cells were titrated with IFN-γ (10–500 U ml$^{-1}$) or TSA (1–250 nM) for 6–72 h and mRNA for CIITA and class II expression were determined by RT-PCR and real-time quantitative RT-PCR. Figure 1 represents CIITA and class II expression at the optimal concentration and time point for each cell line employed. The expression pattern of MPC-11 is typical of murine plasmacytomas. Both CIITA and class II are inducible by TSA but not by IFN-γ (Fig. 1) in MPC-11 and four other plasma cell tumors (J558, P3X63Ag8, XS-63 and XC1.5/51) (data not shown). Another type of response was shown by the mouse SM9-1 trophoblast cell line (Fig. 1), TSA-activated class II but CIITA mRNA was undetectable by real-time RT-PCR. Essentially, identical data were obtained with the human JAR and JEG-3 choriocarcinoma tumors using real-time semi-quantitative RT-PCR and the rat R8RP3 trophoblast cell line employing RT-PCR analysis (data not shown). This pattern of expression is not, however, unique to trophoblast tumors and has been found in a human neuroblastoma (SK-N-MC) and, sporadically, in several other human and mouse tumor types (10) (data not shown). The Colon 26 tumor is a useful model to study MHC expression and demonstrates that a tumor cell can possess both a CIITA-dependent class II response elicited by IFN-γ and a TSA-induced class II response apparently independent of CIITA at 48 h (Fig. 1). Class II expression induced by IFN-γ has been shown, by chromatin immunoprecipitation (ChIP) assay, to correlate with enhanced histone acetylation at the class II promoter (11). That TSA induces acetylation at the class II promoter after TSA treatment was demonstrated by ChIP assays on TSA-treated Colon 26 cells using anti-acetylated histone H3 and H4 antibodies (data not shown).

MHC class II expression in knockout mice and mutant cell lines after deacetylase inhibitor treatment
The above data suggest that, although activation of class II expression by CIITA is important in most cells, in others, such as trophoblast and Colon 26 tumor cells, a pathway for MHC class II induction exists which does not involve CIITA. To further explore the requirement for CIITA in class II expression, we examined mice with defects in CIITA or the pathway for CIITA induction. Exploration of short-term cultures of various tissues from normal mice revealed that kidney cells were responsive to IFN-γ and maintained good viability for 24 h in TSA (250 nM)-treated cultures. We therefore established kidney cultures from CIITA, STAT-1$^+$ and IRF-1 knockout mice. The data in Fig. 2(A) demonstrate that CIITA$^{-/-}$ mouse kidney cultures can be induced to express mRNA for class II by TSA, but not IFN-γ treatment. Since STAT-1 and IRF-1, together with the constitutively expressed E-box factor, USF-1, are required for IFN-γ induction of CIITA, it is noteworthy that kidney cells from STAT-1$^+$ and IRF-1$^+$ mice treated in vitro with TSA expressed significant levels of class II in the absence of CIITA while, as expected, these cells were refractory to IFN-γ (Fig. 2A). The origin of the low levels of constitutive class II found in the kidney culture of STAT-1$^+$ animals is unclear but is consistent with a previous report of class II expression in STAT-1$^+$ animals (28). These transcripts were shown to be class II by sequence analysis and mice are indeed STAT-1$^{-/-}$ as shown by the failure of IFN-γ to elicit IRF-1 (data not shown) or enhance class II in the kidney cultures (Fig. 2A). Also, the overall expression of class II analyzed by flow cytometry was lower in the STAT-1$^{-/-}$ spleen cells when compared with wild-type mice and the levels were not altered after IFN-γ treatment (data not shown).

In addition to the experiments with knockout mice, we extended these studies to several mutant cell lines with established deficiencies in the IFN-γ signaling pathway. As shown in Table 1, TSA can elicit class II expression in the absence of JAK-1, STAT-1 and IRF-1, each of which are signaling molecules critical to IFN-γ induction of class II. Also, human RJ2.2.5 B cells that lack functional CIITA can be
Fig. 2. TSA induced expression of class II in knockout mice and a mutant cell line with defects in CIITA and class II regulatory pathways. (A) CIITA-independent MHC class II expression in primary cultures from CIITA, IRF-1 and STAT-1 knockout mice. Kidney cells were harvested from C57BL/6 and the specified knockout mice and treated with 100 U ml$^{-1}$ IFN-$\gamma$ or 250 nM TSA for 24 h. RT-PCR was performed for analysis of CIITA, MHC class II and GAPDH mRNA expression. IFN-$\gamma$ elicited both CIITA and class II expression only in normal kidney cultures, while in cultures from the wild type and all three knockouts, TSA induced class II but not CIITA expression. Untreated STAT-1$^{-/-}$/ kidney cells display a low level of class II expression which was not enhanced by IFN-$\gamma$. (B) BRG-1, the ATPase subunit of the SWI/SNF chromatin-remodeling complex, is not required for the induction of MHC class II expression by TSA. Class II expression in the BRG-1-deficient SW-13 cells was compared with BRG-1-transfected SW-13 by RT-PCR. Cells were untreated (lanes 1 and 4), treated with 100 U ml$^{-1}$ IFN-$\gamma$ (lanes 2 and 5) or 100 nM TSA (lanes 3 and 6) for 24 h. Negative (no reverse transcriptase) and positive (Raji human B cell line) controls are shown in lane 7 and lane 8, respectively. (C) The same samples were assayed by real-time quantitative RT-PCR for HLA-DR expression. Fold change was calculated by normalization to GAPDH levels followed by comparison with an untreated sample.

induced by TSA to express both class II mRNA (10) and cell-surface HLA-DR (data not shown). Since IFN-$\gamma$ induction of CIITA is dependent on BRG-1 (29), and BRG-1 is recruited to the class II promoter upon activation by IFN-$\gamma$ (30), we queried whether BRG-1 was required for TSA induction of class II. The RT-PCR results shown in Fig. 2(B) demonstrated class II induction by TSA in SW-13 cells deficient in the BRG-1 subunit required for SWI/SNF activity. The induction of class II by IFN-$\gamma$ was rescued when a wild-type BRG-1 expression vector was transfected into SW-13. Real-time quantitative RT-PCR analy-

Table 1. Induction of MHC class II expression by TSA treatment in mutant cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Defect</th>
<th>Untreated</th>
<th>IFN-$\gamma$</th>
<th>TSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SW-13</td>
<td>BRG-1</td>
<td>–</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>RJ2.2.5</td>
<td>CIITA</td>
<td>–</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>J558</td>
<td>JAK-1</td>
<td>–</td>
<td>–</td>
<td>+++</td>
</tr>
<tr>
<td>P19</td>
<td>IRF-1</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
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</table>

Levels of class II expression were determined by real-time quantitative RT-PCR following treatment with IFN-$\gamma$ and TSA and for untreated cells. The plus signs used in the columns correspond to fold changes in mRNA relative to the untreated control: (−) = ≤5-fold, (+) = 5- to 10-fold, (+++) = 10$^{-2}$- to 10$^{-3}$-fold.

Expression of a dominant negative CIITA reveals the CIITA-dependent and -independent pathways of class II expression in a single cell

Because of the low levels of CIITA required to activate class II expression, additional experiments was constructed to determine if the presence of small amounts of functional CIITA were responsible for the TSA-induced class II in Colon 26. Previous reports have identified mutant CIITA constructs that can function as dominant negative repressors of class II expression (25, 34). A dominant negative CIITA (EdCIITA) construct was transiently transfected into Colon 26 cells. This construct contains a truncated human CIITA gene and produces a CIITA protein lacking a functional N-terminal transactivation domain (25). The dominant negative CIITA transfected Colon 26 cells were treated with either TSA or IFN-$\gamma$ and the RNA harvested for RT-PCR analysis. The data, in Fig. 3(A) and (B), demonstrate that the dominant negative CIITA (human CIITA) inhibited MHC class II induction by IFN-$\gamma$ but did not alter the class II TSA response. We obtained similar results using another DN-CIITA (34). These results demonstrate that, within a single cell, two pathways of class II induction can be distinguished: one activated by IFN-$\gamma$ and dependent on CIITA and the other elicited by TSA independent of CIITA.
component of the IFN-γ response in certain cells such as HeLa. Since each of the cell lines examined has a different basal level and capacity for induction by IFN-γ and TSA, for ease of comparison between various cell lines, the data (Table 2) are presented as plus signs over a range of values based on the relative changes in real-time PCR Ct-levels (see Methods). The data demonstrate that TSA selectively activates the B cell PIII-CIITA promoter in the J558 plasmacytoma, the L1210 pro-B cell and the A20 immature B cell. In none of the cells examined here (listed in Table 2) did TSA treatment significantly elevate levels of the PIV promoter. Of interest, is the observation that in HeLa cells TSA elicited low levels of PIIICITA with little or no PIV- or PI-CIITA while IFN-γ elicited high levels of PIV-CIITA and moderate PIII-CIITA (Table 2). In addition to activating PIII-CIITA in B cells, TSA also elicited PI-CIITA, but exclusively in the macrophage IC-21 and the JAWSII DC-like cell lines. Similar to HeLa cells, IFN-γ treatment activated both PIV and, to a lesser extent, PIII-CIITA in IC-21. In Colon 26, TSA did not induce detectable CIITA of any type while IFN-γ elicited PIV-CIITA. The specificity of CIITA promoter activation was also shown in transient transfection assays of PIII and PIV reporter constructs in Colon 26 cells. The PIII reporter employed contained only the proximal promoter region and is not IFN-γ inducible (39). The data in Fig. 4 demonstrate that TSA treatment enhanced CIITA promoter III activity while IFN-γ treatment activated only the PIV promoter. This suggests that TSA either activates or enhances a transcription factor binding uniquely to the PIII promoter or that some chromatin structure does exist on these transient promoters which differ for PIII- and PIV-CIITA.

HDACs may be involved in both activation and repression of CIITA and class II
Exposure of Colon 26 to IFN-γ elicits phosphorylated STAT-1 and a high level of IRF-1 proteins (Fig. 5A) both of which are required for PIV-CIITA activation. In contrast, TSA treatment does not induce significant IRF-1 or phosphorylated STAT-1 proteins in Colon 26 (Fig. 5A). Consistent with these observations, cyclohexamide inhibited IFN-γ activation of class II mRNA in Colon 26 but not the TSA induction of class II mRNA (data not shown).

It has been reported that activation of STAT-1 signaling involves the phosphorylation of STAT-1α (Tyr 701) by JAK kinases and requires the activity of HDAC (8). To assess the requirement for HDAC activity in class II induction by IFN-γ, Colon 26 cells were treated with TSA before IFN-γ or in a combination of both agents. The data in Fig. 5(B) demonstrate that, while IFN-γ alone was able to induce CIITA, pre-treatment with TSA suppressed the level of induction of total CIITA and PIV-CIITA expression. The requirement for HDAC activity in the JAK–STAT pathway was also shown by the decrease of phosphorylated STAT-1α (Tyr 701) in Colon 26 cells pre-treated with TSA (Fig. 5C). In contrast to the enhancing effect on IFN-γ-induced class II expression when TSA is added together (Fig. 5D) or after IFN-γ (11), pre-treatment with TSA is repressive. Thus, the down-regulation of CIITA expression observed in the TSA-pre-treated Colon 26 appears to result from a repression of the JAK–STAT signaling which is required for PIV-CIITA activation by IFN-γ and

Fig. 3. Existence of both CIITA-dependent and -independent MHC class II expression pathways in Colon 26 cells. Colon 26 cultures were transiently transfected with the dominant negative EdCIITA plasmid. Cells were treated with IFN-γ (100 U ml⁻¹) or TSA (100 and 250 nM) 4 h after transfection. RNA was harvested for RT-PCR (A) and real-time quantitative RT-PCR (B) analysis 48 h after the treatments.
(A) Expression of the dominant negative human CIITA, endogenous mouse CIITA, IAα and GAPDH. Lane 1, untreated; lane 2, 100 U ml⁻¹ IFN-γ; lane 3, 100 nM TSA and lane 4, 250 nM TSA. The human B cell line Raji and mouse B cell line A20 were included as positive controls.
(B) Real-time quantitative RT-PCR analysis of class II expression in the EdCIITA-transfected and untransfected Colon 26. Fold changes were calculated by normalization to GAPDH levels followed by comparison with an untreated sample.

The cell type specific CIITA promoters are differentially regulated
The experiments outlined in Fig. 1 suggest a basic difference in the deacetylase inhibitor regulation of MHC class II in plasma cells compared with trophoblasts with regard to the role of CIITA. Since the CIITA gene is known to be activated in a tissue-specific manner, we further examined promoter-specific CIITA expression patterns after TSA treatment of several cell types. The CIITA gene is controlled by three distinct promoters (PI, PIII and PIV) that result in transcripts with different first exons (35). PI is active primarily in macrophages and DCs, while PIII and PIV direct B cell and IFN-γ-inducible expression, respectively (35, 36). However, recently all three promoters were shown to be inducible by IFN-γ in different cell types (36-38). An IFN-γ-responsive element upstream of the proximal promoter has been demonstrated to direct activation of PIII in response to IFN-γ stimulation (37, 38). Thus, PIII-CIITA, in addition to PIV can be an important
deacetylase inhibitors can activate or inhibit MHC class II depending on the treatment conditions.

**Antigen presentation by epigenetically altered tumor cells**

To determine the potential functional relevance of epigenetic induction of class II, we studied the ability of the J558 plasmacytoma treated in vitro with TSA to present antigen to histocompatible T cells. We used the J558 plasmacytoma because TSA-treated J558 tumor cells have been shown to activate tumor immunity in vivo in a well-established tumor model (24). Titration of J558 cells with various concentrations of TSA showed that 25 nM elicited maximal expression of class II on viable cells (Fig. 6A). J558 cells were therefore treated with 25 nM TSA and studied in mixed-lymphocyte tumor cell cultures. The antigen presentation assays employed whole ova protein, an immunodominant ova-peptide322–338 specific to MHC class II (H-2d), and the control (E333A) ova-peptide322–338 defective in TCR binding.

As shown in Fig. 6(B), J558 cells treated with 25 nM TSA for 24 h were able to present ova and ova-peptide322–338 and stimulated significant proliferation of antigen-specific T cells (net c.p.m. 12919 and 10573, respectively) compared with untreated J558 (net c.p.m. 2476 and 309, respectively). The SI of T cell proliferation stimulated by 25 nM TSA-treated J558 cells with ova-peptide322–338 was 18 while pmac had an SI of 8. Similar but slightly lower SIs were obtained in 3-day assays (data not shown). The presentation of ova-peptide322–338 to stimulate CD4+ T cell proliferation, by either 25 nM TSA-treated J558 cells or control pmac cells was epitope specific as both groups of cells were unable to activate T cells when incubated with the (E333A) ova-peptide322–338. For intact ova protein presentation, the SI was 17 for TSA-treated J558 cells compared with an SI of 11 for the pmac. These results demonstrate that treatment with optimal concentrations of TSA generates tumor cells that are effective in presenting class II-restricted peptides and in processing and presenting whole-protein antigens.

**Discussion**

Several types of CIITA and MHC class II responses to deacetylase inhibitor treatment were observed in the various tumors selected for study. One pattern was identified in five murine plasmacytomas in which CIITA was refractory to IFN-γ induction and TSA selectively up-regulated PIII-CIITA and class II. In a second pattern, four trophoblast cell lines failed to express CIITA, class II in response to IFN-γ, yet TSA induced class II in each of the lines. A third pattern was seen with Colon 26 in which IFN-γ elicited PIV-CIITA and class II. In this cell, TSA induced levels of class II equivalent or higher than those elicited by IFN-γ but no CIITA transcripts of any type were detected following TSA treatment. In several ‘classical’ IFN-γ-induced cells, such as HeLa, both PIII- and PIV-CIITA are important components of the IFN-γ response. Evidence for a CIITA-independent pathway of class II expression activated by TSA was also obtained in studies of STAT-1, IRF-1 and CIITA knockout mice. The induction by TSA of class II in kidney cells detected following TSA treatment. In various cases, CIITA was activated in the absence of IFN-γ, indicating that CIITA

<table>
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<th>Cell line</th>
<th>IFN-γ</th>
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<td>IC-21</td>
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<tr>
<td>JAWSII</td>
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<sup>a</sup>Levels of CIITA promoter (type PI, PIII and PIV) expression were determined by real-time quantitative RT-PCR following treatment with IFN-γ and TSA and for untreated cells. The plus signs used in the columns correspond to fold changes in mRNA relative to control (see Methods): (−) = ≤5-fold, (+) = 5 to 10-fold, (+++) = 10<sup>2</sup>-fold, (+++) = 10<sup>2</sup>- to 10<sup>3</sup>-fold, ND = not determined.

**Fig. 4.** CIITA promoter-specific activation by IFN-γ and TSA treatment. Colon 26 cells were transfected with reporter constructs containing the PIII- (PIII-LUC) or PIV- (PIV-LUC) CIITA promoter. Cells were treated with IFN-γ (100 U ml<sup>−1</sup>) or TSA (100 nM) for 48 h and cell extracts were harvested for luciferase activity assay. For each plasmid transfection, the change in luciferase activity was calculated by comparing the RLUs with the untreated samples.
also occurs in non-transformed cells. We also show that cells with defects in JAK-1, IRF-1 and BRG-1 can be induced to express class II by TSA but not by IFN-γ. Other reported data have also suggested that, in certain cells and under specific experimental conditions, class II can be expressed in the absence of CIITA (10, 34, 40, 41).

The mechanisms by which the class II genes are activated by TSA in the absence of CIITA are not yet clearly defined. Earlier studies of histone acetylation at the HLA-DR a promoter demonstrated H3 and H4 acetylation patterns and their association with class II expression (11, 42). The predominance of histone acetyltransferase (HAT) at the class II promoter would shift the balance from histone deacetylation toward acetylation and activation. Deacetylase inhibitors could potentially assume the function of three HATs (CBP/p300, PCAF and CIITA) normally found in the class II promoter enhanceosome. Additionally, TSA could induce the downstream acetylation pattern normally established by the endogenous MHC class II LCR-like element (5). There is, as mentioned in the Introduction, an extensive interplay between several types of covalent histone modifications and TSA could enhance H3K4 methylation and S10 phosphorylation and inhibit H3K9 methylation, a combination of covalent modifications which has been observed in the activation of several genes (43). The histone acetylation induced by TSA can affect chromatin changes and alter the access and recruitment of enzymes, such as histone-specific kinases and methyltransferases, to the promoter. A reasonable hypothesis is that H3K9 methylation creates a binding site for HP-1 at the class II promoter and TSA disrupts HP-1 binding, nucleosome positioning and heterochromatin formation (44). However, histone methylation and HP-1 also occurs in focal areas in euchromatin targeted by Rb (45) and Krab transcription factors (46). Therefore, the ability to re-activate silenced genes by HDAC inhibitors does not clearly distinguish heterochromatic from euchromatic silencing and we do not know whether in certain cells (e.g. trophoblasts) the CIITA and MHC genes are buried in heterochromatin.

The Colon 26 PIV-CIITA promoter, like that of HeLa, may be open to the binding of transcription factors that are induced by IFN-γ (e.g. phosphorylated STAT-1 a and IRF-1). The induced CIITA facilitates the formation and activity of the class II enhanceosome which requires the cooperative binding of the RFX and NF-Y trimeric complexes and the CREB transcription factors together with cofactors including several HATs. Enhanceosomes are dynamic and their composition changes with time (47). An extensive study of the temporal order of events is needed to provide a comprehensive understanding of the mechanisms by which TSA can induce expression of class II genes in the absence of CIITA.
factor recruitment at the MHC class II promoter, after IFN-γ treatment (32), demonstrated that the SWI/SNF components (BRG-1 and Brm) were recruited to class II subsequent to a wave of acetylation which correlated with the onset of elongation. Recent work shows that BRG-1 binds to RFX-AP, but not other class II box-binding proteins, which recruits BRG-1 to the class II promoter (33). This report and others (32, 48) emphasized the role of prior acetylation of histones in BRG-1 recruitment to promoters. We show here that in the SW-13 cell deficient in BRG-1 (and Brm) TSA induces class II. This suggests that the SWI/SNF remodeling complex is not an absolute requirement for class II expression if acetylation is provided. Whether this has any physiological relevance is uncertain. TSA has been reported to enhance elongation (49) and the acetylation levels obtained after TSA treatment may replace the function of HATs and possibly, at least in part, the chromatin-remodeling complexes normally involved in transcriptional elongation (49).

Our studies showing that CIITA activation by IFN-γ can be enhanced or inhibited by TSA, depending on the order of treatment, are consistent with observations that both acetylation and deacetylation of histone lysines can be associated with active transcription. For example, certain STAT-1 responses require HDACs and TSA can inhibit IFN-induced STAT-1 phosphorylation as shown here and in recent reports (8, 50). The precise mechanism by which HDACs function in the class II pathway has not been defined but may involve direct binding of HDAC to STAT-1 and the recruitment of HDACs to the promoter motifs in CIITA (50).

In addition to HP-1, histone methylation and deacetylation at specific lysines, gene silencing is often accompanied by DNA methylation. Previously reported analyses of the PIV-CIITA promoter in the JAR and JEG-3 human trophoblast tumors have demonstrated methylated CpG islands and the failure of these cells to respond to IFN-γ has been attributed to DNA methylation (51, 52). In this regard, TSA has been shown to demethylate certain genes in Neurospora (3). An important component of silencing by DNA methylation is via CpG-binding proteins which recruit HDACs that in turn promote chromatin silencing (53). Thus, TSA could potentially elicit MHC class II expression via inhibition of CpG–HDAC complexes at the level of the PIV-CIITA promoter in trophoblast cells or Colon 26. However, here we show that TSA did not induce CIITA transcripts in trophoblasts. Therefore, in our studies of trophoblast cells, as well as Colon 26, activation of class II by TSA is not attributable to demethylation of the CIITA promoter. There may also be a contribution from direct activation by TSA of the class II promoter in plasma cell as shown for Colon 26 and trophoblasts. These data also raise the issue of whether TSA might inhibit DNA methylation directly at the class II promoter. However, analysis of mouse genomic DNA shows that, in contrast to the CpG-rich PIV-CIITA promoter, no CpG islands were found in the promoter regions of the Iα or Iβ genes (54). It remains, however, a formal possibility that critical site-specific demethylation at the class II promoter is induced by TSA.

To determine whether promoter structure defined its response to TSA, we assayed the activity of promoter-specific reporter constructs transiently transfected into the Colon 26 cells which allowed the comparison of expression levels in a cell which responds to both IFN-γ and TSA. Although care must be taken in the interpretation of results from reporter assays (55), the reporters employed would not be expected to attain a sufficient chromatin structure to explain the differences observed in the activation of PIII- versus PIV-CIITA. Therefore, with this caveat, the ability of TSA to induce the PIII reporter, but not the PIV reporter, suggests that TSA may elicit the expression or enhance the activity of transcription factors and/or cofactors that activate the PIII-CIITA promoter. The failure of TSA to induce any of the type-specific endogenous CIITA genes in Colon 26 indicates that mechanisms other than, or in addition to, histone acetylation are operative in silencing the type-specific CIITAs in this cell type. These data also demonstrate that TSA in most cells does not activate endogenously silenced PIV-CIITA or the transcription factors involved in PIV-CIITA expression and that the expression of PIV-CIITA is tightly regulated by the IFN-γ pathway as previously proposed (35). Although we have not studied a PI-CIITA reporter, our findings show that the TSA activation of the endogenous PI-CIITA is restricted to macrophage–dendritic lineage cells and...
does not extend to other cells including those having IFN-γ-induced PIV-CIITA pathways (e.g., Colon 26 and HeLa). This suggests that the chromatin mechanisms for silencing PI and PIV are different or that TSA induces or enhances the activity of PI-specific transcriptional factors or cofactors in macrophage–DCs.

An alternative mechanism, alluded to the above, by which TSA might activate class II is by the induction of a shift in the HAT/HDAC balance of the cells resulting in the acetylation of one or more of the transcription factors and/or cofactors involved in class II transcription. A recent report demonstrates that the activation of MHC class II is enhanced by mono-ubiquitination of CIITA and, importantly, the HAT CBP/p300 as well as TSA enhances CIITA ubiquitination (56). However, in other genes, transcription factor for acetylation can inhibit as well as extend to other cells including those having IFN-γ-induced PIV-CIITA pathways (e.g., Colon 26 and HeLa). This suggests that the chromatin mechanisms for silencing PI and PIV are different or that TSA induces or enhances the activity of PI-specific transcriptional factors or cofactors in macrophage–DCs.

HDAC inhibitors have been reported to mediate anti-tumor effects via their ability to induce growth arrest, cell differentiation and apoptotic death (reviewed in 60). HDAC inhibitors, administered systemically, have also been found to induce tumor regression in animal models and phase I clinical trials with several inhibitors (SAHA, depsipeptide and MS-275) are currently in progress (60). Our work introduces a new dimension and suggests an alternative or additional mechanism by which TSA may induce an anti-cancer effect. We suggest that, in addition to the potential effects systemic HDAC inhibitors may have on tumor growth, they may have an effect on immunity either on host cells or directly on tumor cells, and the regulation of immune molecules which have been altered during tumor evolution and immune escape. The demonstration of enhanced expression of MHC and the acquisition of antigen presentation, after TSA treatment, suggest the possibility that TSA-treated tumor cells may behave in vivo analogous to the tumor immunity reported when MHC class II and B7 are transfected into tumor cells (23). In this regard, we have recently shown that TSA-treated J558 plasmacytoma cells also up-regulate CD40 and B7 in addition to MHC class II and that TSA-treated tumor cells are less tumorigenic and elicit CTLs and immunity to rechallenge with wild-type tumors (24). These studies suggest that deacetylase inhibitors can be activators of immune genes and generators of immunity and their potential as therapeutic agents should be further explored in cancer treatment protocols.

**Supplementary data**

Supplementary data are available at *International Immunology* Online.

**Acknowledgements**

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**Abbreviations**

- APC: antigen presenting cell
- ChIP: chromatin immunoprecipitation
- CIITA: class II transactivator
- c.p.m.: counts per minute
- DC: dendritic cell
- GAPDH: glyceraldehyde-3-phosphate dehydrogenase
- GFP: green fluorescent protein
- HAT: histone acetyltransferase
- HDAC: histone deacetylase
- HP-1: heterochromatin protein-1
- LCR: locus control region
- ova: ovalbumin
- p300: peritoneal macrophage
- PRL: raw luminescence unit
- RT: reverse transcription
- SI: stimulation index
- TSA: trichostatin A

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