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CIITA is silenced by epigenetic mechanisms that prevent the recruitment of transactivating factors in rhabdomyosarcoma cells

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Abstract

Rhabdomyosarcomas (RMS) are highly malignant pediatric sarcomas. We have discovered that the gene encoding the major histocompatibility complex class II transactivator, *CIITA*, is silenced in cells representing both major subtypes of RMS. Silencing of *CIITA* prevents the IFN- γ inducible expression of *MHC* class II genes in these cells. Overexpression of *CIITA* in these cells can restore MHC expression. We have found that IFN- γ signaling is intact in these cells, but pSTAT1 and IRF1 do not bind to the *CIITA* PIV promoter. The *CIITA* promoter is not hypermethylated in RD (ERMS) cells, but does show a modestly enhanced methylation status in SJRH30 (ARMS) cells. We have found that histone acetylation, which normally increases on the *CIITA* PIV promoter following IFN- γ treatment, is blocked in both types of RMS cells. In RD cells, treatment with a histone deacetylase inhibitor (TSA) reverses the silencing of *CIITA*. In SJRH30 cells, treatment with DNA methyltransferase inhibitors and TSA cooperatively restores *CIITA* expression. Surprisingly, we have also shown that the expression of two components of the immunoproteasome, which are embedded in the class II locus, is stimulated by IFN- γ in certain RMS cells in the absence of stimulation by *CIITA*. *CIITA* overexpression can also activate the expression of these genes, indicating that the immunoproteasome genes *LMP2* and *LMP7* can be activated by both *CIITA* dependent and *CIITA* independent pathways.

Introduction

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma arising in children, adolescents and young adults ¹. RMS tumors share a myogenic phenotype and are thought to arise from skeletal muscle precursors ². RMS tumors are characterized by the expression of the myogenic regulatory factors, including myogenin and MyoD ^{3,4}. While the expression of MyoD and myogenin typically correlates with terminal differentiation, RMS cells typically fail to arrest or differentiate into normal muscle. There are two major histological categories of RMS, the embryonal and alveolar subtypes ⁵. The more common form of the disease is the embryonal subtype (ERMS), characterized by loss of heterozygosity at the 11p15 locus, a region which harbors insulin-like growth factor 2 (IGF2) ⁶. Alveolar RMS

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(ARMS), the more aggressive form of RMS, is characterized by a t(2;13)(q35;q14) translocation in many of the tumors. The translocation results in a chimeric transcript that fuses the 5' portion of PAX3, including an intact DNA binding domain, to the transactivation domain of a forkhead transcription factor, creating a novel PAX3-FKHR fusion protein^{7, 8}.

Several cell lines have been derived from RMS tumors and the cell lines exhibit many of the characteristics of RMS tumors. These lines include RD (ERMS), RD2 (ERMS), SJRH30 (ARMS) and Rh28 (ARMS) cell lines. The RMS cell lines express Myf5, MyoD and myogenin, but the proteins appear non-functional⁹. When MRF responsive reporters are transfected into RD cells, little activity is detected^{10, 11}. Ectopic expression of the MRFs does not rescue the block to differentiation⁹, although it has recently been shown that expression of MyoD and an E protein in RD cells can promote differentiation¹².

Interferon gamma (IFN- γ) is an inflammatory cytokine that was first identified as an antiviral factor. IFN- γ is a pleiotropic cytokine that regulates different immune responses and influences many physiological processes. IFN- γ has a potent anti-fibrotic activity that has been used in numerous clinical trials. IFN- γ signaling both activates and represses large numbers of genes. Importantly, IFN- γ stimulates the expression of several components of the immunoproteasome, or antigen processing machinery complex (APM), that are required for processing MHC class I antigens and protection against oxidative stress^{13, 14}. Thus, altered signaling by IFN- γ is highly implicated in altered immunosurveillance of specific tumors¹⁵⁻¹⁷. IFN- γ signals through the JAK-STAT pathway. When IFN- γ binds to its receptor, the receptor associated protein tyrosine kinases Janus kinase I (JAK1) and JAK2 are activated. This leads to the phosphorylation of STAT1 which then dimerizes, translocates to the nucleus and activates its target promoters, including the pIV promoter of *CIITA*¹⁸.

The MHC class II transactivator, *CIITA*, is required for both constitutive and IFN- γ inducible expression of *major histocompatibility complex (MHC)* class II genes and has an ancillary role in class I gene expression^{19, 20}. *CIITA* lacks DNA binding activity, but is recruited to proximal promoters by interactions with sequence specific DNA binding factors. *CIITA* has been shown to interact with a variety of transcription factors and co-activators²⁰. *CIITA* is encoded by one gene that contains four separate promoters that generate four isoforms²¹. *CIITA* expression is stimulated by IFN- γ primarily through two of the four promoters, promoters III and IV²².

CIITA has also been shown to be critical for IFN- γ induced repression. IFN- γ suppresses a large family of genes that includes genes required for cell proliferation and cell differentiation. *Collagen*, *IL-4*, *IL-10* and *cathepsin E* have all been shown to be targets for IFN- γ mediated *CIITA* repression^{23, 24}. Intriguingly, IL-4R, the receptor for IL-4, has been found to be upregulated in RMS²⁵. IL-4 treatment promotes dedifferentiation and inhibition of IL-4R signaling extends survival in mouse models of RMS²⁵.

CIITA is constitutively expressed in B cells, but is expressed in response to IFN- γ in several cell types, including astrocytes, fibroblasts and aortic smooth muscle cells²⁶⁻²⁸. Recent evidence suggests that anti-tumor immunity is mediated by class II expression on non professional antigen presenting cells. Thus, *CIITA* is proposed to be an important mediator of immunosurveillance and tumor recognition²⁹. Consistent with this role, *CIITA* has been found to be silenced in many tumor cells^{30, 31}. It has been proposed that silencing *CIITA* is an effective immune evasion strategy for malignant cells. The down regulation of expression of major histocompatibility molecules is frequently noted in tumor cells. It is thought that

the lack of expression of MHC molecules hampers cellular immune recognition and prevents an efficient T-cell mediated tumor eradication.

We have recently discovered that *CIITA* functions in skeletal muscle to both activate *MHC* class II expression and repress myogenic differentiation by acting as an inhibitor of myogenin³². Here, we sought to understand if *CIITA* was active in rhabdomyosarcoma cells. We found that in both immortalized and primary cell lines from human and mouse origins that represent both subtypes of RMS, *CIITA* and *MHC* gene expression are silenced. The silencing of *CIITA* appears to be mediated by histone deacetylation in the two cell lines studied, although DNA methylation also contributes to the silencing in SJRH30 cells. We have also shown that class II gene expression can be restored by treatment with a histone deacetylase inhibitor (TSA), or by the over expression of *CIITA*. Thus, this work offers potential therapeutic insight into how *CIITA* silencing in rhabdomyosarcoma cells can be reversed to perhaps restore immune recognition and aid in tumor eradication.

Materials and Methods

Cell culture

RD and SJRH30 cells (ATCC) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone) according to standard protocols³³. RD2 and Rh28 were obtained from Denis Guttridge, Ohio State University, and grown as described above. HeLa cells (ATCC) were grown in DMEM supplemented with 5% fetal bovine serum (Hyclone). C2C12 cells (ATCC) were grown in DMEM supplemented with 10% fetal bovine serum (Hyclone). Mouse primary cell cultures (U33915 and U48484) were established from murine ERMS and ARMS tumor samples, respectively. The Pax7CreER, Ptch1, p53 conditional model of spindle ERMS/UPS has recently been described³⁴. The Myf6Cre, Pax3:Fkhr, p53 conditional mouse model of ARMS has also been described previously³⁵. Tumors were minced into small pieces and digested in collagenase (10mg/ml) overnight at 4°C. The dissociated cells were then grown in DMEM with 10% fetal bovine serum (FBS).

IFN- γ stimulation

RD, SJRH30 and HeLa cells were treated with human IFN- γ (Peprotech). C2C12, U33915 and U48484 cells were treated with murine IFN- γ (Peprotech). Cells were typically stimulated with 100 units/ml IFN- γ (0.01 μ g/ml). Cells were harvested for RNA or protein at defined time points after the IFN- γ stimulation. IFN- γ was added to the media and replenished every time the media was changed. All stimulations were performed at least twice to confirm results.

Gene Expression Analysis

Gene expression was monitored as previously described³². Primers are listed in Supplemental Table 1. When possible, intron spanning primers were used. All quantitative PCR was performed in triplicates and three independent RNA samples were assayed for each time point. qPCR gene expression data are shown using two formats. For measurements of relative gene expression (fold stimulation), a fold change was calculated for each sample pair and then normalized to the fold change observed at *HPRT*. For relative measurements of mRNA expression levels, gene expression levels were quantitated using a calibration curve based on known dilutions of concentrated cDNA. Each mRNA value was normalized to that of *HPRT*. Fold change was calculated by dividing the mRNA expression values of each sample pair.

Flow cytometry

Cells were harvested, washed with PBS containing 5% FBS and 10% bovine serum albumin, and stained for direct immunofluorescence detection of HLA-DR and HLA-A,B,C using PE conjugated mAb (BD Pharmingen). The acquisition was performed on a Cell Lab Quanta SC Flow Cytometer (Beckman Coulter), using Cell lab Quanta SC MPL analysis software (Beckman Coulter).

Western Blots

Cells were lysed in radio-immunoprecipitation assay buffer (RIPA) supplemented with protease inhibitors (Complete protease inhibitor, Roche Diagnostics) and protein concentrations were determined by Bradford's assay (Bio-Rad). 30 µg of protein was loaded on SDS PAGE gels, transferred onto PVDF membrane (Bio-Rad) and handled according to standard protocols. Signal was detected with chemiluminescent substrate according to manufacturer's protocol (SuperSignal, Pierce) and visualized by autoradiography. The antibodies used include anti-STAT1 (E-23, Santacruz Biotechnology (SCBT)), anti-pSTAT1 (Tyr701, Cell Signaling) and anti-GAPDH (Chemicon).

Chromatin Immunoprecipitation (ChIP) assays

ChIP assays were performed and quantified as described previously³⁶ with the following modifications: 1×10^7 cells were used for each immunoprecipitation and protein A agarose beads (Invitrogen) were used to immunoprecipitate the antibody:antigen complexes. Antibodies against the following proteins were used: STAT1 (E-23, SCBT), pSTAT1 (Tyr701, Cell Signaling), IRF1 (H-8, SCBT), histone H3 Ac 9,18 (gift of S.Y.R. Dent, U.T. M.D. Anderson Cancer Center), histone H3 C-term (ab1791, Abcam) and RNAPII (H-224, SCBT). Rabbit IgG (SCBT) was used as a non-specific control. Primers spanning the PIV promoter of human *CIITA* and a gene desert region of *Chr19* were used to detect promoter enrichment and normalize values. Values of $[\Delta][\Delta]Ct$ were calculated using the following formula based on the comparative Ct method: $[\Delta]Ct$, template (antibody)- $[\Delta]Ct$, template (IgG)= $[\Delta][\Delta]Ct$. Fold enrichments were determined using the formula : $2^{-[\Delta][\Delta]Ct}$ (experimental)/ $2^{-[\Delta][\Delta]Ct}$ (reference, *IgH*). Standard error from the mean was calculated from replicate $[\Delta][\Delta]Ct$ values. All ChIP assays shown are representative of at least three individual experiments.

Generation of Stable Cell lines

Stable RD and SJRH30 cell lines over expressing CIITA were made by transfecting RD and SJRH30 cells with linearized myc-CIITA plasmid (gift of J. Boss, Emory University) or the empty vector, linearized pcDNA3.1, and selecting for geneticin (400 µg/ml) resistant colonies. Individual clones were isolated and propagated.

Isolation of genomic DNA and analysis of methylation status

Genomic DNA was isolated from cells by phenol chloroform extractions following treatment with proteinase K. DNA was precipitated with isopropanol and spooled using a glass pipet. Purified DNA was bisulfite treated (EZ DNA Methylation kit, Zymo Research). The PIV promoter of *CIITA* was amplified with primers as previously described³⁰. The PCR products were TOPO cloned into pCR4 (TOPO TA cloning kit, Invitrogen). Ten independent colonies were sequenced (McLab).

Treatment with TSA and 5-aza-2'-deoxycytidine

Toxicity curves were generated for each drug in both cell lines tested before examining the effects on gene expression. Based on these initial studies, cells were grown to 70% confluence, treated with IFN-γ for 24 hours, and then incubated with trichostatin A (TSA,

Sigma) and or 5-aza-2'-deoxycytidine (Sigma) for the indicated number of days. Cells were incubated with 300 nM TSA for 24 hours prior to harvesting RNA. Cells were incubated with concentrations varying between 1.0 and 5 μ M 5-aza-2'-deoxycytidine for four days, with fresh 5-aza-2'-deoxycytidine added each day of culture. For the combined treatment, cells were treated with 5.0 μ M 5-aza-2'-deoxycytidine for three days and 300 nM TSA added for the final day in culture. All assays were performed at least twice to confirm results.

Results

IFN- γ signaling is blocked in RMS cells

As part of our studies on the role of *CIITA* in muscle cells, we tested the effects of IFN- γ on rhabdomyosarcoma cells. The immortal cell lines used for this analysis were RD (ERMS subtype), RD2 (ERMS subtype), SJRH30 (ARMS subtype) and Rh28 (ARMS subtype). We also probed cells from primary mouse tumor models, U33915 and U48484 that were characterized as ERMS or ARMS type tumors, respectively. As C2C12 cells are of mouse origin and the majority of our cells were of human origin, we used both C2C12 cells and human HeLa cells as controls for these experiments. HeLa cells were chosen as it is known that IFN- γ efficiently stimulates *CIITA* in HeLa cells³⁷. Cells were stimulated with 100, 500 and 1,000 units of IFN- γ and assayed for expression of *CIITA*. No significant up regulation of *CIITA* in any of the RMS cell lines was observed at any concentration of IFN- γ while robust stimulation was observed in C2C12 cells and HeLa cells. Shown are the results for 500 units of IFN- γ in Figure 1. We also assayed for the expression of the *MHC* class II genes. As expected, we saw no significant up regulation of the *MHC* class II genes in the RMS lines (Figure 1B). As *CIITA* also contributes to *MHC* class I expression, we examined the expression of *MHC* class I genes and observed that class I expression was also down regulated, although some stimulation with IFN- γ was observed in most cell lines (Figure 1C). It was noted that baseline expression levels of class I expression varied widely between the cell types and these baseline differences are reflected in the mRNA expression calculations used in Figure 1.

To determine if the silencing of *CIITA* was responsible for the lack of expression of class II molecules, we overexpressed exogenous *CIITA* in cells representing both major subtypes of RMS, RD and SJRH30 cells. We found that class II genes were highly expressed in the *CIITA* over expression cell lines, confirming that class II gene expression is repressed in RMS cells due to the lack of induced *CIITA* (Figure 2A). We also noted that class I gene expression was modestly stimulated in the *CIITA* over expression line. To confirm the role of *CIITA* on class I expression, we also assayed for expression in C2C12 cells with *CIITA* knockdowns (Supplemental Figure 1). We found that the presence of *CIITA* stimulated class I expression, but the induction by IFN- γ was independent of *CIITA*. These data support our finding that *CIITA* stimulates the expression of class I molecules, but the data do suggest that *CIITA* is likely not directly required for the IFN- γ mediated induction of class I molecules in RMS cells. We also assayed for the presence of MHC molecules on the surface on the cells by flow cytometry. SJRH30 cells and SJRH30 cells expressing exogenous *CIITA* were assayed with or without the addition of IFN- γ and sorted on the basis of class I and class II expression by FACS analysis (Figure 2B). Cell surface expression of class I and II molecules required both *CIITA* and IFN- γ , as no shift was present if the cells were not treated with IFN- γ (Figure 2B). The presence of class I molecules on the cell surface could also be detected by immunohistochemistry (Supplemental Figure 2). Similar data was obtained for RD cells (data not shown).

CIITA does not alter muscle specific gene expression in RMS cells

As we have recently shown that CIITA inhibits muscle specific gene expression in normal myoblasts³², we were interested in the effect that CIITA would have on muscle gene expression in RMS cells. We have observed that in normal cells, CIITA binds to myogenin and inhibits the activity of myogenin, leading to a down regulation of muscle specific genes. To determine if CIITA could alter muscle specific gene expression in RMS cells, we compared the gene expression profiles in cells over expressing CIITA to cells transfected with an empty vector. No change in muscle specific gene expression was observed (Supplemental Figure 3). This result was consistent with the prior evidence that has suggested that myogenin is inactive in RMS cells.

IFN- γ signaling is intact in RMS cells but pSTAT1 does not bind to the *CIITA* promoter

To determine what step might be blocked in the activation of CIITA, we next assayed for the presence of the IFN- γ stimulated JAK/STAT1 pathway in RMS cells. We first assayed for expression of the IFN- γ receptor and found that the IFN- γ receptor transcript was robustly expressed in RD and SJRH30 cells (Figure 3A). We next asked if STAT1 signaling was intact in RMS cells. First, we assayed for the presence of STAT1 by western blotting and found that STAT1 was robustly expressed in RD, RD2, SJRH30 and Rh28 cells (Figure 3B). To determine if STAT1 could be phosphorylated in response to IFN- γ , these cells were stimulated with IFN- γ and assayed for the presence of phosphorylated STAT1. We observed IFN- γ inducible phosphorylated STAT1 in RMS cells (Figure 3C). The phosphorylation of STAT1 is robust in RD, SJRH30 and Rh28 cells, but we did note that RD2 cells had reduced levels of pSTAT (Figure 3C) compared to the other cell lines.

To determine if phosphorylated STAT binds to the pIV promoter of *CIITA*, chromatin immunoprecipitation assays were performed in RD, SJRH30 and HeLa cells following IFN- γ stimulation. These assays established that pSTAT1 does not bind to the promoter of *CIITA* in RMS cells (Figure 3D).

We were also interested in the status of interferon regulatory factor 1 (IRF1) which is also induced by IFN- γ and acts as a transactivator for CIITA³⁸. Expression of IRF1 was assayed in RMS cells and control cells with or without IFN- γ stimulation. We observed that IRF1 was robustly induced in C2C12 cells upon IFN- γ stimulation. A modest induction was also observed in HeLa cells and the two ARMS lines tested, SJRH30 and Rh28. No induction of IFN- γ was observed in the two ERMS cell lines, RD and RD2. We next assayed for binding of IRF1 to the PIV promoter of *CIITA* by ChIP assays. Cells were stimulated with IFN- γ or vehicle control and assayed for IRF1 binding. We found that IRF1 was recruited to the *CIITA* promoter in C2C12 cells, but this recruitment was not observed in RMS cells (Figure 3F.). Thus, our data show that the recruitment of both pSTAT1 and IRF1 to the *CIITA* PIV promoter was blocked in RMS cells.

DNA methylation is modestly enhanced in SJRH30 cells, not RD cells

Methylation of the *CIITA* promoter has been observed in several tumor types including T cell leukemias, B cell lymphomas, teratocarcinoma, choriocarcinoma, neuroblastoma, erthroleukemia and small lung cancer^{37, 39-41}. DNA methylation at a CpG island in the USF-1 binding site (site 4, Figure 4A) is known to be refractory to USF-1 binding¹⁸. STAT1 binds cooperatively with USF-1, so blocking USF-1 binding would be predicted to block STAT1 binding as well. Genomic DNA was bisulfite treated and sequenced to detect DNA methylation events. We found that the promoter did not appear to be hypermethylated in either cell line when compared to the methylation events in HeLa cells, where CIITA activation is not impaired (Figure 4B). Moreover, no enhanced methylation of site 4 was observed in either cell line. The SJRH30 cell line, representing the ARMS subtype, did show

enhanced DNA methylation as compared to the RD cell line, representing the ERMS subtype, which showed very little DNA methylation.

Histone acetylation is blocked in RMS cells

We next asked if histone acetylation was disrupted in RMS cells. Cells were treated with IFN- γ and assayed for changes in histone acetylation and RNAPII occupancy at the PIV promoter of *CIITA* by ChIP assays. We also assayed for the presence of histone H3 to confirm that any changes observed were due to changes in acetylation states, not histone depletion. As anticipated, we found that the PIV promoter of *CIITA* was highly acetylated upon stimulation by IFN- γ in HeLa cells. RNAPII, which is not detected prior to the stimulation by IFN- γ , was also detected at the PIV promoter following stimulation (Figure 4C). However, in RD and SJRH30 cells, no change was observed in the histone acetylation levels following IFN- γ stimulation. RNAPII was not detected at the promoter and the overall histone levels did not change (Figure 4D and E). Thus, it appears that in both cell types, IFN- γ stimulated histone acetylation of the PIV promoter of *CIITA* is blocked.

CIITA expression can be restored by inhibiting histone deacetylases

To determine the contribution of histone deacetylation and DNA methylation to the silencing of *CIITA* in RMS cells, cells were treated with the histone deacetylase inhibitor, trichostatin A (TSA) and an inhibitor of DNA methylation, 5-aza-2'-deoxycytidine. Cells were treated with both of the drugs independently and in combination. In RD cells, TSA robustly reversed the silencing of *CIITA* in these cell lines, while treatment with a wide range of 5-aza-2'-deoxycytidine concentrations did not activate *CIITA* (Figure 5A). The combined treatment did not activate *CIITA* to a higher degree than TSA alone and in fact showed a reduced activation. In SJRH30 cells, we observed a cooperation between TSA and 5-aza-2'-deoxycytidine. *CIITA* activation was observed when cells were treated with TSA or 5-aza-2'-deoxycytidine, but addition of both drugs enhanced the activation (Figure 5B). These data suggest that both DNA methylation and histone deacetylation contribute to the silencing of *CIITA* in SJRH30 cells, while only histone deacetylation contributes to *CIITA* silencing in RD cells. To establish that epigenetic changes at the promoter promote *CIITA* expression, we performed ChIP assays on SJRH30 cells treated with both TSA and 5-aza-2'-deoxycytidine for acetylated H3 and pSTAT1 binding (Figure 5C). After treatment, the levels of acetylated H3 increased at the promoter and pSTAT1 binding could be detected. Thus, the data show that the lack of histone acetylation at the *CIITA* promoter is indeed refractory to transactivating factor recruitment.

The immunoproteasome is present in many RMS cells, but absent in HeLa cells

We also examined the expression of two immunoproteasome components in RMS cells, *LMP2* and *LMP7*, in the panel of RMS lines. These IFN- γ stimulated immunoproteasome components are embedded in the *MHC* class II genes (Figure 6A), so we anticipated that these genes would be regulated by *CIITA* and not be stimulated in RMS cells. To our surprise, we found that *LMP2* and *LMP7* were stimulated in certain RMS lines to levels comparable to normal muscle cells (Figure 6B). RD, SJRH30 and Rh28 cells all showed robust induction of *LMP2* and *LMP7* following IFN- γ stimulation. This was also observed in U33915, the primary mouse cell line derived from an ERMS tumor. However, in contrast, we observed no induction of *LMP2* and *LMP7* in U48484, the primary mouse cell line derived from an ARMS tumor. Strikingly, RD2 cells, which showed reduced levels of pSTAT1, only showed only a 2 fold induction of the immunoproteasome components. Surprisingly, we also found that the immunoproteasome components were not stimulated by IFN- γ in HeLa cells, where *CIITA* signaling is intact. We did observe that base line expression of *LMP2* and *LMP7* varies widely among the cell lines, which can be observed in the graphs. RD, RD2, Rh28 and HeLa cells contain significant levels of the transcripts,

while SJRH30 and C2C12 cells show very low levels without IFN- γ stimulation. We were surprised to see that *CIITA* did not appear to be required for up regulation of the immunoproteasome components in RMS cells, so we also asked if *LMP2* and *LMP7* were up regulated in the *CIITA* over expression line. We found that indeed, *CIITA* could stimulate *LMP2* and *LMP7* in RD and SJRH30 cells (Figure 6C). We also assayed for *LMP2* and *LMP7* expression in a panel of shRNA constructs targeting *CIITA* in C2C12 cells, a murine myoblast cell line. We observed that the level of down regulation of *LMP2* and *LMP7* correlates with the degree of *CIITA* depletion in these cells (Supplemental Figure 4). Thus, our data suggest that while *CIITA* does activate *LMP2* and *LMP7*, these genes can also be activated by IFN- γ in a non-*CIITA* dependent pathway. Our results also demonstrate that many RMS cells maintain an up regulation of immunoproteasome components in response to IFN- γ , where HeLa cells have lost this ability.

Discussion

In this study, we have shown that rhabdomyosarcoma cell lines representing both common tumor subtypes do not express *MHC* class II molecules because of the silencing of *CIITA*. We have also shown that the binding of pSTAT1 and IRF1 is blocked in these cells and that the *CIITA* promoter does not become hyperacetylated upon IFN- γ treatment. Silencing of *CIITA* can be reversed by inhibiting histone deacetylases, implicating this class of enzymes in the silencing. Our data demonstrate that the rhabdomyosarcoma cells studied here join the large class of cancer cells that exhibit silencing of *CIITA*, suggesting that this is a common feature in oncogenic transformation. It has been suggested that the lack of *MHC* expression is frequently observed in early developmental or embryonically derived cancer cells³⁷. The origin of rhabdomyosarcomas is still controversial, but data suggest that they arise from mesenchymal stem cells that can be pushed to the skeletal muscle lineage⁴² or directly from skeletal muscle cells⁴³. However, in the study that showed the correlation between *MHC* silencing and embryonically derived cancer cells³⁷, DNA methylation was strongly correlated with the silencing of *CIITA*. While we did see some contribution of DNA methylation to the silencing of *CIITA* in one ARMS cell line examined, both ARMS and ERMS cells appeared blocked in histone acetylation of the *CIITA* promoter.

Our data suggest that the increase in histone acetylation observed at the *CIITA* promoter following IFN- γ stimulation in normal cells is required for *CIITA* activation and correlates with pSTAT1 binding. These data argue that a balance of histone acetyltransferases (HATs) and histone deacetylases (HDACs) mediate the activation of *CIITA*. Rhabdomyosarcoma cells do not exhibit the increase in IFN- γ mediated histone acetylation, indicating that HAT activity is somehow blocked at this promoter. Alternately, the data could also indicate that HDAC activity cannot be displaced from the promoter as treatment with a histone deacetylase inhibitor reverses the silencing of *CIITA*, implicating a role for histone deacetylases in maintaining the repressed state of the promoter.

We have found that DNA methylation also contributes to *CIITA* silencing in the one example of an ARMS cell line examined here, SJRH30 cells. In this example, it appears that both histone deacetylation and DNA methylation contribute to the silencing of *CIITA*, as the effects of these drugs were additive. The ARMS subtype of RMS is the more aggressive form of the disease and it is intriguing that the more malignant form of RMS is correlated with two distinct forms of silencing *CIITA*. It is surprising that SJRH30 cells show a dependence on DNA methyltransferases for the silencing of *CIITA*, as our DNA methylation analysis of PIV does not show an obvious hypermethylation when compared to HeLa cells, which can activate *CIITA*. It may be that the small changes in the methylation status observed in our study can affect *CIITA* activation. It is also possible that additional

sites, not included in our assay, can be methylated or that changes in genome wide DNA methylation indirectly activate *CIITA*.

Overexpression of *CIITA* has confirmed that loss of *CIITA* is responsible for the lack of antigen presentation, but consistent with RMS cells not being professional antigen processing cells, IFN- γ is also required to induce cell surface presentation of the class I and II proteins.

In this work, we have also observed that immunoproteasome components are induced normally in many RMS cell lines. In exception to this, we have shown that RD2 cell line, which also exhibits reduced pSTAT1 levels and no IRF1 induction, does not efficiently activate immunoproteasome components following IFN- γ stimulation. This suggests to us that in some percentage of RMS cells, IFN- γ signaling may be blocked at upstream steps of the pathway. Indeed, this is also suggested by the primary data as the cells from the ERMS tumor (U33915) showed a robust activation of the immunoproteasome genes, while the cells from the ARMS tumor (U48484) showed no IFN- γ stimulation of these genes, suggesting that U48484 may be blocked at an upstream step of the IFN- γ signaling pathway. Surprisingly, we also observed that *LMP2* and *LMP7* are not induced in HeLa cells, which induce *CIITA* and *MHC* class II molecules robustly. These results make several important implications. First, the *MHC* class II embedded immunoproteasome subunits can be stimulated by *CIITA* or IFN- γ in a *CIITA* independent pathway. The *CIITA* independent pathway is likely to involve IFN- γ stimulated transcription factors such as IRF1, as a role for IRF1 in *LMP2* expression has been previously reported⁴⁴. Second, loss of *LMP2* and *LMP7* expression has been observed in several types of cancer, including head and neck squamous cell carcinoma (HNSCC) and colorectal carcinomas^{17, 45}. Our results suggest that certain RMS tumors and HeLa cells can be included in this class of tumor cells. Thus, the lack of antigen presentation may be a highly prevalent tumor phenomenon as either *CIITA* silencing or immunoproteasome deregulation could lead to altered antigen processing. Alterations in antigen processing allow cancer cells to evade detection by the immune system and thus, may be one of the crucial steps in oncogenic transformation.

The silencing of *CIITA* would also shield RMS cells from potential immunotherapy approaches as we have shown that RMS cells are likely to be defective in antigen presentation. Immunotherapy approaches have been employed in therapies for rhabdomyosarcoma, but these approaches have shown limited success⁴⁶⁻⁴⁸. ARMS cells, the more aggressive form of the disease, are characterized by a translocation that fuses a myogenic transcription factor (PAX3) to a forkhead transcription factor (FKHR). The unique fusion of these gene products allows for potential design of targeted immunotherapy to detect and destroy cells expressing this fusion protein. While this has been attempted, the approaches have again shown limited success^{49, 50}. We suggest that the reactivation of *CIITA* might improve the success of immunotherapy approaches for rhabdomyosarcoma by improving the antigen presentation of these cells. Here, we have shown that *CIITA* expression can be modulated by histone deacetylase inhibitors and DNA methyltransferase inhibitors in rhabdomyosarcoma cells, offering a potential therapeutic strategy for reactivating *CIITA* in RMS tumors. Reactivation of *CIITA* would restore *MHC* class I and II expression and perhaps allow eradication of tumor cells by the immune system or improve immunotherapy strategies to cure this childhood cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. IFN- γ does not stimulate *CIITA* and *MHC* class I and II gene expression in RMS cells
 A. IFN- γ does not stimulate *CIITA* expression in RMS cells. The indicated cell lines were stimulated with 500 U IFN- γ for 24 hours (+). Untreated samples are indicated with a negative sign. Gene expression was assayed for *CIITA*. B. IFN- γ does not stimulate *MHC* class II expression in RMS cells. Cell lines were stimulated as in A. Gene expression was assayed for *HLA-DRA* for human samples and *H2-Ea* for murine samples. C. IFN- γ does not stimulate *MHC* class I expression in RMS cells. Cells were stimulated as in A. Gene expression was assayed for *HLA-B* for human samples and *H2-K1* for murine samples.

Figure 2. MHC expression can be restored by CIITA over expression

A. CIITA over expression restores *MHC* class I and II expression. Stable cell lines over expressing CIITA were assayed for the expression of *CIITA*, *HLA-DRA* or *HLA-B*. For A., error bars reflect standard deviation from the mean from the replicated samples. B. *MHC* class I and II presentation is restored by over expression of CIITA. SJRH30 cells with and without CIITA were assayed for cell surface expression of HLA-A,B,C and HLA-DR with or without IFN- γ stimulation by FACS analysis. The black curves represent the control cells and the grey curve represents the CIITA overexpression cells.

Figure 3. IFN- γ can stimulate pSTAT1 in RMS cells, but pSTAT1 does not bind DNA

A. The IFN- γ receptor is expressed in RMS cells. Gene expression was assayed with primers against *IFNGR* and represented as expression levels relative to the levels found in HeLa cells normalized to *HPRT* expression levels. Error bars reflect standard deviation from the mean from the replicated samples. B. STAT1 is present in RMS cells. Protein extracts from the indicated cell lines were used for western blot analysis. Blot was probed with anti-STAT1 and anti-GAPDH antibodies. C. STAT1 is phosphorylated in response to IFN- γ in RMS cells. Indicated cell lines were stimulated with 500 u IFN- γ for 24 hours and harvested for protein and used for western blot analysis. Blot was probed with anti-phospho STAT and anti-GAPDH antibodies. D. Indicated cells were cross linked and used for ChIP assays with antibodies against phospho-STAT1 and IgG and primers against the pIV promoter of *CIITA*. Data shown are fold change between signal detected at the *CIITA* PIV promoter for the pSTAT1 antibody minus the signal detected for IgG. Standard error from the mean was calculated from replicate $[\Delta][\Delta]$ Ct values. A region of Chr19 was used to normalize the fold enrichments for the individual promoters. E. IRF1 is induced by IFN- γ in ARMS cell, but not ERMS cells. Cells were stimulated with IFN- γ or vehicle control for 24 hours and gene expression was monitored with primers against *IRF1*. F. IRF1 does not bind the PIV promoter of *CIITA*. ChIP assays on cells treated as in E. with antibodies against IRF1 and primers against the PIV promoter of *CIITA* are shown.

Figure 4. The *CIITA* promoter is not hypermethylated but IFN- γ stimulated histone acetylation and RNAPII recruitment are blocked in RMS cells

A. Schematic representation of the *CIITA* PIV region. CpG nucleotides are indicated in bold and numbered. Known transcription factor binding sites are boxed and labeled. B. Bisulfite sequencing of the template strand of genomic DNA from RD, SJRH30 and HeLa cells. Numbers refer to the CpG nucleotide numbers in A. At least ten individual *CIITA*-PIV amplicons were examined in each case. Each sequenced amplicon is represented by a horizontal row of boxes. Methylated CpGs are indicated by black boxes and unmethylated CpGs are indicated by white boxes. C. ChIP assays on IFN- γ stimulated and unstimulated HeLa cells with antibodies against RNAPII, histone H3 9,18 acetylation, histone H3 and IgG. Data shown are fold change between signal detected at the *CIITA* PIV promoter for each antibody minus the signal detected for IgG. A gene desert region of Chr19 was used to normalize the data. D. As in C., except RD cells were used. E. As in C., except SJRH30 cells were used.

Figure 5. Inhibition of histone deacetylases activates *CIITA* and class II expression in RD cells and partially reactivates *CIITA* in SJRH30 cells

A. Histone deacetylase inhibitors stimulate *CIITA* expression in RD cells. Cells were treated with the histone deacetylase inhibitor TSA or with 5-aza-2'-deoxycytidine. 5-aza-2'-deoxycytidine was used at 1 μ M, 2 μ M and 5 μ M as indicated on the graph. Gene expression of *CIITA* was assayed by qRT-PCR. Data are shown as fold stimulation of treated samples vs. untreated samples. B. Histone deacetylase inhibitors and methyltransferase inhibitors cooperatively activate *CIITA* expression in SJRH30 cells. Data as in panel A, except SJRH30 cells were used. C. TSA and 5-aza-2'-deoxycytidine treatment increases the level of H3Ac and induces the binding of pSTAT1. ChIP assays were performed on SJRH30 cells treated with IFN- γ or vehicle control with antibodies against H3K9,18Ac and pSTAT1 and primers against the pIV promoter of *CIITA*.

Figure 6. Immunoproteasome components are stimulated by IFN- γ and CIITA in certain RMS cells

A. Schematic representation of the locus containing *LMP2* and *LMP7*. Shown is the mouse locus. B. Immunoproteasome components are stimulated by IFN- γ in many RMS cell lines. RD2, U48484 and HeLa cells do not significantly activate these genes in response to IFN- γ . Cell lines were stimulated with 100 u/ml IFN- γ and gene expression assayed for *LMP2* and *LMP7*. Transcript levels were determined by qRT-PCR and normalized to the mRNA levels of *HPRT*. Numbers indicate induction factors relative to untreated samples. C.

Overexpression of CIITA in RMS lines also induces *LMP2* and *LMP7*. Cell line overexpressing CIITA were assayed for expression of *LMP2* and *LMP7*. Transcript levels were determined by qRT-PCR and normalized to the mRNA levels of *HPRT*. Numbers indicate induction factors relative to vector only samples.