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A Novel Role for Histone Deacetylase 6 in the Regulation of the Tolerogenic STAT3/IL-10 Pathway in APCs

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APCs are critical in T cell activation and in the induction of T cell tolerance. Epigenetic modifications of specific genes in the APC play a key role in this process, and among them histone deacetylases (HDACs) have emerged as key participants. HDAC6, one of the members of this family of enzymes, has been shown to be involved in regulation of inflammatory and immune responses. In this study, to our knowledge we show for the first time that genetic or pharmacologic disruption of HDAC6 in macrophages and dendritic cells results in diminished production of the immunosuppressive cytokine IL-10 and induction of inflammatory APCs that effectively activate Ag-specific naive T cells and restore the responsiveness of anergic CD4+ T cells. Mechanistically, we have found that HDAC6 forms a previously unknown molecular complex with STAT3, association that was detected in both the cytoplasmic and nuclear compartments of the APC. By using HDAC6 recombinant mutants we identified the domain comprising amino acids 503–840 as being required for HDAC6 interaction with STAT3. Furthermore, by re-chromatin immunoprecipitation we confirmed that HDAC6 and STAT3 are both recruited to the same DNA sequence within the Il10 gene promoter. Of note, disruption of this complex by knocking down HDAC6 resulted in decreased STAT3 phosphorylation—but no changes in STAT3 acetylation—as well as diminished recruitment of STAT3 to the Il10 gene promoter region. The additional demonstration that a selective HDAC6 inhibitor disrupts this STAT3/IL-10 tolerogenic axis points to HDAC6 as a novel molecular target in APCs to overcome immune tolerance and tips the balance toward T cell immunity.

acterization of HDAC6 assigned its localization and function to the cytosolic compartment (6, 13); however, recent reports have shown that HDAC6 is also present in the nucleus (14, 15). In the cytoplasm, HDAC6 is recognized as a key regulator of cytoskeletal, cell migration, and cell–cell interactions (16) given its effect on the acetylation status of α-tubulin, Hsp90, and cortactin (17). In the nucleus, HDAC6 has been implicated in the regulation of specific transcription factors and gene promoters (14, 15, 18–20). Importantly, accumulating evidence also points to HDAC6 as playing an important role in regulation of inflammatory and immune responses, in particular at the level of the APC/T cell immune synapse (21), regulatory T cell function (22), macrophage responses (23), and aberrant airway inflammation (24).

HDACs are the targets of a family of compounds known as HDAC inhibitors (HDACIs) (12). Most of the HDACs currently available, including those already approved for the treatment of cancer patients (25–27), target multiple HDACs (pan-HDACIs). Recently, we found that treatment of APCs with the pan-HDAC1 LAQ824 or LBH589 inhibits the production of the anti-inflammatory cytokine IL-10. This effect enabled the APCs to effectively prime naive Ag-specific CD4+ T cells and restore the responsiveness of tolerant T cells isolated from tumor-bearing mice (28). These findings suggest that specific HDACs likely participate in regulation of IL-10 gene transcription and could be putative targets in the observed HDAC3-mediated IL-10 inhibition. Supporting this concept, earlier studies in which different HDACs were genetically disrupted in murine and human APCs led us to demonstrate that HDAC11 is a negative regulator of IL10 gene transcriptional activity (29). Those earlier studies also suggested that another member of the HDAC family, HDAC6, could be involved in regulation of IL10 gene transcriptional activity. In this study, to our knowledge we show for the first time that HDAC6 is indeed required for the production of IL-10 by macrophages and dendritic cells (DCs). Mechanistically, we have found that HDAC6 forms a previously unknown molecular complex with STAT3, association that was detected in both the cytoplasmic and nuclear compartments of the APC. Of note, genetic or pharmacologic inhibition of HDAC6 in APCs resulted in decreased STAT3 phosphorylation—but no changes in STAT3 acetylation—and showed that another member of the HDAC family, HDAC6, could be involved in regulation of IL10 gene transcriptional activity. To evaluate protein expression.

Materials and Methods

Mice

Male BALB/c or C57BL/6 mice (6 to 8 wk old) were obtained from the National Institutes of Health (Frederick, MD). TCR transgenic mice expressing an αβ TCR specific for antigenic acids 110–120 from influenza hemagglutinin (HA) presented by I-ED were a gift of H. von Boehmer (30). TCR transgenic mice (OT-II) expressing an αβ TCR specific for peptide 323–339 from OVA presented by MHC class II, I-Ab (31) were provided by Dr. W. Heath (Walter and Eliza Hall Institute of Medical Research, Parkville, VIC, Australia). HDAC6 knockout (KO) mice (H-2b) were provided by Dr. P. Matthias (32) (Friedrich Miescher Institute for Bio-medical Research, Basel, Switzerland). Experiments involving the use of mice were performed in accordance with protocols approved by the Animal Care and Use Committee of the University of South Florida College of Medicine.

Cell lines

The macrophage cell line RAW264.7 has been described previously (29, 33). Cells were cultured in vitro in RPMI 1640 media supplemented with 10% FCS, penicillin/streptomycin (50 U/ml), 1-glutamine (2 mM), and 2-ME (50 μM) (complete media) and grown at 37°C and 5% CO2.

Isolation of macrophages and DCs

BALB/c (H-2b), C57BL/6 (H-2b), or HDAC6 KO (H-2b) mice were injected i.p. with 1 ml thiglycollate (Difco Laboratories, Detroit, MI). Four days later, peritoneal elicited macrophages (PEMs) were isolated by peritoneal lavage as previously described (34). DCs were generated from murine bone marrow cultures using RPMI 1640 medium supplemented with 10% FCS, 20 ng/ml murine rGM-CSF, and 10 ng/ml IL-4 (Research Diagnostics, Flanders, NJ). The cultures were maintained at 37°C in 5% CO2. On day 3 of culture, floating cells were gently removed and fresh medium with cytokines was replaced. On day 5, cells were collected and DCs were enriched by centrifugation on a metrizamide gradient (Accurate Chemical and Scientific, Westbury, NY). Bone marrow–derived macrophages (BMDMs) were generated as described previously (33). Briefly, bone marrow–derived macrophages differentiated in RPMI 1640 supplemented with 20% FBS, 100 U/ml mouse M-CSF, 100 U/ml penicillin/streptomycin, and 2 mM l-glutamine. Cells were seeded on petri dishes and incubated at 37°C in 5% CO2. Four days later an extra 10 ml fresh medium was added to the plate and incubated for an additional 3 d. To harvest BMDMs, the plate was washed once with sterile PBS. Then, 10 ml ice-cold PBS was added to each plate and incubated at 4°C for 10 min. Cells were detached by gentle pipetting. The isolated cells were then centrifuged at 200 × g for 5 min and suspended in 10 ml BMDM media (RPMI 1640, 10% FBS, 20 U/ml mouse M-CSF, and 2 mM l-glutamine). Cells were cultured in tissue culture plates for 12 h before further experimental procedures.

Reagents

LPS (Escherichia coli 055:B5, catalog no. L-2880) and short hairpin RNA (shRNA) lentiviral particles were purchased from Sigma-Aldrich (St. Louis, MO). rIL-10 was purchased from BD Biosciences (San Jose, CA). Lipofectamine 2000 and Lipofectamine LTX were from Invitrogen (St. Louis, MO). rIL-10 was purchased from BD Biosciences (San Jose, CA). The overexpression plasmid for IL-10 was a gift from Dr. W.A. Boisvert (35). HDAC6 mutants were provided by Dr. X. Zhang’s laboratory at the University of South Florida (36).

Abs and immunoblotting

Anti-HDAC6 (C0226) Ab was purchased from Assay Biotech. Anti-FLAG (F1804) Ab was from Sigma-Aldrich. Anti-GAPDH (sc-25778), anti-tubulin (sc-32293), and anti-acetylated tubulin (sc-23950) Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-acetylated H3 (06-599), anti-STAT3 (06-596), anti-JAK2 (06-255), and anti–p-JAK2 (04-1098) Abs were purchased from Millipore (Billerica, MA). Anti–p-STAT3 (Tyr705) (9138), anti–p-STAT3 (Tyr705) (sc-727) (9136), and anti–acetyl-STAT3 (5253) Abs were from Cell Signaling Technology (Danvers, MA). Anti-TYK2 (PA5-34497) and anti-Lamin B (PA5-19468) Abs were from Thermo Scientific.

Overexpression and knocking-down experiments

Adenoviruses and plasmids with FLAG-tag were used as vectors for HDAC6 and STAT3 overexpression as previously described (29). shRNA lentiviral transduction particles for murine HDAC6 (NM010413, TRCN0000006415) and nontarget (NT) shRNA (SHC002V) were obtained from Sigma-Aldrich. Sequences for their respective targets are available from the supplier. For transduction of lentiviral particles for shRNA HDAC6, we followed the protocol provided by the manufacturer using a final multiplicity of infection of 75. Lentiviral particles for NT control were random sequences not present in the human or mouse genome. All assays were carried out in triplicate, and Western blots were performed to evaluate protein expression.

Overexpression of full length or mutants for HDAC6 was performed in RAW264.7 cells transfected with plasmids encoding for the FLAG-tagged version of the respective proteins. Twenty-four hours later, cells expressing each protein variant were lysed and the respective recombinant proteins purified by anti-FLAG affinity columns. Cell extracts isolated from PEMs or BMDMs were incubated with the FLAG-tagged proteins previously purified from sinusoidal macrophages (Sinusoidal macrophages were extracted and analyzed by immunoblot). To overexpress IL-10 in HDAC6KD RAW264.7 cells, we used an expression vector coding for the murine IL-10 (35) or control vector. Cells were transfected using Lipofectamine 2000 reagent for 24 or 48 h according to the manufacturer’s protocol (Invitrogen, Grand Island, NY).

Confocal studies

RAW264.7 cells or primary macrophages were stimulated with either LPS (1 μg/ml) or IL-10 (10 ng/ml). Fixed cells were subjected to immuno-
fluorescent staining using primary Abs against HDAC6 (1:200, CO226, Assay Biotech) and STAT3 (1:25, 9139, Cell Signaling Technology) and secondary Abs Alexa Fluor 488 donkey anti-mouse IgG (1:200, A-21202, Invitrogen) and Alexa Fluor 594 donkey anti-rabbit IgG (1:200, A-21207, Invitrogen), respectively. Samples were viewed with a Leica DMi6000 inverted microscope, TCS SP5 confocal scanner, and a ×63/1.40 numerical aperture Plan Apochromat oil immersion objective (Leica Microsystems, Wetzlar, Germany). Argon, 405 Diode, and HeNe 594 laser lines were applied to excite the samples, and a tunable AOBS was adjusted to minimize crosstalk between fluorochrome emissions. Gain, offset, and pinhole settings were identical for all samples within the treatment group. Images were captured with photomultiplier detectors and prepared with LAS AF software version 2.5.0 (Leica Microsystems).

Phenotypic and functional analysis of APCs
The expression of B7.2 in macrophages was determined by staining with FITC-conjugated anti-CD68 (GL1 Ab, BD Biosciences). Ten thousand gated events were collected on a FACScan (Becton Dickinson) and analyzed using FlowJo software. PEMS were treated with HDAC6 inhibitor (tubastatin A [Tub-A]) alone, LPS alone, or a combination thereof for 24 h. Supernatants were then collected and production of IL-10 was determined by ELISA (R&D Systems, Minneapolis, MN). Similarly, the production of IL-10 by macrophages or DCs from HDAC6 KO mice in response to stimulation with OVA was determined by ELISA. The expression of IL-10 mRNA by macrophages or DCs from HDAC6 KO mice in response to stimulation with RIL-10 was determined by quantitative RT-PCR.

Ag-presentation studies
PEMs (1 × 10^6/well) from the different experimental groups were treated with LPS alone or with LPS plus increasing concentrations of Tub-A for 24 h. Cells were then washed and 5 × 10^5 purified naive Ag-specific CD4+ T cells (isolated from the spleen of OVA TCR transgenic mice) or a similar number of tolerized Ag-specific CD4+ T cells were added to the macrophage monolayer with and without OVA peptide 323–339 ([QGLLTV]8A). The expression of OVA-specific Ag was measured by ELISPOT (24 h) for total Ag-specific CD4+ T cells and by division-type ELISPOT for the Ag-specific T cell response. The peptide specific Ag-specific CD4+ T cells were added to the macrophage monolayer with and without OVA peptide (233–339) ([OVA(233-339)]) for 24 h. Cells were then washed and 5 × 10^5 purified naive CD4+ T cells from OVA TCR transgenic mice or a similar number of tolerized Ag-specific CD4+ T cells were added to the macrophage monolayer with and without OVA peptide 323–339 ([QGLLTV]8A) for 24 h.

Quantitative real-time PCR analysis
RAW264.7 cells, primary murine macrophages, or DCs were plated at 2 × 10^5 cells per 35-mm well and cultured under conditions detailed under each experiment. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) with treatment with or without LPS for 24 h. These cells were then cultured with OVA peptide and/or HDAC6 KO monolayer and/or HDAC6 KO with and without SLE peptide 323–339 ([QGLLTV]8A) for 24 h. Cells were then washed and 5 × 10^4 purified naive CD4+ T cells from OVA TCR transgenic mice or a similar number of tolerized Ag-specific CD4+ T cells were added to the macrophage monolayer with and without OVA peptide 323–339 ([QGLLTV]8A) for 24 h. Cells were then washed and 5 × 10^4 purified naive CD4+ T cells from OVA TCR transgenic mice or a similar number of tolerized Ag-specific CD4+ T cells were added to the macrophage monolayer with and without OVA peptide 323–339 ([QGLLTV]8A) for 24 h. Cells were then washed and 5 × 10^4 purified naive CD4+ T cells from OVA TCR transgenic mice or a similar number of tolerized Ag-specific CD4+ T cells were added to the macrophage monolayer with and without OVA peptide 323–339 ([QGLLTV]8A) for 24 h. Cells were then washed and 5 × 10^4 purified naive CD4+ T cells from OVA TCR transgenic mice or a similar number of tolerized Ag-specific CD4+ T cells were added to the macrophage monolayer with and without OVA peptide 323–339 ([QGLLTV]8A) for 24 h. Cells were then washed and 5 × 10^4 purified naive CD4+ T cells from OVA TCR transgenic mice or a similar number of tolerized Ag-specific CD4+ T cells were added to the macrophage monolayer with and without OVA peptide 323–339 ([QGLLTV]8A) for 24 h. Cells were then washed and 5 × 10^4 purified naive CD4+ T cells from OVA TCR transgenic mice or a similar number of tolerized Ag-specific CD4+ T cells were added to the macrophage monolayer with and without OVA peptide 323–339 ([QGLLTV]8A) for 24 h.

Chromatin immunoprecipitation (ChIP) studies
Chromatin immunoprecipitation assays (ChIP) were performed as previously described (38). Briefly, 2.5 × 10^6 naive CD4+ T cells specific Abs as detailed under each experiment. Quantification is expressed in arbitrary units, and target mRNA levels were normalized to the input signal using the method of Pfaffl (37). All ChIP experiments were repeated twice starting from the crosslinking, and final quantitative real-time PCR was done in triplicates.

The rechromatin immunoprecipitation or sequential ChIP (re-ChIP) for HDAC6 and STAT3 was performed using the same experimental procedure described above, only adding an extra clearance step between the sequential immunoprecipitations. Briefly, the eluted fraction after the first immunoprecipitation was precleared by incubation with protein Ag/Ag-agarose (Santa Cruz Biotechnology) for 2 h at 4˚C. After centrifugation at 1000 × g for 5 min, the supernatant was collected and immunoprecipitated using specific Abs as detailed under each experiment.

Tolerance model
For induction of anergy of HA-specific CD4+ T cells, we used a well-established experimental model of A20HA tumor→induced T cell tolerance (38). Briefly, 2.5 × 10^6 naive CD4+ transgenic T cells specific for an MHC class II epitope of influenza HA were injected i.v. into A20HA lymphoma-bearing mice. Twenty-one days after T cell transfer, animals were sacrificed and T cells were harvested from their spleens as previously described (38). Cytokine production by clonotypic CD4+ T cells in response to HA peptide 110–120 presented by WT, HDAC6KD RAW264.7 cells, or NT controls was then determined as described above. Flow induction of anergy of OVA-specific CD4+ T cells, we used a model of high-dose i.v. injection of OVA peptide into C57BL/6 mice as previously described (39).

Phosphatase activity assay
The tyrosine phosphatase activity was determined using a tyrosine phosphatase assay system (Promega, Madison, WI) following the manufacturer’s protocol. Briefly, cell lysates from PEMS isolated from WT or HDAC6 KO mice were removed of endogenous phosphate by spin column. In parallel, 100 µl PPaS 5X reaction buffer and 5 µl 1 mM mophospo peptide were added to each well in triplicate in a 96-well plate and incubated at 37˚C for 3 min. Then, 57 µl sample or standard was added to the wells and the plate was incubated at 37˚C for 8 min. The reaction was stopped by adding 50 µl molybdate dye additive mixture to each well, and the plate was incubated at room temperature for 20 min. Absorbance was then measured at 600 and 630 nm using the Benchmark Plus microplate reader (Bio-Rad).

Statistical analysis
All experiments were repeated at least twice unless indicated otherwise. Unpaired t tests were performed using Microsoft Excel software (Microsoft, Redmond, WA) with significance at p < 0.05.
Results

Genetic disruption of HDAC6 inhibits IL-10 gene expression and induces inflammatory APCs

To better understand the role of HDAC6 in the regulation of Il10 gene expression, we first transduced the macrophage cell line RAW264.7 with adenovirus carrying a FLAG- and GFP-tagged version of HDAC6, and with control adenovirus carrying only GFP. Cells infected with control GFP adenovirus displayed minimal levels of IL-10 mRNA in the absence of in vitro stimulation with LPS (Fig. 1A, GFP, LPS–). LPS stimulation upregulated the expression of IL-10 mRNA in these control cells (Fig. 1A, GFP, LPS+), and overexpression of HDAC6 further enhanced LPS-induced IL-10 mRNA (Fig. 1A, HD6, LPS+). Given these results, we next asked whether an opposite effect would be observed when the expression of HDAC6 is stably knocked down (HDAC6KD) in RAW264.7 cells by using lentiviral particles carrying specific shRNA for HDAC6. As compared with control cells transfected with NT shRNA (Fig. 1B, NT, LPS+), HDAC6KD abrogated the response to LPS in RAW264.7 cells (Fig. 1B, shRNAHD6, LPS+). The efficiencies of the aforementioned genetic manipulations were confirmed by Western blot analysis. As expected, HDAC6KD resulted in increased tubulin acetylation (Supplemental Fig. 1A).

Next, we determined the phenotype and Ag-presenting capabilities of RAW264.7 cells lacking HDAC6. In response to increasing concentrations of LPS (0.1–5.0 µg/ml), HDAC6KD cells produce minimal amounts of IL-10 protein as compared with WT or NT control cells (Fig. 1C). Additionally, HDAC6KD cells displayed an enhanced expression of the costimulatory molecule B7.2 (Fig. 1D). No changes in the expression of other costimulatory molecules were observed on these cells (data not shown). To assess the Ag-presenting capabilities of HDAC6KD cells, we cocultured naive CD4+ T cells specific for an MHC class II-restricted epitope of influenza HA with HDAC6KD RAW264.7, NT RAW264.7, or WT RAW264.7 cells in the presence of HA peptide. As shown in Fig. 1E, clonotypic T cells encountering HA peptide presented by cells lacking HDAC6 are better activated as evidenced by their increased production of IFN-γ relative to clonotypic T cells recognizing Ag on WT or NT control cells. It is noteworthy that this enhancement in the APC function of HDAC6KD cells was more pronounced when they were stimulated with LPS (HDAC6KD, gray bars).

Previously we demonstrated that adoptive transfer of naive anti-HA transgenic CD4+ T cells into mice bearing tumors expressing HA as a model tumor Ag resulted in the induction of Ag-specific CD4+ T cell tolerance (38). In this system, reisolated T cells were found to be anergic given their failure to be primed in vivo as well as by their diminished IFN-γ production in response to in vitro restimulation with cognate HA peptide. As shown in Fig. 1F, in vitro incubation of the same tolerant T cells (reisolated from tumor-bearing mice) with HDAC6KD cells resulted in restoration of their ability to produce IFN-γ in response to cognate Ag (Fig. 1F, HDAC6KD). In sharp contrast, anergic T cells encountering peptide on WT or NT cells remained unresponsive (Fig. 1F, WT and NT).

![FIGURE 1](http://www.jimmunol.org/) Phenotypic and functional analysis of macrophages lacking HDAC6. RAW264.7 cells were transduced in vitro with adenovirus carrying GFP-HDAC6 (HD6) or GFP alone (A), or with lentiviral particles carrying specific shRNA (HDAC6KD) or NT shRNA control (NT) (B). Forty-eight hours later cells were treated with or without LPS (1 µg/ml) for an additional 2 h. Then, total RNA was isolated and expression of IL-10 and GAPDH was measured by quantitative real-time RT-PCR. The results are expressed as percentage over control cells transduced with GFP alone (A) or NT shRNA (B) and data were normalized by GAPDH expression. Three experiments were performed with similar results. Error bars represent SD from triplicates. (C) WT RAW264.7 (WT), NT, or HDAC6KD (shRNA HDAC6) cells were stimulated with increasing concentrations of LPS (0.1–5.0 µg/ml) for 24 h. Then, IL-10 production was determined by ELISA. Two experiments were performed with similar results. Error bars represent SD from triplicates. (D) The expression of B7.2 on LPS-stimulated macrophage clones was determined by flow cytometry. In a parallel experiment, 1 × 10⁶/well RAW264.7 cells lacking HDAC6 or control cells were treated with or without LPS (1 µg/ml) for 24 h (open bars, LPS–; gray bars, LPS+). Then, 5 × 10⁵ purified naive (E) or tolerized (F) anti-HA CD4+ T cells were added to the cultures in the presence of 12.5 µg/ml cognate HA peptide 110–120 (SFERFEIFPKE). After 48 h, IFN-γ production was determined by ELISA. Three experiments were performed with similar results. Error bars represent SD from triplicates.
Macrophages and DCs from HDAC6 KO mice are better activators of T cells in vitro and in vivo

To confirm the above findings in primary APCs, we next determined the production of IL-10 by PEMs or DCs isolated from mice with genetic disruption of HDAC6 (HDAC6 KO mice) (32). As shown in Fig. 2A, PEMs (left panel) and DCs (right panel) from HDAC6 KO mice produce less IL-10 in response to increasing concentrations of LPS (0.1–5.0 μg/ml) as compared with APCs from WT mice. Furthermore, they trigger a better activation of Ag-specific CD4+ T cells (Fig. 2B) as naive CD4+ T cells specific for an MHC class II-restricted epitope of OVA encountering OVA peptide on PEMs (left) or DCs (right) from HDAC6 KO mice (H-2b) produced significantly higher levels of IFN-γ relative to OVA-specific CD4+ T cells recognizing Ag on APCs from WT mice.

To determine whether this lack of IL-10 production by HDAC6-deficient APCs could be observed in response to stimuli other than LPS, we treated PEMs and DCs with rIL-10, which has been shown to induce Il10 gene expression in an autocrine fashion (40). As shown in Fig. 2C, minimal IL-10 mRNA was observed in HDAC6 KO PEMs or DCs treated with recombinant murine IL-10 as compared with WT APCs. Similarly, no IL-10 mRNA expression was observed in HDAC6 KO APCs stimulated with IL-6, a cytokine that also induces Il10 gene expression (data not shown). Taken together, HDAC6 is required for Il10 gene transcriptional activity in APCs in response to different stimuli.

To assess whether the observed inhibition of IL-10 production represents the major explanation for the improved APC function of PEMs and DCs devoid of HDAC6, we next transduced HDAC6 KO cells with a plasmid expressing IL-10 or with an empty vector control (35). In the absence of LPS stimulation, cells transduced with plasmid IL-10 produced higher levels of IL-10 (350–550 pg/ml) relative to cells transduced with empty vector control (data not shown). HDAC6 KO PEMs or DCs with or without empty vector trigger effective activation of CD4+ T cells (Fig. 2D, black bars, none and empty vector). When these same APCs were transduced with a plasmid IL-10, they lost their ability to trigger IFN-γ production by CD4+ T cells (Fig. 2D, HDAC6 KO, IL-10). This abrogation of IFN-γ production may have been the result of the significantly higher levels of IL-10 produced by APCs transduced with the IL-10 plasmid. However, the production of IFN-γ by T cells was also diminished when DCs from HDAC6 KO mice were cultured with OT-II CD4+ T cells in the presence of lower concentrations of rIL-10 (range, 10–1000 pg/ml) (Supplemental Fig. 1B). These concentrations of IL-10 were within the range of IL-10 detected in the supernatants of LPS-stimulated WT APCs (range, 50–400 pg/ml). Given the known autocrine effects of IL-10 produced by the APC, we next determined the phenotypic and functional changes in APCs lacking HDAC6 or control cells when they were cultured in the presence of neutralizing anti-IL-10 Abs. As shown in Fig. 2E (left panel), addition of IL-10-neutralizing Abs to control APCs resulted in increased B7.2 expression. However, this effect was not seen in HDAC6KD macrophages (Fig. 2E, right panel). We also evaluated the production of IFN-γ by T cells cocultured with macrophages from WT or HDAC6 KO mice in the presence of anti-IL-10 Abs. As expected, IFN-γ production by T cells cultured with WT macrophages was enhanced in the presence of IL-10-neutralizing Abs (Fig. 2F, open bars). However, this effect was not observed when T cells were cocultured with HDAC6 KO APCs (Fig. 2F, filled bars). Taken together, these findings suggest that the improved T cell activating function of APCs lacking HDAC6 is mainly mediated by the regulatory effect of this HDAC upon IL-10 production.

Next, we determined the in vivo T cell activating function of APCs lacking HDAC6. DCs from HDAC6 KO or WT mice were pulsed in vitro with OVA peptide and stimulated with LPS for 24 h. Then, these DCs were injected i.v. into WT C57BL/6 mice that had previously received Ag-specific OT-II CD4+ T cells. Animals were sacrificed after 5 d, and T cells were isolated from their spleens. As shown in Fig. 2G, a higher expansion of Ag-specific CD4+ T cells was observed when these cells were exposed in vivo to HDAC6 KO DCs as compared with WT DCs (4.56 versus 1.36%, respectively). Furthermore, Ag-specific CD4+ T cells exposed to HDAC6 KO DCs were better primed in vivo because they produced higher amounts of IL-2 (Fig. 2H, left) and IFN-γ (Fig. 2H, right) upon in vitro restimulation with cognate OVA peptide.

Treatement of APCs with an isotype-specific HDAC6 inhibitor in vitro and in vivo also improves T cell activation via decreased IL-10 production

Given the above results, we next asked whether similar effects could be observed when APCs are treated with the isotype-selective HDAC6 inhibitor Tub-A (41). First, Tub-A did not affect the viability of APCs (data not shown) and it induced acetylation of α-tubulin but not of histones H3 or H4 (Fig. 3A), indicating that at the concentrations used, Tub-A selectively inhibits HDAC6. Second, reminiscent of the changes observed in APCs lacking HDAC6, treatment of PEMs from WT mice with Tub-A resulted in a dose-dependent inhibition of IL-10 production in response to LPS (Fig. 3B). Tub-A–treated macrophages also displayed an enhanced expression of the costimulatory molecules B7.2 (Fig. 3C).

Next, we evaluated the kinetics of IL-10 mRNA expression in PEMs treated with LPS, Tub-A, or LPS plus Tub-A. In response to LPS stimulation, IL-10 mRNA expression peaked at 2 h, followed by a rapid decline, and was back to baseline by 12 h (Fig. 3D, ▴). However, in PEMs treated with LPS plus Tub-A there was a decrease in IL-10 mRNA induction (Fig. 3D, ■). No changes in IL-10 mRNA levels were observed in PEMs treated with Tub-A alone (Fig. 3D, ○). Therefore, pharmacologic inhibition of HDAC6 resulted in decreased Il10 gene transcriptional activity in response to LPS.

To assess the Ag-presenting capabilities of Tub-A–treated PEMs, these cells as well as untreated PEMs were cultured in vitro with naive or tolerant anti-HA CD4+ T cells in the presence (or not) of cognate HA peptide. Compared to untreated PEMs, PEMs treated with Tub-A triggered a better activation of naive HA-specific CD4+ T cells given their increased production of IFN-γ (Fig. 3E, left). More importantly, Tub-A–treated PEMs were able to restore the responsiveness of tolerant CD4+ T cells isolated from tumor-bearing mice (Fig. 3E, right).

Utilizing an adoptive transfer system of Ag-specific CD4+ T cells into C57BL/6 mice, we previously demonstrated that vaccination of these mice with recombinant vaccinia encoding OVA (vacc-OVA) resulted in Ag-specific T cells that fulfill several functional criteria indicative of effective priming by APCs in vivo: clonal expansion and differentiation into effector cells capable of producing IFN-γ (38, 42). We therefore used this system to determine whether treatment of C57BL/6 mice with Tub-A might increase function of APCs and their response to vacc-OVA immunization in vivo. Briefly, on day 0 C57BL/6 mice were adoptively transferred with Ag-specific OT-II CD4+ T cells. Mice were then treated with Tub-A (25 mg/kg) or with vehicle control from day 0 to +9. On day +9 mice were given 107 PFU vacc-OVA given s.c. Six days later, animals were sacrificed and T cells were isolated from their spleen and their function was evaluated. First, Ag-specific CD4+ T cells isolated from vaccinated C57BL/6 mice...
FIGURE 2. APCs from HDAC6 KO mice are better activators of T cells in vitro and in vivo. (A) PEM (left) or bone marrow–derived DCs (right) were isolated from HDAC6 KO or WT mice and stimulated with increasing concentrations of LPS (0.1–5.0 μg/ml) for 24 h. The production of IL-10 was then determined by ELISA. (B) PEMs or DCs (1 × 10^5/well) from HDAC6 KO or WT mice were treated with or without LPS (1.0 μg/ml) for 24 h. Then, 5 × 10^4 purified naive anti-OVA CD4+ T cells were added to the cultures in the presence of 3 μg/ml cognate OVA peptide 323–339. After 48 h, IFN-γ was measured by ELISA. The results are expressed as fold over nontreated control cells normalized by GAPDH expression. Three independent experiments were performed with similar results. (C) PEMs or DCs as described in (B) were treated with or without IL-10 (10 ng/ml) for 2 h. Then, total RNA was isolated and expression of IL-10 mRNA and GAPDH was measured by quantitative RT-PCR. The results are expressed as fold over nontreated control cells normalized by GAPDH expression. Three independent experiments were performed with similar results. (D) The Ag-presenting function of HDAC6 KO macrophages or DCs transfected with a plasmid expressing IL-10 was evaluated as described in (B). Data are from a representative experiment of two independent experiments with similar results. (E) Expression of B7.2 on WT (left) or HDAC6KD (right) macrophages in the presence (or not) of anti–IL-10-neutralizing Abs. (F) Production of IFN-γ by T cells cocultured with macrophages from WT or HDAC6 KO mice in the presence of anti–IL-10-neutralizing Abs or isotype control (IgG). Data (E and F) are from a representative experiment of two independent experiments with similar results. (G) DCs were isolated from WT and HDAC6 KO mice and pulsed with OVA peptide for 24 h, then stimulated with LPS (1.0 μg/ml) for an additional 24 h. These DCs were then injected i.v. into C67BL/6 recipients that had previously received Ag-specific OT-II CD4+ T cells (2.5 × 10^6 given i.v. 1 d before the injection of DCs). On day 5 after DC transfer, mice
produced higher levels of IFN-γ (Fig. 3F, No Tx, vacc-OVA) relative to unvaccinated mice (No Tx) or mice treated with Tub-A alone following in vitro restimulation with OVA peptide. Second, the magnitude of this Ag-specific T cell response was further augmented in mice treated with Tub-A in combination with vacc-OVA. Animals were sacrificed 6 d later and T cells were purified from their spleens as indicated in Materials and Methods. T cells were then cultured with splenocytes from C57BL/6 mice in the presence of 3 × 10⁶ anti-OVA TCR transgenic CD4⁺ T cells given i.v. (day 0). Animals were then treated with either Tub-A (25 mg/kg) or vehicle control given i.p. for 10 d (day 0 to +9). On day +9 half the mice in each group were immunized s.c. with 1 × 10⁷ PFU vacc-OVA. Animals were sacrificed 6 d later and T cells were purified from their spleens as indicated in Materials and Methods. T cells were then cultured with splenocytes from C57BL/6 mice in the presence of 3 μg/ml OVA peptide 323–339. After 48 h, supernatants were collected and assayed for IFN-γ production by ELISA. Data represent means ± SE of triplicate cultures from eight mice in each group and are representative of two independent experiments with similar results. Values for T cells cultured without OVA peptide were below the limit of detection.

**Figures**

**FIGURE 3.** Phenotype and function of APCs treated with the isotype-selective HDAC6 inhibitor Tub-A. (A) PEMs were treated with increasing concentrations of Tub-A for 24 h. Protein extracts were prepared and subjected to SDS-PAGE and immunoblotting with anti-HDAC6, anti-tubulin, anti-acetylated tubulin, anti–histone 4 (H4), anti–acetylated H4, anti–histone 3 (H3), anti–acetylated H3–specific Abs. (B) PEMs (1 × 10⁵/well) were treated with LPS (1 μg/ml) alone or LPS plus increasing concentrations of Tub-A for 24 h. Then, supernatants were collected and the production of IL-10 was determined by ELISA. (C) Expression of B7.2 on macrophages treated with Tub-A (5 μM) was determined by flow cytometry. (D) PEMs (3 × 10⁵/well) were treated with 1 μg/ml LPS (A), 5 μM Tub-A (B), or the combination thereof (C). Cells were harvested at 0, 2, and 12 h and total RNA was isolated. The IL-10 expression by quantitative RT-PCR is expressed as fold over nontreated control cells and normalized by GAPDH expression. Data (D–E) are from a representative experiment of three independent experiments with similar results. (E) PEMs (1 × 10⁷/well) were treated as in (B). Then, cells were washed and 5 × 10⁴ purified naive (left) or tolerized (right) anti-HA CD4⁺ T cells were added to the cultures in the presence of 12.5 μg/ml cognate HA peptide 110–120. After 48 h, supernatants were collected and assayed for IFN-γ production by ELISA. Data are from a representative experiment of three independent experiments with similar results. (F) C57BL/6 mice were adoptively transferred with 2.5 × 10⁶ anti-OVA TCR transgenic CD4⁺ T cells given i.v. (day 0). Animals were then treated with either Tub-A (25 mg/kg) or vehicle control given i.p. for 10 d (day 0 to +9). On day +9 half the mice in each group were immunized s.c. with 1 × 10⁷ PFU vacc-OVA. Animals were sacrificed 6 d later and T cells were purified from their spleens as indicated in Materials and Methods. T cells were then cultured with splenocytes from C57BL/6 mice in the presence of 3 μg/ml OVA peptide 323–339. After 48 h, supernatants were collected and IFN-γ production was determined by ELISA. Data represent means ± SE of triplicate cultures from eight mice in each group and are representative of two independent experiments with similar results. Values for T cells cultured without OVA peptide were below the limit of detection.

**Previous Studies**

Previous studies have shown the important role of STAT3 in the regulation of Il10 gene expression and in tolerance induction mediated by APCs (39, 40). The demonstration by Yuan et al. (8) that acetylation of STAT3 regulates its function and that STAT3 can partner with HDACs to influence gene expression (43, 44) led us therefore to explore whether HDAC6 might influence Il10 gene transcriptional activity through regulation of STAT3 function in the APC. As shown in Fig. 4A (row 1), STAT3 protein expression was similar in HDAC6KD clones and in NT controls. However, no differences in STAT3 acetylation were found among HDAC6KD macrophages and NT control cells (Fig. 4A, row 2). These results led us to explore the phosphorylation status of STAT3 in these cells. Whereas phosphorylation of STAT3 Y705 was observed in control cells after 4 h of LPS stimulation, notably less phosphorylation was observed in macrophages lacking HDAC6 (Fig. 4A, row 3). Unlike tyrosine phosphorylation, analysis of serine 727 phosphorylation revealed no differences among cells lacking HDAC6 and NT controls (Fig. 4A, row 4). To assess the potential participation of HDAC6 upon activation of upstream components of the STAT3 pathway, we evaluated the phosphor-

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were sacrificed and T cells were purified from their spleens. The percentage of the double-positive CD4 and V-β 5.1 TCR population was determined by flow cytometry. (H) In parallel, 5 × 10⁶ purified T cells from these mice were cultured with splenocytes from C57BL/6 mice in the presence of 3 μg/ml OVA peptide 323–339. After 48 h, supernatants were collected and IL-2 (left panel) and IFN-γ (right panel) production were determined by ELISA.
FIGURE 4. Genetic or pharmacologic disruption of HDAC6 decreased phosphorylation but not acetylation of STAT3 in APCs. (A) NT or HDAC6KD RAW264.7 macrophages were stimulated with LPS (1.0 μg/ml). After the indicated time points, cells were lysed and immunoblotted using specific Abs against STAT3, acetylated STAT3, p-STAT3 Y705, p-STAT3 S727, JAK2, p-JAK2, TYK2, p-TYK2, and GAPDH. (B) PEMs were isolated from WT or HDAC6 KO mice and stimulated or not with LPS (1.0 μg/ml). After 24 h, cells were lysed and immunoblotted using Abs against HDAC6, tubulin, acetylated tubulin, STAT3, p-STAT3 Y705, acetylated STAT3, and GAPDH. (C) PEMs from HDAC6 KO or WT mice were treated with LPS as in (B) and then subjected to ChIP analysis using anti-STAT3 and anti-p-STAT3 Abs. (D) NT or HDAC6KD RAW264.7 cells were stimulated with IL-10 (10 ng/ml). After the indicated time points, cells were lysed and immunoblotted using specific Abs as indicated on (A). (E) PEMs isolated from WT or HDAC6 KO mice were stimulated with IL-10 or left untreated. After 30 min, cells were lysed and immunoblotted using the indicated Abs. (F) PEMs isolated from C57BL/6 mice were treated with Tub-A for 24 h. Cells were then lysed and immunoblotted using the indicated Abs. (G) PEMs from HDAC6 KO as well as WT mice were stimulated with LPS (1.0 μg/ml) for 2 h or remained untreated. Then, total RNA was isolated and analyzed by quantitative real-time PCR for expression of Socs3, Vegf, Il1a, and Il6 genes. GAPDH was used as control. The results are expressed as fold over nontreated control cells normalized by GAPDH expression according to the Pfaffl equation (37). Three experiments were performed with similar results. (H) Production of IL-10 by NT or STAT3KD macrophages in response to LPS. Data displayed (A–H) are from a representative experiment of three independent experiments with similar results.
ylation status of JAK2 and TYK2, two well-known activators of STAT3 signaling (45). As shown in Fig. 4A, no differences in the expression of total JAK2, p-JAK2, total TYK2, and p-TYK2 were observed between NT and HDAC6KD cells, suggesting that disruption of HDAC6 is not affecting these two upstream components of the STAT3 pathway in the APC.

The results above were then confirmed in PEMS isolated from HDAC6 KO mice in which we observed a diminished STAT3 Y705 phosphorylation (Fig. 4B, row 5), but no changes in STAT3 acetylation relative to PEMS from WT mice (Fig. 4B, row 6). Additionally, in these HDAC6 KO macrophages we found a decreased recruitment of STAT3 and p-STAT3 to the Il10 gene promoter (Fig. 4C). This impairment in STAT3 phosphorylation and its recruitment to the Il10 gene promoter could explain the diminished IL-10 production observed in HDAC6 KO macrophages in response to LPS (Fig. 2A).

To determine whether this decrease in STAT3 phosphorylation also occurred in response to stimuli other than LPS, macrophages lacking HDAC6 and NT controls were treated with IL-10, a cytokine that induces STAT3 phosphorylation within minutes of binding to its receptor (46). As shown in Fig. 4D, phosphorylation of STAT3 Y705 in NT controls occurs at 5 min after stimulation and peaks by 30–60 min (Non-Target, row 2). In contrast, Y705 phosphorylation was both delayed and diminished in HDAC6KD cells (HDAC6KD, row 2). Analysis of S727 phosphorylation and STAT3 acetylation revealed no differences (rows 3 and 4, respectively). Reminiscent of our findings with LPS stimulation, the phosphorylation status of JAK2 and TYK2 was similar in HDAC6KD and control macrophages treated with IL-10 (Fig. 4D, rows 5–8). Studies in PEMS from HDAC6 KO mice or control mice treated with IL-10 in vitro confirmed that STAT3 Y705 phosphorylation, but not STAT3 acetylation, is diminished in the absence of HDAC6 (Fig. 4E, rows 5 and 6).

To assess whether similar changes in STAT3 phosphorylation could be seen with pharmacologic inhibition of HDAC6, we treated PEMS with Tub-A. No changes in HDAC6, tubulin, and STAT3 proteins were observed in untreated or Tub-A–treated PEMS (Fig. 4F, rows 1, 2, and 3, respectively). However, treatment with Tub-A was associated with an increased in tubulin acetylation (row 4) and almost complete absence in STAT3 Y705 phosphorylation (Fig. 4F, row 5).

Next, we determined the impact of the absence of HDAC6 upon downstream STAT3-regulated genes other than Il10. Macrophages lacking HDAC6 display decreased mRNA expression of Cbfa1, Cmyc, Pim1, Cfos, P21, and Cdc25 in response to LPS (Supplemental Fig. 1C). Interestingly, immunologically relevant genes known to influence the T cell activating properties of APCs, such as Socs3, Vegf, Il1a, and Il6, were also downregulated in response to LPS in macrophages lacking HDAC6 (Fig. 4G). However, no significant differences were observed in the expression of Socs3, Il1a, and Il6 at the basal state (absence of LPS stimulation) among control and HDAC6 KO macrophages.

Finally, we asked whether disruption of STAT3 in macrophages (STAT3KD) would recapitulate the effects of HDAC6 knockdown upon IL-10 production. Reminiscent of our findings in STAT3 KO PEMS (39), STAT3 KD PEMS produce significantly lower levels of IL-10 in response to LPS as compared with NT PEMS (Fig. 4H). Taken together, we have found that HDAC6 is required for STAT3 phosphorylation and activation of STAT3 downstream target genes in macrophages.

**HDAC6 physically interacts with STAT3 in the cytoplasm and nuclei of the APC**

Next, we asked whether a physical interaction between HDAC6 and STAT3 is required for their effects upon Il10 gene expression in APCs. First, PEMS (Fig. 5A, top) or RAW264.7 cells (Fig. 5A, bottom) transfected with a plasmid encoding FLAG-tagged HDAC6 were subjected to immunoprecipitation and immunoblot analysis. Endogenous STAT3 coimmunoprecipitated with FLAG-HDAC6 in the absence of LPS stimulation (Fig. 5A, LPS”) and this interaction was further increased in response to LPS stimulation (Fig. 5A, LPS”). Similarly, HDAC6 was identified in the immunoprecipitated fraction after the reverse immunoprecipitation was performed with a FLAG-tagged STAT3 construct (Fig. 5B). This STAT3/HDAC6 interaction was also demonstrated in primary macrophages stimulated with IL-10 when either a FLAG-tagged STAT3 (Fig. 5C, top) or a FLAG-tagged HDAC6 (Fig. 5C, bottom) were used.

To determine the subcellular fraction of the APC in which the STAT3/HDAC6 interaction might be occurring, we first evaluated the presence of STAT3 and HDAC6 in immunoblots of cytosolic and nuclear extracts from macrophages treated with IL-10. In the absence of stimulation, both STAT3 and HDAC6 were detected in the cytoplasm (Fig. 5D, Input, −IL-10). In the nuclear fraction of untreated cells, HDAC6 was almost undetectable, whereas HDAC6 was detected. In response to IL-10 treatment, a significant increase in STAT3 was observed in the nucleus, whereas cytosolic STAT3, cytosolic HDAC6, and nuclear HDAC6 remain unchanged (Fig. 5D, Input, +IL-10). Anti-GAPDH and anti-Lamin B Abs were used as controls for cytosolic and nuclear localization, respectively (Fig. 5D). Next, coimmunoprecipitation of endogenous STAT3 from cytosolic and nuclear fractions of macrophages treated with IL-10 demonstrated that STAT3 and HDAC6 interact in both subcellular fractions; however, this association seems to be increased in the nuclear compartment of the APC (Fig. 5D, IP: STAT3).

To further assess the role of this nuclear STAT3/HDAC6 interaction upon Il10 gene expression, we asked whether HDAC6 and STAT3 might be recruited to the same DNA sequence within the Il10 promoter. We therefore performed a re-ChIP for HDAC6 and STAT3. Re-ChIP is a two-step sequential chromatin immunoprecipitation, where the first Ab is directed against HDAC6 and the second Ab against STAT3, and vice versa (47). As shown in Fig. 5E, both proteins were recruited to the Il10 gene promoter in response to LPS stimulation (black bars, ChIP STAT3 and ChIP HDAC6, respectively). Following this first ChIP, and after proper preclearance, samples were subjected to a second immunoprecipitation with Ab against the second protein. Regardless of the order of the Abs, both re-ChIP approaches demonstrated that STAT3 and HDAC6 are recruited to the same DNA sequence within the Il10 gene promoter (Fig. 5E, black bars, ChIP STAT3/re-ChIP HDAC6 and ChIP HDAC6/re-ChIP STAT3, respectively). These results suggest that in addition to its role in regulating STAT3 phosphorylation, HDAC6 might also function as a transcriptional cofactor of STAT3 at the level of the Il10 gene promoter.

Finally, confocal microscopy studies performed in RAW264.7 cells and PEMS stimulated with either LPS (Supplemental Fig. 2A) or IL-10 (Supplemental Fig. 2B) also showed that HDAC6 and STAT3 colocalize in both the cytoplasmic and nuclear compartment of these cells.

**HDAC6 domain comprising amino acids 503–840 is required for its interaction with STAT3 and IL-10 production**

Next, we investigated which segment of the HDAC6 protein is necessary for its physical interaction with STAT3. We overexpressed two FLAG-tagged HDAC6 constructs (HDAC61–840 or HDAC61–503) and full-length HDAC61–1215 (Fig. 6A, top) in macrophages. Fig. 6A (middle) shows the electrophoretic migration of...
these HDAC6 proteins on Western blot (WB) probed with anti-FLAG Ab. Following purification, these recombinant mutants were incubated in vitro with protein extracts from primary murine macrophages to determine the presence of STAT3 in the immunoprecipitated fraction. As shown in Fig. 6A (bottom), STAT3 from murine macrophages coimmunoprecipitated with full-length HDAC6$_{1-1215}$ and HDAC6 mutant 1–840 (lanes 1 and 2), but not with HDAC6 mutant 1–503 (lane 3). These results point to the segment comprising amino acids 503–840 of HDAC6 as being required for its physical interaction with STAT3.

To further explore the functional role of the HDAC6 segment 503–840 in regulation of IL-10 production, we asked whether this cytokine would be produced when macrophages expressing endogenous STAT3, but lacking HDAC6, were transfected with the HDAC6 mutants described above. Briefly, HDAC6 KO macrophages were transfected with empty vector, full-length HDAC6, or mutant HDAC6. HDAC6 KO macrophages transfected with empty vector produced minimal amounts of IL-10 (Fig. 6B, Empty), whereas those cells transfected with full-length HDAC6 or HDAC6 mutant 1–840 produced significant amounts of this cytokine. However, in cells transfected with the mutant 1–503, the production of IL-10 was minimal and similar to the levels observed in empty vector–transfected cells. These findings indicate that the HDAC6 domain comprising aa 503–840 is required for its direct interaction with STAT3 and regulation of IL-10 production by macrophages.

Discussion

Accumulating evidence indicates that epigenetic modifications of specific genes play a key role in influencing the inflammatory status of the APC and T cell activation versus T cell anergy (48, 49). The immunosuppressive action of the cytokine IL-10 is critical in the generation of APCs with tolerogenic properties (50, 51) and in the prevention of self-tissue damage (52–54). In this study, to our knowledge we have shown for the first time that genetic or pharmacologic disruption of HDAC6 in macrophages and DCs resulted in diminished production of the immunosuppressive cy-
clear role for HDAC6 in influencing activator of Il10 in APCs by influencing the function of STAT3, a transcriptional properties, we have found that HDAC6 regulates IL-10 production inflammatory and immune responses (21–24). Expanding on these 18–20). Of note, HDAC6 has also been found to regulate in-

nucleus (14, 15) where it can function as cofactor of specific transcription factors to influence gene promoter activity (14, 15, 18–20). Of note, HDAC6 has also been found to regulate inflammatory and immune responses (21–24). Expanding on these properties, we have found that HDAC6 regulates IL-10 production in APCs by influencing the function of STAT3, a transcriptional activator of Il10 gene activity. First, HDAC6 forms a previously unknown molecular complex with STAT3. Such a complex was found in both the cytoplasm and nucleus, with a stronger protein interaction occurring in the nuclear compartment of stimulated APCs. Second, by re-ChIP, we demonstrated that HDAC6 and STAT3 are both recruited to the same DNA sequence within the Il10 gene promoter. Third, disruption of this complex, by either knocking down HDAC6 or by studies in HDAC6 KO APCs, showed a significant impairment in STAT3 phosphorylation—but no changes in STAT3 acetylation—as well as diminished recruitment of STAT3 to the Il10 gene promoter region in response to stimulation with either IL-10 or LPS.

Our observation that the impairment of STAT3 phosphorylation in APCs lacking HDAC6 occurs within minutes after IL-10 stimulation suggests a proximal (cytosolic) role for HDAC6 in STAT3 regulation. However, the findings that HDAC6 and STAT3 become more nuclear in WT APCs after similar stimulation, together with the demonstration that both proteins are recruited to the same DNA sequence within the Il10 gene promoter, also suggest a nuclear role for HDAC6 in influencing Il10 gene promoter activity. Therefore, the diminished IL-10 production observed in APCs lacking HDAC6 could be explained by the impairment in STAT3 phosphorylation and its recruitment to the Il10 gene promoter and by the absence of this HDAC6 in the nuclei where it could be functioning as a transcriptional cofactor of STAT3 at the level of the Il10 gene promoter (as suggested by the re-ChIP data). We are currently determining which one of these two mechanisms could be the dominant one in influencing IL-10 production by the APC.

Regardless of the mechanisms, the above findings provided the rationale to target HDAC6 with specific inhibitors (41, 55, 56) and determine whether such an approach could also disrupt the tolerogenic STAT3/IL-10 axis in the APC. Proof of concept was provided in APCs treated with the HDAC6 selective inhibitor Tub-A in which we observed a diminished production of IL-10 in response to LPS stimulation. These APCs were also rendered more inflammatory in vitro and in vivo and were able to effectively activate naive Ag-specific CD4+ T cells. However, our data point to the regulatory effects of HDAC6 upon inflammatory phenotype and improved T cell priming elicited by APCs lacking HDAC6. First, transfection of HDAC6 KO APCs with a plasmid expressing IL-10 resulted in lost of their improved T cell priming function. Second, adding back rIL-10 to cultures of APCs lacking HDAC6 with Ag-specific T cells also yielded similar results, that is, diminished IFN-γ production by T cells. Third, the addition of anti–IL-10-neutralizing Abs attenuated IL-10 production, B7.2 upregulation, and the T cell priming function of WT APCs, but it had minimal effects on APCs lacking HDAC6.

Although previous reports have indicated that the deacetylase activity of type I HDACs, in particular HDAC1 and HDAC3, plays a role in regulating the function and nuclear trafficking of STAT3 (8, 44, 59), we did not find any changes in STAT3 acetylation in the absence of HDAC6. Instead, to our knowledge our studies are the first linking HDAC6 with regulation of STAT3 phosphorylation. Given its lack of kinase activity, it is plausible that HDAC6 could influence the function of upstream tyrosine kinases known to regulate STAT3 phosphorylation. However, we did not find significant differences in the expression of total JAK2, p-JAK2, total TYK2, and p-TYK2 between WT or APCs lacking HDAC6. Alternatively, HDAC6 might influence the function of phosphatases that regulate phosphorylation of STAT3. Supporting this possibility, Gupta et al. (60) reported that treatment of malignant cells with the pan-HDACi LBH589, which inhibits the enzymatic activity of HDAC6 among other HDACs, resulted in increased

![Figure 6](http://www.jimmunol.org/)
protein phosphatase activity and Akt dephosphorylation. More recently, McKinsey and colleagues (61) have shown that class I HDAC inhibitors suppress ERK1/2 signaling through the induction of an ERK-specific phosphatase, DSP5. Furthermore, Tøgø et al. (59) found that HDAC3 interacts with the phosphatase PP2A to regulate phosphorylation of target proteins. The particular association of HDAC6, HDAC1, and HDAC10 with phosphatases has been previously reported in transformed cells (62). Of note, treatment of these cells with the pan-HDACi trichostatin A disrupted the association of HDACs with phosphatases, suggesting that their enzymatic activity is necessary for this association. In line with these reports, we have recently found an important increase in the global Tyr phosphatase activity in HDAC6 KO PEMS when compared with WT PEMS (Supplemental Fig. 2B), suggesting that the diminished STAT3 phosphorolysis observed in these APCs could be a consequence of an enhanced phosphatase activity in the absence of HDAC6. Experiments to identify which STAT3-specific phosphatases might be regulated/associated with HDAC6 are currently ongoing in our laboratory.

In summary, we have unveiled a previously unknown mechanism for the transcriptional regulation of IL10 that involves HDAC6 and STAT3. To our knowledge, this is the first demonstration of such an interaction that is necessary for STAT3 phosphorylation and IL10 gene expression. A dynamic interaction between HDAC6 and STAT3 that occurs both in the cytoplasmic and nuclear compartiment of the APC and, as a result, dynamic changes in the expression of the tolerogenic IL-10 cytokine might explain the intrinsic plasticity of these cells to determine T cell activation versus T cell tolerance, a critical decision with important implications to the autoimmunity, cancer immunity, and transplantation fields.

Disclosures

The authors have no financial conflicts of interest.

References

10. Yang, X. J., and E. Seto. 2008. The Rpd3/Hda1 family of lysine deacetylases: the particular association of an ERK-specific phosphatase, DUSP5. Furthermore, Tøgø et al. (59) found that HDAC3 interacts with the phosphatase PP2A that involves HDAC6 and heat shock protein 90 control the functions of the APC and, as a result, dynamic changes in the expression of the tolerogenic IL-10 cytokine might explain the intrinsic plasticity of these cells to determine T cell activation versus T cell tolerance, a critical decision with important implications to the autoimmunity, cancer immunity, and transplantation fields.

The authors have no financial conflicts of interest.

References


