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Lysosome-dependent p300/FOXP3 degradation and limits T_{reg} cell functions and enhances targeted therapy against cancers

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Abstract

p300 is one of several acetyltransferases that regulate FOXP3 acetylation and functions. Our recent studies have defined a complex set of histone acetyltransferase interactions which can lead to enhanced or repressed changes in FOXP3 function. We have explored the use of a natural p300 inhibitor, Garcinol, as a tool to understand mechanisms by which p300 regulates FOXP3 acetylation. In the presence of Garcinol, p300 appears to become disassociated from the FOXP3 complex and undergoes lysosome-dependent degradation. As a consequence of p300's physical absence, FOXP3 becomes less acetylated and eventually degraded, a process that cannot be rescued by the proteasome inhibitor MG132. p300 plays a complex role in FOXP3 acetylation, as it could also acetylate a subset of four Lys residues that repressively regulate total FOXP3 acetylation. Garcinol acts as a degradation device to reduce the suppressive activity of regulatory T cells (T_{reg}) and to enhance the in vivo anti-tumor activity of a targeted therapeutic anti-p185^{her2/neu} (ERBB2) antibody in MMTV-neu transgenics implanted with neu transformed breast tumor cells. Our studies provide the rationale for molecules that disrupt p300 stability to limit T_{reg} functions in targeted therapies for cancers.

Keywords

Acetyltransferase; Lysosome degradation; FOXP3; Garcinol; p300; Regulatory T cells; T_{reg}

Introduction

FOXP3 (Forkhead box P3) is a member of the forkhead/winged-helix family of transcription regulators regulatory T (T_{reg}) cells' development and function (Ziegler, 2006). While mouse has only one isoform, human FOXP3 includes two isoforms, FOXP3a and FOXP3b (Allan et al., 2005; Ziegler, 2006). Both the expression level and stability of the FOXP3 protein play crucial roles in T_{reg} differentiation and suppressive functions (Li and Greene, 2007; Wan and Flavell, 2007; Williams and Rudensky, 2007). Loss of FOXP3 function limits T_{reg}

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Conflict of interest statement

There is no conflict of interest to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.yexmp.2013.04.003>.

cell functionalities and can promote lethal auto-aggressive lymphoproliferation, and overexpression of FOXP3 limits immune responses (Hori et al., 2003; Merlo et al., 2009).

Post-translational modifications, such as phosphorylation, acetylation, methylation, ubiquitination and sumoylation have been shown to occur on many transcriptional factors and affect transcriptional activity by regulating protein stability, subcellular localization, oligomerization, or DNA binding (Li and Greene, 2007). FOXP3 lysine ϵ -acetylation is an important post-translational modification that regulates FOXP3 expression levels and functions in T_{reg} cells (Beier et al., 2011; Zhang et al., 2012b). At least two histone acetyltransferases (HAT), including Tat-interactive protein (Tip60, also known as KAT5) and p300, promote FOXP3 acetylation (Li et al., 2007; van Loosdregt et al., 2010; Xiao et al., 2010). Acetylated forms of FOXP3 appear to be more resistant to polyubiquitination and proteasomal degradation, and move from the nucleoplasm to the chromatin (Li et al., 2007; Samanta et al., 2008; van Loosdregt et al., 2010).

Garcinol, a polyisoprenylated benzophenone derivative from the fruit rind of *Garcinia indica*, has been claimed to represent a natural inhibitor of p300 and possibly other enzymes (Balasubramanyam et al., 2004). Garcinol inhibits p300 auto-acetylation as well as p300-mediated p53 acetylation (Arif et al., 2007; Mantelingu et al., 2007). We now discover that Garcinol induces physical degradation of p300, not through the proteasome but the lysosomal degradation processes. The study of Garcinol and FOXP3 has revealed that degradation of p300 leads to more dramatic effects on phenotypic changes in T_{reg} cells beyond the post transcriptional modification of FOXP3.

Materials and methods

Reagents

Garcinol and Bafilomycin A1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Chloroquine diphosphate salt and MG-132 were obtained from Sigma-Aldrich (St. Louis, MO) and EMD Millipore (Billerica, MA), respectively. FITC-conjugated anti-mouse CD4 and PE-conjugated anti-CD25 were purchased from BD Biosciences (San Jose, Ca). APC-conjugated anti mouse CD45RB was purchased from BioLegend (San Diego, CA).

Cell culture and transfection

H2N113 is a mouse breast tumor cell line derived from 20- to 23-week-old female BALB/c MMTV-ErbB-2/neuT transgenic mice (Stagg et al., 2008). H2N113 and the human embryonic kidney cell line 293T were cultured in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Scientific, Waltham, MA), 100 units/ml penicillin, 100 μ g/ml streptomycin and 2 mM L-glutamine (Gibco, Grand Island, NY). H2N113 was further supplemented with 1 $\times$ sodium pyruvate, Glutamax and MEM NEAA (all from Gibco). Cells were maintained at 37 °C in a 95% humidified incubator containing 5% CO₂. For transfection, cells were transfected with expression plasmids (3 μ g each) in a 10-cm dish using the Fugene 6 transfection reagent (Promega, Madison, WI) following the manufacturer's instructions. Cells were subjected to treatment with inhibitors 24 h after transfection.

Immunoprecipitation and Western blot analysis

Cells were washed twice with ice-cold PBS and lysed by lysis buffer (20 mM Tris-Cl, pH 7.5, 1% Nonidet P-40, 2 mM EDTA, 420 mM NaCl, 400 nM TSA, 10 mM Nicotinamide, 1 mM Na₃VO₄, 1 mM NaF, 1 mM DTT, and 1 mM PMSF) in the presence of protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), followed by immunoprecipitation with Acetylated-Lysine antibody (Cell Signaling Technology, Danvers, MA) and protein G

Dynabeads (Invitrogen/Life Technologies, Grand Island, NY). For co-immunoprecipitation with the anti-FLAG mAb, whole cell lysates were first pre-cleared using control IgG and Protein A/G-Agarose (Santa Cruz Biotechnology, Inc.) after rotation at 4 °C for 0.5 h and centrifugation at 3000 rpm at 4 °C for 1 min. Pre-cleared cell lysates were immunoprecipitated with anti-FLAG mAb M2 (Sigma) and Protein A/G-Agarose. Total lysates and immunoprecipitated samples were separated on 6% or 10% glycine SDS-PAGE gel and transferred to nitrocellulose membrane. For Western blot analysis, anti-HA-HRP antibody (Roche), anti-Flag-HRP (cloneM2) and Anti- β -actin-HRP antibody (Sigma-Aldrich), Acetylated-Lysine antibody (Cell Signaling Technology) were used at dilutions suggested by the manufacturers. The antigen-antibody complex was revealed with Millipore Immobilon Western Chemiluminescent HRP substrate (Millipore Corporation, MA).

Cell proliferation assay

To measure cell proliferation, we used the modified 3-[4,5-dimethylthiazol-2-yl]- 2,5-diphenyl tetrazolium bromide (MTT) assay as previously described (Masuda et al., 2006). Briefly, H2N113 cells were seeded at a density of 5000 cells/well in 96-well plates and treated with different concentration of 7.16.4 antibody and/or Garcinol for 3 days at 37 °C in a humidified 5% CO₂ atmosphere. Old culture medium was replaced with 100 μ L fresh medium and incubated for 4 h at 37 °C. 25 μ L of MTT solution (5mg/mL in PBS) were added to each well. After 2 h of incubation at 37 °C, 100 μ L of extraction buffer (20% w/v SDS, 50% N,N-dimethyl formamide, pH 4.7) was added. After an overnight incubation at 37 °C, the absorbance was measured at 570 nm using an ELISA reader.

In vivo tumor studies

All mouse procedures were performed according to the guidelines and protocols approved by the IACUC of University of Pennsylvania. 6–8 week old female MMTV-neu/FOXP3-GFP mice, which express the oncogenic neu under the MMTV promoter, were used as the syngeneic host of the H2N113 tumor line. Tumors were developed by s.c. injection of 1×10^6 H2N113 cells in the flank region of mice. After 10 days, mice were treated with Garcinol and/or 7.16.4 antibody by intraperitoneal injection twice weekly. Control mice were injected with PBS. Tumor size was measured with a Vernier caliper, and tumor volume was calculated by the formula: $3.14 \times \text{length} \times \text{width} \times \text{height} / 6$.

In vitro T_{reg} suppression assay

CD4⁺ T cells were enriched by MACS CD4⁺ T cell isolation kit II (Miltenyi) from the spleen of C57BL/6 mice. CD4⁺CD25⁻CD45RB^{high} effector T cells (T_{eff}) and CD4⁺CD25^{high} regulatory T cells (T_{reg}) of spleen were isolated by FACS Aria II, yielding a purity of ~97% for both types of cells. T_{eff} were labeled with CFSE (Invitrogen) and co-cultured with different ratio of T_{reg}, anti-CD3/CD28 beads (Invitrogen), with or without Garcinol (2 or 4 μ M), in RPMI supplemented with 10% FBS, 1 $\times$ non-essential amino acids (Invitrogen), 2 mM sodium pyruvate (Invitrogen) and 50 μ M β -mercaptoethanol (Sigma). After 3 days of co-culture, cells were harvested and in vitro proliferation of lymphocytes was analyzed by FACSCanto Flow Cytometry.

Results

Inhibition of p300 by Garcinol leads to degradation of p300 and influences FOXP3 acetylation

Since both cellular FOXP3 level and transcriptional activity are affected by acetylation (Li et al., 2007; Song et al., 2012) and p300 is known to regulate FOXP3 acetylation (van Loosdregt et al., 2010), we investigated the effects of the p300 inhibitor Garcinol on FOXP3

activity. HEK 293T cells were transfected with HA-tagged FOXP3 (HA-FOXP3a) and Flag-tagged p300 (Flag-p300). Cells were treated with different doses of Garcinol (0–25 μ M) for 4 h. Acetylation of FOXP3 and p300 were examined by immunoprecipitation (IP) of acetylated proteins from cell lysate using an anti-acetyl lysine antibody and blotted with anti-HA and anti-Flag antibody for FOXP3 and p300, respectively.

As shown in Fig. 1, FOXP3 acetylation was minimal in the absence of p300. Co-expression with p300 enhanced acetylation as well as the protein abundance of FOXP3. Acetylation of both FOXP3 and p300 was reduced by Garcinol in a dose dependent manner (Fig. 1A, Ac-K IP).

Unexpectedly, the p300 expression level, as determined by anti-FLAG blot from the total lysate, was also clearly reduced by Garcinol. p300 proteins in the lysate were reduced quantitatively by 15 μ M of Garcinol and became undetectable after treatment with 25 μ M of Garcinol. No reduction of the FOXP3 protein level was observed at 15 μ M of Garcinol. In the presence of 25 μ M of Garcinol, when p300 was maximally degraded, the FOXP3 level was reduced and became comparable with that of control transfection (pFLAG, no ectopically expressed p300, no Garcinol treatment). Clearly Garcinol induces p300 degradation and subsequently influences both FOXP3 acetylation levels and stability.

We studied the interaction between p300 and FOXP3 by co-immunoprecipitation (co-IP). Lysates from cells with co-expression of p300 and FOXP3 were first precipitated with the anti-Flag antibody for p300 and probed with the anti-HA antibody for FOXP3. As shown in Fig. 1B, Garcinol limited the interaction between p300 and FOXP3. At 15 μ M Garcinol, the amount of precipitated p300 was comparable with that in the control (no Garcinol treatment), but less FOXP3 proteins were associated with p300. At 25 μ M Garcinol, there were still some p300 proteins in the co-IP but FOXP3 became indiscernible. Garcinol promotes the disassembly of the FOXP3-p300 complex while driving p300 into degradation.

Garcinol-mediated degradation of p300 is prevented by Chloroquine

FOXP3 degradation is thought to be mediated primarily through proteasomal pathways (van Loosdregt et al., 2010). To determine whether Garcinol-induced p300 degradation is promoted by proteasomal processes or by other mechanisms, we examined several inhibitors of protein degradation, including MG132, Chloroquine, E-64, pepstatin A, and PMSF (Fig. S1). Chloroquine, but not MG132 or any other inhibitors, efficiently blocked Garcinol-mediated p300 degradation. As shown in Fig. 2A, Chloroquine dose-dependently reversed the effect of Garcinol on both p300 and FOXP3 in terms of both protein and acetylation levels.

FOXP3 acetylation was clearly observed when 5 μ M or more Chloroquine was present as compared with cells treated with 25 μ M Garcinol alone. Complete blocking of p300 degradation was observed with 20 μ M Chloroquine. Interestingly, although Garcinol was able to prevent p300 from acetylating substrates (Balasubramanyam et al., 2004), it did not affect auto-acetylation of p300. As shown in Fig. 2A, acetylation of Garcinol treated-p300 was detectable once p300 degradation was blocked by Chloroquine.

The effect of MG132 on FOXP3 degradation was complex. At 5 μ M, a level that is routinely used to block proteasome-mediated protein degradation (van Loosdregt et al., 2010), MG132 was not able to counteract Garcinol on FOXP3 acetylation and stability (Fig. 2A). At higher concentrations (>20 μ M), MG132 promoted FOXP3 degradation, as well as the degradation of the control protein β -actin, indicating a broad, non-specific outcome that may be related to the presence of both Garcinol and MG132. Interestingly, acetylation of FOXP3

was increased at higher MG132 concentrations, despite the reduced FOXP3 protein levels. Acetylated forms of FOXP3 appeared to be more stable.

Clearly, these studies indicate that Garcinol promotes the entry of p300 into a lysosome-mediated protein degradation pathway, indirectly leading to reduced acetylation of FOXP3. The influence of Garcinol on the FOXP3 protein level is limited as compared with that on p300. Although massive destruction of FOXP3 was not observed in the presence of Garcinol, the reduction of FOXP3 protein levels accompanying the loss of p300 also appeared to be lysosomally related.

To further confirm the role of Chloroquine in the degradation of p300, we utilized bafilomycin A1. Bafilomycin A1 inhibits the initial localization of Chloroquine to acidic vesicles, thus blocking Chloroquine from disrupting lysosomal function (Boya et al., 2003). Bafilomycin A1 alone slightly promoted p300 degradation (Fig. 2B, lane 2 vs lane 7). While Chloroquine prevented Garcinol-induced p300 degradation (lane 2B vs lane 2), bafilomycin A1 attenuated the effect of Chloroquine (Fig. 2B, lane 5), leading to reduced acetylated p300 signals noted after anti-acetyl lysine immunoprecipitation as well as reduced total p300 protein levels in the lysate (Fig. 2B).

Degradation of FOXP3 after Garcinol treatment is not dependent on acetylation of FOXP3 by ectopic p300

We next examined if the Garcinol effect was only dependent on ectopic expression of p300. For this purpose, HEK 293T cells were transfected with HA-FOXP3a only. As shown in Fig. 3A, Garcinol was able to reduce FOXP3 acetylation in a dose dependent manner, and Chloroquine reversed the Garcinol effect on FOXP3 (Fig. 3A). This is consistent with our observation that FOXP3 can be acetylated by endogenous acetyltransferases but to a lesser extent than with ectopically over-expressed p300.

Previously we have identified K250/K252 in human FOXP3 as an important acetylation site for p300 (Song et al., 2012). Mutations on these two sites greatly reduce FOXP3 acetylation by p300. We investigated the effect of Garcinol on the FOXP3 K250R/K252R mutant. As shown in Fig. 3B, although the acetylation level of this mutant was reduced as compared with the wild type FOXP3 (Fig. 3A), Garcinol was still able to reduce acetylation of this mutant, and degradation of FOXP3 by Garcinol was prevented by the addition of Chloroquine. For both the wild type FOXP3 (Fig. 3A) and the K250R/K252R mutant (Fig. 3B), MG132 at 5 μ M further increased Garcinol-induced FOXP3 degradation.

Acetylation of the FOXP3 K250R/K252R mutant indicates that there exist additional acetylation sites for p300. It is also possible that other endogenous acetyltransferases are involved in the acetylation of FOXP3, and Garcinol can also inhibit these acetyltransferases. We have observed synergistic effects of p300 and TIP60 on FOXP3 (Xiao et al., in preparation).

Garcinol as a broad spectrum acetyltransferase inhibitor

Garcinol was reported to inhibit p300 and P/CAF (also known as KAT2B) with comparable activity (IC₅₀: 7 and 5 μ M, respectively) (Mantelingu et al., 2007). Garcinol was also observed to inhibit TIP60 at comparable levels to anarcadic acid (IC₅₀: Garcinol = 12 μ M, anarcadic acid = 6 μ M) (Arun Dutta, personal communication). We compared the inhibitory activity of Garcinol on several acetyltransferases in our cell-based system and examined the acetylation level by immunoprecipitation with the anti-acetyl lysine antibody.

As shown in Fig. 4, Garcinol inhibited all enzymes (p300, CBP (also known as CREBBP), TIP60 and P/CAF) to some extent. Among these enzymes, p300 was the most sensitive to

Garcinol inhibition. At 15 μ M, Garcinol clearly inhibited both protein level and acetylation level of p300. TIP60 was also sensitive to Garcinol, but only the acetylation level was reduced by 15 μ M Garcinol. At 25 μ M, Garcinol reduced only P/CAF acetylation and barely affected the protein level. CBP was the most resistant to Garcinol, with both acetylation and protein levels still readily detectable in 25 μ M Garcinol.

We had shown earlier that 25 μ M Garcinol completely abolished FOXP3 acetylation levels (Fig. 1). At this concentration, Garcinol affected both p300 and TIP60, but not CBP and P/CAF levels. Taken together, this study indicates that both p300 and TIP60 may be more relevant to regulation of FOXP3 than P/CAF and CBP.

Mutations on FOXP3 that increase acetylation and stability render FOXP3 more susceptible to Garcinol-induced degradation

We have identified four Lys residues that affect acetylation of FOXP3: K31, K179, K227, and K268. When any of these Lys residues was substituted with the same positively charged Arg residue, acetylation of FOXP3 as well as the total protein levels were increased (Fig. 3C). Although protein levels of these mutants were not significantly affected by Garcinol, acetylation of these FOXP3 mutants was much more susceptible to inhibition by Garcinol (15 μ M) than that of the wild type FOXP3 (Fig. 3D). Treatment of Garcinol induced more significant reduction in acetylation levels in these FOXP3 mutants, especially the K31R mutant. Our study concluded that these 4 Lys play a negative role in the overall acetylation levels of FOXP3. The mutants might be much more accessible to acetyltransferases and thus had much higher acetylation levels. However, the elevated acetylation levels were sustained by endogenous acetyltransferases such as p300 and TIP60. When these enzymes were inhibited and degraded by Garcinol, the acetylation level of FOXP3 was then quickly reduced.

Inhibition of T_{reg} functions by Garcinol

We next examined whether Garcinol affected T_{reg} suppressive function. Mouse CD4⁺CD25^{high} T_{reg} cells were isolated from C57BL/6 mice and used to study their activity to suppress the proliferation of carboxyfluorescein diacetate succinimidyl ester (CFSE)—labeled primary CD4⁺CD25⁻CD45RB^{high} T cells (representing effector T cells, T_{eff}) (Fig. 5). T_{eff} and T_{reg} cells were mixed at different ratios. The proliferative population of T_{eff} were compared between all treatments. T_{reg} cells inhibited the proliferation of T_{eff} cells in a dose-dependent manner.

Modest intrinsic inhibitory activity of Garcinol on T_{eff} was noted even in the absence of T_{reg} cells. Effector cells were slightly less proliferative when incubated with Garcinol (data not shown). The population of proliferative T_{eff} cell was reduced from 54% in the control to 48.3% and 38.4% in the presence of 2 μ M and 4 μ M Garcinol, respectively. Nevertheless, after the population of dividing T_{eff} under different conditions was normalized to that of T_{eff} cells treated only by Garcinol (Fig. 5), it was obvious that Garcinol treatment reduced the suppressive activity of T_{reg} cells and led to more proliferative T_{eff} cells. The Garcinol effect was more obvious when there were less T_{reg} cells in the mixture, e.g. at the T_{eff}/T_{reg} ratio of 16:1 and 8:1.

Synergistic activity of Garcinol and anti-p185^{her2/neu} in the treatment of tumors in vivo

p185^{her2/neu} is a member of the ERBB family of receptor tyrosine kinases and has been validated as a clinical target for breast and stomach cancers. Monoclonal antibodies to the oncoprotein of rat origin were developed to establish the foundation for targeted therapies to solid tumors (Drebin et al., 1984, 1986). One of the monoclonal antibodies, mAb7.16.4, has a shared epitope with trastuzumab, a FDA approved therapeutic agent in clinical use (Zhang

et al., 1999). 7.16.4 is active on *ErbB2/neu* transformed rodent and human tumors in a variety of assays (Cai et al., 2013; Zhang et al., 1999). 7.16.4 has been used in many labs around the world in transgenic animal models of tumors induced by the *neu* oncogene (Katsumata et al., 1995; Park et al., 2010; Stagg et al., 2011).

Recent studies defined a role for CD8+ IFN- γ -secreting cells and NK ADCC mediating cells as contributory elements in 7.16.4 therapy of implanted tumor model of *neu* transformed cells (Park et al., 2010; Stagg et al., 2011). It is expected that both active innate and adaptive immune cells that translocate and reside in the tumors contribute to the anti-cancer activity of 7.16.4 and trastuzumab. In addition we have noted the presence of infiltrated FOXP3+ T cells in tumor tissues (unpublished) and others have noted that FOXP3+ T cells may affect the growth of transplanted *neu* transformed cells in rodent model of tumor evasion (Kerkar and Restifo, 2012; Zhang et al., 2012a).

We examined whether Garcinol, which promotes degradation of p300 and contributes to FOXP3 loss, could enhance 7.16.4 anti-tumor activity. The target tumor cell line H2N113 was established from the tumor of MMTV-*neu* transgenic mice (Stagg et al., 2011). In vitro proliferation assay demonstrated that 7.16.4 could inhibit H2N113 in a dose-dependent manner (Fig. 6A). We did note that Garcinol slightly inhibited the proliferation of H2N113. However, Garcinol was not able to enhance the activity of 7.16.4 in this in vitro assay.

In vivo activity of Garcinol was studied in a transplant model. We implanted H2N113 tumor cells into BALB/c-MMTV-*neu*/FOXP3-GFP mice as reported previously (Stagg et al., 2011). Treatment began when tumors were apparent (10 days after tumor inoculation). Mice were treated with 7.16.4, at either low dose (1.8 mg/kg) or high dose (9 mg/kg), twice weekly. As shown in Fig. 6B, the high dose 7.16.4 treatment alone significantly reduced the tumor growth.

The growth of tumor appeared to be only modestly reduced by the low dose 7.16.4 treatment. While Garcinol (43.6 mg/kg) had little discernible effects on the growth of tumors, the combination of Garcinol and low dose 7.16.4 led to enhanced inhibition of tumor growth. These data indicate that the inhibitory activity of Garcinol on T_{reg} function has a positive effect on anti-p185^{her2/neu} antibody targeted therapy.

Discussion

Garcinol-induced p300 degradation leads to FOXP3 hypo-acetylation

FOXP3 plays a key role in maintaining T_{reg} cells' functions and represents a therapeutic target to regulate T_{reg} cells. Our laboratory identified acetylation as one mechanism to regulate FOXP3 stability. Clearly enzymes that are involved in the acetylation or deacetylation of FOXP3 represent targets that may lead to manipulation of FOXP3 functions. For example, the histone deacetylase (HDAC) inhibitor VPA has been studied as an enhancer for T_{reg} function (de Zoeten et al., 2010, 2011; Saouaf et al., 2009).

p300 and TIP60 are two acetyltransferases that synergistically affect FOXP3 acetylation levels (Xiao et al., in preparation). To understand if FOXP3 can be regulated by inhibiting acetyltransferases, we have studied Garcinol, a natural compound that was initially identified as an inhibitor for p300.

Garcinol leads to rapid degradation of p300 via a lysosome dependent mechanism, as Chloroquine but not MG132 can prevent the Garcinol induced degradation. After Garcinol-mediated degradation was blocked by Chloroquine, p300 acetylation was easily detected.

Our studies indicate that lack of acetylation is not the mechanism to sort p300 for degradation via the lysosome pathway.

p300 degradation has been investigated previously (Chen and Li, 2011). Changes in acetylation as well as phosphorylation of p300 have been shown to associate with the degradation of p300 (Jain et al., 2012; Poizat et al., 2005). We report here for the first time the involvement of the lysosome pathway in the degradation of p300.

FOXP3 acetylation levels are affected by p300 degradation (Fig. 1). In the presence of 15 μ M Garcinol, the FOXP3 protein level is not affected but the acetylation level is reduced. The FOXP3 level itself is eventually reduced by higher concentration of Garcinol (25 μ M). Loss of FOXP3 acetylation precedes degradation, which is consistent with our observation that acetylation of FOXP3 increases its stability (van Loosdregt et al., 2010) (Xiao et al., manuscript in preparation).

The acetyltransferase activity is not critical for p300 to interact with FOXP3

This study reveals that Garcinol achieves its effect on FOXP3 independently of inhibiting the enzymatic activity of p300. When the degradation of p300 is arrested by Chloroquine treatment, FOXP3 acetylation is readily detected even in the presence of Garcinol and inactivated p300 enzymatic activity (Fig. 2). Chloroquine does not interfere with p300 enzymatic activity, as it only inhibits the lysosomal degradation process.

FOXP3 acetylation and stability is dependent on the presence of the intact p300 protein but not linked to p300 enzymatic activity. Our own work and that of others have established that p300 interacts with FOXP3 (van Loosdregt et al., 2010). Our co-immunoprecipitation studies (Fig. 1B) indicate that Garcinol interferes with the interaction between p300 and FOXP3. In addition, in separate studies we observed that the acetyltransferase-deficient p300 could interact with FOXP3 as well as the wild type p300 did in co-immunoprecipitation (Fig. S2).

The role of lysosome in the regulation of p300/FOXP3

Proteasome mediated degradation is one mechanistic operation to regulate FOXP3 levels, as the proteasome inhibitor MG132 modifies the steady state FOXP3 levels (van Loosdregt et al., 2010). MG132 could not rescue Garcinol-mediated p300 or FOXP3 degradation. At higher concentrations (20–50 μ M), MG132 even increases FOXP3 degradation (Fig. 2). We do not think that the effect of the high dose MG132 is specific to FOXP3, as the protein levels of β -actin were also reduced, indicating a general cytotoxicity related to high MG132 concentration in the presence of Garcinol. Interestingly, the acetylation level of FOXP3 is increased despite the reduced protein level, reflecting that the acetylated form is more stable than the non-acetylated FOXP3. Our study reveals for the first time that lysosome also plays an important role to maintain FOXP3, primarily by regulating p300 levels.

A repressive model of regulatory Lys residues for FOXP3 acetylation

Kwon and colleagues reported three acetylation sites in mouse Foxp3: K31, K262 and K267 (Kwon et al., 2012). p300 and the deacetylase SIRT1 appeared to regulate acetylation on K31 and K262, as the presence of either p300 or the SIRT1 specific inhibitor Ex-527 enhanced acetylation on these sites. The corresponding sites in human FOXP3 are K31, K263, and K268, respectively. Consistently, we also observed elevated stability and acetylation levels for K31R and K268R FOXP3 mutants. In addition, two other sites (K179 and K227) may also play similar roles in the regulation of FOXP3 stability (Fig. 3).

It was suggested that these Lys residues might also be ubiquitination sites on FOXP3 (Kwon et al., 2012). Acetylation of Lys or substitution with Arg would prevent ubiquitination and subsequent proteasome degradation of FOXP3. However, this “competitive” model is not consistent with our findings. If these sites were equally accessible to ubiquitination, we would not expect to observe such a dramatic increase in FOXP3 protein level with a single Arg substitution.

In addition, if the ubiquitination sites were eliminated by Arg substitution, we would expect to observe reduced ubiquitination levels in mutants. In contrast, the K227R and K268R FOXP3 mutants were shown to have increased ubiquitination accompanying elevated total FOXP3 levels (Fig. S3). Acetylation on these Lysines may be repressive and prevent acetylation on additional Lys sites that are more directly involved in FOXP3 stability. As shown in Fig. 3, mutation of any of these sites leads to a significant increase in total FOXP3 acetylation.

p300 alone may not be sufficient to acetylate FOXP3. In studies to be published separately it is the formation of heteroacetylase complexes which determine the acetylation patterns per se. p300 may be involved in FOXP3 stability as a chaperone-like binding partner, although its enzymatic activity is also required for its cooperation with TIP60 to regulate some FOXP3 functionalities (Xiao et al., manuscript in preparation).

Garcinol as a potential inhibitor of FOXP3 and T_{reg} functions for cancer therapies

Garcinol affects the FOXP3 stability by causing lysosome-dependent p300 degradation and the loss of p300 protein may reflect Garcinol's mechanism of action. Garcinol by reducing p300 protein levels enhances the *in vivo* activity of 7.16.4 in the inhibition of tumor growth, in which T_{reg} plays a negative role (Fig. 6B). We are encouraged by this study and have started to design p300 inhibitors that are more specific for p300–FOXP3 interaction, an approach that can circumvent the non-specific toxicity of Garcinol due to multiple functions of p300 (Chen and Li, 2011) and the broad effects of Garcinol on many types of targets (Ahmad et al., 2012; Padhye et al., 2009; Saadat and Gupta, 2012).

Conclusions

Garcinol induces p300 degradation via the lysosome pathway. Since p300 is involved in the regulation of FOXP3, Garcinol treatment leads to FOXP3 hypoacetylation and degradation. Garcinol treatment is associated with reduced Treg suppressive activity and helps improve the anti-tumor activity of targeted antibody therapy to p185^{her2/neu}. In summary, Garcinol as an inhibitor for p300 has provided a unique tool to understand how p300 regulate FOXP3 acetylation and stability. This knowledge may lead to new strategies to develop therapeutic agents for cancers and other diseases that T_{reg} cells are involved in.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
CFSE	Carboxyfluorescein succinimidyl ester
CREB	cAMP response element-binding protein
DMEM	Dulbecco's modified Eagle's medium
RPMI	Roswell Park Memorial Institute medium
EGFR	epidermal growth factor Receptor
FDA	Food and Drug Administration
FOXP3	Forkhead box P3
HAT	histone acetyltransferase
HDAC	histone deacetylase
HRP	Horseradish peroxidase
IFN	interferon
IP	Immunoprecipitation
mAb	Monoclonal Antibody
NK	Natural killer cell
PCAF	p300/CBP-associate factor
Treg	Regulatory T Cell
TSA	Trichostatin

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Fig. 1.

Garcinol induces p300 and FOXP3 degradation. HEK293T cells were transfected with plasmids to express HA-FOXP3 and/or Flag-p300 (pFLAG-p300). pFLAG and PIPHA2 were control plasmids for the tagged expression vector. Cells were treated for 4 h with 15 or 25 μ M Garcinol. Dose-dependent inhibition of protein acetylation (A) and p300-FOXP3 interaction (B) were studied by immunoprecipitation (IP) with anti-acetyl-lysine antibody (A) and anti-FLAG antibody M2 (B), respectively. Tagged proteins in precipitants and lysates were studied by Western blot (WB) using anti-HA (for HA-tagged FOXP3) or anti-FLAG antibodies (for FLAG-tagged p300). Lysates from different treatments were also blotted for β -actin as the loading control.

Fig. 2.

Effects of Garcinol on p300 and FOXP3 are prevented by Chloroquine. HEK293T cells were first transfected with HA-FOXP3 and/or Flag-p300. 24 h after transfection, cells were exposed to the lysosome specific inhibitor Chloroquine or the proteasome specific inhibitor MG132 for 1 h, and subsequently incubated with 25 μ M Garcinol for 4 h (A) or with 1 μ M Bafilomycin A1 together with 50 μ M Chloroquine (B). IP and blot were performed as in Fig. 1. A. Chloroquine but not MG132 prevented Garcinol-induced changes in p300 and FOXP3. B. Bafilomycin A1 dampens Chloroquine's protective effect on p300.

Fig. 3.

Effect of Garcinol on FOXP3 mutants. A & B: Reduction of FOXP3 acetylation by Garcinol is not dependent on acetylation by p300 on K250/K252. HEK 293T cells were transfected with only HA-FOXP3a (A) or HA-FOXP3a K250R/K252R mutant (B). Acetylation and degradation of FOXP3 were studied as in Fig. 1. In the absence of ectopic p300, Garcinol could still induce reduction of acetylation in either wild type or the K250/K252 FOXP3 mutant. C & D: Mutations on FOXP3 increase acetylation and stability but render protein more susceptible to Garcinol-induced degradation. HEK 293T cells were transfected with only HA-FOXP3a mutants (K179R, K268R, K227R, or K31R). Expression level of these mutants (C) and response to 15 μ M Garcinol (D) were compared with the wild type FOXP3. Note: Since the wild type FOXP3 had a much lower expression level than mutants, wild type lysates were used at twice that amount of mutants for IP and blot (2 $\times$).

Fig. 4.

Activity of Garcinol as a broad spectrum acetyltransferase inhibitor. HEK 293T cells were transfected with FLAG-tagged p300, CBP, TIP60 or P/CAF. Acetylation and protein levels of these FLAG-tagged proteins were analyzed as in Fig. 1. Garcinol showed varied activity to inhibit the acetylation and induce degradation for these acetyltransferases.

Fig. 5.

Garcinol reduces T_{reg} function. A. CFSE-labeled T_{eff} cells were co-cultured with T_{reg} cells and Garcinol for 3 days before FACS analysis for proliferating cells. The proliferative population of cells in each treatment was normalized using the T_{eff} cells treated only with Garcinol (no T_{reg}).

Fig. 6.

Garcinol enhances the in vivo, not in vitro, anti-tumor activity of 7.16.4. (A) Effect of Garcinol and the 7.16.4 antibody on the proliferation of H2N113 cells. 5000 H2N113 cells were plated in 96-well plates and incubated with different concentrations of Garcinol (0.5–1 µM) and/or 7.16.4 antibody (1 µg/ml–10 µg/ml) for 72 h. Cell viability was determined by MTT assay as described in Materials and methods. (B) In vivo activity of Garcinol and/or the 7.16.4 antibody on tumor growth. Established MMTV-neu/FOXP3-GFP transgenic mice were subcutaneously injected into the flank with 1×10^6 H2N113 cells. 10 days after tumor cell injection, mice received intraperitoneal injection of Garcinol and/or 7.16.4 antibody at

the indicated dose two times per week, for 3 weeks. Mean values of tumor volume (in mm³) at the end of the treatment are presented.