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Histone Deacetylase Inhibitor LAQ824 Augments Inflammatory Responses in Macrophages through Transcriptional Regulation of IL-10


APCs are important in the initiation of productive Ag-specific T cell responses and the induction of T cell anergy. The inflammatory status of the APC at the time of encounter with Ag-specific T cells plays a central role in determining such divergent T cell outcomes. A better understanding of the regulation of proinflammatory and anti-inflammatory genes in its natural setting, the chromatin substrate, might provide novel insights to overcome anergic mechanisms mediated by APCs. In this study, we show for the first time, to our knowledge, that treatment of BALB/c murine macrophages with the histone deacetylase inhibitor LAQ824 induces chromatin changes at the level of the IL-10 gene promoter that lead to enhanced recruitment of the transcriptional repressors HDAC11 and PU.1. Such an effect is associated with diminished IL-10 production and induction of inflammatory cells able of priming naive Ag-specific T cells, but more importantly, capable of restoring the responsiveness of anergized Ag-specific CD4+ T cells. The Journal of Immunology, 2011, 186: 3986–3996.

The potency of an immune response is dictated in large part by the potency of the APC and its ability to optimally prime the T cell response. This, in turn, is influenced by such factors as the particular APC type as well as the context, inflammatory versus non-inflammatory, in which the APC acquires the Ag for processing and presentation to Ag-specific T cells (1, 2). Not surprisingly, APCs isolated from a noninflammatory tumor microenvironment are relatively inefficient at priming protective responses, inducing instead T cell anergy (3–5).

During the past several years, numerous studies in experimental models as well as in humans have provided sufficient evidence supporting the conclusion that the induction of T cell anergy to tumor Ags represents a significant barrier to harness antitumor immunity (5–9). Important lessons learned from these studies point to manipulation of the inflammatory status of the APC as an enticing strategy to overcome anergic mechanisms in cancer (10–13). A better understanding of the molecular/signaling mechanism(s) regulating pro- and/or anti-inflammatory genes in the APC would likely provide important insights into how these cells influence T cell responses and might unveil novel targets to overcome anergy to tumor Ags.

Recently, a significant effort has been devoted to better understand the regulation of pro- and anti-inflammatory genes in their natural setting, the chromatin substrate (14). Chromatin modification by acetylation/deacetylation of histone tails is an important mechanism of regulation of gene transcription, including genes involved in the inflammatory response (15). In general, histone acetylation mediated by histone acetyl transferases results in transcriptionally active chromatin. In contrast, histone deacetylation mediated by histone deacetylases (HDACs) leads to an inactive chromatin and gene repression (16).

HDACs exist as large multimeric complexes and are recruited to gene promoters by corepressors or by multiprotein transcriptional complexes. Eighteen HDACs have been identified, and they have been grouped into four principal classes (17, 18). HDACs are the molecular target of several structurally diverse compounds known as histone deacetylase inhibitors (HDIs). Existing HDIs inhibit proliferation of malignant cells in vitro by inducing cell cycle arrest and apoptosis, and some of them have already demonstrated significant antitumor activity in cancer patients (19, 20). In contrast to their well-known effects upon cancer cells, little is still known about the immunological effects of HDIs. Although some studies have shown that HDIs have anti-inflammatory properties (21, 22), promote the expression of the suppressive factor IDO in dendritic cells (DCs) (23), and diminish the morbidity and mortality of graft-versus-host disease (24), others have highlighted the proinflammatory effects of these compounds. For instance, Tomasi’s group (25, 26) has shown that treatment of melanoma cells with HDIs augments their Ag-presenting capabilities, leading to activation of IFN-γ-secreting T cells via the class I pathway. Vo et al. (27) have recently demonstrated that in vivo treatment of tumor-bearing mice with the hydroxamic acid analog pan-HDI LAQ824 significantly enhances the antitumor activity of adoptively transferred Ag-specific T cells. Needless to say, the underlying molecular mechanism(s) by which HDIs influence inflammatory responses remain to be fully elucidated.

In this study, we show that the pan-HDI LAQ824 induces several chromatin changes in macrophages that resulted in enhanced recruitment of the transcriptional repressors HDAC11 and PU.1...
to the IL-10 gene promoter. Such an effect is associated with inhibition of IL-10 production and induction of cells able of priming naive Ag-specific T cells and capable of restoring the responsiveness of anergized CD4+ T cells.

Materials and Methods

Mice
Male BALB/c mice (6–8 wk old) were obtained from the National Institutes of Health (Frederick, MD). TCR-transgenic mice expressing an αβ TCR specific for aa 110–120 from influenza hemagglutinin (HA) presented by 1-β2 were a generous gift of H. von Boehmer (28). All 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) and the B-cell lymphoma cell line A20 was obtained from the American Type Culture Collection. A20HA was generated by electroporation-mediated plasmid transfection, and transfected cells were selected as previously reported (3, 6). Cells were cultured in vitro in RPMI 1640 media, supplemented with 10% FBS, penicillin/streptomycin (100 U/ml), l-glutamine (0.2 mM), and 2-ME (50 M) (complete media), and grown at 37°C and 5% CO2. A20 media was also supplemented with additional sodium pyruvate (1 mM) and 2-ME (50 M) (complete media), and grown at 37°C and 5% CO2 for 2 h to allow macrophages to adhere to the plate. Nonadherent cells were then removed with two washes of cold PBS. Splenic macrophages were isolated, washed with PBS, and then suspended in PBS. Splenic macrophages were then treated with HDI alone, LPS alone, or a combination thereof. Then supernatants were collected on an FACScan (BD Biosciences) and analyzed using FlowJo software (Tree Star). In a parallel plate, PEMs were treated with HA peptide110–120 presented by untreated, LPS-treated, or PEM-treated A20HA lymphoma-bearing mice and were then analyzed by staining with biotin-conjugated anti-CD86 (GL1 Ab; BD Pharmingen, San Jose, CA) and followed by strepavidin-PE (Caltag Laboratories, Carlsbad, CA) Abs. Ten thousand gated events were collected on an FACScan, and target mRNA levels were normalized to GAPDH expression using the method of Pfaff (31).

ChIP assays
ChIP studies were performed as previously described (32). The primers used were: IL-10 (proximal region) sense, 5'-GGAGGAGGACCT-GAATAAAC-3' and antisense, 5'-CTGTTCCCTGGTCCCTTTTT-3'; IL-12 (distal region) sense, 5'-AACCTGGCTGACTACGACCC-3' and antisense, 5'-GCCCTCTCCTCCTGACACTTC-3'; and IL-12 sense, 5'-GGGGCC-AAAACGGAGGTT-3' and antisense, 5'-GACCTGTCGAACTCCAGTTA-3'. All samples and inputs were quantified using MyiQ single-color real-time PCR detection system (Bio-Rad) and iQ SYBR green Supermix (Bio-Rad). Single-product amplification was confirmed by melt curve analysis, and primer efficiency was ~100% in all the experiments performed. Quantification is expressed in arbitrary units, and target mRNA levels were normalized to GAPDH expression using the method of Pfaff (31).

Statistical analysis
Unpaired t tests were performed using Microsoft Excel (Microsoft) with significance at p < 0.05.

Results
Phenotypic and functional changes in macrophages treated with the HDI LAQ824
First, we asked whether treatment of macrophages with the hydroxamic acid analog pan-HDI LAQ824 would result in changes in histone acetylation and the induction or not of inflammatory cells. As shown in Fig. 1A, treatment of PEM with LAQ824 was associated with increased acetylation of histones H3 and H4.
relative to their acetylated status in untreated PEM (None). No changes in the expression of MHC molecules or costimulatory molecules were observed in PEM treated with LAQ824 alone (data not shown). However, higher expression of B7.2 was observed in PEMs treated with LPS plus LAQ824 as compared with cells treated with LPS alone (Fig. 1B). Furthermore, treatment of PEMs with LPS in the presence of increasing concentrations of LAQ824 resulted in a dose-dependent inhibition of the anti-inflammatory cytokine IL-10 (Fig. 1C). Of note, this inhibitory effect was accompanied by an increased production of the pro-inflammatory cytokine IL-12 (Fig. 1D). It should be pointed out that LAQ824 was not toxic to PEMs because these cells were viable (as determined by trypan blue exclusion) and capable of producing IL-12 even when they were exposed to the highest concentration of HDI (100 nM) used in our experiments.

To further confirm the proinflammatory effects of LAQ824, we determined next the production of cytokines/chemokines by PEMs treated with HDI. As shown in Fig. 2, PEMs treated with LPS and LAQ824 produced higher levels of IL-1α, IL-1β, IL-6, RANTES, TNF-α, and GM-CSF as compared with PEMs treated with LPS alone. Of note, LAQ824 also inhibits the production of IL-10 by macrophages isolated from the spleen of tumor-bearing mice (Fig. 3A). No changes in IL-12 production were observed in tumor-bearing macrophages treated with HDI plus LPS as compared with macrophages treated with LPS alone (Fig. 3B).

**Pan-HDIs LBH589 and TSA, but not the class I selective HDI MS-275, inhibit IL-10 production by macrophages**

HDIs represent a family of structurally diverse compounds that inhibit the enzymatic function of HDACs. LAQ824 as well as LBH589, TSA, and SAHA are pan-HDIs that belong to the hydroxamic acid family of compounds. To determine whether the other members of this family share the proinflammatory effects of LAQ824, we treated PEMs with either LBH589 or TSA. Reminiscent of our findings with LAQ824, LBH589 and TSA also inhibit in a dose-dependent manner the production of IL-10 by PEMs in response to LPS (Fig. 4A, 4B, left panel). Such an inhibition was associated with increased production of IL-12 (Fig. 4A, 4B, right panel). Similar changes, although of a lesser magnitude, were observed in PEMs treated with SAHA (data not shown).

In contrast to the above results, treatment of PEMs with a more selective HDI, compound MS-275, which inhibits the enzymatic function of class I HDACs (HDAC 1, 2, 3, and 8) did not result in inhibition of IL-10 or augmentation of IL-12 production by treated cells (Fig. 4C). These findings argue against a role for class I HDACs in regulating IL-10 production in PEMs and point to class II and/or class IV HDACs as potential molecular targets involved in the inhibitory effect on IL-10 mediated by pan-HDIs.

**LAQ824-treated PEMs efficiently prime naive Ag-specific T cells and restore the responsiveness of anergized T cells**

Next, we determined whether the acquisition of inflammatory properties by LAQ824-treated macrophages renders these cells better activators of Ag-specific CD4+ T cells. PEMs were therefore treated with LAQ824, LPS, or a combination of LPS plus LAQ824 for 24 h. Following this treatment, naive CD4+ T cells specific for an MHC class II-restricted epitope of influenza HA were added to the PEM monolayer and stimulated, or not, with cognate HA peptide. First, clonotypic T cells encountering cognate peptide on untreated PEM (None) or on PEM treated with LAQ824 (HDI alone) produce similar levels of IL-2 (Fig. 5A, top panel). A slightly increased production of IL-2 was observed in T cells encountering Ag in LPS-treated PEMs (Fig. 5A, top panel, LPS alone). IL-2 production was further enhanced in CD4+ T cells that encountered HA peptide in PEMs treated with LPS plus increasing concentrations of LAQ824 (Fig. 5A, top panel, LAQ+LPS). LAQ824-treated PEMs also triggered an enhanced effector function of clonotypic CD4+ T cells, as determined by their capacity to produce higher levels of IFN-γ in response to cognate peptide (Fig. 5A, bottom panel).

Using a TCR-transgenic model, we have previously demonstrated that CD4+ T cells specific for an MHC class II epitope of influenza HA are rendered anergic during the growth of a B cell lymphoma expressing HA as a model tumor Ag (A20HA). Iso-

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**FIGURE 1.** Phenotypic and functional changes in macrophages treated with the HDI LAQ824. A. PEMs were treated or not with LAQ824 (12.5 nM) for 24 h. Then, cell lysates were obtained, and acetylation of histones H3 and H4 was determined by immunoblotting using anti-acetyl H3 and H4 Abs, respectively. B. PEMs were treated with LPS (2 μg/ml) or with LPS plus LAQ824 (12.5 nM) for 24 h. Cells were then stained with an anti-B7.2 Ab or an isotype control. In a parallel experiment, PEMs were treated with LPS alone (2 μg/ml) or LPS plus increasing concentrations of LAQ824 as indicated. After 24 h, supernatants were collected and the levels of IL-10 (C) and IL-12 (D) were determined by ELISA. Shown is a representative experiment of five experiments with similar results. *p < 0.05, **p < 0.01.
lation of these clonotype-positive T cells from tumor-bearing mice followed by their in vitro restimulation with HA peptide plus APCs demonstrated that these cells were anergic given their inability to produce IL-2 or IFN-γ (3, 6). However, in vitro incubation of these same anergic T cells with LAQ824-treated PEMs resulted in restoration of T cell responsiveness (Fig. 5B).

Indeed, anergized Ag-specific CD4+ T cells encountering cognate Ag on PEMs treated with LAQ824 and LPS regained their ability to produce IL-2 (Fig. 5B, top panel) and IFN-γ (Fig. 5B, bottom panel). In contrast, anergic T cells encountering cognate Ag on untreated (None), HDI alone, or LPS-treated PEM were unable to produce IL-2 (Fig. 5B, top panel) and produced minimal amounts of IFN-γ (Fig. 5B, bottom panel). Therefore, LAQ824-treated PEMs effectively prime naive Ag-specific CD4+ T cells and restore the responsiveness of anergic CD4+ T cells.

To better define the role of IL-10 inhibition induced by LAQ824 in the augmentation of the APC function of macrophages, we cultured PEMs with naive Ag-specific CD4+ T cells in the presence of recombinant murine IL-10. As shown in Fig. 5C, T cells produced less IFN-γ in response to cognate Ag presented by LPS-treated macrophages when rIL-10 was added (Fig. 5C, LPS alone, black bar versus gray bar). Similarly, the enhanced APC function displayed by macrophages treated with 12.5 nM LAQ824+LPS (Fig. 5C, LAQ+LPS, gray bar) was abrogated when rIL-10 was added back to the cultures (Fig. 5C, LAQ+LPS, black bar). This result was not surprising given the well-known ability of IL-10 to inhibit Th1-type responses (33). Given that the inhibition of IL-10 induced by LAQ824 at the dose of 12.5 nM is incomplete (Fig. 1C), we asked next whether neutralization of the remaining secreted IL-10 with anti–IL-10 Abs could further augment the APC function.

**FIGURE 2.** Production of proinflammatory mediators by macrophages treated with HDI LAQ824. PEMs were treated with LPS (2 μg/ml), LAQ824 (12.5 nM), or LPS (2 μg/ml) plus increasing concentrations of LAQ824 as indicated. After 24 h, supernatants were collected, and cytokine/chemokine levels were determined by ELISA. Shown is a representative experiment of three experiments with similar results. *p < 0.05, **p < 0.01.

**FIGURE 3.** LAQ824 inhibits IL-10 production by macrophages from tumor-bearing mice. A, Splenic macrophages from A20-bearing mice were treated with LPS (2 μg/ml), LAQ824 alone (12.5 nM), or LPS (2 μg/ml) plus LAQ824 for 24 h. The supernatants were collected and production of IL-10 (A) and IL-12 (B) were determined by ELISA. Shown is a representative experiment of two experiments with similar results. **p < 0.01.
function of LAQ824-treated macrophages. As shown in Fig. 5D, IL-10 blockade was insufficient to enhance the APC function of LPS-treated macrophages to augment IFN-γ production by CD4+ T cells (Fig. 5D, LPS alone, black bar versus gray bar), and it was not able to significantly enhance the effect of LAQ824 treatment (Fig. 5D, LAQ+LPS, black bar versus gray bar). Taken together, these data point to a contributory role of IL-10 inhibition in the enhanced APC function displayed by LAQ824-treated macrophages because this effect was reversed when rIL-10 was added back to the cultures. However, neutralizing the remaining IL-10 protein that could have still been produced by LAQ824-treated macrophages did not result in further enhancement of their APC function.

**IL-10 mRNA expression and histone acetylation of the IL-10 gene promoter in PEMs treated with LAQ824**

IL-10 is an anti-inflammatory cytokine that plays an important role in the establishment and maintenance of T cell anergy (34, 35). One of the most striking and consistent effects of pan-HDIs was their ability to inhibit IL-10 production (Figs. 1C, 3, 4). To better understand the mechanism(s) underlying this inhibitory effect, we evaluated next the kinetics of IL-10 mRNA expression in macrophages treated with LPS, LAQ824, or LPS plus LAQ824. In response to LPS stimulation, a rapid increase in IL-10 mRNA was observed at 1 h, followed by a rapid decline, and, after 6 h, the IL-10 mRNA levels were back to baseline (Fig. 6A, open triangles). In PEMs treated with LPS plus LAQ824, a decreased induction of IL-10 mRNA was observed at 1 h (Fig. 6A, closed circles). This initial response was followed by a progressive decline, and, by 6 h, IL-10 mRNA levels were back to baseline levels. No changes in IL-10 mRNA levels were observed in PEMs treated with LAQ824 alone (Fig. 6A, open squares). Therefore, treatment of PEMs with LAQ824 resulted in decreased IL-10 gene transcriptional activity in response to LPS stimulation.

Next, we determined the acetylation status of histones at the level of the IL-10 promoter. First, by using a ChIP assay, we assessed the kinetics of acetylation of histones H3 and H4 in PEMs treated with LPS alone. As seen in Fig. 6B, acetylation changes occurred within a particular time window, with a peak acetylation occurring at 60 min. This was followed by a progressive decline, and, by 4 h, H3 and H4 acetylation levels were almost back to baseline. Given that the peak acetylation was observed 1 h after LPS stimulation, in the next set of experiments, PEMs were pretreated, or not, with LAQ824 for 24 h and then stimulated or not with LPS and replenished LAQ824 as appropriate to maintain pretreatment conditions for an additional 1 h. By ChIP assay, we...
found that in response to LPS, histones H3 and H4 display increased acetylation (Fig. 6C, Control LPS) relative to untreated PEMs (Fig. 6C, Control w/o LPS). When PEMs were treated with HDI alone, no significant changes in acetylation of H3 and H4 were observed (Fig. 6C, LAQ w/o LPS). However, treatment of PEMs with LPS in the presence of HDI resulted in decreased H3 and H4 acetylation (Fig. 6C, LAQ LPS). This effect was specific for IL-10 because a similar analysis of H3 and H4 acetylation at the level of the IL-12 promoter revealed no differences between PEMs treated with LPS alone and PEMs treated with LPS plus HDI (Fig. 6D, Control LPS versus LAQ/LPS). Taken together, treatment of PEMs with LAQ824 resulted in decreased IL-10 gene transcriptional activity and diminished H3 and H4 acetylation at the level of the IL-10 gene promoter.

**Chromatin changes in the IL-10 gene promoter induced by the HDI LAQ824**

Previously, we have reported the sequence of chromatin modifications that occur at the level of the IL-10 gene promoter in macrophages stimulated with LPS (32). Briefly, phosphorylation of Ser10 on H3 is an early event that occurs 30 min after LPS stimulation. This is followed by increased acetylation of H3 and H4 at 60 min, subsequent recruitment to the IL-10 gene promoter of the transcriptional activators Sp1 and STAT3, and, ultimately, IL-10 gene expression. This sequence of events was affected in RAW264.7 macrophage cells pretreated with LAQ824 for 24 h and then stimulated with LPS (Fig. 7).

First, phosphorylation of Ser10 on H3 at the IL-10 promoter has been shown necessary for transcriptional activation (36). No differences in the kinetics of phosphorylation of H3 at the Ser10 position were observed among macrophages treated with LPS alone (Fig. 7A, solid lines) or LAQ824 (12.5 nM) in the presence or not of recombinant murine IL-10 (C) or neutralizing anti-mouse IL-10 Ab (D) for 24 h. Then, cells were washed, with warmed RPMI 1640 and 5 x 10^5 naive anti-HA transgenic CD4^+ T cells were added to the PEMs in the presence of cognate HA peptide (12.5 μg/ml). Recombinant murine IL-10 (C) or neutralizing anti-mouse IL-10 Ab (D) were replenished, or not, in the macrophages/T cell cultures to maintain the respective pretreated conditions, but without LPS. After 48 h, supernatants were collected, and IFN-γ production was measured by ELISA. *p < 0.05, **p < 0.01.
by determining the binding of PolII to the IL-10 gene promoter. Unlike macrophages treated with LPS alone, only a minimal and transient increase in PolII binding was observed in macrophages treated with LAQ824 plus LPS (Fig. 7D).

Sp1 and STAT3 are known IL-10 transcriptional activators that are recruited to the proximal region of the IL-10 gene promoter in macrophages stimulated with LPS (37–39). As shown in Fig. 7E, recruitment of Sp1 peaks at 1 h after LPS stimulation and is then followed by a rapid decline at 2 h. Similarly, STAT3 binding to the IL-10 promoter is evident within 60 min of LPS stimulation. This is followed by a progressive decline to basal levels by 3 h (Fig. 7F). No such kinetics of recruitment of either Sp1 or STAT3 was observed in cells treated with LAQ824 and LPS (Fig. 7E, 7F, dashed lines).

PU.1 is a transcriptional repressor that interacts with the distal IL-10 gene promoter region (40, 41). Recently, we have demon-
strated that HDAC11, by interacting with the distal segment of the IL-10 gene promoter, negatively regulates the expression of this cytokine in murine and human APCs (32). As shown in Fig. 7G and 7H, ChIP analysis of the IL-10 gene distal promoter in macrophages treated with LAQ824 revealed some interesting findings. Recruitment of PU.1 to the IL-10 gene promoter in cells treated with LPS reached a peak within 2 h and remained elevated for the duration of the analysis (Fig. 7G, solid line). In macrophages treated with LAQ824, recruitment of HDAC11 reached a higher peak (relative to macrophages treated with LPS alone) within 2 h, and it was followed by a rapid return to baseline levels at 3 h after LPS stimulation (Fig. 7H, dashed line).

Therefore, treatment of macrophages with LAQ824 is associated with several chromatin changes at the level of the IL-10 gene promoter, among them an enhanced recruitment of the transcriptional repressors PU.1 and HDAC11.

Discussion
In this study, we have demonstrated that the pan-HDI LAQ824, by inhibiting IL-10 and increasing the expression of B7.2 and the production of several proinflammatory mediators, induced inflammatory macrophages that effectively activate Ag-specific CD4+ T cells and restore the responsiveness of anergic T cells.
Among the above changes, the most striking effect of LAQ824 was its ability to inhibit the production of the immunosuppressive cytokine IL-10. Such an effect was also displayed by other members of the hydroxamic acid family like LBHS89, TSA, and SAHA, but not by the more specific HDI MS-275, which mainly target class I HDACs. The central role of IL-10 in the establishment and maintenance of T cell anergy (34, 35, 42–44) prompted us to further investigate the underlying mechanism(s) by which these particular HDIs inhibit IL-10 production in macrophages.

The dynamic production of pro- and anti-inflammatory mediators at the site of Ag encounter has been shown to shape the initiation, magnitude, and duration of an immune response (45). IL-10 plays a key role in negatively regulating these dynamic changes to prevent self-tissue damage that might otherwise occur if an ongoing immune response is not kept in check (39). This protective property of IL-10 imposes, however, a significant barrier to our efforts to effectively harness antitumor immune responses. Indeed, production of this cytokine at the tumor site by tumor cells themselves or infiltrating cells, such as APCs or regulatory T cells, creates a microenvironment that is conducive to T cell anergy rather than T cell activation (5). A better understanding of the genetic and/or epigenetic mechanisms regulating IL-10 production therefore has important implications to manipulate the inflammatory status of APCs and their intrinsic ability to prime, or not, Ag-specific T cell responses.

Recent studies have shown that IL-10 production is regulated at the chromatin level by changes in the acetylation status of the gene promoter (46, 47). For instance, changes in the chromatin structure of the IL-10 promoter in T cells differentiated into the Th1 or Th2 phenotype closely regulate IL-10 expression (48). In macrophages, increased acetylation of the IL-10 promoter has been associated with enhanced transcriptional activity (46). Conversely, we have recently demonstrated that decreased acetylation of the IL-10 promoter is associated with decreased IL-10 transcriptional activity in murine and human APCs (32). Given the above, we expected that treatment of macrophages with HDIs would result in increased histone acetylation and increased IL-10 production. Although an increased global acetylation of histones H3 and H4 was observed in cells treated with LAQ824 (Fig. 1A), the opposite outcome was observed at the level of the IL-10 gene promoter of HDI-treated cells. To our surprise, diminished H3 and H4 acetylation at the IL-10 promoter was observed at all evaluated time points in treated macrophages (Fig. 7B, 7C). This decrease in histone acetylation, which occurs early, might explain the decreased recruitment of the transcriptional activators STAT3 and Sp1 to the IL-10 gene promoter (Fig. 7E, 7F). It is plausible that a more compacted chromatin due to diminished histone acetylation in the IL-10 promoter region might block the access of these transcriptional activators to the promoter region resulting in the decreased IL-10 gene transcriptional activity observed in LAQ824-treated cells.

However, we are left to explain how HDIs induce decreased histone acetylation at the level of the IL-10 gene promoter in the first place. Kinetic analysis of H3 and H4 acetylation provided some hints. In macrophages pretreated with LAQ824 and then stimulated with LPS and replenished LAQ824, we observed an initial acetylation of H3 and H4 that reaches its peak at 1 h poststimulation. However, the magnitude of these changes was significantly lower than in macrophages treated with LPS alone. Following this peak acetylation, we observed a rapid abrogation of such a response in cells treated with HDI, suggesting either a lack of stimuli to support H3 and H4 acetylation and/or the induction of counterregulatory mechanism(s) that attenuated the degree of H3 and H4 acetylation observed in cells treated with LPS alone.

Of particular interest is the finding that following stimulation, enhanced recruitment of two transcriptional repressors to the distal promoter of IL-10 was observed, a process that occurs faster for PU.1 than for HDAC11 (Fig. 7G, 7H). It is plausible therefore that recruitment of PU.1 and HDAC11 to the IL-10 gene promoter might represent a counterregulatory mechanism triggered by HDIs to diminish H3 and H4 acetylation and block the sequence of events that lead to IL-10 gene transcriptional activation. Supporting the above, we have recently demonstrated that overexpression of HDAC11 in the macrophage cell line RAW264.7 resulted in decreased H3 and H4 acetylation of the IL-10 gene promoter and inhibition of IL-10 gene transcriptional activity (32). Interestingly, Bradbury et al. (49) have found that treatment of myeloid leukemic blasts with TSA, a member of the hydroxamic acid family of HDIs, resulted in a 60–200-fold induction of HDAC11 mRNA expression. Similarly, we have also found that RAW264.7 cells treated with either TSA or LAQ824 display increased HDAC11 mRNA and protein expression (data not shown). Such an effect of HDIs upon HDAC11 expression might explain, at least in part, the increased recruitment of this particular HDAC to the IL-10 gene promoter in cells treated with LAQ824. The mechanism(s) by which HDI increases the expression of HDAC11 in macrophages, however, remains to be elucidated.

An additional observation in macrophages treated with LAQ824 is their enhanced expression of B7.2 and increased production of several proinflammatory mediators, in particular IL-12. It is plausible that these effects are secondary to the inhibition of IL-10, a cytokine that has been shown to downregulate the expression of costimulatory molecules and the production of IL-12 in APCs (50, 51). This switch from an IL-10– to an IL-12–producing and B7.2-expression state is critical in polarizing T cells toward Th1 cell-mediated immunity. In our system, the demonstration that H3 and H4 acetylation in the IL-12 promoter was neither inhibited nor enhanced in cells treated with LAQ824 (Fig. 6D) suggests that the enhanced production of this proinflammatory mediator in treated macrophages might be secondary to the abrogation of IL-10 production by LAQ824. Our experiments using rIL-10 also point to a contributory role of IL-10 inhibition (induced by LAQ824) in the enhanced APC function displayed by treated macrophages because this effect was reversed when rIL-10 was added back to the cultures. It remains to be demonstrated whether the upregulation of B7.2 is a direct or indirect effect of LAQ824. It is noteworthy to mention that B7.2 was preferentially upregulated in treated macrophages, whereas no changes were observed in various other costimulatory molecules (data not shown). Although the exact mechanism for this selectivity has yet to be elucidated, our results are consistent with observations in human DCs whereby IL-10 mediated downregulation of B7.2 but not B7.1 (50).

Our findings are at odds with the studies by Reddy et al. (23), who recently found that the HDI SAHA actually attenuated inflammatory responses in DCs through IDO-dependent mechanisms. Injection of DCs treated ex vivo with SAHA also decreased the severity of graft-versus-host disease in their murine allogeneic bone marrow transplantation model. Several differences between Reddy’s study (23) and ours may explain these seemingly conflicting data. First, in our in vitro system measuring cytokine secretion, we used macrophages that were treated with HDI and LPS given at the same time. In their study, DCs primarily, but in one experiment macrophages, were pretreated with SAHA prior to stimulation with TLR agonist. Second, they found that SAHA treatment did not induce significant changes in the production of IL-10 by DCs. Supporting their observation, we have also found that among all of the members of the hydroxamic family of HDI, SAHA is the weakest inhibitor of IL-10 production in macro-
phages (data not shown). It is plausible therefore that differences among members of the same family of HDI, with LAQ824 and LBH589 being more potent inhibitors of IL-10 production than SAHA, might explain in part at least the divergent effects of these HDI upon the inflammatory status of DCs and/or macrophages. Of note, the overall potencies of HDIs must also be reconciled with the relative potencies, as pan-HDIs do not inhibit all members of the HDAC family at equimolar concentrations, which may explain the differing effects on a single target such as IL-10 (52). Our findings are, however, consistent with reports by others indicating that HDIs can potentiate inflammatory and antitumor responses in vitro and in vivo (25–27). Among the latter, Vo et al. (27) has demonstrated an enhanced T cell-mediated immunity in tumor-bearing mice treated with LAQ824. Whether the preferential expansion of adoptively transferred Ag-specific T cells observed in this model is the result of T cell interaction with inflammatory cells can potentially tip the balance toward T cell anergized T cells from tumor-bearing mice. LAQ824 by inducing maims to be explored.

Taken together, LAQ824-treated macrophages cannot only more potently activate naive T cells, but also restore the reactivity of anergized T cells from tumor-bearing mice. LAQ824 by inducing inflammatory cells can potentially tip the balance toward T cell activation rather than T cell anergy, an effect that holds promise for inflammatory cells and T cell anergy, an effect that holds promise for the use of HDIs in the adjunct setting to ultimately improve the efficacy of cancer immunotherapy.

Disclosures

The authors have no financial conflicts of interest.

References


